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Peroxisomal lignoceroyl-CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy

(peroxisomal acyl-CoA ligase/peroxisomal fatty acid oxidation/lignoceric acid/cultured skin fibroblasts)

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ABSTRACT We previously reported that in childhood adrenoleukodystrophy (C-ALD) and adrenomyeloneuropathy (AMN), the peroxisomal β-oxidation system for very long chain (>C_{22}) fatty acids is defective. To further define the defect in these two forms of X chromosome-linked ALD, we examined the oxidation of [1-^{14}C]lignoceric acid (*n*-tetracosanoic acid, C24:0) and [1-^{14}C]lignoceroyl-CoA (substrates for the first and second steps of β-oxidation, respectively). The oxidation rates of lignoceric acid in C-ALD and AMN were 43% and 36% of control values, respectively, whereas the oxidation rate of lignoceroyl-CoA was 109% (C-ALD) and 106% (AMN) of control values, respectively. On the other hand, the oxidation rates of palmitic acid (*n*-hexadecanoic acid) and palmitoyl-CoA in C-ALD and AMN were similar to the control values. These results suggest that lignoceroyl-CoA ligase activity may be impaired in C-ALD and AMN. To identify the specific enzymatic deficiency and its subcellular localization in C-ALD and AMN, we established a modified procedure for the subcellular fractionation of cultured skin fibroblasts. Determination of acyl-CoA ligase activities provided direct evidence that lignoceroyl-CoA ligase is deficient in peroxisomes while it is normal in mitochondria and microsomes. Moreover, the normal oxidation of lignoceroyl-CoA as compared with the deficient oxidation of lignoceric acid in isolated peroxisomes also supports the conclusion that peroxisomal lignoceroyl-CoA ligase is impaired in both C-ALD and AMN. Palmitoyl-CoA ligase activity was found to be normal in peroxisomes as well as in mitochondria and microsomes. This normal peroxisomal palmitoyl-CoA ligase activity as compared with the deficient activity of lignoceroyl-CoA ligase in C-ALD and AMN suggests the presence of two separate acyl-CoA ligases for palmitic and lignoceric acids in peroxisomes. These data clearly demonstrate that the pathognomonic accumulation of very long chain fatty acids in C-ALD and AMN is due to a deficiency of peroxisomal very long chain (lignoceric acid) acyl-CoA ligase.

The peroxisomal disorders represent a newly characterized group of inherited diseases (1, 2). In the adrenoleukodystrophies (ALD), three forms are recognized: childhood ALD (C-ALD; X chromosome-linked), adult ALD [adrenomyeloneuropathy (AMN); X chromosome-linked], and neonatal ALD (autosomal recessive). C-ALD is the most common form (3, 4) and usually appears between the ages of 4 and 8 years. It is characterized by central nervous system demyelination and adrenal cortical insufficiency. Death occurs during the first or second decade. AMN occurs mainly in adults, progresses more slowly, and affects the adrenal cortex, spinal cord, and peripheral nerves (5). The occurrence of both C-ALD and AMN within the same kindred suggests that these forms of ALD are different clinical manifestations of the same mutation (4). The identification of an identical biochemical defect in both would confirm this assumption. The neonatal form of ALD is a severe disorder that is evident in early childhood and involves a wide range of tissues (6, 7).

All three forms of ALD are characterized by excessive accumulation of very long chain (VLC; >C_{22}) fatty acids as a constituent of cholesterol esters and other lipids (4, 8). Our laboratory (9–11) and others (12–15) have previously demonstrated that the accumulation of these fatty acids in different forms of ALD is due to defective oxidation of VLC fatty acids. However, the oxidation of the long chain fatty acid palmitic acid is normal. VLC fatty acids are mostly oxidized in peroxisomes (11). Peroxisomes in the liver of C-ALD (11) and AMN (16) are normal in number and size, whereas in neonatal ALD they are smaller and fewer in number (16, 17). Based on these observations, it was postulated that the deficiency of VLC fatty acid oxidation in neonatal ALD may be due to the lack of peroxisomes, whereas in chromosome X-linked ALD (C-ALD and AMN), deficient oxidation of VLC fatty acids may be due to an enzymatic defect in the peroxisomal β-oxidation system. The present study was undertaken to identify the specific enzyme of the peroxisomal fatty acid oxidation system that may be impaired in X-linked ALD (C-ALD and AMN).

