

2,4-Dienoyl-Coenzyme A Reductase Deficiency: A Possible New Disorder of Fatty Acid Oxidation

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Abstract

Several inherited disorders of fatty acid β -oxidation have been described that relate mainly to saturated precursors. This study is the first report of an enzyme defect related only to unsaturated fatty acid oxidation and provides the first in vivo evidence that fat oxidation in humans proceeds by the reductase-dependent pathway.

The patient was a black female, presenting in the neonatal period with persistent hypotonia. Biochemical studies revealed hyperlysinemia, hypocarnitinemia, normal organic acid profile, and an unusual acylcarnitine species in both urine and blood. The new metabolite was positively identified by mass spectrometry as 2-*trans*,4-*cis*-decadienoylcarnitine, derived from incomplete oxidation of linoleic acid. In spite of dietary therapy, the patient died of respiratory acidosis at four months of age. Samples of liver and muscle from the autopsy were assayed for 2,4-dienoyl-coenzyme A reductase activity. Using the substrate 2-*trans*,4-*cis*-decadienoylcoenzyme A, the reductase activity was 40% of the control value in liver and only 17% of that found in normal muscle. It is suggested that unsaturated substrates should be used for in vitro testing to cover the full range of potential β -oxidation defects and that acylcarnitine species identification be used for in vivo detection of this disorder. (*J. Clin. Invest.* 1990. 85:1703–1707.) acylcarnitine • hypotonia • reductase deficiency • tandem mass spectrometry • beta oxidation

Introduction

Several new inherited disorders of fatty acid β -oxidation have been recognized in recent years. Most notably, deficiencies of each of the three acyl-coenzyme A dehydrogenases that are coupled to electron transfer flavoprotein, the first step of β -oxidation, have been documented (1). More recently, a deficiency of NAD-dependent L-3-hydroxyacyl-coenzyme A dehydrogenase has been reported (2). This group of disorders is

of particular interest owing to the association with Reye-like episodes and sudden infant death. They are rather difficult to detect, since there is a lack of specific biochemical indicators except during clinical episodes. Usually, these disorders are recognized from abnormalities in the urine organic acid and acylcarnitine profiles or by in vitro tests showing reduced oxidation rates of saturated precursors, such as palmitic acid. Direct enzyme assays are normally required for confirmation of the enzyme deficiency.

This report constitutes the first description of a patient with a disorder of unsaturated fatty acid oxidation, verified by enzyme assay as a deficiency of 2,4-dienoyl-coenzyme A reductase.

Methods

Urine and plasma collections from the patient were stored without preservatives at -20°C before analysis. Urinary organic acids were analyzed as ethoxime-trimethylsilyl derivatives by capillary gas chromatography/mass spectrometry (GC/MS)¹ as previously described (3). Carnitine assays (free and acylated) were performed by radioenzymatic assay using the method of Brass and Hoppel (4). Acylcarnitines were identified in urine and plasma using fast atom bombardment with tandem mass spectrometry (FAB-MS/MS) using the methods described by Millington et al. (5). Acetylcarnitine and 2,4-decadienoylcarnitine were quantified by measurement of molecular ion abundances relative to internal standards using tandem mass spectrometry (5). The internal standards, acetyl-[$^2\text{H}_3$]carnitine and [$^2\text{H}_9$]octanoylcarnitine; were added to the urine before extraction. It was assumed that the molar responses of 2,4-decadienoylcarnitine and its internal standard were equal for the purpose of quantification. In selected urine samples, acylcarnitines were further characterized by capillary column GC/MS analysis of the acids liberated by mild alkaline hydrolysis from extracts purified by ion-exchange chromatography (6). The acids were converted to methyl esters using excess diazomethane, then separated on a nonpolar capillary GC column (DB-1, 30 m \times 0.25 mm i.d., 0.25- μm film thickness; J & W Scientific, Rancho Cordova, CA) using an oven temperature program from 60 $^{\circ}$ to 290 $^{\circ}\text{C}$ at 4 $^{\circ}$ /min after a 4-min initial hold. The injector temperature was 260 $^{\circ}\text{C}$.

