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The Cerebrohepatorenal (Zellweger) Syndrome: An Improved Method for the Biochemical Diagnosis and its Potential Value for Prenatal Detection[1]

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ABSTRACT. The sequence of reactions involved in plasmalogen biosynthesis has been evaluated in cultured fibroblasts of patients with the cerebrohepatorenal syndrome. A double-label, double-substrate incubation using [1-^{14}C] hexadecanol and 1-0-[9′, 10′-^{3}H]hexadecylglycerol was performed to monitor the relative rates of peroxisomal and microsomal biosynthesic steps. [^{14}C] radioactivity associated with 1′-alkenyl groups of plasmalogens was found to be drastically reduced in fibroblasts of affected patients whereas [^{3}H] incorporation was apparently normal. This finding is specific for cerebrohepatorenal syndrome fibroblasts since cell lines of patients with childhood adrenoleukodystrophy and neuronal ceroidlipofuscinosis utilized the lipid precursors of plasmalogen biosynthesis at normal rates. The results show that the defect in plasmalogen synthesis in the cerebro-hepato-renal syndrome is restricted to the peroxisomal steps. The finding of normal microsomal biosynthetic steps was exploited to devise a novel diagnostic assay in fibroblasts and amniocytes based on the comparison of [^{3}H/^{14}C] isotope ratios within aldehydes released from plasmalogens by acid hydrolysis. The procedure can be completed with a minimal amount of cells since it renders quantitative analyses unnecessary. Therefore, this technique appears ideally suited for the sensitive and safe prenatal diagnosis of the cerebro-hepato-renal syndrome. (*Pediatr Res* 19: 930–933, 1984)

Abbreviations

ALD, adrenoleukodystrophy
CHRS, cerebro-hepato-renal syndrome
DHAP, dihydroxyacetonephosphate
NCL, neuronal ceroidlipofuscinosis
VLCFA, very-long-chain fatty acids

The CHRS (Zellweger) is a rare autosomal recessive disorder, clinically characterized by abnormalities in liver and kidney function, severe hypotonia, psychomotor retardation, and dysmorphic features (1, 2).

This syndrome is accompanied by a variety of biochemical disturbances such as hyperpipecolic acidemia, hepatic and cerebral glycogen storage (2), elevated VLCFA (3, 4), abnormal bile acids (5, 6), dicarboxylic aciduria, and diminished levels of plasmalogens (7, 8). Most of the abnormalities may relate to the absence of peroxisomes and/or to severely impaired mitochondrial functions in patients (9, 10).

Since the infants usually die within the 1st yr of life, an early and precise biochemical diagnosis is important for providing adequate genetic counseling. The study of VLCFA, particularly of hexacosanoic acid ($C_{26:0}$) and hexacosenoic acid ($C_{26:1}$) in amniotic fluid and in cultured amniocytes has been proposed for prenatal diagnosis (3, 11). Alternately, diagnosis based on measurement of DHAP-acyltransferase—the first enzyme in the pathway leading to plasmalogen biosynthesis (12)—has been suggested (13). However, the complete biosynthetic pathway leading to plasmalogens has not been investigated so far in CHRS. In this study we used [1-^{14}C]hexadecanol and [9′,10′-^{3}H] hexadecylglycerol to investigate both the peroxisomal and microsomal steps of plasmalogen biosynthesis. The finding of normal microsomal steps of plasmalogen formation was exploited to devise a novel technique for the biochemical diagosis of CHRS.

MATERIALS AND METHODS

[1-^{14}C]hexadecanol was purchased from Amersham (England) and diluted with hexadecanol to a specific activity of 11.4 mCi/mmol. [9′,10′-^{3}H]-*sn*-hexadecylglycerol (specific radioactivity 5.5 mCi/mmol) was prepared by chemical synthesis (14) (on request it is available from the authors). Unlabeled phospholipid standards were prepared in our laboratory by conventional chromatographic procedures.

