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EFFECTS OF SODIUM 2-[5-(4-CHLOROPHENYL)PENTYL]-OXIRANE-2-CARBOXYLATE (POCA) ON FATTY ACID OXIDATION IN FIBROBLASTS FROM PATIENTS WITH PEROXISOMAL DISEASES

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Abstract—The effects of sodium 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA), a potent inhibitor of carnitine palmitoyltransferase I, on fatty acid oxidation were investigated using fibroblasts from control subjects and from patients with peroxisomal disorders. [1-^{14}C]Palmitate oxidation was inhibited by 8% of the control value when 15 μM POCA was added to the medium. The inhibition by POCA was significantly ($P < 0.05$) stronger in fibroblasts from patients with Zellweger syndrome or with neonatal adrenoleukodystrophy, in which peroxisomes and peroxisomal β-oxidation enzymes were absent. However, the inhibition in fibroblasts from patients with X-linked adrenoleukodystrophy, in which a specific defect of peroxisomal lignoceroyl-CoA synthetase was speculated, was similar to that in the controls. [1-^{14}C]Lignocerate oxidation was not influenced by the addition of POCA, in samples from the controls and from the patients. These results indicate that peroxisomes account for a small but demonstrable proportion of palmitate oxidation, and add new evidence to the concept that lignocerate is oxidized exclusively in the peroxisomes. Our findings also support the hypotheses that the activity of palmitoyl-CoA synthetase and the enzymes of β-oxidation cycle in peroxisomes are normal in patients with X-linked adrenoleukodystrophy and that a specific defect of lignoceroyl-CoA synthetase is responsible for the accumulation of very long chain fatty acids in these patients.

Analysis of the metabolic effects of mitochondrial fatty acid oxidation inhibitors is important if one is to estimate the contribution of the peroxisomal β-oxidation system to degradation of long chain fatty acids and to evaluate the pathophysiology of fatty acid oxidation in patients with peroxisomal disorders such as Zellweger syndrome [1], neonatal adrenoleukodystrophy (ALD)‡ [2], X-linked ALD [3] and a single deficiency of peroxisomal β-oxidation enzymes [4–6].

Zellweger syndrome and neonatal ALD are fatal autosomal recessive diseases characterized by profound hypotonia, psychomotor retardation, hepatomegaly and facial dysmorphism. Absence of peroxisomes [7, 8] and multiple defects of peroxisomal β-oxidation enzymes [9, 10] and other enzymes [11] are present in patients with these diseases. Defects in β-oxidation enzymes are considered to be a core biochemical abnormality since clinical findings of patients with a single deficiency of peroxisomal β-oxidation enzymes are similar to those of patients without peroxisomes. In patients with X-linked ALD, there is neurological deterioration and an accumulation of very long chain fatty acids (VLCFA). A specific defect of peroxisomal lignoceroyl-CoA synthetase is speculated to be the primary etiology [12].

The peroxisomal β-oxidation system, first discovered by Lazarow and DeDuve in 1976 [13], differs from that of the mitochondria. Enzyme proteins of the former differ from those of the latter [14]; the peroxisomal system is cyanide insensitive because it does not couple with electron transport systems, and is carnitine independent. Carnitine palmitoyltransferase I (CPT-I) is essential for fatty acyl-CoA to enter the mitochondrial β-oxidation cycle. The peroxisomal system does not require CPT-I and mainly oxidizes VLCFA such as lignocerate (C24:0) and cerotate (C26:0) [15, 16]. Accumulation of VLCFA in patients with peroxisomal disorders has been attributed to a defect in the peroxisomal fatty acid oxidation system.

Potassium cyanide has been used to block the electron transport system, and to measure peroxisomal fatty acid oxidation activity [17]; however, this compound cannot be used for viable cells. Sodium 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA or B807-27) is a potent inhibitor of mitochondrial fatty acid oxidation at the stage of CPT-I, by the tight binding of POCA–CoA to this enzyme [18]. POCA inhibits the oxidation of [U-^{14}C]palmitate in cultured fibroblasts and there is

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‡ Abbreviations: ALD, adrenoleukodystrophy; POCA, sodium 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate; VLCFA, very long chain fatty acids; CPT-I, carnitine palmitoyltransferase I; MEM, Minimal Essential Medium; and FBS, fetal bovine serum.

a decrease in the production of $^{14}CO_2$ and ^{14}C-labeled acid-soluble products [18].