MATERIALS AND METHODS

Malate, FAD, NAD^+, L-carnitine, and α-cyclodextrin were purchased from Sigma. ATP and CoA free acid, CoA-SH, were obtained from P-L Biochemicals. [1 – ^{14}C]Palmitic acid (58.7 mCi/mmol; 1 Ci = 37 GBq) and [$K^{14}CN$] (52.0 mCi/mmol) were purchased from New England Nuclear. Nycodenz was obtained from Accurate Chemical and Scientific (Westbury, NY). [1-^{14}C]Lignoceric acid was synthesized by treatment of *n*-tricosanoyl bromide with [$K^{14}CN$] as described (18). [1-^{14}C]Lignoceroyl-CoA was synthesized as described (19).

Fractionation of Cultured Skin Fibroblasts by Differential and Density Gradient Centrifugation. Fibroblast cell lines (from The National Institute of General Medical Sciences Human Genetics Mutant Cell Repository, Camden, NJ and Dr. William Rizzo, Virginia Medical College, Richmond, VA) were cultured as described (20). Cells from 30 or more confluent flasks (75 cm^2) were collected by centrifugation (5 min at 500 × *g*) and incubated for 1 hr as a suspension in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum at 37°C. After centrifugation, cell pellets were washed with homogenization medium (0.25 M sucrose/1 mM EDTA/0.5 μg of antipain, 0.05 μg of leupeptin, and 0.7 μg of pepstatin per ml/0.2 mM phenylmethylsulfonyl fluoride/0.1% ethanol/3 mM imidazole buffer, pH 7.4) at 4°C and then resuspended in 1.0 ml of homogenization medium. Cells were lysed by using four to six hand strokes in a Teflon–glass

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Abbreviations: C-ALD, childhood adrenoleukodystrophy; VLC, very long chain; AMN, adrenomyeloneuropathy.

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Table 1. Rates of oxidation of fatty acids and their acyl-CoA derivatives in the postnuclear fraction of cultured skin fibroblasts

	Oxidation rate, nmol oxidized per hr per mg of protein		
	Control	C-ALD	AMN
Palmitic acid	1.79 ± 0.39	1.77 ± 0.35	1.87 ± 0.29
Palmitoyl-CoA	1.99 ± 0.30	2.15 ± 0.45	2.07 ± 0.41
Lignoceric acid	0.14 ± 0.03	0.06 ± 0.01	0.05 ± 0.01
Lignoceroyl-CoA	0.68 ± 0.28	0.74 ± 0.17	0.72 ± 0.19

Enzyme activities are expressed as the means ± SD derived from six control, five C-ALD, and four AMN different cell lines. Significant differences between the control and the C-ALD and AMN cells were observed only for the oxidation of lignoceric acid ($P < 0.001$).

homogenizer until ≈90% of the cells were broken. This step was monitored by phase-contrast microscopy. The homogenate was centrifuged at 500 × *g* for 5 min, and the supernatant (postnuclear fraction) was further fractioned by isopycnic equilibrium centrifugation in continuous Nycodenz gradients.

Tubes (39 mm) for a JV-20 Beckman rotor were layered with 4 ml of 35% Nycodenz and then 28 ml of a continuous gradient consisting of 0–30% Nycodenz in homogenization medium as described above. Samples (5 ml) of the postnuclear fraction were placed on top of the gradients. The tubes were sealed and then centrifuged at 33,700 × *g* for 60 min at 8°C in a J2-21 Beckman centrifuge with low acceleration and deacceleration. The gradient was collected from the bottom, each fraction was analyzed for marker enzyme activities, and the densities of gradient fractions were determined with a hand refractometer (Atago type 500).

