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Research Article

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Rhizomelic Chondrodysplasia Punctata

Deficiency of 3-Oxoacyl-Coenzyme A Thiolase in Peroxisomes and Impaired Processing of the Enzyme

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Abstract

The rhizomelic form of chondrodysplasia punctata (RCDP) is a peroxisomal disorder characterized biochemically by an impairment of plasmalogen biosynthesis and phytanate catabolism. We have now found that the maturation of peroxisomal 3-oxoacyl-CoA thiolase is impaired in fibroblasts from RCDP patients.

To establish the subcellular localization of the 3-oxoacyl-CoA thiolase precursor protein, cultured skin fibroblasts were fractionated on a continuous Nycodenz gradient. Only a small amount of 3-oxoacyl-CoA thiolase activity was present in the catalase-containing (peroxisomal) fractions of RCDP fibroblasts in comparison with control fibroblasts. Moreover, the amount of thiolase protein in immunoblots of the catalase-containing fractions was below the limit of detection. Finally, the β -oxidation of [¹⁴C]palmitoyl-CoA was found to be reduced in these fractions.

We conclude that the mutation in RCDP leads to a partial deficiency of 3-oxoacyl-CoA thiolase activity in the peroxisomes and, concomitantly, an impairment in the ability to convert the precursor of this protein to the mature form. The reduction of 3-oxoacyl-CoA thiolase activity results in a decrease in the rate of peroxisomal β -oxidation of palmitoyl-CoA. However, the capacity of the peroxisomes to oxidize very-long-chain fatty acids must be sufficient to prevent excessive accumulation of these compounds in vivo. (*J. Clin. Invest.* 1990. 86:126–130.) Key words: β -oxidation • Zellweger syndrome • organelle biogenesis • inborn errors • lipid metabolism

Introduction

During the last decade it has become increasingly evident that peroxisomes play an indispensable role in the metabolism of mammalian cells. The importance of peroxisomes is stressed by the existence of a number of genetic disorders in man in which one or several peroxisomal functions are impaired (for a review see references 1–3).

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The peroxisomal disorders known today can be subdivided into three categories. Diseases such as the cerebro-hepato-renal (Zellweger) syndrome (ZS),¹ neonatal adrenoleukodystrophy, and infantile Refsum disease are characterized by a decreased number or absence of morphologically distinguishable peroxisomes in liver and other tissues. Multiple biochemical abnormalities, including impaired β -oxidation of very-long-chain fatty acids (VLCFA), decreased oxidation of phytanic acid and pipecolic acid, impaired plasmalogen synthesis, and accumulation of intermediates in the biosynthesis of bile acids, are observed in these diseases of peroxisome biogenesis (1–3).

In another group of disorders, including X-linked adrenoleukodystrophy (4, 5), hyperoxaluria type 1 (6), acyl-CoA oxidase deficiency (7), bifunctional enzyme deficiency (8), and 3-oxoacyl-CoA thiolase deficiency (9), peroxisome structure is normal and the mutation involves a single peroxisomal enzyme.

In a third group of disorders the activity of more than one peroxisomal enzyme is deficient, but the peroxisomal structure appears to be intact. The rhizomelic form of chondrodysplasia punctata (RCDP) is one of two examples of such disorders with an impairment of some, but not all, peroxisomal functions (10–15). RCDP patients have an impairment in their ability to synthesize plasmalogens due to a deficiency of acyl-CoA: dihydroxyacetonephosphate acyltransferase (DHAP-AT) and alkyl dihydroxyacetonephosphate synthase (Alkyl-DHAP synthase), the enzymes required for the introduction of the ether bond in etherphospholipids. Furthermore, there is an accumulation of phytanic acid in body fluids, presumably due to a deficiency of one of the components of the phytanic acid oxidase system. Finally, recent immunoblotting studies have revealed that peroxisomal 3-oxoacyl-CoA thiolase is present in the unprocessed 44-kD form in liver (13) and fibroblasts (14), as is the case in the disorders of peroxisome biogenesis; peroxisomal thiolase, in contrast to most peroxisomal proteins, is synthesized as a larger precursor (16, 17). However, peroxisomes are present in RCDP, as indicated by the fact that the catalase activity in fibroblasts is particle bound (10–14). In RCDP patients there is no accumulation of VLCFA in body fluids, suggesting that peroxisomal β -oxidation of fatty acids is normal.

