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J Gustafsson

J Clin Invest. 1985;75(2):604-607. <https://doi.org/10.1172/JCI111737>.

Research Article

Hydroxylation of 5 beta-[7 beta-3H]cholestane-3 alpha, 7 alpha-diol was studied in mitochondrial preparations from human fetal livers. The livers were obtained at legal abortions between weeks 14 and 24. In addition to hydroxylation in the 26-position, 5 beta-cholestane-3 alpha, 7 alpha-diol was hydroxylated in the 12 alpha-position. In one experiment, mitochondrial protein was solubilized and partially purified. Material with such chromatographic properties as those of cytochrome P450 showed 12 alpha-hydroxylase activity when combined with adrenodoxin and adrenodoxin reductase from bovine adrenal mitochondria. Because adrenodoxin and adrenodoxin reductase are components specific for mitochondrial hydroxylase systems, the results exclude microsomal contamination as the origin of this 12 alpha-hydroxylase activity. Further, there was no hydroxylase activity when NADPH-cytochrome P450 reductase from rat liver microsomes was added instead of adrenodoxin and adrenodoxin reductase. The microsomal fraction of fetal liver was also shown to possess 12 alpha-hydroxylase activity. Microsomal and mitochondrial hydroxylase activities per milligram of protein towards 5 beta-cholestane-3 alpha, 7 alpha-diol were of the same order of magnitude. The occurrence of an efficient sterol nucleus hydroxylase activity in liver mitochondria appears to be unique for fetal liver.

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Bile Acid Synthesis During Development

Mitochondrial 12 α -Hydroxylation in Human Fetal Liver

Jan Gustafsson

Departments of Pediatrics and Pharmaceutical Biochemistry, Uppsala University, Uppsala, Sweden

Abstract

Hydroxylation of 5 β -[7 β -³H]cholestane-3 α ,7 α -diol was studied in mitochondrial preparations from human fetal livers. The livers were obtained at legal abortions between weeks 14 and 24. In addition to hydroxylation in the 26-position, 5 β -cholestane-3 α ,7 α -diol was hydroxylated in the 12 α -position. In one experiment, mitochondrial protein was solubilized and partially purified. Material with such chromatographic properties as those of cytochrome P₄₅₀ showed 12 α -hydroxylase activity when combined with adrenodoxin and adrenodoxin reductase from bovine adrenal mitochondria. Because adrenodoxin and adrenodoxin reductase are components specific for mitochondrial hydroxylase systems, the results exclude microsomal contamination as the origin of this 12 α -hydroxylase activity. Further, there was no hydroxylase activity when NADPH-cytochrome P₄₅₀ reductase from rat liver microsomes was added instead of adrenodoxin and adrenodoxin reductase. The microsomal fraction of fetal liver was also shown to possess 12 α -hydroxylase activity. Microsomal and mitochondrial hydroxylase activities per milligram of protein towards 5 β -cholestane-3 α ,7 α -diol were of the same order of magnitude. The occurrence of an efficient sterol nucleus hydroxylase activity in liver mitochondria appears to be unique for fetal liver.

Introduction

Knowledge concerning bile acid synthesis during fetal life is limited (1). The presence of bile acids in the fetal gall bladder indicates capacity for bile acid synthesis (1). No studies in vitro on bile acid synthesis in the human fetus have been reported so far (1).

Both microsomal and mitochondrial cytochromes P₄₅₀ are involved in the hydroxylations of bile acid precursors in adult liver (2). Microsomal enzymes catalyze nuclear and side-chain hydroxylations, whereas the mitochondria only catalyze hydroxylation of the sterol side-chain (2).

This work reports studies on the hydroxylation of 5 β -cholestane-3 α ,7 α -diol in fetal liver. It is shown that fetal liver mitochondria, in addition to side-chain hydroxylase activity, also possess sterol nucleus hydroxylase activity, i.e., 12 α -hydroxylase activity.

Methods

Labeled compounds. 5 β -[7 β -³H]cholestane-3 α ,7 α -diol (sp act, 500 μ Ci/ μ mol) was prepared as described previously (3).

Received for publication 17 February 1984 and in revised form 18 October 1984.

J. Clin. Invest.

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0021-9738/85/02/0604/04 \$1.00

Volume 75, February 1985, 604-607

Unlabeled compounds. 5 β -cholestane-3 α ,7 α ,12 α -triol and 5 β -cholestane-3 α ,7 α ,26-triol were prepared as described previously (3).