Marker Enzyme Assays. The gradient fractions were analyzed for the following subcellular enzyme markers: cytochrome *c* oxidase for mitochondria (21), NADPH-cytochrome *c* reductase for microsomes (22), and catalase for peroxisomes (23). Protein concentration was determined by the procedure of Bradford (24).

Enzyme Assay for Activation and Oxidation of 1-^{14}C-Labeled Fatty Acids to Acetate (Water-Soluble Products). Acyl-CoA ligase activity was measured by our procedure as reported (25). Enzyme activity for the oxidation of 1-^{14}C-labeled fatty acids to acetate was measured as described (11) except that the fatty acid substrate was solubilized with α-cyclodextrin and was added to the assay medium. The enzyme reaction was stopped with 1.25 ml of 1 M potassium hydroxide in methanol, and the denatured protein was removed by centrifugation. The supernatant was incubated at 60°C for 1 hr, neutralized with acid, and partitioned by the procedure of Folch *et al.* (26). All studies with palmitic acid were done with this procedure. The amount of radioactivity in the upper phase is an index of the amount of 1-^{14}C-labeled fatty acid oxidized to acetate. For solubilization of the fatty acid substrate with α-cyclodextrin, the fatty acid (20 × 10^6 dpm) was first dried in a tube under nitrogen and resuspended in 3.5 ml (20 mg/ml) of α-cyclodextrin by sonication for 1 hr at 4°C. For 1-^{14}C-labeled acyl-CoA oxidation, the assay medium was the same except for the omission of ATP, $MgCl_2$, and CoA-SH.

Effect of Antisera to Palmitoyl-CoA Ligase on Lignoceroyl-CoA Ligase Activity. Antisera raised against purified palmitoyl-CoA ligase (27) was used to inhibit the peroxisomal palmitoyl-CoA ligase as follows: Antisera (700 μg) were incubated with peroxisomal proteins (40–70 μg) solubilized with 0.05% Triton X-100 in 20 mM Mops·HCl buffer (pH 7.8) at 4°C in a total volume of 0.25 ml. At the end of 1 hr, 50 μl of a 15% suspension of protein-Sepharose A was added, and incubation was continued for another 40 min with constant shaking. The mixture was centrifuged for 10 min at 2000 rpm in a Microfuge, and lignoceroyl-CoA ligase activity was measured in the supernatant.

RESULTS

Oxidation of 1-^{14}C-Labeled Fatty Acids and Their CoA-SH Derivatives in the Postnuclear Fraction of Cultured Skin Fibroblasts. The oxidation of lignoceric acid (substrate for the first step of β-oxidation) was impaired (43% of the control value) in the postnuclear fraction of cultured skin fibroblasts from C-ALD patients, whereas lignoceroyl-CoA and palmitoyl-CoA (substrates for the second step of the β-oxidation) were oxidized normally (Table 1). Moreover, a preliminary communication from this laboratory (20) also reported the normal oxidation of 1-^{14}C-labeled β-unsaturated lignoceroyl-CoA (substrate for the third step of β-oxidation) in C-ALD fibroblasts. The oxidation of different derivatives of fatty acids was also examined in AMN fibroblasts. As found with C-ALD fibroblasts, the oxidation of lignoceric acid in AMN fibroblasts was only 36% of the control values, whereas the oxidation of lignoceroyl-CoA was normal (Table 1). These results showing the normal oxidation of the substrate for the second step (lignoceroyl-CoA) in the β-oxidation system and the reduced oxidation of the substrate for the first step (lignoceric acid) in C-ALD and AMN fibroblasts suggest that the β-oxidation of lignoceric acid is impaired at the first step of β-oxidation (activation of lignoceric acid to lignoceroyl-CoA) in both of these forms of adrenoleukodystrophy.

