Lack of 3β-Hydroxy-Δ⁵-C_{27}-Steroid Dehydrogenase/Isomerase in Fibroblasts from a Child with Urinary Excretion of 3β-Hydroxy-Δ⁵-Bile Acids

A New Inborn Error of Metabolism

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Abstract

Cultured fibroblasts were shown to be capable of catalyzing the conversion of 7α-hydroxy-cholesterol to 7α-hydroxy-4-cholesten-3-one, an important reaction in bile acid synthesis. The apparent K_m was ~ 7 μmol/liter and V_{max} varied between 3 and 9 nmol/mg protein per h under the assay conditions used.

The assay was used to investigate fibroblasts from a patient who presented with a familial giant cell hepatitis and who was found to excrete the monosulfates of 3β,7α-dihydroxy-5-cholenoic acid and 3β,7α,12α-trihydroxy-5-cholenoic acid in urine (Clayton, P. T., J. V. Leonard, A. M. Lawson, K. D. R. Setchell, S. Andersson, B. Egestad, and J. Sjövall. 1987. *J. Clin. Invest.* 79:1031–1038). In addition 7α-hydroxy-cholesterol was found to accumulate in the circulation. Cultured fibroblasts from this boy were completely devoid of 3β-hydroxy-Δ⁵-C_{27}-steroid dehydrogenase/isomerase activity. Fibroblasts from his parents had reduced activity, compatible with a heterozygous genotype.

The results provide strong evidence for the suggestion that this patient's liver disease was caused by a primary defect in the 3β-hydroxy-Δ⁵-C_{27}-steroid dehydrogenase/isomerase involved in bile acid biosynthesis. (*J. Clin. Invest.* 1990. 86:2034–2037.) Key words: fibroblasts • bile acid biosynthesis

Introduction

Until recently, the only known inborn errors of metabolism affecting the bile acid synthesis involve defects of the degradation of the cholesterol side chain (for review see reference 1). The primary defect in cerebrotendinous xanthomatosis, characterized by xanthomatous deposits and progressive neurological dysfunction, is a lack of the mitochondrial 26-hydroxylase (2). In the Zellweger syndrome, as well as in some other peroxisomal disorders, there is a lack of several peroxisomal enzymes. Among the enzymes lacking are those responsible for the β-oxidation of the C_{27}-steroid side chain. As a consequence, C_{27}-bile acids with an incompletely oxidized side-chain accumulate in serum and bile (1, 3).

Recently, two disorders, both affecting transformations in the steroid nucleus, have been described. Two identical twins with neonatal hepatitis were shown to synthesize 7α-hydroxy-3-oxo-4-cholenoic acid and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (4). It was suggested that this was due to a primary defect in the Δ⁴-3-oxo-steroid 5β-reductase involved in the normal biosynthesis of bile acids. There is, however, an accumulation of 3-oxo-Δ⁴-bile acids although to a smaller degree, also in other severe liver diseases such as, e.g., hepatitis B and autoimmune chronic active hepatitis (5). In view of this, it remains to be established if there is a primary defect of the Δ⁴-3-oxosteroid 5β-reductase. In 1987, a 3-mo-old boy with cholestatic jaundice was reported (6). He excreted monosulfates of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids and their glycine conjugates as major bile acids in urine. The metabolic pattern and the family history indicated a hereditary defect of the 3β-hydroxysteroid dehydrogenase/isomerase involved in bile acid biosynthesis. The natural substrate for the 3β-Δ⁵-C_{27} steroid dehydrogenase/isomerase is 7α-hydroxy-cholesterol (7), and in accordance with the above hypothesis, the concentration of unesterified 7α-hydroxy-cholesterol in serum was markedly higher than normal (unpublished observation). However, no enzyme studies were done and further evidence was required before it could be stated with certainty that this case represented an inborn error of 3β-hydroxysteroid dehydrogenase/isomerase.