The GC/MS analyses were performed with a VG TRIO-1 quadrupole mass spectrometer (VG Instruments, Inc., Danvers, MA) equipped with electron ionization (EI) and chemical ionization (CI) ion sources. In the EI mode, the electron energy was 70 eV, trap current 100 μA , source temperature 180 $^{\circ}\text{C}$, interface temperature 260 $^{\circ}\text{C}$, and the spectrometer was scanned cyclically from m/z 50 to

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1. Abbreviations used in this paper: CI, chemical ionization; EI, electron ionization; FAB-MS/MS, fast atom bombardment with tandem mass spectrometry; GC/MS, gas chromatography/mass spectrometry.

500 in 0.95 s with 0.15 s interscan delay. The reactant gas for CI was methane, scan conditions were the same as for EI. Fast atom bombardment-MS/MS was carried out on a VG TRIO-3 triple-quadrupole mass spectrometer and accurate mass determination of the molecular cation (m/z 326) of the new acylcarnitine was carried out by peak matching at a resolution of 8,000 in FAB-MS mode on a VG 70-S double focussing mass spectrometer (VG Analytical, Manchester, UK). The reference ion was the molecular ion of the methyl ester of octanoylcarnitine (m/z 302.2331), added to the matrix as an internal standard.

2-*trans*,4-*trans*-decadienal was obtained from Aldrich Chemical Co. (Milwaukee, WI). ICN Pharmaceuticals (Cambridge, MA) was the source of 4-*cis*-decenal. Catalase, acyl-CoA oxidase, and all standard biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation and characterization of decadienoic acid methyl esters. Aliquots of ~ 75 mg of the seed oil from the Chinese tallow tree, *Sapium sebiferum*, were applied to LK6 thin-layer plates (Whatman, Inc., Clifton, NJ). The plates were developed with hexane/ethyl ether/acetic acid 70:30:2 (vol/vol/vol) to separate triglycerides from the tetraester triglycerides (7). The tetraester triglyceride was refluxed with 5% anhydrous HCl in methanol for 1 h. Methyl esters were isolated and fractionated by silver nitrate thin layer chromatography using 10% AgNO₃ in silica gel G (wt/wt). The solvent system was hexane/ethyl ether 70:30 (vol/vol). The fraction containing methyl 2-*trans*,4-*cis*-decadienoate was then fractionated by high-performance liquid chromatography using a 9.4 mm by 25 cm Zorbax ODS column (DuPont & Co., Wilmington, DE). The column was eluted with acetonitrile/water 70:30 (vol/vol) at a flow rate of 4.7 ml/min. The column effluent was monitored at 265 nm and the appropriate compound was collected in a screw cap vial. The acetonitrile was removed under a stream of nitrogen and the methyl 2-*trans*,4-*cis*-decadienoate was recovered by extraction with diethyl ether.

Analysis of this material by GC/MS gave the following information (mass to charge ratio-followed by percent relative abundance in parentheses): 182(32)-molecular ion; 151(18); 113(33); 111(100); 81(75); 79(40); 67(47); 66(31). The NMR spectrum was consistent with the assigned structure. Two-dimensional COSY (8) experiments enabled assignment of the olefinic protons as follows: C-2: 5.88 ppm (multiplet, unresolved from C-5 proton); C-3 7.61 ppm (multiplet); C-4: 6.12 ppm (multiplet); C-5: 5.88 ppm (multiplet, unresolved from C-2 proton).

2-*trans*,4-*trans*-decadienal and 4-*cis*-decenal were oxidized with Ag₂O to 2-*trans*,4-*trans*-decadienoic acid and 4-*cis*-decenoic acid, respectively, according to the general procedure of Thomason and Kubler (9). The dienoic acid was converted to the methyl ester by reaction with excess ethereal diazomethane for 1 min at 0°C. The mass spectrum of this material by GC/MS exhibited the molecular ion (m/z 182) and the following major fragments (percent relative abundance in parentheses): 182(29); 151(18); 113(20); 111(100); 81(50); 79(28); 67(30); 66(20). The NMR spectrum was consistent with the assigned structure. Two-dimensional COSY (8) experiments enabled the olefinic protons to be assigned as follows: C-2: 5.79 ppm (doublet, $J = 15.7$ Hz); C-3: 7.27 ppm (multiplet); C-4 and C-5: 6.15 ppm (unresolved multiplet).