Oxidation and Activation of Fatty Acids in Isolated Subcellular Organelles from Cultured Skin Fibroblasts. To deter-

Table 2. Specific enzyme activities of marker enzymes and enzyme activities for oxidation and activation of palmitic and lignoceric acids in fibroblast homogenates and their recovery in the postnuclear fraction

	Enzyme activities in fibroblast homogenates			% recovery in the postnuclear fraction		
	Control	C-ALD	AMN	Control	C-ALD	AMN
Marker enzymes, milliunit per mg of protein						
Catalase	5.5 ± 1.3	4.8 ± 1.8	5.4	84.1 ± 4.0	86.3 ± 2.5	88.6
Cytochrome c oxidase	0.9 ± 0.2	0.8 ± 0.2	0.8	74.3 ± 7.4	69.3 ± 5.8	75.5
NADPH cytochrome c reductase	4.5 ± 1.3	4.2 ± 0.9	4.2	81.7 ± 10.5	75.8 ± 6.1	80.1
Activation and oxidation activities, nmol/hr per mg of protein						
Palmitoyl-CoA ligase	8.4 ± 1.5	9.3 ± 1.1	8.2	87.8 ± 6.2	92.1 ± 6.4	88.1
Palmitic acid oxidation	1.5 ± 0.5	1.4 ± 0.4	1.5	91.1 ± 7.7	94.9 ± 9.1	87.9
Lignoceroyl-CoA ligase	0.27 ± 0.02	0.26 ± 0.04	0.25	74.8 ± 14.1	70.6 ± 11.3	71.0
Lignoceric acid oxidation	0.08 ± 0.01	0.03 ± 0.01	0.02	78.3 ± 11.4	74.4 ± 14.3	72.2
Protein				76.7 ± 6.2	75.4 ± 5.4	77.9

Enzyme activities are expressed as the means ± SD from different control (four), C-ALD (three), and AMN (two) cell lines. Significant differences between the control and C-ALD and AMN were observed only for the oxidation of lignoceric acid ($P < 0.001$). Units of enzyme activities for mitochondria (21), microsomes (22), and catalase (23) were calculated as described.

