Lignoceroyl-CoASH ligase: enzyme defect in fatty acid β-oxidation system in X-linked childhood adrenoleukodystrophy

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We have previously reported that the peroxisomal β-oxidation system for very long chain fatty acids is defective in X-linked childhood adrenoleukodystrophy [(1984) Proc. Natl. Acad. Sci. USA 81, 4203–4207]. In order to elucidate the specific enzyme defect, we examined the oxidation of [1-^{14}C]lignoceric acid, [1-^{14}C]lignoceroyl-CoA and (1-^{14}C)-labelled α,β-unsaturated lignoceroyl-CoA (substrates for the 1st, 2nd, and 3rd steps of the β-oxidation cycle, respectively). These studies suggest that the pathognomonic accumulation of very long chain fatty acids in X-linked childhood ALD may be due to the defective activity of peroxisomal very long chain (lignoceroyl-CoA) acyl-CoA ligase.

Adrenoleukodystrophy *Peroxisome* *Fatty acid* *β-Oxidation* *Lignoceric acid* *Lignoceroyl-CoA* *Lignoceroyl-CoA ligase*

1. INTRODUCTION

X-linked adrenoleukodystrophy (ALD) is a childhood disorder characterized by progressive demyelination and impaired function of the adrenal cortex [1]. It is associated with the pathognomonic accumulation of cholesterol esters of saturated very long chain (VLC) fatty acids ($> C_{22}$) in the brain and adrenals [2]. In other tissues the accumulation of VLC fatty acids is found in glycerolipids and sphingolipids [3]. Other forms of ALD include adrenomyeloneuropathy (AMN) and ALD (autosomal recessive). Although accumulation of VLC fatty acids is the observed hallmark in all forms of ALD, the childhood form differs from the neonatal in that peroxisomes are present in the liver of childhood ALD whereas they are very much reduced in size and number in the neonatal ALD [4]. Due to the excessive accumulation of VLC fatty acids in the cholesterol ester fraction, initial studies to delineate the specific enzyme defect focused on the metabolism of cholesterol esters. However, no defect in ALD was observed [5,6]. Studies of normal cholesterol ester metabolism and the accumulation of VLC fatty acids in lipids other than cholesterol esters suggested that the defect in childhood ALD may be in the metabolism of VLC fatty acids. Tsuji et al. [7] reported an increased rate of chain elongation in ALD fibroblasts as compared to normal cells. These findings suggested that the defect in ALD may be in the synthesis of VLC fatty acids. However, the excessive accumulation of VCL fatty acids in ALD fibroblasts as compared to control cells when [1-^{14}C]lignoceric acid [8] and [1-^{14}C]-cerotic acid [9] were incubated with fibroblast cultures indicated that catabolism of these fatty acids could be defective.

We examined the degradation of fatty acids of different chain lengths (C_{16}–C_{26}) and reported that the cause of excessive accumulation of VLC fatty acids in ALD may be in the degradation of VLC

The following desginations are used: palmitic acid, *n*-hexadecanoic acid; lignoceric acid, *n*-tetracosanoic acid; α,β-unsaturated lignoceric acid, *n*-tetracosenoic acid

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(C_{24},C_{26}) fatty acids whereas oxidation of palmitic acid (C_{16}) was normal [10–12]. The studies of Jaffe et al. [13] and Rizzo et al. [14] also demonstrated a defect in the degradation of VLC fatty acids in ALD. Although it is not known whether both synthesis and degradation of VLC fatty acids are impaired in ALD, a defect in degradation of VLC fatty acids has been confirmed in 4 different laboratories [8–14]. This study was undertaken to delineate the specific enzymatic step of the β-oxidation system which may be impaired in X-linked childhood ALD.

2. MATERIALS AND METHODS

2.1. *Materials*

Malate, FAD, NAD, L-carnitine and α-cyclodextrin were purchased from Sigma, St. Louis, MO. ATP and CoASH were obtained from PL-Biochemicals, Milwaukee, WI. [1-^{14}C]Palmitic acids and K^{14}CN were purchased from New England Nuclear, Boston, MA.