Preparation of substrates for enzyme assays. The CoA derivatives of 2-*trans*,4-*trans*-decadienoic acid and 4-*cis*-decenoic acid were prepared by the mixed anhydride method of Goldman and Vagelos (10). The concentrations of acyl-CoA thioesters were determined by the method of Ellman (11) after cleaving the thioester bond with 1 M hydroxylamine at pH 7. 4-*cis*-decenoyl-CoA was converted enzymatically to 2-*trans*,4-*cis*-decadienoyl-CoA in the presence of acyl-CoA oxidase and catalase as described by Yang et al. (12).

Assay of 2,4-dienoyl-CoA reductase. 2,4-dienoyl-CoA reductase was assayed spectrophotometrically at 340 nm with a recording spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, OH). The assay mixture contained 0.2 M potassium phosphate (pH 7), 0.1 mM NADPH, 15 μ M 2-*trans*,4-*trans*-decadienoyl-CoA or 15 μ M 2-*trans*,4-*cis*-decadienoyl-CoA and tissue extract to give

an absorbance change between 0.01 and 0.025 A/min. The reaction was started by the addition of decadienoyl-CoA substrate.

Preparation of a soluble tissue extract. Liver and muscle (psoas) tissue from three children who died of sudden infant death syndrome (< 6 mo of age) were used as controls for the enzyme assays. Tissues and fluids from the control cases had been evaluated with both organic acid and acylcarnitine analyses and showed no evidence of abnormal fatty acid catabolism. The control tissue samples had been maintained at -80°C for 1 yr before the analyses.

Pieces of human liver or psoas muscle were finely cut with chilled sharp scissors and placed into ice-cold STE buffer (0.25 M sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM [ethylenebis(oxyethylenitrilo)] tetraacetic acid). The minced tissue was washed several times with cold STE buffer to remove all soluble extracellular proteins. Minced liver (0.3–0.7 g) in ice-cold STE buffer (2–4 ml) was homogenized with a Potter-Elvehjem tissue grinder, whereas psoas muscle was homogenized with a Polytron homogenizer (Polytron; Brinkmann Instruments, Inc., Westbury, NY). The resultant homogenates were sonicated eight times for 7 s each with a Branson sonifier (model W-185; Branson Sonic Power Co., Danbury, CT) equipped with a microtip. The sonicated homogenates were centrifuged for 1 h at 100,000 g and the supernatants were used for assaying 2,4-dienoyl-CoA reductase. The protein concentrations of the supernatants were determined by the method of Lowry et al. (13) and found to be between 1 and 5 mg/ml.

Case report

The patient was a 2,200-g, term black female infant born to noncongenitously parents. She was hypotonic initially but by 2 d was taking formula and discharged. She was readmitted 6 h after discharge with possible sepsis. *Streptococcus pneumoniae* was cultured from her blood and treated. Feeding difficulties persisted and she remained diffusely hypotonic. A small ventricular septal defect was also noted. At 2 mo of age she was irritable, hypotonic, maintained deep tendon reflexes, and was microcephalic. Cranial ultrasound revealed ventriculomegaly with bands. The EEG was a normal wake/sleep study for age. Additional physical findings included short trunk, arms, and fingers with small feet and a large face. Karyotype was 46 xx.

She continued to be a poor feeder with poor weight gain, and occasional vomiting. Gastrostomy and Nissen fundoplication were performed but her nutritional status was not improved. Metabolic studies revealed hyperlysinemia (908 and 1,052 μ M, nl:81–213 μ M), carnitine deficiency (plasma total 16 μ M, free 10 μ M, acylcarnitines 6 μ M), normal urinary organic acids (GC/MS), but an abnormal urine acylcarnitine profile; the dominant species corresponded to decadienoylcarnitine.