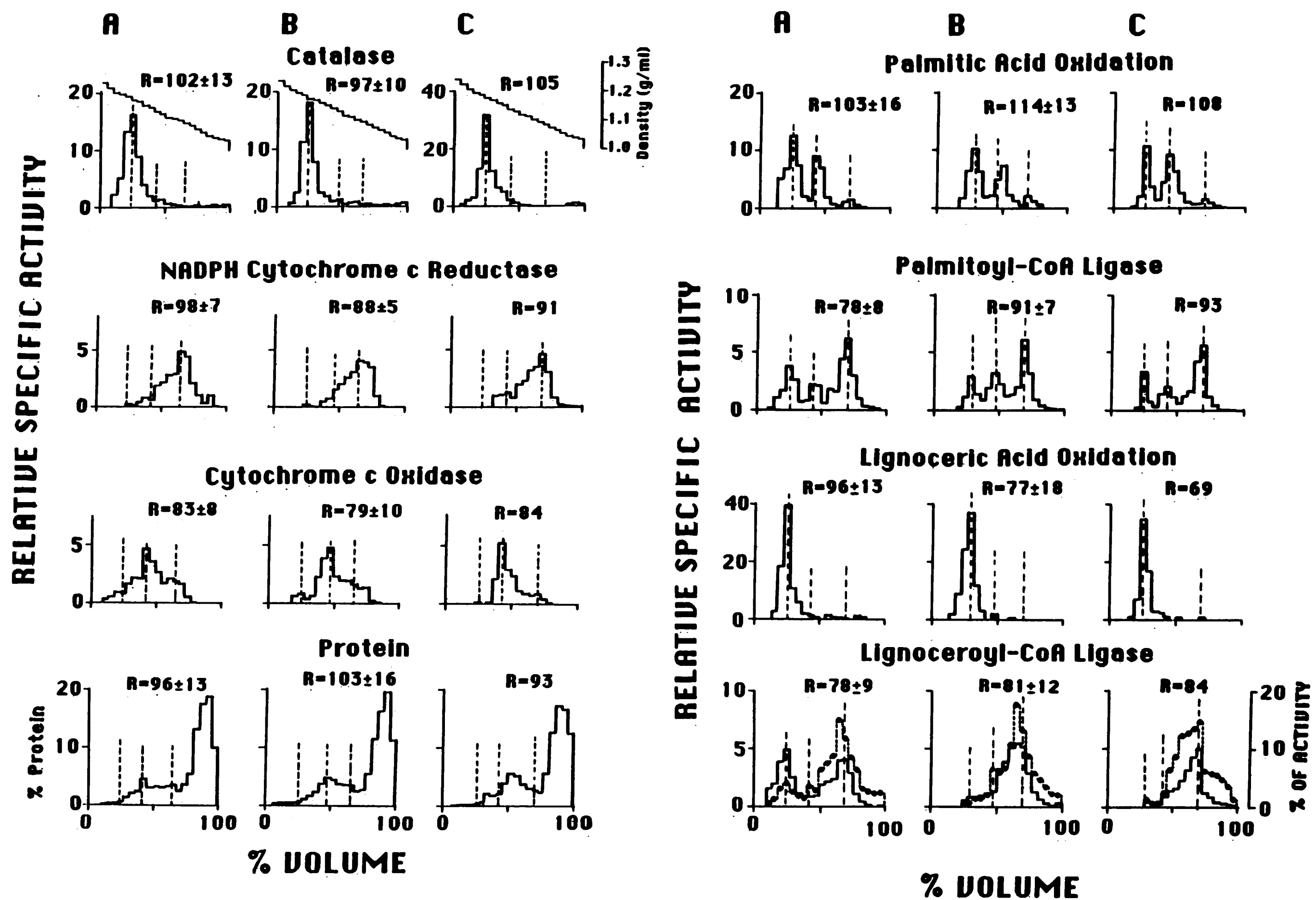

FIG. 1. Fractionation of the postnuclear fraction from cultured skin fibroblasts by Nycodenz isopycnic gradient. The postnuclear fractions from control (*A*), C-ALD (*B*), and AMN (*C*) fibroblasts were further fractionated by isopycnic continuous Nycodenz gradient as described. The distribution patterns of marker enzymes (*Left*) and enzyme activities for activation and oxidation of fatty acids (*Right*) are presented. The relative specific activity (ordinate) plotted against cumulative volume (abscissa) is an average from six control, four C-ALD, and two AMN gradients. Different cell lines were used for each experiment. R is the percent recovery of each enzyme activity in the gradient of the postnuclear fraction ± SD. The density profile of the gradients is also shown. The recovery of protein in gradients of control, C-ALD, and AMN fractions was 96.15 ± 13, 103 ± 16, and 93, respectively. Solid lines represent the relative specific activity, and dotted lines represent the percentage of lignoceroyl-CoA ligase activity. The vertical discontinuous lines represent the peaks of marker enzymes for peroxisomes, mitochondria, and microsomes.

mine if lignoceroyl-CoA ligase was impaired in C-ALD and AMN, the procedure of Santos *et al.* (29) was modified for better separation of subcellular organelles (peroxisomes, mitochondria, and microsomes) by using a Nycodenz gradient.

Table 2 shows the specific enzyme activities in the homogenates of cultured skin fibroblasts from C-ALD, AMN, and control subjects and their recovery in the postnuclear fraction. The specific activities of the marker enzymes and the enzyme activities for the activation of fatty acids and oxidation of palmitic acid in C-ALD and AMN fibroblasts were similar to control values, but the oxidation of lignoceric acid was impaired in C-ALD and AMN. The recovery of all of the enzymes in the fibroblast postnuclear fraction was similar for C-ALD, AMN, and control cells, and these fractions were used for the isolation of subcellular organelles by density gradient centrifugation.

