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Cytochrome P450scc-dependent metabolism of 7-dehydrocholesterol in placenta and epidermal keratinocytes

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ABSTRACT

The discovery that 7-dehydrocholesterol (7DHC) is an excellent substrate for cytochrome P450scc (CYP11A1) opens up new possibilities in biochemistry. To elucidate its biological significance we tested ex vivo P450scc-dependent metabolism of 7DHC by tissues expressing high and low levels of P450scc activity, placenta and epidermal keratinocytes, respectively. Incubation of human placenta fragments with 7DHC led to its conversion to 7-dehydropregnenolone (7DHP), which was inhibited by DL-aminoglutethimide, and stimulated by forskolin. Final proof for P450scc involvement was provided in isolated placental mitochondria where production of 7DHP was almost completely inhibited by 22*R*-hydroxycholesterol. 7DHC was metabolized by placental mitochondria at a faster rate than exogenous cholesterol, under both limiting and saturating conditions of substrate transport, consistent with higher catalytic efficiency (k_{cat}/K_m) with 7DHC as substrate than with cholesterol. Ex vivo experiments showed five 5,7-dienal intermediates with MS spectra of dihydroxy and mono-hydroxy-7DHC and retention time corresponding to 20,22(OH)$_2$7DHC and 22(OH)7DHC. The chemical structure of 20,22(OH)$_2$7DHC was defined by NMR. 7DHP was further metabolized by either placental fragments or placental microsomes to 7-dehydroprogesterone as defined by UV, MS and NMR, and to an additional product with a 5,7-dienal structure and MS corresponding to hydroxy-7DHP. Furthermore, epidermal keratinocytes transformed either exogenous or endogenous 7DHC to 7DHP. 7DHP inhibited keratinocytes proliferation, while the product of its pholytic transformation, pregcalciferol, lost this capability. In conclusion, tissues expressing P450scc can metabolize 7DHC to biologically active 7DHP with 22(OH)7DHC and 20,22(OH)$_2$7DHC serving as intermediates, and with further metabolism to 7-dehydroprogesterone and (OH)7DHP.

1. Introduction

7-Dehydrocholestrol (7DHC) serves as a precursor to both cholesterol, through enzymatic reduction of the B ring by the action of 7DHC Δ-reductase (Miller and Auchus, 2011; Tint et al., 1994), and to vitamin D3 through physicochemical process induced by ultraviolet light B (Holick, 2003). Until recently it was thought that cytochrome P450scc (CYP11A1) used solely cholesterol as its substrate, hydroxylating its side chain at C22 and C20, then cleaving between C20 and C22 to produce pregnenolone, a substrate for steroid hormones (Miller and Auchus, 2011; Tuckey, 2005). Recently, it was demonstrated that CYP11A1 is capable of the oxidation and cleavage of the 7DHC side chain to produce 7-dehydropregnenolone (7DHP) (Slominski et al., 2004, 2009; Guryev et al., 2003), and of hydroxylating the side chain of vitamin D and ergosterol without its cleavage (Slominski et al., 2005a, 2005b, 2006; Guryev et al., 2003; Tuckey et al., 2008a; Nguyen et al., 2009).

A deficiency of 7DHC Δ-reductase, as seen in Smith Lemli Opitz syndrome (SLOS), leads to increased accumulation of 7DHC and production of 7DHP (Nowaczyk and Waye, 2001; Tint et al., 1994; Shackleton et al., 1999). Similarly, in vivo production of 7DHP occurs under physiological conditions during synthesis of equilin (Δ-7-estrone) in horses (Tait et al., 1983), and ex vivo in rat, rabbit, dog and pig adrenal glands (Slominski et al., 2009). 7DHP may then undergo sequential transformation to the hydroxy-derivatives of 5,7-steroidal dienes, as supported by the documented

Abbreviations: cyclodextrin, 2-hydroxypropyl-β-cyclodextrin; COSY, correlation spectroscopy; 7DHP, 7-dehydropregnenolone; 7DHC, 7-dehydrocholesterol; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; pD, pregcalciferol; TOCSY, total correlation spectroscopy; RT, retention time.

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accumulation of 7DHP and its hydroxy-derivatives (including 21-hydroxy-, 17-hydroxy-, 20-hydroxy- and 17,20-dihydroxy-7DHP) in body fluids of SLOS patients (Shackleton et al., 1999, 2002; Marcos et al., 2004), and its ex vivo transformation to the corresponding hydroxy-7DHP or 7-dehydroprogesterone in mammalian adrenal glands. 7DHP, its hydroxy-derivatives and 20(OH)7DHC are biologically active toward skin and leukemia cells (Slominski et al., 2009, 2010), and as indicated by the pathological features of SLOS patients (Nowaczyk and Waye, 2001; Marcos et al., 2004; Tint et al., 1994; Smith et al., 1964).

P450scc shows relatively high expression in placenta where it initiates a local steroidogenic pathway leading to synthesis of progesterone with pregnelone as an intermediate (Tuckey, 2005). While epidermal keratinocytes accumulate significant amount of 7DHC, representing a major source of vitamin D3 for the body (Holick, 2003; Bikle, 2011), they express comparatively low levels of P450scc (Slominski et al., 1996, 2004), however, sufficient to initiate a local steroidogenic pathway (reviewed in Slominski et al., 2008, 2012b). Furthermore, studies with the purified enzyme have shown that 7DHC is a better substrate for P450scc than cholesterol (Slominski et al., 2009). To better define the biological significance of the discovery that 7-dehydrocholesterol (7DHC) is metabolized by cytochrome P450scc (CYP11A1), being an even better substrate than cholesterol, we elucidated ex vivo its metabolism by tissues/cells expressing high (placenta) and low (epidermal keratinocytes) CYP11A1 activities. We tested in detail the ability of human placental fragments incubated ex utero to convert 7DHC to 7DHP, and 7DHP to 7-dehydroprogesterone. We also investigated the capability of keratinocytes to produce 7DHP, including its endogenous production with preliminary assessment of the antiproliferative activity of 7DHP.