Cofactors. NADPH and isocitric acid were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental procedures. Six human fetuses were obtained at legal abortions performed for sociomedical reasons between weeks 14 and 24. Consent was given by the local ethical committee of the University of Uppsala. The abortions were performed by use of prostaglandins or by hysterotomy. Fetal age was determined from data concerning the pregnancies and from fetal height measurements. The fetuses were taken to the laboratory; liver tissue was taken out and chilled in ice-cold buffer solution. Preparation of the homogenates was started within 45-60 min after abortion. Liver homogenates (10%, wt/vol) were prepared in 0.25 M sucrose solution by use of a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle (3). The mitochondrial fraction was obtained from the homogenates by initial centrifugations at 800 g and 20,000 g. The 20,000-g pellet was suspended in 0.25 M sucrose solution and recentrifuged twice at 6,400 g for 20 min (4). The final precipitate was suspended in 0.1 M Tris-Cl buffer, pH 7.4, corresponding to 16-25% of the initial volume of the homogenate. The protein concentration of the mitochondrial fraction was 0.4-2.0 mg/ml when determined according to Lowry et al. (5). In one experiment the activity of urate oxidase, a peroxisomal marker enzyme, was assayed in subcellular fractions obtained from the liver homogenate of a 15-wk-old fetus (6). 50% of the total activity readily sedimented with the 800-g pellet. 20% was found in the mitochondrial fraction, showing that this fraction was contaminated with peroxisomes.

The microsomal fraction was obtained by centrifugation at 100,000 g of the 20,000 g supernatant. The microsomal pellet was finally suspended and homogenized in 0.05 M Tris-acetate buffer, pH 7.4, in a volume corresponding to 25-50% of the initial volume. The protein concentration of the microsomal fraction was 0.5-2.0 mg/ml when determined according to Lowry et al. (5).

Mitochondrial protein corresponding to cytochrome P₄₅₀ was partially purified from one fetal liver by solubilization with sodium cholate and chromatography on octylamine-Sepharose 4B (column 1 \times 10 cm) as described by Imai and Sato (7). The fraction eluted from this column, with such chromatographic properties as those of cytochrome P₄₅₀, was applied to a hydroxylapatite column to remove Emulgen 913 (8). The final material was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The amount of cytochrome P₄₅₀ recovered was too small to permit an accurate quantitation, but an estimation by measuring oxidized cytochrome P₄₅₀ at 416 nm (9) showed that the total amount obtained was ~0.5 nmol. The total amount of protein quantitated by measuring absorption at 280 nm was ~0.5 mg. Adrenodoxin and adrenodoxin reductase were prepared from bovine adrenal mitochondria as described by Wikvall (10). NADPH-cytochrome P₄₅₀ reductase was prepared from liver microsomes of phenobarbital-treated rats as described by Yasukochi and Masters (11).

Incubation procedure and analysis of incubation mixtures. In incubations with the mitochondrial or microsomal fractions, 0.125 μ mol of substrate dissolved in 50 μ l of acetone were incubated with 2.0 ml of subcellular fraction in a total volume of 3 ml. The mitochondrial incubation mixtures were supplemented with 4.6 μ mol of isocitric acid (12). MgCl₂, 30 μ mol was also added (12). The microsomal incubation mixtures were supplemented with 3 μ mol of NADPH.

In the reconstitution experiment, solubilized and partially purified mitochondrial protein, estimated to correspond to ~0.5 nmol of cytochrome P₄₅₀ and 0.5 mg of protein, was divided into two parts. One part was combined with adrenodoxin, 2 nmol, and adrenodoxin reductase, 0.2 nmol. The other part was combined with microsomal NADPH-cytochrome P₄₅₀ reductase, 2 U, and dilauroylglycero-3-phosphorylcholine, 15 µg. Total incubation volume was 1.2 ml. The incubation mixtures were supplemented with 1.2 µmol of NADPH. All incubations were performed at 37°C for 20 min and were terminated by the addition of 5 ml of 96% (vol/vol) ethanol. After acidification and ether extraction, the incubations were analyzed by thin-layer chromatography and radio gas chromatography (3). In some instances, the material from thin-layer chromatography corresponding to the product was subjected to crystallization to constant specific radioactivity after addition of unlabeled 5β-cholestane-3α,7α,12α-triol. In some experiments, the product was converted into trimethylsilyl ether and subjected to gas chromatography-mass spectrometry using an LKB 9000 instrument (LKB Produkter, Uppsala, Sweden) with an SE-30 capillary column.