The subcellular distribution of marker enzymes and the enzyme activities for the oxidation and activation of palmitic and lignoceric acids are shown in Fig. 1 *Left* and *Right*, respectively. The specific activities of the respective peak

Table 3. Rates of activation and oxidation of lignoceric acid in different subcellular organelles from cultured skin fibroblasts

	Lignoceroyl-CoA ligase activity, nmol/hr per mg of protein			Rate of oxidation of lignoceric acid, nmol/hr per mg of protein		
	Peroxisomes	Mitochondria	Microsomes	Peroxisomes	Mitochondria	Microsomes
Control	0.87 ± 0.10	0.31 ± 0.02	0.78 ± 0.12	0.17 ± 0.02	0.00	0.00
C-ALD	0.15 ± 0.04	0.32 ± 0.04	0.78 ± 0.20	0.02 ± 0.01	0.00	0.00
AMN						
Exp. I	0.15	0.28	0.90	0.02	0.00	0.00
Exp. II	0.14	0.31	0.70	0.02	0.00	0.00

The oxidation and activation of lignoceric acid were measured as described in the text. The results are expressed as the means ± SD in different control (six), C-ALD (four), and AMN (two) cell lines. Significant differences between control and C-ALD and AMN were observed only for the oxidation of lignoceric acid and the activity of lignoceroyl-CoA ligase in peroxisomes ($P < 0.001$).

Table 4. Rates of activation and oxidation of palmitic acid in different subcellular organelles from cultured skin fibroblasts

	Palmitoyl-CoA ligase activity, nmol/hr per mg of protein			Rate of oxidation of palmitic acid, nmol/hr per mg of protein		
	Peroxisomes	Mitochondria	Microsomes	Peroxisomes	Mitochondria	Microsomes
Control	22.9 ± 3.5	16.0 ± 2.2	40.7 ± 5.2	15.4 ± 3.2	10.8 ± 2.1	1.1 ± 0.6
ALD	19.8 ± 0.9	20.3 ± 5.9	44.7 ± 3.3	14.8 ± 3.8	11.6 ± 3.0	2.0 ± 0.6
AMN						
Exp. I	22.4	14.2	37.0	15.9	14.8	1.3
Exp. II	21.3	17.3	41.0	13.7	10.2	1.6

The oxidation and activation of palmitic acid were measured, and the results are expressed as the means ± SD in different control (six), C-ALD (four), and AMN (two) cell lines. There are no significant differences in these values between control and C-ALD and AMN in any of the subcellular organelles.

fractions are summarized in Tables 3 and 4. In agreement with previous studies, lignoceric acid was oxidized only in the peroxisomes purified from control fibroblasts, and little oxidation (11.8% of the control value) was found in the peroxisomes from either C-ALD and AMN fibroblasts (Fig. 1 *Right* and Table 3). However, the oxidation of palmitic acid was normal in peroxisomes isolated from C-ALD and AMN fibroblasts (Fig. 1 *Right* and Table 4). In addition, oxidation of lignoceroyl-CoA in isolated peroxisomes from C-ALD and AMN fibroblasts was equivalent to control cells (Table 5). This further indicates that the defect is at the peroxisomal lignoceroyl-CoA ligase level. The specific activity of lignoceroyl-CoA ligase in the control fibroblasts (Table 3) was higher in peroxisomes and microsomes than in mitochondria. However, lignoceroyl-CoA ligase activity in C-ALD (17.2%) and AMN (16.7%) fibroblasts was deficient compared with the control value in the peroxisomes but was normal in mitochondria and microsomes (Fig. 1 *Right* and Table 3). This residual activity was eliminated when peroxisomes were treated with antibody against palmitoyl-CoA ligase (Fig. 2). Palmitoyl-CoA ligase activity was normal in peroxisomes as well as in mitochondria and microsomes from both C-ALD and AMN fibroblasts (Fig. 1 *Right* and Table 4). This shows that the lack of oxidation of lignoceric acid in C-ALD and AMN fibroblasts is due to a deficiency of peroxisomal lignoceroyl-CoA ligase activity (Figs. 1 *Right* and 2, and Tables 3 and 5).