Dietary changes included a low-lysine formula and, later, portagen plus carnitine supplement. Although there was some weight gain on this diet, she developed respiratory acidosis that did not respond to treatment and died at 4 mo of age. Autopsy was performed and revealed pulmonary vascular congestion and bilateral ventricular hypertrophy in addition to the multiple congenital anomalies and microcephaly described previously. Small amounts of liver and psoas muscle were obtained for biochemical studies but ultrastructural studies were not done.

Results

The key diagnostic metabolite was observed in the patient's urine using fast atom bombardment with tandem mass spectrometry, a relatively new mass spectrometric technique adapted for the analysis of acylcarnitines (5). The method detects the molecular ions of intact acylcarnitines with very high sensitivity and specificity. Analysis of the patient's urine by this technique revealed an unusual, prominent signal at mass-to-charge ratio (m/z) 326 (Fig. 1 A). The elemental composition of this ion was determined by high resolution mass spec-

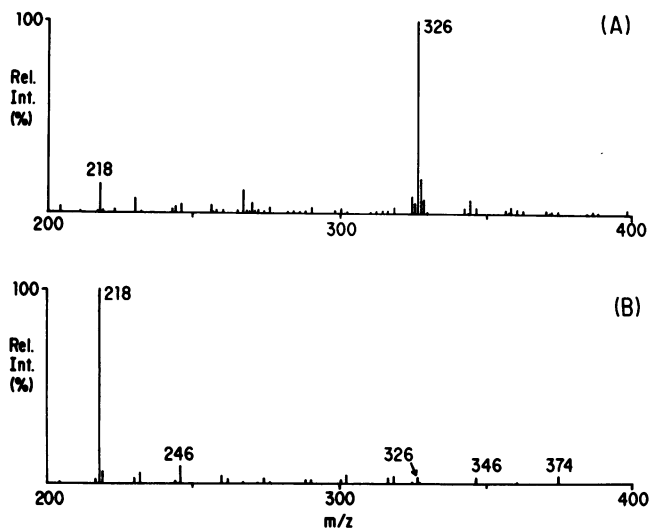


Figure 1. Urine acylcarnitine profiles from affected patients showing predominance of m/z 326, corresponding to decadienoylcarnitine (A). After the dietary change to MCT-supplement, the profile is essentially normalized (B) with the signal for acetylcarnitine (m/z 218) predominant.

trometry, which gave a measured mass of 326.2335 corresponding to $C_{18}H_{32}NO_4$ (calculated mass 326.2331). This is consistent with an acylcarnitine methyl ester having a 10-carbon doubly unsaturated acyl group ($C_{10:2}$). The m/z 326 signal was consistently detected in urine samples collected over a 6-wk period (118 nmol/mg creatinine compared with 41 nmol/mg creatinine for acetylcarnitine). Its association with dietary long-chain unsaturated fat was strongly supported by the change to a new formula, enriched with medium-chain triglyceride but with a very low unsaturated fat content, which resulted in a marked reduction of the putative $C_{10:2}$ acylcarnitine (33 nmol/mg creatinine) in favor of acetylcarnitine (2270 nmol/mg creatinine), the normal metabolite ($M^+ = m/z$ 218, Fig. 1 B). The m/z 326 signal was also consistently observed in plasma samples but was not detected in either the postmortem liver sample or the muscle samples.

Further characterization of the novel acylcarnitine was accomplished by analysis of the acids liberated by mild alkaline hydrolysis from the acylcarnitines recovered from the patient's urine by solid-phase (ion-exchange) extraction (6). The liberated acids were analyzed as methyl esters by capillary GC/MS. The most prominent component (peak 1, Fig. 2 A) exhibited a mass spectrum consistent with a $C_{10:2}$ methyl ester (Fig. 2 B). The molecular weight (m/z 182) was confirmed by repeating the GC/MS analysis under CI conditions, which produced a protonated molecular ion (m/z 183) as the base peak. The EI and CI mass spectra and retention time of this component were indistinguishable from those of an authentic sample of methyl *2-trans,4-cis*-decadienoate. The authentic *2-trans,4-trans* isomer exhibited a similar mass spectrum but it eluted later on the GC column. The chromatographic properties of the two standards are compared in Fig. 2, C and D. The presence of a peak corresponding to the *2-trans,4-trans* isomer in both the patient's chromatogram (peak 2, Fig. 2 A) and in the *2-trans,4-cis*-isomer (Fig. 2 C) might result from partial rearrangement to the more thermodynamically stable isomer. The identity and purity of the reference materials were checked by

