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Kinetics of vitamin D3 metabolism by cytochrome P450scc (CYP11A1) in phospholipid vesicles and cyclodextrin

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ABSTRACT

Vitamin D3 can be hydroxylated sequentially by cytochrome P450scc (CYP11A1) producing 20-hydroxyvitamin D3, 20,23-dihydroxyvitamin D3 and 17,20,23-trihydroxyvitamin D3. The aim of this study was to characterize the ability of vitamin D3 to associate with phospholipid vesicles and to determine the kinetics of metabolism of vitamin D3 by P450scc in vesicles and in 2-hydroxypropyl-β-cyclodextrin (cyclodextrin). Gel filtration of phospholipid vesicles showed that the vitamin D3 remained quantitatively associated with the phospholipid membrane. Vitamin D3 exchanged between vesicles at a rate 3.8-fold higher than for cholesterol exchange and was stimulated by N-62 StAR protein. The K_m of P450scc for vitamin D3 in vesicles was 3.3 mol vitamin D3/mol phospholipid and the rate of conversion of vitamin D3 to 20-hydroxyvitamin D3 was first order with respect to the vitamin D3 concentration for the range of concentrations of vitamin D3 that could be incorporated into the vesicle membrane. 20-Hydroxyvitamin D3 was further hydroxylated by P450scc in vesicles, producing primarily 20,23-dihydroxyvitamin D3, with K_m and k_{cat} values 22- and 6-fold lower than those for vitamin D3, respectively. 20,23-Dihydroxyvitamin D3 was converted to 17,20,23-trihydroxyvitamin D3 with even lower K_m and k_{cat} values. Vitamin D3 and cholesterol were metabolized with comparable efficiencies in cyclodextrin, but the K_m for both showed a strong dependence on the cyclodextrin concentration, decreasing with decreasing cyclodextrin. This study shows that vitamin D3 quantitatively associates with phospholipid vesicles, can exchange between membranes, and can be hydroxylated by membrane-associated P450scc but with lower efficiency than for cholesterol hydroxylation. The k_{cat} values for metabolism of vitamin D3 in vesicles and 0.45% cyclodextrin are similar, but the ability to solubilize vitamin D3 at a concentration higher than its K_m makes the cyclodextrin system more efficient for producing the hydroxyvitamin D3 metabolites for further characterization.

1. Introduction

Cytochrome P450scc catalyzes the conversion of cholesterol to pregnenolone which is the first catalytic step in the synthesis of steroid hormones (Tuckey, 2005). The reaction involves removal of the side chain of cholesterol via production of enzyme-bound intermediates, 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol (Hume et al., 1984; Lambeth et al., 1982; Tuckey, 1990, 2005). Cytochrome P450scc can also metabolize vitamin D3 (D3) and vitamin D2 (D2), as well as their precursors 7-dehydrocholesterol and ergosterol (Guryev et al., 2003; Slominski et al., 2004, 2005a,b, 2006; Tuckey et al., 2008). Our most recent study shows that P450scc can hydroxylate D3 at carbons 17, 20 and 23 (Tuckey et al., 2008). The major pathway of metabolism

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involves initial hydroxylation at C20 followed by C23 and C17, producing 20-hydroxyvitamin D3 (20(OH)D3), 20,23-dihydroxyvitamin D3 (20,23$(OH)_2$D3) and 17,20,23-trihydroxyvitamin D3 (17,20,23$(OH)_3$D3) as the major products. Minor products result from the hydroxylations occurring in a different order. 20(OH)D3 and 20,23$(OH)_2$D3 accumulate indicating that these derivatives can escape from the active site of the enzyme. It should be noted that earlier reports incorrectly identified 20,23$(OH)_2$D3 as 20,22$(OH)_2$D3 (Guryev et al., 2003; Slominski et al., 2005b).