2.2. *Synthesis of substrates*

[1-^{14}C]Lignoceric acid was synthesized from K^{14}CN and tricosanoyl bromide [15]. (1-^{14}C)-labeled α,β-unsaturated lignoceric acid was synthesized from [1-^{14}C]lignoceric acid by α-bromination followed by dehydrobromination [16]. [1-^{14}C]Lignoceroyl-CoA and α,β-unsaturated lignoceroyl-CoA were prepared as in [17].

2.3. *Cell cultures*

Fibroblast cell lines from X-linked childhood ALD patients and controls were grown in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's minimum essential medium supplemented with 15% fetal calf serum. Cells were harvested 3–4 days after confluence by trypsinization and the cell pellets washed 3 times with Hank's balanced salt solution.

2.4. *Enzyme assay*

Enzyme activity for oxidation of (1-^{14}C)-labeled fatty acids to acetate (water-soluble product) was measured as described [12]. Briefly, the reaction mixture (0.5 ml) contained 12 μM (1-^{14}C)-labeled fatty acid coated on celite [11], 20 mM Mops-HCl buffer, pH 7.9, 30 mM KCl, 1 mM $MgCl_2$, 8.5 mM ATP, 0.25 mM NAD, 0.17 mM FAD, 2.5 mM L-carnitine, 0.08 mM CoASH, and 1 mg α-cyclodextrin. Cells were homogenized by 20 passes with a hand-driven teflon-coated homogenizer in 0.06% NaCl. The reaction was started by the addition of enzyme and stopped with 0.5 ml ice-cold 0.6 N perchloric acid. The supernatant fraction was transferred to another tube, partitioned by the procedure of Folch et al. [18], and the radioactivity in the upper layer measured. The amount of radioactivity is an index of the amount of (1-^{14}C)-labeled fatty acid oxidized to acetate. Protein was measured by the procedure of Bradford [19].

3. RESULTS AND DISCUSSION

Fatty acid β-oxidation takes place in both mitochondria and peroxisomes. Palmitic acid is oxidized in mitochondria at 3–5-times the rate of that in peroxisomes [20–22] whereas lignoceric acid is mostly and possibly exclusively oxidized in peroxisomes [12]. Our previous studies of childhood ALD demonstrated that peroxisomal β-oxidation of palmitic acid is normal but that of lignoceric acid is defective [10–12]. Since peroxisomes in childhood ALD are normal in size and number, a specific enzyme defect is suggested [12]. The peroxisomal β-oxidation enzyme system consists of a sequence of 5 steps: (1) activation by acyl-CoA ligase; (2) α,β-unsaturation by acyl-CoA oxidase; (3) hydration by acyl-CoA hydratase; (4) dehydrogenation by β-hydroxyacyl-CoA dehydrogenase and (5) formation of acetate by β-ketoacyl-CoA thiolase. To elucidate the specific enzyme defect we synthesized [1-^{14}C]lignoceric acid, [1-^{14}C]lignoceroyl-CoA, (1-^{14}C)-labeled α,β-unsaturated lignoceroyl-CoA and examined their rate of oxidation as described [12]. Since the procedure for isolation of peroxisomes from cultured fibroblasts has not yet been developed, these studies were performed with total cellular homogenates. In agreement with our previous studies [10–12] and others [8,9,13,14], there was no difference in the oxidation of palmitic acid between ALD and control fibroblasts. However, the degradation of [1-^{14}C]lignoceric acid to water-soluble product (acetate) in ALD was 32% of control fibroblasts (table 1). The ratio of the relative rate of degradation of palmitic acid to lignoceric acid was 3.18-times higher for ALD than the con-

Table 1

Oxidation of (1-^{14}C)-labeled fatty acids and their acyl-CoA derivatives

	Control (mean ± SE)	Childhood ALD (mean ± SE)
Palmitic acid	88534 ± 6548	95027 ± 8026 ($p = 0.634$)
Palmitoyl-CoA	229427 ± 10263	238938 ± 13694 ($p = 0.148$)
Lignoceroyl acid	10242 ± 586	3255 ± 439 ($p < 0.001$)
Lignoceroyl-CoA	41530 ± 3708	44853 ± 8243 ($p = 0.585$)
α,β-unsaturated lignoceroyl-CoA	77237 ± 13927	76881 ± 11889 ($p = 0.568$)