Among the different enzymes involved in bile acid biosynthesis (for a review see reference 7), only the 26-hydroxylase and the 3β-hydroxysteroid dehydrogenase/isomerase active on 7α-hydroxy-cholesterol is present in cultured skin fibroblasts (Björkhem, I., S. Skrede, and J. I. Pedersen, unpublished results).

In view of this, we have used cultured skin fibroblasts to evaluate if the patient excreting 3β-hydroxy-Δ⁵-bile acids has a deficiency of 3β-hydroxysteroid dehydrogenase/isomerase. Evidence is provided in the present work that the patient has such a defect.

Methods

Case history. The boy is the fifth child of Saudi Arabian parents who are first cousins. One sister and one brother died as a result of an aggressive hepatitis progressing to cirrhosis at the age of 10 mo and 3 yr, 9 mo, respectively. The patient was born at term after a normal pregnancy and weighed 4 kg. At the age of 3 mo he was referred and presented with moderate jaundice and hepatomegaly. Developmental assessment was normal and there were no dysmorphic features. The most notable biochemical finding was the excretion of 3β,7α-dihydroxy-5-cholenoic acid 3-sulfate and 3β,7α,12α-trihydroxy-5-cholenoic acid monosulfate in urine (6). Chenodeoxycholic acid and cholic acid were undetectable in urine and plasma.

The parents did not consent to liver biopsy and the patient was without medical attention until the age of 2 yr, 9 mo. He then weighed

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13.6 kg (25th percentile) and had a height of 82.5 cm (below fifth percentile). He had signs of rickets and hepatomegaly. According to abdominal ultrasound, the liver was slightly enlarged but echogenicity appeared normal. Gall bladder and bile ducts were normal. Serum total bilirubin: 2.4 mg/dl, direct 2.1 mg/dl; serum glutamate pyruvate transaminase: 56 U/liter; serum glutamate oxaloacetate transaminase: 57 U/liter; albumin: 4.7 g/liter; total proteins: 6.7 g/liter. Supportive therapy with vitamins, especially A, K, E, and high doses of vitamin D was started. The patient's rickets improved.

The patient was then given chenodeoxycholic acid (Chendol, C. P. Pharmaceuticals, Wrexham, UK). After 2 mo on this treatment, the patient felt better, itching was less, and the urine normalized in color. A detailed description of the effect of this treatment will be given later. The patient is now in good condition at age 5 yr, 9 mo.

Fibroblast cultures and preparation of cell suspensions. Skin biopsies were obtained from the patient and his parents. The fibroblast cultures were established in Norway. The controls were patients from other metabolic studies, without liver disease. The cells were grown and maintained as monolayers in tissue culture flasks (75 cm^2) (Costar, Cambridge, MA) in MEM supplemented with 17% calf serum. Penicillin (100 U/liter), streptomycin (100 μg/ml), amphotericin (2 U/liter), and L-glutamine (1.5 μmol/ml) were added. The cells were harvested with trypsin after 5–10 passages, washed three times in isotonic salt solution, and stored as pellets at −70°C until assay. The cells were then resuspended in potassium phosphate buffer 0.1 M, pH 7.5, and subjected to ultrasound waves 40 W for 3 s 10 times to obtain a smooth cell suspension with disrupted cells. The activity of the actual enzyme was not affected by moderate ultrasound treatment (results not shown).

Assay of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase. The assay was performed essentially as described previously (8). Cell suspensions containing 1 mg cell protein were incubated for 20 min with [7β-^{3}H]7α-hydroxycholesterol (0.1 mmol/liter; sp act 4,000 dpm/nmol), NAD (1.5 mmol/liter), and phosphate buffer (0.1 mol/liter, pH 7.5) in a total volume of 0.5 ml. The incubations were stopped by adding 2 ml of ethanol, followed by 3 ml of water. Extractions were performed twice with *n*-hexane/ethyl acetate (1:1; vol/vol).

All incubations were performed in fibroblasts frozen and thawed once.