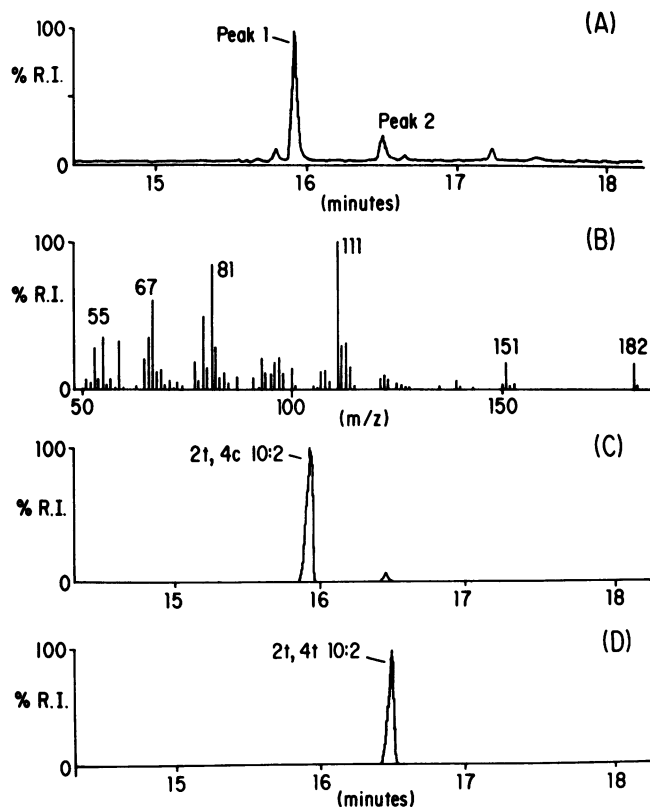


Figure 2. Characterization of the methylated decadienoic acids liberated from corresponding acylcarnitines by hydrolysis of patient urine. Partial GC/MS profile (A) shows a major component (peak 1), the EI spectrum of which (B) is identical with that of *2-trans,4-cis*-decadienoic acid methyl ester. The GC/MS profiles for this standard (C) and the *2-trans,4-trans* isomer (D) confirm the identities of peaks 1 and 2 in A.

nuclear magnetic resonance (NMR) spectroscopy in addition to GC/MS (see Methods).

Since *2-trans,4-trans*-decadienoyl-CoA was shown to be a good substrate of NADPH-dependent *2,4*-dienoyl-CoA reductase (14), also referred to as *4-enoyl*-CoA reductase (EC 1.3.1.34) and since it can be synthesized more easily than its

Table 1. *2,4*-Dienoyl-CoA Reductase Activity in Liver and Psoas Muscle of a Patient with a Suspected Deficiency in the Oxidation of Polyunsaturated Fatty Acids

Tissue and source	2,4-Dienoyl-CoA reductase activity	
	2t,4c*	2t,4t†
	nmol/min/mg of protein	
Liver, control	13±2.6 (100%)	8.4±2.2 (100%)
Liver, patient	5.1 (40%)	5.5 (65%)
Muscle, control	4.6±0.8 (100%)	3±0.28 (100%)
Muscle, patient	0.8 (17%)	1.24 (43%)

Control values are means±SD obtained with three different samples. For experimental details see Methods.

* Reductase activity with *2-trans,4-cis*-decadienoyl-CoA as substrate.

† Reductase activity with *2-trans,4-trans*-decadienoyl-CoA as substrate.