Culture medium, enzyme solutions, and additions for the culture media were from Serva (Heidelberg, FRG), bovine serum albumin from Armour Pharmaceutical Co. (Phoenix, AZ). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture. Three of the cell strains were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ): CHRS1 (GM 0228), CHRS 2 (GM 4340), and ALD 3 (GM 4933). ALD 1 and ALD 2 were kindly donated by M. Vetterlein, Institute for Tumor Biology and Cancer Research, University of Vienna. Fibroblast samples of CHRS-heterozygotes were friendly provided by H. Moser, John F. Kennedy Institute, Baltimore, MD.

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All other cell strains were initiated at the Paediatric Tissue Culture Unit, University of Graz, Medical School.

Control skin biopsies were taken on the occasion of minor surgery (herniotomia) from otherwise healthy children. Diagnosis of ALD was based on neurological findings and was confirmed by increased $C_{26:0}$ fatty acid levels in leukocytes and fibroblasts (15). Patients with NCL were diagnosed by the typical clinical and ultrastructural findings (16) and the patient with CHRS was recognized by the typical clinical characteristics (2) and by the presence of hyperpipecolic acidemia.

Stock cultures of human fibroblasts were grown in Eagle's basal medium supplemented with Earle's salts, 10% fetal calf serum, and 2 mM glutamine as previously described (17). Subcultures, initiated with 1×10^6 cells in 60-mm dishes (Falcon Plastics, CA), were grown to confluency and the standard medium was renewed the day before experiments were carried out.

Incubations. Incubations were carried out with fibroblast cultures that had been equilibrated for 30 min at 37° C in incubation medium consisting of serum-free Dulbecco's minimal essential medium containing 0.05% bovine albumin. Stock solutions of radiolabeled substrates were prepared in absolute ethanol and diluted with the incubation medium to give the desired amount of [^{3}H-^{14}C] lipid mixture. The final ethanol concentration did not exceed 0.1%. This solution was sonicated for 20 s, and a 2-ml aliquot containing 0.3 μCi (26 nmol) of [1-^{14}C]hexadecanol and 3 μCi (600 nmol) of [9′,10′-^{3}H]hexadecyl-*sn*-glycerol was added to the fibroblast monolayers. At the times indicated cells were washed twice with phosphate-buffered saline at 4° C, scraped off with a rubber policeman, and collected by centrifugation.

Analyses. Fibroblast pellets were disintegrated by sonication in 1 ml of water and total cellular lipids were then extracted according to Folch *et al.* (18). The plasmalogen content of the phosphatidylethanolamine and phosphatidylcholine fractions was determined by two-dimensional thin-layer chromatography (19). Silica gel PF 60 plates were first developed in chloroform-methanol-water (65:25:4, by vol), dried, exposed to fumes of concentrated HCl, and finally developed in the second dimension with the same solvent. Phospholipid phosphorus was quantitated according to the procedure of Rouser *et al.* (20). When radioactively labeled substrates were supplemented to the cultures, the separation of labeled aldehydes released from plasmalogens by acid catalyzed hydrolysis was accomplished by thin-layer chromatography using a dual-development technique according to Paltauf (21). Aliquots of the lipid extracts were applied to thin-layer plates coated with 0.5 mm silica gel H (Merck, Darmstadt, FRG). To each spot 0.2 mg of ox brain phosphatidylethanolamine and 0.2 mg hexadecylglycerol were added to carriers. The plate was first developed with diethylether/H_2O (100/0.25, by vol) to remove radioactively labeled neutral lipids, then exposed to HCl fumes for 10 min, and developed twice with light petroleum/diethylether (95/5, by vol) to a distance of 10 cm from the origin. Spots corresponding to aldehydes were visualized by brief exposure to iodine vapor, signed, and decolorized under a stream of air. Radioactivity was measured after the spots were transferred directly into counting vials, using a xylene-based scintillation cocktail.

Isolation of fatty acids, esterification, and analysis of fatty acid methylesters was performed as reported previously (15).

RESULTS AND DISCUSSION

Recent findings demonstrate that CHRS fibroblasts exhibit abnormally elevated levels of VLCFA similar to those found in ALD (4, 15, 22). In this study (Table 1) concentrations of hexacosanoic acid ($C_{26:0}$) were consistently higher in CHRS fibroblasts (0.38 ± 0.17 μg/mg lipids) and ALD fibroblasts (0.46 ± 0.18 μg/mg lipid) as compared to control cell strains (0.14 ± 0.04 μg/mg lipid). $C_{26:0}/C_{22:0}$ ratio was elevated in CHRS and ALD fibroblasts as well. The higher $C_{26:0}/C_{22:0}$ ratio in CHRS than in ALD cells was due to the relatively low $C_{22:0}$ levels in CHRS fibroblasts (data not shown). The VLCFA levels and $C_{26:0}/C_{22:0}$ ratios in fibroblasts of patients with NCL were within the normal range.