Chromatography and measurement of radioactivity. Extracts obtained as described above were redissolved in toluene/ethyl acetate (1:1, vol/vol). This was also the mobile phase for preparative thin-layer chromatography. The appropriate zones of the gel were scraped into counting vials and assayed for radioactivity.

Labeled steroids. [7β-^{3}H]7α-hydroxycholesterol and [7β-^{3}H]5β-cholestane-3α,7α,12α-triol were synthesized as described earlier (8, 9). The compounds were purified by HPLC before use. ^{3}H-Pregnenolone, ^{3}H-dehydroepiandrosterone, and ^{14}C-cholesterol were bought from Radiochemical Centre (Amersham, UK).

Other reagents. NAD was purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were standard commercial high-purity materials.

Identification of product. The product of the incubation was isolated by thin-layer chromatography (c.f. above) and converted into the trimethylsilyl ether. The derivatized material was subjected to combined gas chromatography–mass spectrometry (GC/MS), using an model 2091 instrument (LKB Instruments, Inc., Gaithersburg, MD) equipped with a 30 m × 0.25 mm DB-1301 column. The material gave only one peak with the same retention time and mass spectrum as the derivative of authentic 7α-hydroxy-4-cholesten-3-one. The same results were obtained with the methyloxime-trimethylsilyl ether derivatives of the product and authentic 7α-hydroxy-4-cholesten-3-one.

Results

Enzymatic properties of the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in control fibroblasts. During incubation under standard conditions, one product, slightly less polar than the substrate was obtained. This product was identified as 7α-hydroxy-4-cholesten-3-one by GC/MS. No other products were seen.

Fig. 1 *A* shows the time course of the conversion of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one. The conversion was found to be linear with time to at least 60 min at standard incubation procedures. With higher protein concentration the conversion was linear for a shorter time. The reason for this was the further metabolism of the reaction product. The enzyme itself was stable under the conditions employed.

Fig. 1 *B* shows that the activity was linear with protein concentration to ~ 2 mg/ml.

The pH optimum under identical conditions was between 9 and 9.5 in glycine buffer (Fig. 1 *C*), which is close to that reported by Wikvall for the purified enzyme when tested in Tris-Cl buffer (10). The pH optimum was assayed in rat liver microsomes as well, and was also here found to be between 9 and 9.5. The pH chosen for the assay was 7.5, which is lower than the demonstrated pH optimum in the experiments shown in Fig. 1 *C*. However, all previous studies published on this enzyme (8, 10) have been performed at physiological pH. The enzyme activity in the fibroblasts was satisfactory at this pH.

In Fig. 1 *D* the effect of the substrate concentration on the enzyme activity is shown. By using a computer program for calculation of the Michaelis-Menten kinetics, we found an apparent K_m of 6.5±0.5 μmol/liter (mean±SD). V_{max} was 5.1 nmol/mg protein per h. For comparison we assayed the activity of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in a human liver homogenate, and found a similar value for K_m, but a significantly higher V_{max} (Table I).

The standard conditions for the enzyme assay was thus as described in Methods. These conditions were always used during the experiments.

Characteristics of the enzyme. The substrate specificity was tested with ^{3}H-pregnenolone, ^{3}H-dehydroepiandrosterone, and ^{14}C-cholesterol. When incubated at standard conditions, no product was formed.

The addition of DTT or EDTA did not influence the activity of the reaction.

The 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity was also assayed in freshly isolated lymphocytes from normal controls. The lymphocytes were isolated on sodium metrizoate/Ficoll by standard procedures. We were not able to demonstrate any activity in these cells.

To eliminate possible interfering substances, gel filtration of the cell suspension was performed. However, the enzyme activity was lost as well. Attempts to completely solubilize the enzyme by sonication were not successful.

Enzyme activity in fibroblasts from controls, patient and parents. Table II shows the mean activity of the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in fibroblasts from controls, the patient and his parents. The enzyme activity of the controls was not age dependent. The enzyme activity of the fibroblasts from the mother was in the lower control range. The fibroblasts from the father, however, had an activity that was significantly reduced as compared to the control mean. The fibroblasts from the patient were shown to be completely devoid of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity.