2-*trans*,4-*cis*-isomer, it was used to assay the reductase. Although the reductase activities in the patient's liver and muscle were lower than in control tissues, the residual activity in the patient was relatively high with this substrate (see Table I). Since the fatty acid metabolite identified in the urine of the patient was 2-*trans*,4-*cis*-decadienoylcarnitine, 2-*trans*,4-*cis*-decadienoyl-CoA was prepared and used for assaying the reductase. The residual reductase activity in the patient's muscle with the 2-*trans*,4-*cis* substrate was only 17% of the activity found in control muscle in contrast to liver where the patient's activity was 40% of the control (Table I).

Since the patient was hyperlysinemic, the effect of lysine on the activity of 2,4-dienoyl-CoA reductase was evaluated. The presence of lysine in the reductase assay resulted in only a slight inhibition ($\leq 20\%$) which remained virtually unchanged when the lysine concentration was increased from 1 to 10 mM.

Discussion

Recent studies of fatty acid oxidation have led to a revision of the pathway by which polyunsaturated fatty acids are degraded (for a review, see reference 15 and Fig. 3). It is by now generally accepted that β -oxidation of unsaturated fatty acids with *cis* double bonds extending from even-numbered carbon atoms results in the formation of 2-*trans*,4-*cis*-dienoyl-CoA intermediates, which in mammals are reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-enoyl-CoA thioesters. The latter compounds, after their isomerization to 2-enoyl-CoA's, can be completely degraded via the well known β -oxidation cycle. According to the revised pathway, β -oxidation of linoleic acid yields 2-*trans*,4-*cis*-decadienoyl-CoA as an intermediate that, in the presence of NADPH, can be reduced by 2,4-dienoyl-CoA reductase and then further degraded by β -oxidation. If 2,4-dienoyl-CoA reductase is completely or partially missing, 2-*trans*,4-*cis*-decadienoyl-CoA would be expected to accumulate in mitochondria and be excreted as 2-*trans*,4-*cis*-decadienoylcarnitine, as has been observed in the patient presented here.

It should be noted that 2,4-dienoyl-CoA reductase activities are present in mitochondria as well as in peroxisomes (16) and that the reductase assay does not distinguish between the peroxisomal and mitochondrial activities. However, in the absence of conditions that promote proliferation of peroxisomes and the induction of peroxisomal β -oxidation enzymes, the measured reductase activity, especially in muscle, is mostly due to the mitochondrial enzyme (16). In fact, the residual reductase activity detected in the patient may reflect the part of the activity associated with peroxisomes.

A deficiency of 2,4-dienoyl-CoA reductase in *Escherichia coli* rendered the organism unable to grow on petroselinic acid (6-*cis*-octadecenoic acid) without affecting growth on oleic acid (17). Thus, in *E. coli* 2,4-dienoyl-CoA reductase is required for the *in vivo* degradation of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms. This study leads to the conclusion that the degradation of linoleic acid in humans also requires the involvement of 2,4-dienoyl-CoA reductase. Hence, the β -oxidation of linoleic acid *in vivo* proceeds by the pathway presented in Fig. 3. This case represents the first *in vivo* evidence that fatty acid oxidation in humans proceeds by the reductase-dependent pathway (15).