DISCUSSION

The VLC fatty acids are oxidized in peroxisomes and their oxidation in C-ALD and AMN fibroblasts is impaired (11). Therefore, the excessive accumulation of VLC fatty acids in C-ALD and AMN may be due to impairment of an enzyme(s) in the peroxisomal β-oxidation system. This pathway consists of five different steps catalyzed by four different enzymes: (*i*) activation of fatty acids to acyl-CoA by acyl-CoA ligase; (*ii*) α,β-unsaturation of acyl-CoA to enoyl-CoA by acyl-CoA oxidase; (*iii*) hydration of enoyl-CoA to β-hydroxyacyl-CoA by a bifunctional enzyme; (*iv*) dehydrogenation of β-hydroxyacyl-CoA to β-ketoacyl-CoA by bifunctional enzyme; and (*v*) hydrolysis of β-ketoacyl-CoA to acetyl-CoA and acyl-CoA by β-ketoacyl-CoA thiolase (28, 30).

Table 5. Rate of oxidation of lignoceroyl-CoA in homogenates and purified peroxisomes from cultured skin fibroblasts

Exp.		Rate of oxidation of lignoceroyl-CoA, nmol/hr per mg of protein: Homogenate	Peroxisomes
I	Control	0.65	4.81
	C-ALD	0.64	4.58
II	Control	0.61	4.49
	C-ALD	0.62	4.97
III	Control	0.64	4.97
	AMN	0.68	5.10

Lignoceroyl-CoA oxidation was measured, and results are from different control (three), C-ALD (two), and AMN (one) cell lines. There are no significant differences between control and C-ALD and AMN values.

The normal rate of oxidation of lignoceroyl-CoA (substrate for the second step of β-oxidation) but the impaired oxidation of lignoceric acid (substrate for the first step) suggests that the defect in C-ALD and AMN involves the first step (activation of lignoceric acid to lignoceroyl-CoA) in the β-oxidation of VLC fatty acids. Since the substrates for the second and third steps are catabolized normally to acetate (Table 1; ref. 20), the enzymes for the fourth (bifunctional enzyme) and fifth steps (thiolase) should be normal. Similarly, this normal oxidation of lignoceroyl-CoA contrasted with the oxidation of lignoceric acid has been recently confirmed by other laboratories (15, 31). This suggests that the enzymatic step for the activation of lignoceric to lignoceroyl-CoA may be impaired in C-ALD and AMN. However, the near normal activity of lignoceroyl-CoA ligase observed in total homogenates from C-ALD fibroblasts (ref. 20, Table 2) would suggest that, like palmitoyl-CoA ligase (32–35), lignoceroyl-CoA ligase activity may also be distributed in different subcellular organelles. Therefore, deficient activity in one organelle may not result in an appreciable change in total activity in the homogenate.