We investigated the effects of POCA on palmitate and lignocerate oxidation in fibroblasts from normal controls and from patients with peroxisomal diseases in an attempt to elucidate the contribution of peroxisomes to degradation of fatty acids and to the pathophysiology of defects in fatty acid oxidation in these diseases. We found that a small portion of palmitate oxidation occurs in peroxisomes and that lignocerate is oxidized exclusively in the peroxisomes. The pathogenesis of X-linked adrenoleukodystrophy is also discussed.

MATERIALS AND METHODS

Patients. Cultured skin fibroblasts from five infants with Zellweger syndrome, four with neonatal ALD, three with X-linked ALD, and five control subjects were used. Clinical and biochemical findings of the patients with Zellweger syndrome have been reported [19]. A Japanese girl with neonatal ALD was diagnosed by the biochemical findings described by Chen *et al.* [10]. Three cell lines from neonatal ALD were the gift of Prof. Hugo W. Moser (J. F. Kennedy Institute, Johns Hopkins University, Baltimore, MD, U.S.A.). Three boys with X-linked ALD were diagnosed by the typical clinical findings and by the accumulation of VLCFA in serum sphingomyelin.

Cell culture and [1-^{14}C]*fatty acid oxidation.* Skin fibroblasts were grown in 25 cm^2 flasks containing Eagle's Minimal Essential Medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Whittaker Bioproducts, Walkersville, MD, U.S.A.). The cells were harvested with 0.05% trypsin and seeded to 8 cm^2 dishes 24 hr prior to experiments (about 2×10^5 cells per dish). The medium was changed into MEM with or without 15 μM POCA (Byk Gulden Pharmazeutika, Konstanz, F.R.G.) and the preparation preincubated for 4 hr at 37° for the binding of POCA to CPT-I. The cells were further preincubated for 1 hr using FBS-free MEM at the same concentration of POCA in order to omit the effect of fatty acids in FBS. For analysis of the inhibitory effect of POCA, 5, 15 and 50 μM POCA were added to the medium and the preparation incubated. Fatty acid oxidation reaction was initiated by adding 4 nmol [1-^{14}C]palmitate (55.5 mCi/mmol, CEA, Gif-Sur-Yvette, France) or 4 nmol [1-^{14}C]lignocerate (46.7 mCi/mmol, CEA, Gif-Sur-Yvette, France) to the freshly prepared FBS-free MEM (1 mL), with or without POCA. [1-^{14}C]Palmitate or [1-^{14}C]lignocerate dissolved in benzene was evaporated under a stream of nitrogen and dissolved in a solution containing 0.1 M Tris–HCl, pH 8.5/10 mM α-cyclodextrin [20]. The reaction was let run for 1 hr, and then the dishes were placed on ice; 0.15 mL of 10% bovine serum albumin and 0.2 mL of 3 N perchloric acid were added to the medium and the preparation was incubated for 30 min on ice. The medium was centrifuged and the unreacted fatty acid in the supernatant was removed with 5 mL of *n*-pentane, with three extractions. Radioactive degradation products such as acetate and citrate in the acid-soluble layer were counted.