The active form of D3, 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3), has antiproliferative effects and promotes differentiation in a number of cell types including keratinocytes (Bikle, 2004; Holick, 2003a,b). We have found that the major product of D3 metabolism by P450scc, 20(OH)D3, also inhibits proliferation and stimulates differentiation of keratinocytes with a similar potency to that of 1,25$(OH)_2$D3 (Zbytek et al., in press). Thus, at least one of the products of P450scc action on D3 is biologically active and may have potential therapeutic actions. It may also have physiological actions if it is produced in sufficient amounts in vivo, such as in skin where a low concentration of P450scc is found (Slominski et al., 2004) and D3 is produced (Holick, 2003a,b), or in the adrenal gland, which has a high P450scc content and may receive vitamin D from the bloodstream. Isolated rat adrenal mitochondria produce a number of hydroxyvitamin D derivatives when supplied with exogenous D3 and the synthesis of at least some of these involves P450scc (Slominski et al., 2005b). Metabolism of D3 by P450scc in vivo would require that it compete with cholesterol. To further assess this possibility we have determined the kinetics of D3 metabolism by P450scc incorporated into phospholipid vesicles to mimic the inner-mitochondrial membrane where P450scc resides. The major products, 20(OH)D3 and 20,23$(OH)_2$D3, were purified and tested as substrates to determine the rate of each hydroxylation. We compared the use of 2-hydroxypropyl-β-cyclodextrin (cyclodextrin) and phospholipid vesicles for dissolving D3 to make it available to P450scc. Both of these systems have been used previously with data for cyclodextrin suggesting that D3 and cholesterol are metabolized at similar rates, whereas in phospholipid vesicles, D3 appears to be metabolized more slowly (Guryev et al., 2003; Slominski et al., 2005b). In addition, we tested the ability of D3 to incorporate quantitatively into phospholipid membranes and exchange between membranes.

2. Methods

2.1. Preparation of enzymes and hydroxyvitamin D3 derivatives

Adrenodoxin reductase and P450scc were purified from bovine adrenal mitochondria (Tuckey and Stevenson, 1984a,b). The concentration of cytochrome P450scc was determined from the CO-reduced minus reduced difference spectrum using an extinction coefficient of 91,000 M^{-1} cm^{-1} for the absorbance difference between 450 nm and 490 nm (Omura and Sato, 1964). Adrenodoxin was expressed in *Escherichia coli* and purified as previously described (Woods et al., 1998). N-62 StAR was a gift from Walter Miller (University of California, San Francisco). 20(OH)D3 and 20,23$(OH)_2$D3 were prepared enzymatically from 50 ml incubations of P450scc with D3 and purified by preparative TLC as described before (Slominski et al., 2005b). The purity of these samples was checked prior to use by HPLC (Tuckey et al., 2008). The concentration of hydroxyvitamin D3 was determined using an extinction coefficient of 18,000 M^{-1} cm^{-1} at 263 nm (Hiwatashi et al., 1982).

2.2. Measurement of cytochrome P450scc activity in phospholipid vesicles

Vesicles were prepared from dioleoyl phosphatidylcholine and bovine heart cardiolipin in the ratio 85:15 (mol/mol). Where required, D3, hydroxyvitamin D3 or cholesterol was added to the phospholipid (see Section 3). Buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol and 0.1 mM EDTA was added to 1.25 μmol of phospholipid and the mixture sonicated for 10 min in a bath-type sonicator (Tuckey and Kamin, 1982). Purified P450scc was incorporated into the vesicle membrane by incubation with the vesicles for 20 min at room temperature (Tuckey and Kamin, 1982). The incubation mixture comprised 510 μM phospholipid vesicles, cytochrome P450scc (0.2–2 μM), 15 μM adrenodoxin, 0.2 μM adrenodoxin reductase, 2 mM glucose 6-phosphate, 2 U/ml glucose 6-phosphate dehydrogenase and 50 μM NADPH, in the buffer used for sonication. Samples were preincubated for 8 min, reactions started by the addition of NADPH and incubations carried out at 37 °C in a shaking water bath. Typical incubation volumes were 0.2–1.0 ml. Reactions were stopped by the addition of 2 ml ice-cold dichloromethane. After centrifugation to separate phases, the lower dichloromethane phase was retained and the aqueous phase re-extracted twice more with 2 ml dichloromethane. The dichloromethane was removed under nitrogen and samples dissolved in 64% methanol in water for HPLC analysis.

Incubation times were kept short (3–5 min) in experiments designed to measure the kinetic constants of P450scc to ensure initial rates were measured. Time courses were carried out to confirm initial rates were linear for the incubation times used. Short incubation times avoided the more complex kinetics that occurred later in the incubation resulting from intermediates of D3 metabolism acting as competitive substrates. Products were identified based on their retention times compared to authentic standards, whose structures were identified in our previous report (Tuckey et al., 2008).

2.3. Measurement of P450scc activity with substrates dissolved in cyclodextrin

Incubations were carried out as described for phospholipid vesicles except that the vesicles were replaced by 2-hydroxypropyl-β-cyclodextrin at a final concentration of 0.45–4.5%. Substrates were initially dissolved in 45% cyclodextrin (typically 5–8 mM) (De Caprio et al., 1992). These solutions were diluted as necessary (see Section 3)