To demonstrate the enzyme defect in a specific organelle, it was necessary to examine acyl-CoA ligase activities in different organelles after subcellular fractionation of cultured C-ALD and AMN skin fibroblasts. Metrizamide gradients have been used for the isolation of subcellular organelles from kidney and liver (36–38), but its use in the isolation of organelles from fibroblasts was not successful in completely removing peroxisomes from mitochondria (29). The procedure of Santos *et al.* (29) for the isolation of peroxisomes, the only procedure described for fibroblasts, was not suitable because the distribution pattern of membranous catalase and acyl-CoA oxidase (peroxisomal markers) has a bimodal distribution with a significant percentage in the mitochondrial

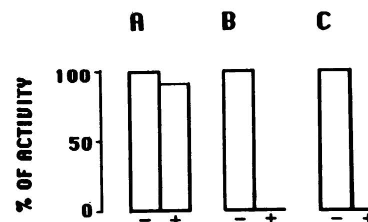

FIG. 2. Effect of antisera to palmitoyl-CoA ligase on lignoceroyl-CoA ligase activity in peroxisomes from control (*A*), C-ALD (*B*), and AMN (*C*) fibroblasts without (–) and with (+) the addition of antibody. Lignoceroyl-CoA ligase activities in control, C-ALD, and AMN peroxisomes in the absence of antibody are expressed as 100%.

fractions (29). They did not report the distribution of microsomes, but in our studies we were unable to resolve mitochondria and peroxisomes from microsomes (data not shown here). Their procedure (29) was modified by using low centrifugal force (500 × *g* for 5 min as compared to 1,000 × *g* for 10 min) for a higher recovery of subcellular organelles of interest in the postnuclear fraction and the use of Nycodenz in 0.25 M sucrose containing protease inhibitors as described for subcellular fractionation. Nycodenz has been previously used for the isolation of peroxisomes from liver (39), and we describe here its use in the isolation of peroxisomes, mitochondria, and microsomes from fibroblasts. We obtained a single peak of membranous catalase in the Nycodenz gradient as compared to the bimodal distribution observed in metrizamide (29). The mitochondrial and microsomal peaks were broader than the sharp peak observed for peroxisomes, but the peaks were well resolved from each other with only slight cross-contamination (Fig. 1 *Left*).

These studies with isolated organelles from cultured skin fibroblasts from C-ALD, AMN, and control subjects clearly show a marked deficiency of lignoceroyl-CoA ligase in only the peroxisomal fraction. This deficient activity of lignoceroyl-CoA ligase compared with the normal activity in mitochondria and microsomes shows that the deficiency of lignoceric acid oxidation in C-ALD and AMN is due to impaired activity of VLC peroxisomal (lignoceric acid) acyl-CoA ligase. The normal oxidation of lignoceroyl-CoA in peroxisomes from C-ALD and AMN fibroblasts supports this conclusion. It is also of interest that palmitoyl-CoA ligase activity was normal in peroxisomes. This normal peroxisomal palmitoyl-CoA ligase activity compared with the deficient activity for lignoceroyl-CoA ligase provides evidence that peroxisomes may have separate acyl-CoA ligases for palmitic and lignoceric acids. This conclusion is further supported by the elimination of the residual lignoceroyl-CoA ligase activity in C-ALD and AMN peroxisomes when treated with antibodies against palmitoyl-CoA ligase. This treatment resulted in only a minor decrease in control lignoceroyl-CoA ligase activity. Our studies with microsomal ligase activities suggest that in microsomal membranes, palmitoyl-CoA and lignoceroyl-CoA may be synthesized by two separate enzymes (27, 40). The palmitoyl-CoA ligases (76-kDa protein) present in mitochondria, peroxisomes, and microsomes are similar with respect to molecular mass and immunoreactivity (35). It should be of interest to determine if the lignoceroyl-CoA ligases in these organelles are the same or different.

The oxidation of lignoceric acid in the control fibroblasts as well as in C-ALD and AMN fibroblasts is confined to peroxisomes (Fig. 1 *Right* and Table 3), thus confirming the previous observations (11). The residual activity (11.8% of control) in peroxisomes from C-ALD and AMN fibroblasts may be the result of activation of lignoceric acid by the normal peroxisomal palmitoyl-CoA ligase. In summary, the results described in this paper clearly show that the basis for the pathognomonic accumulation of VLC fatty acids in both C-ALD and AMN is due to impaired activity of peroxisomal lignoceroyl-CoA ligase.

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