Results

Table I shows results of incubations of 5β-cholestane-3α,7α-diol with the mitochondrial fraction of five different fetal livers. In four experiments, both 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,26-triol were formed as judged by thin-layer chromatography and radio gas chromatography (cf. reference 3). The specific activities of the products were approximately the same as that of 5β-cholestane-3α,7α-diol. In one experiment, nearly only 5β-cholestane-3α,7α,12α-triol was formed. The identity of the product in this experiment was confirmed by gas chromatography-mass spectrometry and by crystallization to constant specific radioactivity after dilution with unlabeled 5β-cholestane-3α,7α,12α-triol (Table II).

When solubilized and partially purified mitochondrial protein with chromatographic characteristics as cytochrome P₄₅₀ was incubated with adrenodoxin, adrenodoxin reductase, and NADPH, significant conversion of 5β-cholestane-3α,7α-diol occurred as judged from thin-layer chromatography (Table III). Crystallization of the product to constant specific radioactivity (Table IV) showed that ~60% was 5β-cholestane-3α,7α,12α-triol. No conversion of 5β-cholestane-3α,7α-diol occurred when the same material was incubated instead with microsomal NADPH-cytochrome P₄₅₀ reductase, dilauroylglycero-3-phosphorylcholine, and NADPH (Table III). The microsomal fraction of fetal liver catalyzed the conversion of 5β-cholestane-3α,7α-diol into 5β-cholestane-3α,7α,12α-triol. Table V shows a comparison between mitochondrial 12α- and

Table I. Hydroxylation of 5β-[7β-³H]Cholestane-3α,7α-diol by Fetal Liver Mitochondria

Product	Gestational week				
	14	15	16	24	24
	<i>pmol/mg protein · min</i>				
12α-hydroxylated	5	3	107	66	67
26-hydroxylated	22	11	<2	39	103

Incubation conditions were as described in Methods.

Table II. Identification by Crystallization to Constant Specific Radioactivity of 5β-cholestane-3α,7α,12α-triol Formed in an Incubation of 5β-[7β-³H]cholestane-3α,7α-diol with the Mitochondrial Fraction from a Liver of a Fetus in Gestational Week 16

Solvent	Number of crystallizations	Weight	Specific radioactivity
	<i>n</i>	<i>mg</i>	<i>counts × min⁻¹ × mg⁻¹</i>
None	0	18.0	14,500
Acetone-water	1	17.4	12,100
Acetone-water	2	17.0	13,400
Acetone-water	3	9.2	14,300
Acetone-water	4	8.6	13,400

The product was isolated by thin-layer chromatography from an extract of the incubation mixture. (cf. Table I.)

26-hydroxylations and microsomal 12α-hydroxylation in livers of two fetuses, 19 and 24 wk old. The mitochondrial and microsomal 12α-hydroxylase activities were of the same order of magnitude.

Discussion

Detection and solubilization of liver mitochondrial cytochrome P₄₅₀ were described recently (13, 14). The monooxygenase activities in liver mitochondria known so far include cholesterol (12) and vitamin D 25-hydroxylase(s) (15), C₂₇-sterol 26-hydroxylase (3, 14), and kynurenin hydroxylase (16).

The present work shows that fetal human liver mitochondria catalyze, in addition to 26-hydroxylation of the C₂₇-sterol side-chain, 12α-hydroxylation. This 12α-hydroxylase activity is dependent upon cytochrome P₄₅₀. The capacity of mitochondria to catalyze 12α-hydroxylation apparently is lost during ontogeny.

In the adult, 12α-hydroxylation of bile acid precursors is catalyzed by microsomal cytochrome P₄₅₀ (2). Because the present results show that fetal liver microsome also catalyze 12α-hydroxylation of 5β-cholestane-3α,7α-diol, the possibility must be considered that the mitochondrial 12α-hydroxylase activity can be due to contaminating microsomal protein. The

Table III. Hydroxylation of 5β-[7β-³H]Cholestane-3α,7α-diol by Solubilized and Partially Purified Liver Mitochondrial Protein of a Human Fetus in Gestational Week 21

Mitochondrial protein plus adrenodoxin and adrenodoxin reductase	Mitochondrial protein plus NADPH-cytochrome P ₄₅₀ reductase
<i>pmol/mg protein · min</i>	<i>pmol/mg protein · min</i>
30	<2

The material had chromatographic properties as cytochrome P₄₅₀ (cf. Methods) and was combined with adrenodoxin and adrenodoxin reductase or NADPH-cytochrome P₄₅₀ reductase and dilauroylglycero-3-phosphorylcholine. NADPH was added in both incubations.