To ascertain the vitality of the cells, two other enzymes were measured. τ-Glutamyl transpeptidase (τ-GT, EC 2.3.2.2)

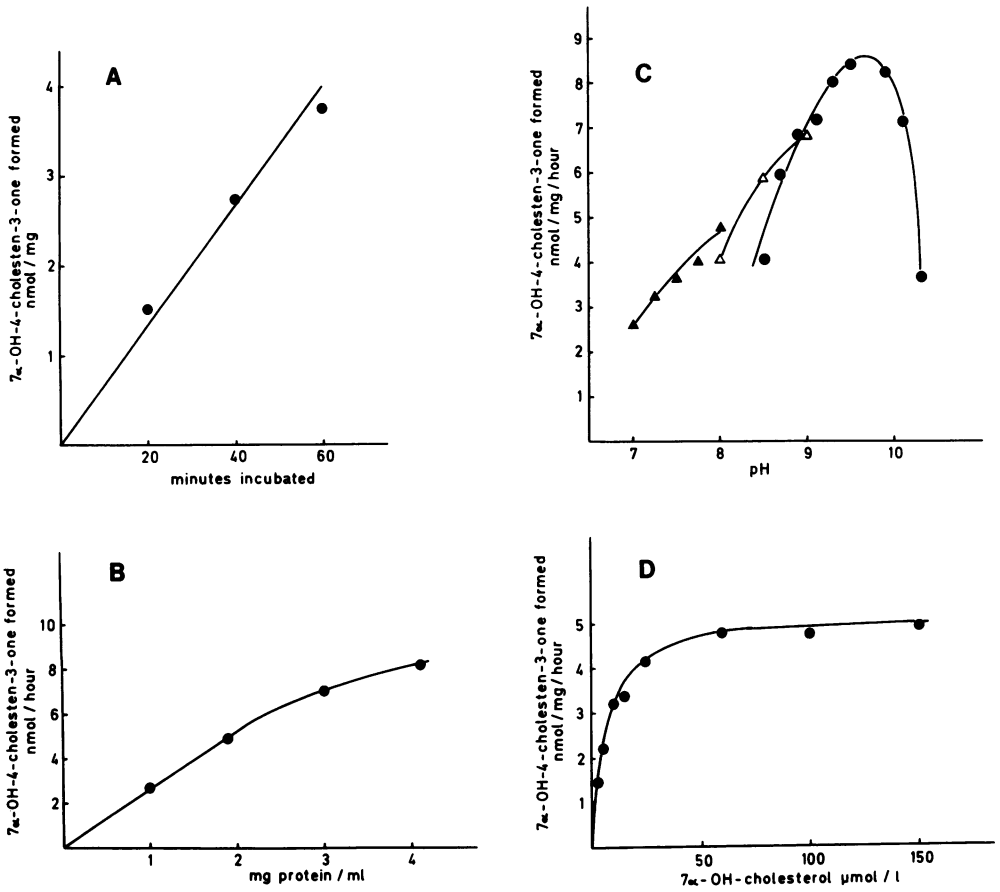

Figure 1. (*A*) Effect of time on 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity. (*B*) Effect of enzyme protein concentration on 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity. (*C*) Effect of pH on 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity. (▲) Potassium phosphate buffer. (●) Glycine buffer. (△) Tris-maleate buffer. (*D*) Effect of substrate concentration on 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity.

is a microsomal enzyme, as is the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase. The activity of this enzyme was not significantly different in fibroblasts from the patient, his mother, and two controls. Likewise the activity of the 26-hydroxylase of the bile acid biosynthesis was assayed. The activity was the same in fibroblasts from the controls, patient, and mother (Table III).