The aim of the study described in this paper was to obtain more information about the nature of the defect in RCDP that

1. *Abbreviations used in this paper:* Alkyl-DHAP synthase, alkyl dihydroxyacetonephosphate synthase; DHAP-AT, acyl-CoA: dihydroxyacetonephosphate acyltransferase; RCDP, rhizomelic chondrodysplasia punctata; VLCFA, very-long-chain fatty acids (C > 22); ZS, cerebro-hepato-renal (Zellweger) syndrome.

leads to a deficient conversion of the precursor of 3-oxoacyl-CoA thiolase to the mature form of the enzyme. Such studies should provide further insight into the mechanisms involved in the biogenesis of peroxisomes. Some of the results have been reported in abstract form (14).

Methods

Patients. Cell lines from 13 patients with clinical and biochemical manifestations characteristic of RCDP were used in these studies (see reference 12 for details).

Culture of fibroblasts. Skin fibroblasts were grown to confluency, harvested by gentle trypsinization, and collected by centrifugation exactly as described by Wanders et al. (18).

Digitonin fractionation of cultured skin fibroblasts. Cultured skin fibroblasts were incubated in isotonic media containing 250 mM sucrose, 20 mM 3-(4-morpholino)propane sulfonic acid (MOPS), pH 7.4, 2 mM EDTA, and 125 μ g/ml digitonin. Soluble and sedimentable fractions were prepared by centrifugation (10,000 g_{av} , 5 min). This concentration of digitonin led to rupture of the plasma membrane, whereas the peroxisomal membrane remained intact, as indicated by the fact that the cytosolic marker lactate dehydrogenase was liberated (> 95%), while most of the catalase activity (> 80%) was still in the sedimentable fraction.

Subcellular fractionation of cultured skin fibroblasts on continuous Nycodenz gradients. Fibroblasts were fractionated on linear Nycodenz gradients (10–40%) exactly as described by Wanders et al. (19).

Biochemical assays. De novo plasmalogen biosynthesis in fibroblasts was measured as described (20). Levels of VLCFA both in plasma and in fibroblasts were measured as described previously (18). Plasma levels of phytanic acid were estimated as described earlier (21).

The amount of particle-bound catalase and the activity of glutamate dehydrogenase and lactate dehydrogenase were measured as described previously (22). In the fractions obtained after density gradient centrifugation, the activity of 3-oxoacyl-CoA thiolase was assayed as described elsewhere (19). To measure [14 C]palmitoyl-CoA β -oxidation, Nycodenz gradient fractions prepared as described above were incubated at 37°C in a medium containing 150 mM Tris, pH 8.5, 2 mM NaCN, 10 mM MgCl₂, 10 mM ATP, 50 μ M FAD, 1 mM CoA, 0.01% Triton X-100, 1 mM NAD, and 100 μ M palmitoyl-CoA. After incubation for 2 h, reactions were terminated by the addition of 500 μ l of 1 mol/liter perchloric acid and 50 μ l of a solution containing 20% (wt/vol) BSA. The [14 C]acetyl-CoA produced was separated from the [14 C]acyl-CoA esters as described for the thiolase assay (19).

Immunoblot analysis. Samples were subjected to electrophoresis on 10% polyacrylamide gels according to the method of Laemmli (23). Immunoblot analyses were performed as described previously (24) with the following modifications. Nonspecific binding sites were blocked for 1 h with 4% (vol/vol) goat serum in PBS, pH 7.2, containing 0.1% Tween 20. Nitrocellulose sheets were incubated overnight with antibodies against 3-oxoacyl-CoA thiolase prepared as described by Miura et al. (25). Antigen-antibody complexes were visualized by incubation with goat anti-rabbit Ig conjugated to peroxidase, followed by color development using tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) as described by the manufacturers.