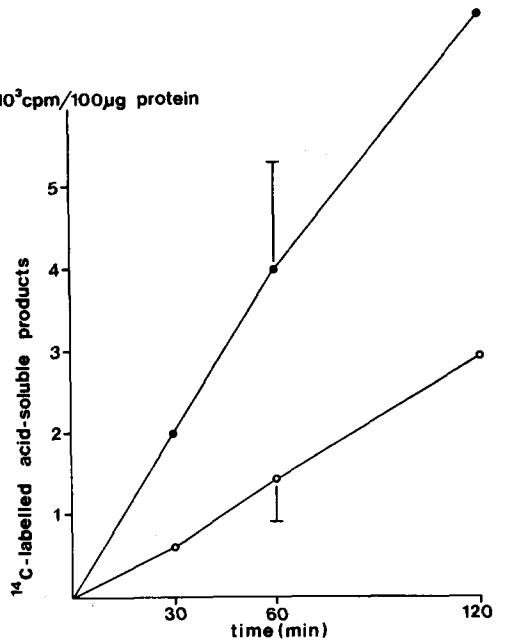

Fig. 1. Time course of [1-^{14}C]palmitate and [1-^{14}C]lignocerate oxidation in the control fibroblasts. ^{14}C-Labeled acid-soluble degradation products were increased linearly up to 2 hr. [1-^{14}C]palmitate oxidation (●) and [1-^{14}C]-lignocerate oxidation (○) activities at 60 min were 3997 ± 1496 and 1415 ± 492 cpm/hr/100 μg protein respectively. Values are means ± SD (N = 5).

RESULTS AND DISCUSSION

The time course of [1-^{14}C]palmitate and [1-^{14}C]-lignocerate oxidation in the control fibroblasts without POCA and FBS is shown in Fig. 1. ^{14}C-Labeled acid-soluble degradation products in the medium increased linearly up to 2 hr, in the case of both [1-^{14}C]palmitate and [1-^{14}C]lignocerate oxidation. The mean rate of oxidation of [1-^{14}C]-lignocerate at 1 hr in five control fibroblasts was 35% that of [1-^{14}C]palmitate.

The effects of FBS on [^{14}C]fatty acid oxidation were analyzed since FBS was considered to have an effect on the efficiency of the reaction. In the presence of 10% FBS, the oxidation of [1-^{14}C]palmitate was nearly the same as that of [1-^{14}C]lignocerate. When FBS was omitted from the medium, [1-^{14}C]palmitate and [1-^{14}C]lignocerate oxidation were increased 9.8- and 3.4-fold respectively. Palmitate in total fatty acids from FBS used in this experiment was about 30-fold greater than lignocerate, as determined by gas chromatography. As this event would result in a greater suppression of [1-^{14}C]palmitate oxidation in the presence of FBS, we did not supplement FBS during the later reactions.

To evaluate the inhibitory effect of POCA on [^{14}C]fatty acid oxidation, reactions with various concentrations of POCA were carried out, using control fibroblasts. At concentrations of 5, 15, and 50 μM POCA in the medium, [1-^{14}C]palmitate oxidation was inhibited by 15.7, 7.7, and 8.0%, respectively, of the control experiment without

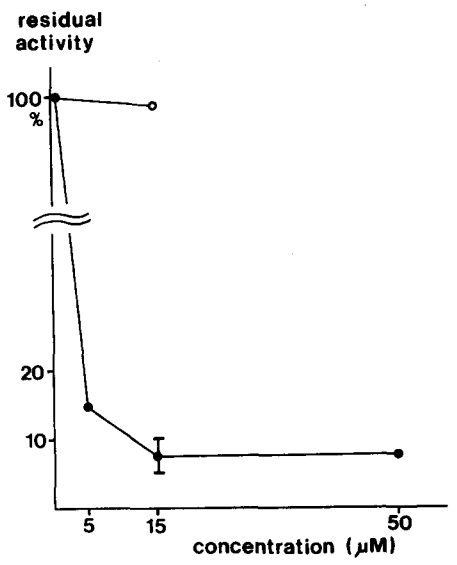

Fig. 2. Effect of POCA on [1-^{14}C]palmitate and [1-^{14}C]-lignocerate oxidation in the control fibroblasts. POCA was added to the medium at final concentrations of 5, 15 and 50 μM as described under Materials and Methods. Residual activities of [1-^{14}C]palmitate oxidation (●) and [1-^{14}C]-lignocerate oxidation (○) at 15 μM POCA were 7.7 ± 2.3 and 97.9 ± 21.7% of the control experiments without POCA respectively. Values are means ± SD (N = 5).

POCA (Fig. 2). On the other hand, [1-^{14}C]lignocerate oxidation was not inhibited by POCA. The residual activity in the palmitate oxidation with POCA was considered to be the activity of the peroxisomal β-oxidation which does not require CPT-I.