Discussion

Early mechanistic work with crude microsomal preparations from rat liver (8) and later studies with apparently homogenous enzyme from rabbit liver microsomes (10) have given strong support for the contention that only one enzyme is involved in the conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholestene-3-one. As judged from studies with the homogenous preparation and with a specific 3β-hydroxy-Δ^5-C_{19}-steroid dehydrogenase inhibitor (11), the enzyme is not identical with the 3β-hydroxy-Δ^5-steroid dehydrogenase active towards C_{19} and C_{21} steroids (11). The enzyme also seems to be different from that involved in oxidation of cholesterol to yield 4-cholesten-3-one (2).

It is well known from other metabolic diseases that, in vivo, pathological metabolites may modulate the activity of many enzymes and thereby obscure the primary defect. Fibroblasts do not synthesize bile acids and metabolites are therefore not present. It may be pointed out that the only other known

Table I. Michaelis-Menten Kinetics of 3β-Hydroxy-Δ^5-C_{27}-Steroid Dehydrogenase/Isomerase

Tissue	Apparent K_m	V_{max}
	μmol/liter	*nmol/mg prot per h*
Fibroblasts	6.5	5.2
Human liver	6.3	14.2

Values were calculated by a computer program.

Table II. 3β-Hydroxy-Δ^5-C_{27}-Steroid Dehydrogenase/Isomerase Activity in Cultured Fibroblasts from Various Sources

	Mean	Range
	nmol/mg prot per h	
Controls (n = 18)	9.38	6.22–21.57
Mother	6.86	
Father	2.50	
Patient	<0.10	

Fibroblasts were cultured from 18 different subjects, healthy as to liver disease. The 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity was assayed at standard conditions, i.e., fibroblasts (1 mg protein) were incubated with 0.1 mmol/liter 7α-hydroxycholesterol, NAD 1.5 mmol/liter, 0.1 M potassium phosphate buffer, pH 7.5 for 20 min.

Table III. γ-Glutamyl-Transpeptidase (γ-GT) and C_{27}-Steroid 26-Hydroxylase Activities in Fibroblasts from Various Sources

	γ-Glutamyl-transpeptidase	C_{27}-Steroid 26-hydroxylase
	nmol/mg per h	*pmol/mg per h*
Control 1	5.20	710
Control 2	2.93	410
Mother	2.05	370
Patient	2.82	440

γ-GT activity was measured in the routine laboratory in a multianalyzer, and the 26-hydroxylase activity as described earlier (12).

inborn error of metabolism affecting nuclear transformations in bile acid biosynthesis, Δ^4-3-oxosteroid 5β-reductase deficiency, is not possible to establish with the use of fibroblasts, as we have failed to demonstrate the expression of this enzyme in fibroblasts from healthy subjects. The present finding that the patient's fibroblasts were completely devoid of the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity, together with the previous in vivo finding of urinary excretion of 3β-hydroxy-Δ^5 bile acids and accumulation of 7α-hydroxycholesterol in the circulation, give strong support for the contention that the primary biochemical defect is located to the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase. The levels of enzyme activity in both parents' fibroblasts were compatible with a heterozygous genotype. Due to the interindividual variation, however, it may be difficult to discriminate completely between normal fibroblasts and fibroblasts from heterozygotes.

The only other enzyme involved in bile acid biosynthesis which is expressed in fibroblasts, the 26-hydroxylase, has a wide distribution in various organs (12–14). The possibility has been discussed that the 26-hydroxylase may have a role different from that of bile acid formation (14). The general properties of the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase present in fibroblasts were similar to those of the hepatic enzyme. At present, there are no indications that the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase has any other function than that associated with bile acid biosynthesis.

The enzyme measured in this assay seems to be specific for 7α-hydroxycholesterol, as it was not active towards C_{19}-, C_{21}-steroids or cholesterol. It is of interest in this connection that there are reports on complete as well as incomplete deficiency of the 3β-hydroxy-Δ^5-steroid dehydrogenase/isomerase catalyzing the oxidation of 3β-hydroxy-Δ^5-steroids in the formation of steroid hormones. The latter deficiencies are not associated with disturbances in bile acid biosynthesis (15).

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