Table 1. *VLCFA in cultured fibroblasts**

	$C_{26:0}$ (μg/mg lipid)	$\frac{C_{26:0}}{C_{22:0}}$
Co_1†	0.17	0.23
Co_2	0.11	0.34
NCL_1	0.10	0.22
NCL_2	0.10	0.43
ALD_1	0.53	0.64
ALD_2	0.60	0.76
ALD_3	0.26	0.91
$CHRS_1$	0.31	1.60
$CHRS_2$	0.26	1.67
$CHRS_3$	0.58	1.90

* Fatty acids of total lipids extracted from fibroblasts were analyzed by gas-liquid chromatography as their methyl esters (15).

† Control.

Table 2. *Plasmalogen content of cultured human fibroblasts**

	Diacyl-GPE† (Phosphatidyl-ethanolamine)	1-Alkenylacyl-GPE (Plasmalogen)
	(% of total phospholipids)	
Co_1‡	12.32	12.83
Co_2	11.77	14.68
NCL_1	12.28	10.28
NCL_2	11.76	11.68
ALD_1	13.22	9.78
ALD_2	13.72	10.93
ALD_3	13.76	12.10
$CHRS_1$	23.20	5.3
$CHRS_2$	18.68	2.5
$CHRS_3$	17.82	3.3

* Total lipids extracted from fibroblasts were analyzed by two-dimensional reaction thin-layer chromatography as described in "Materials and Methods." Only the data obtained for the ethanolamine glycerophospholipid fraction are shown in Table 2 since the plasmalogen content of other phospholipid classes was negligibly low.

† Glycerophosphoethanolamine.

‡ Control.

It has been proposed that ALD and CHRS share some impaired peroxisomal functions although the exact mechanisms underlying the VLCFA accumulations in these two diseases appear to be different (3).

Plasmalogen deficiency in tissues of CHRS is also suspected to be due to peroxisomal abnormalities (7, 8). Recent experiments have shown (13) that acyl coenzyme A:dihydroxyacetonephosphate acyltransferase is virtually absent from CHRS cells (brain, liver, and fibroblasts). So far, however, the specificity of this finding has not been systematically evaluated. For example, a virtual absence of plasmalogens in brain extracts from patients with NCL has been reported (16). Therefore we determined total plasmalogen levels in cultured fibroblasts of patients with CHRS, ALD, and NCL (Table 2). Phospholipid analyses of fibroblast lipid extracts revealed a pronounced reduction of total