Results

Biochemical parameters in plasma and cultured skin fibroblasts

Patients with RCDP show a severe decrease in de novo plasmalogen biosynthesis in cultured skin fibroblasts (10–13), as has also been described for ZS and related disorders (1, 3). This is due to a deficiency of DHAP-AT (10–12, 15) and Alkyl-DHAP synthase (15), the enzymes required for the introduction of the ether bond in ether phospholipids (1). Plasma phy-

tanic acid levels are strongly elevated (10–15) and are comparable to those in ZS (1, 3). In RCDP, in contrast to ZS, normal C26:0/C22:0 fatty acid ratios are found in both plasma (12, 13, 15) and cultured skin fibroblasts (12–14), suggesting that the metabolism of VLCFA is not impaired in these patients (see below, however).

Finally, the amount of particle-bound catalase is normal in cultured skin fibroblasts from RCDP patients (11–14), thus indicating that catalase is contained within particles (peroxisomes) in RCDP fibroblasts.

Immunoblot analysis of peroxisomal 3-oxoacyl-CoA thiolase in cultured skin fibroblasts

Immunoblot experiments were carried out using antibodies against peroxisomal 3-oxoacyl-CoA thiolase isolated from rat liver. The results are shown in Fig. 1. In fibroblasts from control subjects cross-reactive immunological material was present corresponding to the 41-kD mature form of 3-oxoacyl-CoA thiolase (compare reference 24). In RCDP fibroblasts the amount of cross-reactive material was comparable to the amount in control fibroblasts. However, the molecular mass of this protein was \sim 2–3 kD larger than that of the protein in controls, and was identical to that of the precursor of 3-oxoacyl-CoA thiolase (16, 17), as seen in liver (26) and fibroblasts (24) from Zellweger patients (Fig. 1).

Subcellular localization of peroxisomal 3-oxoacyl-CoA thiolase

Digitonin fractionation studies. To obtain information on the localization of peroxisomal 3-oxoacyl-CoA thiolase in RCDP fibroblasts, we determined the amount of sedimentable 3-oxoacyl-CoA thiolase after fractionation of fibroblasts with digitonin. This method is based on the fact that digitonin forms stoichiometric complexes with cholesterol leading to permeabilization of membranes, and that the various cellular membranes differ in cholesterol content so that they can be selectively ruptured by titrating with digitonin (see also references 22 and 27). Incubation of cultured skin fibroblasts with a relatively low concentration of digitonin (125 μ g/ml) ruptured the plasma membrane preferentially (as shown by the liberation [> 95%] of the cytosolic marker enzyme lactate dehydrogenase), while most of the catalase activity (> 80%) was still in

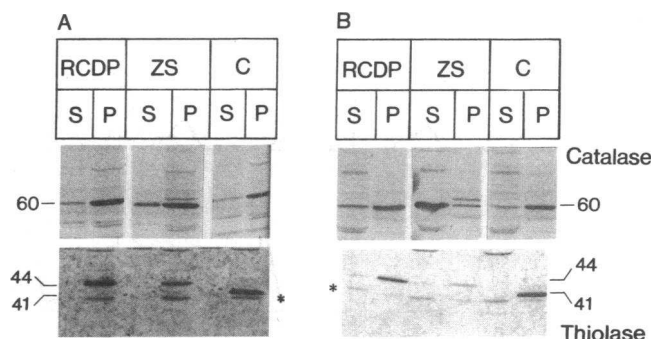


Figure 1. Immunoblot analysis of peroxisomal 3-oxoacyl-CoA thiolase and catalase. Soluble (S) and sedimentable (P) fractions of cultured skin fibroblasts from a control subject (C), a patient with ZS, and a patient with RCDP were prepared by digitonin fractionation and subjected to immunoblot analysis. For experimental details see Methods. A, Incubation without digitonin; B, incubation in the presence of 125 μ g/ml digitonin. *Unrelated protein band.