2. Materials and methods

2.1. Placentas

Term placentas (37–42 weeks) were obtained from King Edward Memorial Hospital for Women located at Crawley, WA, according to protocols approved by the hospital's human ethics committee, or from the MedPlex in Memphis, according to protocols approved by local IRB committee at the UTHSC. The patients were 18–35 years old.

2.2. Incubations with human placental fragments and analysis of products

Placental fragments (parenchyma) were dissected from the membranes, washed in PBS, cut with scissors into small fragments and suspended in buffer (pH 7.4) comprising 33 mM Tris aminomethane, 110 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 and 2 mg/ml glucose, and incubated as described previously (Slominski et al., 2009). In total, 40 placentas were used, with 2–3 placentas being used for each experiment. Briefly, the reactions were started by adding isocitrate (5 mM), and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol; 7DHC) or 7-dehydropregnenolone (3β-hydroxypregna-5,7-dien-20-one; 7DHP) to a final concentration of 1 mM,100 μM or 10 μM as indicated. Tubes were then incubated at 37 °C for various times as indicated in the figure legends. For the majority of experiments, a 10 mM stock solution of 7DHC or 7DHP was prepared in 45% 2-hydroxypropyl-β-cyclodextrin immediately before use. In selected experiments 100 mM stock solutions were prepared in ethanol and added to the incubation mixtures at a 1:200 or 1:250 ratio. There was no difference in the metabolism of these compounds for either solvent. 7DHC was purchased from Sigma Chemical Co. (St. Louis, MO), while 7DHP was chemically synthesized and purified or photochemically transformed to pregcalciferol (pD) as described (Zmijewski et al., 2008). In control experiments, either 7DHC or 7DHP were omitted from the incubation mixture or fragments of placentas were boiled for 5 min before addition of the substrates. The reactions were stopped by placing the tubes on ice and steroids were extracted twice with methylene chloride and dried under nitrogen.

Two different HPLC and mass spectrometry systems were used to analyze incubations of placental extracts and the independent analyses confirmed the major findings of this study. First, extracts were re-dissolved in methanol and analyzed using an API-3000 LC–MS/MS (Applied Biosystems, Toronto, Canada) mass spectrometer equipped with an ESI source with Zorbax Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm) (Agilent Technology, Santa Clara, CA). For initial identification of 7DHP, the elution was carried out isocratically using 85% methanol in water at a flow rate of 0.05 ml/min. The relative amount of 7DHP produced by placenta fragments was based on the intensity of the LC/MS signal (see Figs. 2, 4, 6–9). Both standards and placental extracts were separated and measured and under the same conditions, using a gradient of 85% to 100% methanol at 0.075 ml/min over 20 min.

The second RP-HPLC system used was a dual pump chromatograph (Waters 2695 Alliance, Milford, MA) equipped with a Waters Atlantis dC18 column (100 mm × 4.6 mm, 5 μm particle size). In order to detect the products of 7DHC metabolism, elution was carried out with a gradient of methanol in water (85–100%) at a flow rate 0.5 ml/min (20 min), followed by a wash with 100% methanol (10 min). Fractions were monitored with a photodiode array detector (Waters 996, Milford, MA) and collected manually for further MS analyses. The collected samples were analyzed using an API-4000 LC–MS/MS (Applied Biosystems, Toronto, Canada) mass spectrometer equipped with an ESI source in positive mode with a declustering potential of 80 V, an entrance potential of 10 V and an ion spray voltage of 4.5 kV.

Mass spectra of intermediates generated by purified P450scc were also acquired with a Bruker Esquire-LC/MS system (Bruker Daltonics, Billerica, MA) utilizing an electrospray ionization (ESI) source. Data were collected and processed by ACD mass processor.

2.3. Measurement of 7-dehydrocholesterol and [4-^{14}C]cholesterol metabolism by placental mitochondria

A mitochondrial fraction from the placentas was prepared by differential centrifugation as described previously (Tuckey et al., 1997). Mitochondria isolated by this procedure showed minimal cytoplasmic contamination, containing only 1.9% of the total lactate dehydrogenase activity assayed by the oxidation of NADPH (0.15 mM) in the presence of 1.0 mM pyruvate. Mitochondria (0.88 mg/ml) were incubated in buffer comprising 50 mM HEPES pH 7.4, 0.25 M sucrose, 20 mM KCl, 5 mM MgSO4, 0.2 mM EDTA and 0.5 mg/ml bovine serum albumin (fatty acid free) in a final volume of 0.5 ml. Cyanoketone (8 μM) (Sterling-Winthrop Research Institute, Rensselaer, NY), a 3β-hydroxysteroid dehydrogenase inhibitor (Arthur and Boyd, 1974), was added to prevent metabolism of the pregnenolone or 7-dehydropregnenolone product, as before (Slominski et al., 2009). Mitochondria were preincubated at 37 °C for 8 min then 7-dehydrocholesterol or [4-^{14}C]cholesterol (0.05 μCi) was added to the mitochondria to a final concentration of 200 μM from a 10 mM stock solution in ethanol. Reactions were then started immediately by the addition of isocitrate and $NADP^+$, to final concentrations of 5 mM and 50 μM, respectively. N-62 StAR protein (5 μM) was present for some incubations (see Section 3) and was added at the same time as substrate. The N-62 StAR protein, which was expressed in *Escherichia coli* (Bose et al., 2000), was provided by Walter Miller