Table IV. Identification by Crystallization to Constant Specific Radioactivity of 5 β -Cholestane-3 α ,7 α ,12 α -triol Formed in an Incubation of 5 β -[7 β -³H]Cholestane-3 α ,7 α -diol with Solubilized and Partially Purified Fetal Liver Mitochondrial Protein, Adrenodoxin, and Adrenodoxin Reductase

Solvent	Number of crystallizations	Weight	Specific radioactivity
		mg	counts \times min ⁻¹ mg ⁻¹
None	0	10.4	7,200
Acetone-water	1	8.6	5,100
Acetone-water	2	6.7	4,700
Acetone-water	3	5.5	4,300
Acetone-water	4	4.6	4,400

Cf. Table III.

role of isocitric acid in supporting mitochondrial 12 α -hydroxylation of 5 β -cholestane-3 α ,7 α -diol speaks in favor of a mitochondrial origin of the 12 α -hydroxylase activity inasmuch as isocitric acid acts by generating NADPH intramitochondrially (12).

Mitochondrial and microsomal cytochrome P₄₅₀-dependent hydroxylases differ with respect to their reductase components. The mitochondrial reductase system consists of two components an iron-sulfur protein, adrenodoxin, and a flavoprotein, adrenodoxin reductase (17). The microsomal reductase system consists of one component, NADPH-cytochrome P₄₅₀ reductase (17).

The reconstitution of 12 α -hydroxylase activity from solubilized and partially purified mitochondrial protein by the addition of adrenodoxin and adrenodoxin reductase strongly supports the view that the 12 α -hydroxylase activity is mitochondrial (cf. reference 14). Further, the chromatographic and spectral properties of the isolated protein fraction, as well as the requirement of adrenodoxin and adrenodoxin reductase, strongly indicate that mitochondrial cytochrome P₄₅₀ catalyzes the reaction. The fact that microsomal NADPH-cytochrome P₄₅₀ reductase was inactive in the reconstitution experiment excludes contamination with microsomal cytochrome P₄₅₀ as responsible for mitochondrial C₂₇-sterol 12 α -hydroxylase activity.

Table V. Hydroxylation of 5 β -[7 β -³H]Cholestane-3 α ,7 α -diol by Fetal Liver Mitochondria and Fetal Liver Microsomes

Product	Gestational week	
	19	24
	pmol/mg protein \cdot min	pmol/mg protein \cdot min
Mitochondria		
26-hydroxylated	20	99
12 α -hydroxylated	20	71
Microsomes		
12 α -hydroxylated	22	10

Incubation conditions were as described under Methods.

As mentioned in the experimental section, the mitochondrial fraction from fetal liver was contaminated with peroxisomes. In view of this, the possibility of peroxisomal 12 α -hydroxylation must be taken into account. The preparation of a peroxisomal-enriched fraction from a fetal liver homogenate has been prevented by the small amounts of fetal liver tissue available. However, there are no reports on the existence of peroxisomal cytochrome P₄₅₀-dependent hydroxylases. Further, the dependence of the 12 α -hydroxylase upon adrenodoxin and adrenodoxin reductase favors its mitochondrial origin.

Oftebro et al. (18) recently reported on the occurrence of 5 β -cholestane-3 α ,7 α -diol 12 α -hydroxylase activity in a mitochondrial preparation from the liver of a patient with cerebrotendinous xanthomatosis. Since no solubilization-reconstitution experiment was performed in that study, it was not possible to exclude that the 12 α -hydroxylase activity was due to a contamination with microsomal protein (18). If the 12 α -hydroxylase activity in the experiments by Oftebro et al. (18) was mitochondrial, it would point to "immature" functions in the liver cell organelles of patients with cerebrotendinous xanthomatosis.

It should be pointed out that no significant 12 α -hydroxylase activity has been detected in liver mitochondrial preparations from healthy adults (18, 19). It is not possible to evaluate from the present experiments the relative roles of mitochondrial and microsomal 12 α -hydroxylase activities in bile acid biosynthesis in fetal liver.

Acknowledgments

The skilled technical assistance of Miss Åsa Byström is gratefully acknowledged. I am grateful to Dr. Kjell Wikvall for help in the purification and reconstitution experiments.

This work was supported by the "Expressen" Prenatal Research Foundation and the Swedish Society of Medical Sciences.

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