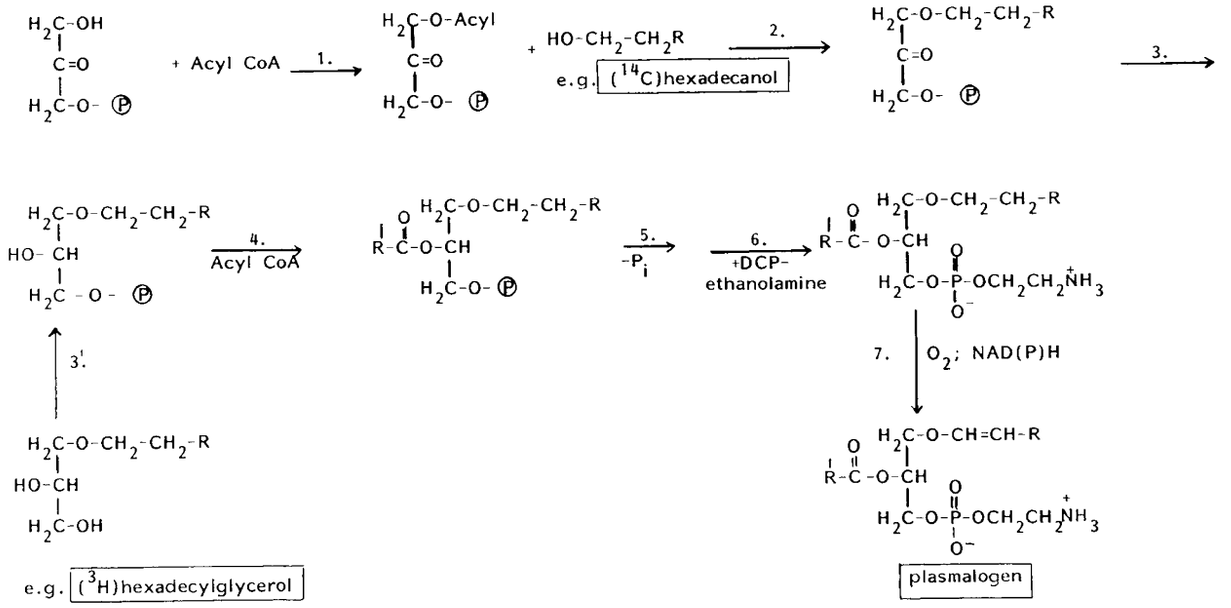

Fig. 1. Sequence of reactions involved in plasmalogen biosynthesis in aerobic cells.

plasmalogen content in CHRS fibroblasts (4.0 ± 1.1% of total lipids *versus* 13.7 ± 1.3% in controls) that is balanced by somewhat increased amounts of phosphatidylethanolamine (19.9 ± 2.9% in CHRS *versus* 12.1 ± 0.4% in controls). No differences in the distribution of the other phospholipids were found (data not shown). The fibroblast phospholipid pattern of patients with ALD and NCL was indistinguishable from normal controls.

The sequence of reactions involved in plasmalogen biosynthesis in aerobic cells is depicted in Figure 1. Formation of acyl DHAP (reaction 1) that is utilized for ether lipid synthesis, and alkyl DHAP synthase (reaction 2) are restricted to peroxisomes (12). The subsequent reactions leading to the formation of 1-0-alkyl-2-acyl glycerophosphoethanolamine are catalyzed by enzymes that are also involved in diacyl glycerophosphoethanolamine synthesis and are localized in the endoplasmic reticulum. Whereas reaction 1 was found to be deficient in CHRS cells (13), so far no data are available concerning the activity of the key enzymes (23, 24) of plasmalogen biosynthesis (reactions 2 and 7) in CHRS cells.

The method for the biochemical diagnosis of CHRS described here was devised assuming that the utilization of long-chain alcohols (*e.g.* [^{14}C]hexadecanol) for plasmalogen synthesis should be impaired in the absence of peroxisomes. On the other hand, the microsomal conversion of alkylglycerols (*e.g.* [^{3}H]hexadecylglycerol) to plasmalogens which proceeds via reactions 3′ to 7 should be normal. Consequently, a double-label, double-substrate incubation using [1-^{14}C]hexadecanol and 1-0-[9′,10′-^{3}H] hexadecylglycerol was performed to monitor relative rates of peroxisomal and microsomal steps of plasmalogen biosynthesis in fibroblasts. As shown in Table 3, incorporation of [^{3}H]hexadecylglycerol into the 1′-alkenyl groups of plasmalogens proceeded at approximately equal rates in CHRS cells and controls. In contrast, utilization of [1-^{14}C]hexadecanol for plasmalogen synthesis was drastically reduced in CHRS cells (0.6 ± 0.3% in CHRS *versus* 15.0 ± 2.9% in controls).

By calculating the [^{3}H/^{14}C] isotope ratio within alkenyl groups of plasmalogens a 15- to 60-fold difference between CHRS and controls was found. This difference was largely independent of the incubation time, although the absolute [^{3}H/^{14}C] ratios increased with time (*e.g.* 0.2–0.9 in controls and 3–15 in CHRS from 6–24 h of incubation). For practical purposes an overnight incubation (16–20 h) was routinely employed.

These results demonstrate that the defect in plasmalogen synthesis in CHRS cells is restricted to peroxisomal steps (reactions 1 and 2 in the scheme), whereas the microsomal reactions obviously proceed at normal rates. Therefore, one could speculate that at least the symptom of plasmalogen deficiency in CHRS might be accessible to supplementation therapy, *e.g.* by the administration of alkylglycerolipids.