the sedimentable fraction (not shown). Fig. 1 shows the results of immunoblot analyses of the soluble and sedimentable fractions of fibroblasts. In Fig. 1 *A* fibroblasts were incubated in the absence of digitonin and the results show that the bulk of catalase and peroxisomal 3-oxoacyl-CoA thiolase are recovered in the pellet fraction. In Fig. 1 *B*, fibroblasts were incubated in the presence of digitonin. In accordance with earlier results (22) catalase was recovered predominantly in the soluble fraction of Zellweger fibroblasts, in line with its cytosolic localization in these cells. In the case of both RCDP and control fibroblasts catalase was found to be present in the pellet fractions. The results in Fig. 1 *B* further show that the mature form of peroxisomal 3-oxoacyl-CoA thiolase is present in the particulate fraction prepared from control fibroblasts. Peroxisomal thiolase is also found in the particulate fraction in RCDP and ZS fibroblasts, but in the mutant fibroblasts it is the precursor form of the enzyme that is present. Note that the band with an M_r of ~ 40 kD is an unrelated contaminant. There is less of the precursor of thiolase present in the particulate fraction from ZS fibroblasts than in that from RCDP fibroblasts.

Nycodenz density gradient fractionation studies. To study the localization of 3-oxoacyl-CoA thiolase in more detail, cultured skin fibroblasts were fractionated on continuous Nycodenz gradients. Fig. 2 shows that peroxisomes and mitochondria were resolved well on gradients, as indicated by the separation of the marker enzymes catalase and glutamate dehydrogenase, respectively, in control fibroblasts. The distribution of catalase was similar in gradients of RCDP fibroblasts and control cells. In contrast, in gradients of ZS fibroblasts

catalase colocalized with the cytosolic marker enzyme lactate dehydrogenase. Fig. 2 *D* shows that we were able to detect some 3-oxoacyl-CoA thiolase activity in peroxisomal fractions from RCDP fibroblasts, but this activity was low compared with that in control fibroblasts. Immunoblot analysis revealed that peroxisomal 3-oxoacyl-CoA thiolase protein was below the limit of detection in peroxisomal fractions of RCDP patients, whereas the mature form of this protein was clearly present in peroxisomal fractions of controls (Fig. 3). A high M_r band unrelated to peroxisomal thiolase is present in fractions 12–14 in both RCDP and control fibroblasts.

Peroxisomal β -oxidation was measured with [14 C]palmitoyl-CoA as substrate in the presence of cyanide (to inhibit as far as possible the mitochondrial β -oxidation of fatty acids). Fig. 2 *E* shows that in peroxisomal fractions of RCDP fibroblasts the β -oxidation activity was strongly reduced compared with that in controls. Some residual cyanide-insensitive palmitoyl-CoA oxidation was found in the mitochondrial fractions.

Discussion

The patients described in this paper exhibited the clinical features of RCDP and manifested the characteristic biochemical features of this disorder as described earlier (10–14), including an impairment in plasmalogen biosynthesis and accumulation of phytanic acid in body fluids. Immunoblot analysis revealed that 3-oxoacyl-CoA thiolase is present in the 44-kD, unprocessed, precursor form in human skin fibroblasts from RCDP