The oxidation of (1-^{14}C)-labeled fatty acids and their acyl-CoA derivatives in homogenates of control and ALD cultured skin fibroblasts was measured as cpm water-soluble product/mg protein per h

trol (table 2). When the acyl-CoA derivative was used as the substrate (substrate for the 2nd step of the β-oxidation cycle) the oxidation of both [1-^{14}C]lignoceroyl-CoA and [1-^{14}C]palmitoyl-CoA was normal (table 1). Therefore, the ratios of relative rates of oxidation of palmitoyl-CoA to lignoceroyl-CoA in ALD and control were 5.12 and 5.7, respectively (table 2). These results suggest that the defect in childhood ALD is in the 1st step of the β-oxidation cycle, which is activation of VLC fatty acids to their acyl-CoA derivatives. Moreover, α,β-unsaturated lignoceroyl-CoA, the substrate for the 3rd step of β-oxidation, was also oxidized normally in fibroblasts from childhood ALD (table 1). Since substrates for the 2nd and 3rd step of the β-oxidation cycle are metabolized normally, the enzymes for the 4th step, β-hydroxyacyl-CoA dehydrogenase, and 5th step, thiolase, are normal. The ratios of the oxidation of palmitoyl-CoA to α,β-unsaturated lignoceroyl-CoA were 3.16 and 3.13 for ALD and control fibroblasts, respectively (table 2). The ratios for the oxidation of lignoceroyl-CoA to α,β-unsaturated lignoceroyl-CoA were also the same for both ALD and control (table 2). Fatty acid β-oxidation is a series of 5 steps in sequence. Our results indicate that activation of lignoceric acid by acyl-CoA ligase, the 1st step in the β-oxidation sequence, is defective while lignoceroyl-CoA and α,β-unsaturated lignoceroyl-CoA, the substrates for the 2nd and 3rd steps, are degraded normally. This identifies lignoceroyl-CoA ligase as the enzyme impaired in childhood ALD.

At the subcellular level, palmitoyl-CoA ligase activity is known to be present in mitochondria, microsomes and peroxisomes [23–25]. Lignoceroyl-CoA ligase activity has also been demonstrated in microsomal [26] and mitochondrial peroxisomal fractions (Singh et al., unpublished). Peroxisomes isolated from human liver contain only 16% palmitoyl-CoA ligase activity, while 21 and 60% of this enzyme activity was localized in mitochondrial and microsomal fractions, respectively [27]. Therefore, direct measurements of lignoceroyl-CoA ligase activity in the homogenates of fibroblasts did not show any significant differences between ALD and controls, (50.3 and 42.8 pmol/mg protein per 5 min for control and

Table 2

Relative rates of oxidation of fatty acids and their acyl-CoA derivatives in homogenates of control and ALD fibroblasts

	Childhood ALD (mean ± SE)	Control (mean ± SE)
[1-^{14}C]Palmitic acid/[1-^{14}C]lignoceric acid	27.24 ± 2.65	8.57 ± 1.15
[1-^{14}C]Palmitoyl-CoA/[1-^{14}C]lignoceroyl-CoA	5.12 ± 0.5	5.7 ± 0.67
[1-^{14}C]Palmitoyl-CoA/(1-^{14}C)-labeled α,β-unsaturated lignoceroyl-CoA	3.16 ± 0.36	3.13 ± 0.5
[1-^{14}C]Lignoceroyl-CoA/(1-^{14}C)-labeled α,β-unsaturated lignoceroyl-CoA	0.61 ± 0.12	0.57 ± 0.07

ALD, respectively). Since lignoceric acid is oxidized mostly and possibly exclusively in peroxisomes [12], the impaired oxidation of lignoceric acid as compared to normal activity with lignoceroyl-CoA in ALD fibroblasts (tables 1 and 2) therefore clearly demonstrates that activation of lignoceric acid to lignoceroyl-CoA is defective in childhood ALD. Palmitoyl-CoA and lignoceroyl-CoA are synthesized by two different enzymes in microsomal membranes [28]. Our previous studies suggested that in peroxisomes there may be two different fatty acid β-oxidation enzyme systems (one for palmitic acid and the other for lignoceric acid [12]). Therefore, it may be possible that peroxisomes like microsomal membranes [28] also have different enzymes for the synthesis of lignoceroyl-CoA and palmitoyl-CoA and that synthesis of lignoceroyl-CoA by peroxisomal lignoceroyl-CoA ligase is impaired in childhood ALD.

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