Cell lines of patients with childhood ALD and NCL were used as pathological controls because of reported peroxisomal dysfunction (4) or proposed plasmalogen deficiency in brain (16).

Table 3. *Relative contribution of lipid precursors to plasmalogen synthesis in cultured human fibroblasts**

	Radioactivity in 1′-alkenylgroups (% of total incorporated)		
	[^{3}H]	[^{14}C]	[^{3}H/^{14}C] Ratio
Co_1†	10.9	17.9	0.61
Co_2	11.2	12.2	0.91
Co_3	10.4	15.0	0.69
NCL_1	10.2	17.0	0.60
NCL_2	11.9	13.1	0.91
ALD_1	9.4	18.6	0.51
ALD_2	4.3	4.6	0.93
ALD_3	8.7	9.9	0.88
$CHRS_1$	13.4	0.86	15.6
$CHRS_2$	12.3	0.31	39.6
$CHRS_3$	12.7	0.61	20.8
CHRS-HE_1‡	8.2	9.9	0.83
CHRS-HE_2	8.3	10.0	0.83
CHRS-HE_3	9.2	14.5	0.63

* Radioactivity associated with aldehydes released after acid treatment of total phospholipids was measured and related to the total radioactivity incorporated into fibroblast lipids. Incubations were for 16 h. Total radioactivity in lipid extracts ([^{3}H] from [9′, 10′-$^{3}H_2$] hexadecyl-*sn*-glycerol; [^{14}C] from [1-^{14}C]hexadecanol) was essentially identical with all types of cells.

† Control.

‡ Heterocygotes.

Cell strains of both diseases showed normal utilization of the two precursors of plasmalogen biosynthesis. This is consistent with the hypothesis that impaired ether lipid biosynthesis is specific for CHRS.

We further show by this technique that ether lipid biosynthesis in fibroblasts of obligate heterozygotes is in the range of controls. This is in agreement with the demonstration of normal acyl CoA DHAP acyltransferase activity in CHRS heterozygote fibroblasts (13). The failure to demonstrate a simple gene dosage relationship suggests that the basic defect in CHRS resides rather in the assembly of functional peroxisomes than in a specific enzyme involved in ether lipid synthesis.

A specific and reliable biochemical method for the diagnosis of CHRS is essential to differentiate this disease from other causes of the "floppy infant syndrome." For practical purposes, the double-label, double-substrate technique reported herein offers several advantages. Since microsomal plasmalogens synthesis from alkyl glycerol precursors is normal in CHRS, [^{3}H]hexadecylglycerol can be utilized to serve as an internal reference. Diagnosis of CHRS can be established simply by comparing the [^{3}H/^{14}C] isotope ratio within aldehydes released from plasmalogens by acid hydrolysis (Table 3). This renders quantitative analyses (*e.g.* of lipids, enzyme activities, or cell protein) unnecessary and allows diagnosis from a limited amount of cells. [^{3}H/^{14}C] isotope ratios similar to those shown in Table 3 (0.5–1.0 for controls, 15–50 for CHRS) were obtained with grossly different amounts (10–200 μg of protein) of normal and CHRS fibroblasts. Values obtained for normal amniocytes (10 μg of protein) did not differ from those of control fibroblasts (data not shown).

The calculation of the [^{3}H/^{14}C] isotope ratio appears to provide a high degree of sensitivity due to the great difference between disease and control (15- to 60-fold). While the existing techniques for the diagnosis of a CHRS child or fetus appear to work well the method described herein may be a useful supplement especially under conditions when high diagnostic sensitivity and safety is mandatory.

Note added in proof. Since submission of this manuscript, Schutgens *et al.* (25) have reported prenatal diagnosis of two fetuses with Zellweger syndrome by measurement of DHAP-acyltransferase and incorporation of [1 – ^{14}C] hexadecanol into plasmalogens of amniotic fluid cells.

Datta *et al.* (26) have measured the activity of various enzymes catalyzing the biosynthesis of glycerol-ether lipids in tissues of Zellweger syndrome patients, corroborating the findings reported here by enzymatic techniques.

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