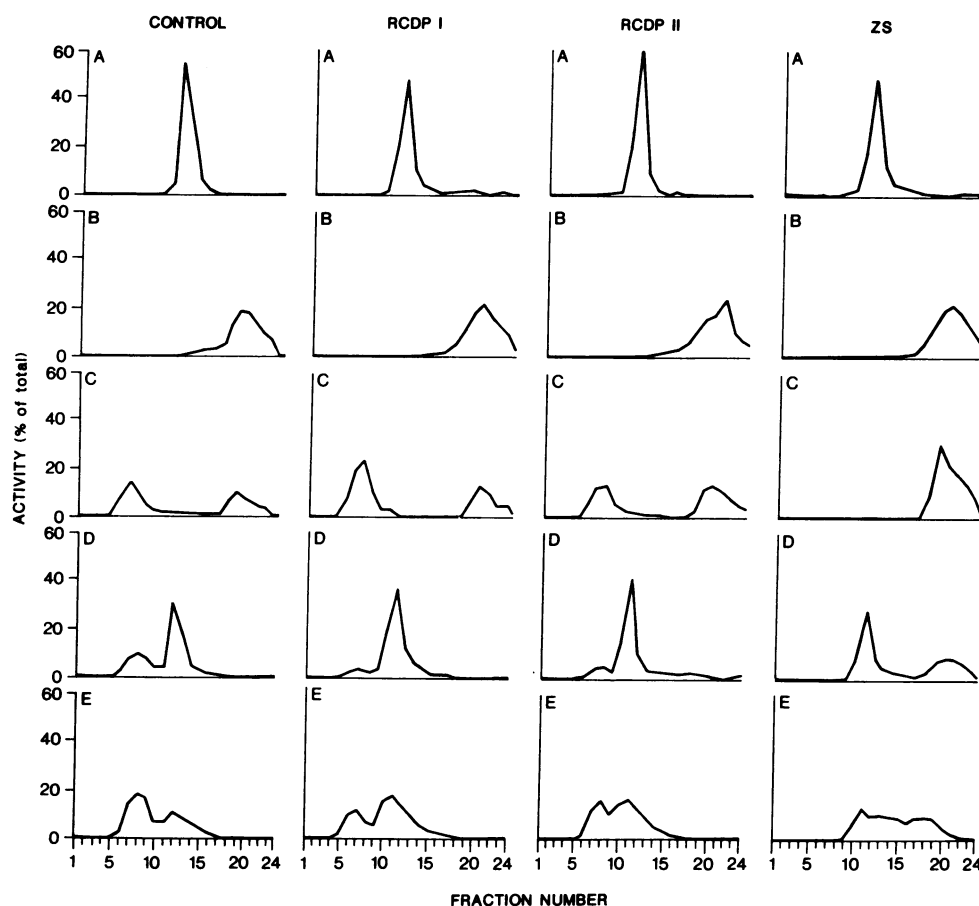


Figure 2. Subcellular fractionation of cultured skin fibroblasts on Nycodenz gradients. Fibroblast homogenates from a control subject, a ZS patient, and two RCDP patients were prepared and fractionated as described in Methods. Fractions were collected from the bottom of the tube and analyzed for the activity of the marker enzymes glutamate dehydrogenase (*A*), lactate dehydrogenase (*B*), and catalase (*C*), for the activity of thiolase (*D*), and for the rate of cyanide-insensitive β -oxidation of [14 C]palmitoyl-CoA (*E*). Activities are expressed as percent of total activity across the gradient.