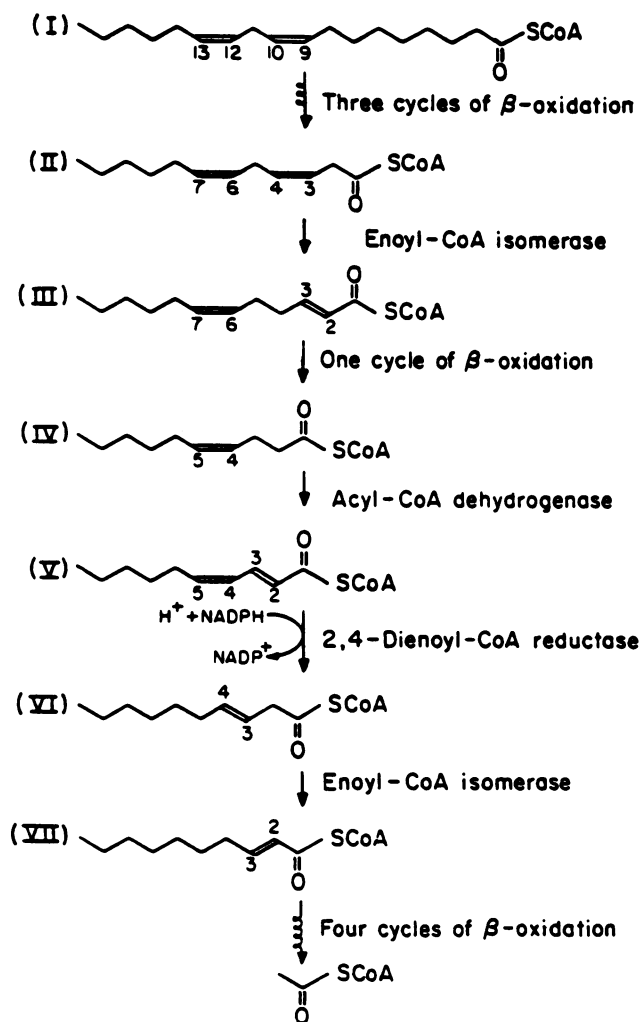


Figure 3. Summarized is the β -oxidation of linoleic acid which after its activation to linoleoyl-CoA (I) can be chain-shortened by three cycles of β -oxidation to 3-*cis*,6-*cis*-dodecadienoyl-CoA (II). Isomerization of the 3-*cis* to a 2-*trans* double bond by *cis*- Δ^3 -*trans*- Δ^2 -enoyl-CoA isomerase yields 2-*trans*,6-*cis*-dodecadienoyl-CoA (III), which can pass once more through the β -oxidation cycle. The 4-*cis*-decenoyl-CoA (IV) is converted by medium-chain acyl-CoA dehydrogenase to 2-*trans*,4-*cis*-decadienoyl-CoA (V), which is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-*trans*-decenoyl-CoA (VI). Isomerization of the 3-*trans* isomer to 2-*trans*-decenoyl-CoA (VII) facilitates its subsequent complete degradation to 5 mol of acetyl-CoA.

As with other inherited disorders of and fatty acid oxidation, this case manifested a secondary carnitine deficiency. Unlike these other disorders, however, the organic acid analysis by GC/MS provided no clues to the presence of a metabolic abnormality. Recognition of the 2,4-dienoyl-CoA reductase deficiency was based solely upon the detection of the 2-*trans*,4-*cis*-decadienoylcarnitine species by tandem mass spectrometry. Identification of unusual acylcarnitine species in urine and blood, therefore, is a valuable tool for detection of disorders involving both saturated and unsaturated fatty acid oxidation.

Disorders involving unsaturated fatty acids would not be recognized easily *in vivo* or *in vitro*. Traditionally, the integrity

of mitochondrial β -oxidation is tested by using palmitate or other saturated species as substrate. These compounds would not require either 2,4-dienoyl-CoA reductase or the 3-enoyl-CoA to 2-enoyl-CoA isomerase. The use of unsaturated substrates for in vitro testing is therefore indicated, to cover the full range of possible enzyme defects.

Following supplementation with L-carnitine and changing the dietary fat to mainly medium-chain triglyceride, the abnormal carnitine species was no longer readily detectable. Medium chain triglyceride oil does not contain significant quantities of fatty acids that would require the 2,4-dienoyl-CoA reductase. Serial studies of plasma and urine following the change in diet and postmortem studies of tissue failed to show the decadienoylcarnitine. There was no biochemical or histologic evidence to suggest significantly altered metabolism.

The association of the hyperlysinemia in this patient with the reductase deficiency is not clear since decadienoylcarnitine is not excreted by other patients with hyperlysinemia and lysine is not a significant inhibitor of the reductase in vitro. The effects of the reductase deficiency on the metabolism of other unsaturated acids, such as prostaglandins and leukotrienes, is unknown but worthy of investigation.

Although this is the first case described, it suggests that this deficiency should be sought in hypotonic infants and attempts at therapy initiated early.

Acknowledgments

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