This hypothesis was supported by data obtained using fibroblasts from patients with peroxisomal disorders. In these patients, defects in the peroxisomal β-oxidation enzymes were diagnosed by the established methods: acyl-CoA oxidase (EC 1.3.99.3), bifunctional enzyme which has the activity of enoyl-CoA hydratase (EC 4.2.1.17) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) were all deficient in Zellweger syndrome [9]; bifunctional enzyme was deficient and 3-ketoacyl-CoA thiolase was present in the precursor form in neonatal ALD [10]. The reaction was carried out under conditions where FBS was not included and 15 μM POCA was added.

There was no significant difference in the [1-^{14}C]-palmitate oxidation without POCA between the controls and the patients with peroxisomal disorders (Table 1). When POCA was added to the medium, [1-^{14}C]palmitate oxidation in the control fibroblasts was suppressed to 7.7% of that seen in the experiment without POCA. In fibroblasts from the patients with Zellweger syndrome and neonatal ALD, suppression by the addition of POCA was greater than that in the control and the activity was often hardly detectable. The difference of residual activities between the control and latter two groups of patients was statistically significant as determined by Student's *t*-test ($P < 0.05$). These results suggest that the residual activities reflect the activities of peroxisomal β-oxidation.

On the other hand, residual activity of palmitate oxidation in fibroblasts from the patients with X-linked ALD was similar to that of the control and significantly higher than those of peroxisome-deficient disorders ($P < 0.05$). This finding suggests that the synthesis of palmitoyl-CoA and its oxidation in the peroxisomal system is not defective in patients with X-linked ALD. These observations support the present hypothesis that the primary etiology of X-linked ALD is a defect of lignoceroyl-CoA synthetase, an enzyme different from long chain fatty acyl-CoA (palmitoyl-CoA) synthetase [12].

[1-^{14}C]Lignocerate oxidation rates in patients with X-linked ALD, neonatal ALD and Zellweger syndrome were significantly lower than that in the control ($P < 0.05$) as described [15, 16]. The ratios of [1-^{14}C]lignocerate oxidation/[1-^{14}C]palmitate oxidation reflected the severity of the disease, though the difference was not clear. No significant difference

Table 1. Effects of 15 μM POCA on fatty acid oxidation in fibroblasts from patients with peroxisomal disorders

	[1-^{14}C]Palmitate oxidation (cpm/hr/100 μg protein)		[1-^{14}C]Lignocerate oxidation (cpm/hr/100 μg protein)	
	POCA(−)	POCA(+)	POCA(−)	POCA(+)
Control (N = 5)	3997 ± 1496	308 ± 93** (7.7%)	1415 ± 492*** (35.4%)	1399 ± 673 (35.0%)
X-linked ALD (N = 3)	2829 ± 384	328 ± 58 ** (11.6%)	386 ± 49 * (13.6%)	315 (N = 1) (11.1%)
Neonatal ALD (N = 4)	2842 ± 1121	39 ± 27 (1.4%)	160 ± 88 (5.6%)	†
Zellweger (N = 5)	3415 ± 1031	59 ± 45 (1.7%)	93 ± 70 (2.7%)	37 ± 54 (1.1%)

Values are means ± SD. Percentage of the [1-^{14}C]palmitate oxidation without POCA is indicated in parentheses under the values.

* Statistically significant by Student's *t*-test ($P < 0.05$).

† Not examined.

was observed between lignocerate oxidation with POCA and that without POCA, which suggests that lignocerate is oxidized exclusively in peroxisomes [15, 21]. Deficient lignocerate oxidation and normal palmitate and lignoceroyl-CoA [12] oxidation in X-linked ALD support our hypothesis that a specific defect of lignoceroyl-CoA synthetase is a primary etiology of X-linked ALD.

Thus, POCA-insensitive palmitate oxidation can be made use of when investigating peroxisomal fatty acid oxidation in various species of viable cells and is applicable for the prenatal and early postnatal diagnoses of peroxisomal disorders.

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