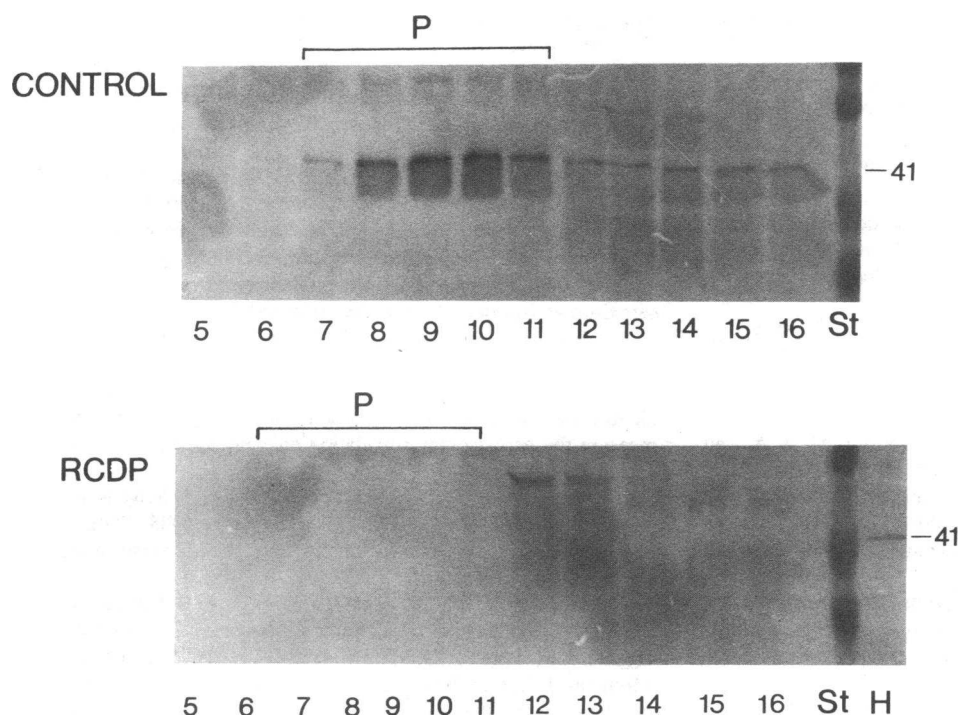


Figure 3. Immunoblot analysis of fractions obtained after Nycodenz fractionation of fibroblast homogenates. Cultured skin fibroblasts from an RCDP patient and from a control subject were fractionated as in Fig. 2 and subjected to immunoblot analysis by use of antibodies against rat liver 3-oxoacyl-CoA thiolase as described in Methods. The bar labeled *P* indicates fractions enriched in the peroxisomal marker enzyme catalase. High *M_r* bands unrelated to 3-oxoacyl-CoA thiolase are observed in fractions 12–14 (which contain the mitochondrial marker) both in control and RCDP fibroblast homogenates. *H*, Total homogenate of control fibroblasts; *St*, standards.

patients (14), in accordance with recent results obtained in post-mortem liver tissue by Hoefler et al. (13).

Wiemer et al. (28) have presented evidence that in rat hepatocytes the proteolytic processing of the precursor of 3-oxoacyl-CoA thiolase occurs after association of the enzyme with the particulate fraction. They concluded that part, if not all, of the proteolytic processing of 3-oxoacyl-CoA thiolase takes place within the peroxisomal compartment. Schram et al. (29) showed that the conversion of the 44-kD precursor of 3-oxoacyl-CoA thiolase to the mature protein does not take place in fibroblasts from patients with disorders of peroxisome biogenesis. They concluded that functional peroxisomes are required for the proteolytic processing of the precursor of 3-oxoacyl-CoA thiolase.

Fibroblasts from RCDP patients exhibit structure-linked latency of catalase, indicating that peroxisomes are present in these cells. Experiments with density gradient fractionation showed that in RCDP fibroblasts 3-oxoacyl-CoA thiolase protein was below the limit of detection in the catalase-enriched (peroxisomal) fractions (Fig. 3). We could detect some 3-oxoacyl-CoA thiolase activity in these fractions, although this was much lower than in the corresponding fractions from control fibroblasts (Fig. 2 D).

Our results lead us to suggest that the primary lesion in RCDP patients is at the level of a protein required for the import into peroxisomes of a specific group of peroxisomal proteins, including DHAP-AT, alkyl-DHAP synthase, a component of phytanic acid oxidase, and either prothiolase or a putative prothiolase protease. Another possibility is that one of the four enzymes is mutated and forms an abortive complex with a specific receptor, thus preventing transport into peroxisomes of a distinct set of peroxisomal enzyme proteins. The fact that in ZS the import defect involves not only these proteins but also a variety of other peroxisomal proteins suggests that in ZS a more universal component of the import machinery is affected than in RCDP.

Digitonin fractionation studies revealed that in RCDP fibroblasts the 3-oxoacyl-CoA thiolase protein is associated with the particulate fraction, as in control fibroblasts. The nature of the particulate fraction(s) in which the 3-oxoacyl-CoA thiolase is found in RCDP is at present unclear.

The β -oxidation of palmitoyl-CoA was found to be partially deficient in peroxisomal fractions from RCDP fibroblasts. However, the residual β -oxidation capacity in RCDP patients appears to be sufficient to prevent excessive accumulation of VLCFA in vivo (11–14).

The question remains of why there is only a partial deficiency of the activity of 3-oxoacyl-CoA thiolase in peroxisomes of RCDP. One of the possibilities is that the component of the import machinery impaired in RCDP is only partially defective. Another possibility worth investigating is that 3-oxoacyl-CoA thiolase is imported into the peroxisomes by a bypass of the component affected in RCDP.

Finally, the precursor of 3-oxoacyl-CoA thiolase appears to be more stable in RCDP patients than in ZS patients. The reason for this difference in stability is not clear at present.

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Note added in proof. Balfe et al. (30) have recently reported that the subcellular localization of peroxisomal 3-oxoacyl-CoA thiolase is aberrant both in rhizomelic chondrodysplasia punctata and in the Zellweger syndrome.

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