Analysis of Fatty Acid Oxidation Intermediates in Cultured Fibroblasts to Detect Mitochondrial Oxidation Disorders

Morteza Pourfarzam,1,2,3 Jochen Schaefer,2 Douglass M. Turnbull,2 and Kim Bartlett1

We describe a method for the diagnosis of mitochondrial fatty acid oxidation disorders that is based on the analysis of acylcarnitine and acyl-coenzyme A (acyl-CoA) esters generated during fatty acid oxidation by permeabilized skin fibroblasts. This method requires only small amounts of cultured fibroblasts with minimal preparation, and no isolation of mitochondrial fractions is necessary. During oxidation of [U-14C]hexadecanoate, normal human fibroblasts produce a characteristic pattern of acylcarnitine and acyl-CoA ester intermediates. Incubations of fibroblasts from patients with fatty acid oxidation defects show a completely different pattern of intermediates, and in each case the observed profile reflects the site of the defect. The diagnosis and likely site of a mitochondrial fatty acid oxidation defect can be made readily from two 80-cm2 culture flasks of fibroblasts with this technique.

Indexing Terms: inborn errors of metabolism/acylcarnitines/acyl-coenzyme A esters/enzyme activity/chromatography, reversed-phase/radioassay

In humans, long-chain fatty acids constitute a major source of energy, especially for skeletal muscle, heart, and liver. In skeletal muscle, fatty acid oxidation is the major source of energy both in the resting state (1) and during prolonged exercise. In the myocardium, long-chain fatty acids are the preferred substrate in the resting state (2). In the liver, during fasting, fatty acid oxidation produces ketone bodies, which are oxidized by extrahepatic tissues (3), thereby sparing glucose for those tissues such as brain and erythrocytes, which have obligatory requirements for glucose as a metabolic fuel. Fatty acids are predominately metabolized in mitochondria by β-oxidation. Mitochondrial β-oxidation of long-chain fatty acids involves activation to their corresponding acyl-coenzyme A (acyl-CoA) esters, which are transported into the mitochondrial matrix by the concerted action of carnitine palmitoyltransferase I (CPT I), carnitine–acylcarnitine translocase, and carnitine palmitoyltransferase II (CPT II).4 Fatty acid oxidation then proceeds by a repeated sequence of flavoprotein-linked dehydrogenation, hydration, NAD+-linked dehydrogenation, and thiolyis to generate acetyl-CoA. There are two or more enzymes with overlapping chain-length specificities for each of the reactions of β-oxidation. Thus, there are thought to be four acyl-CoA dehydrogenases (short-chain, medium-chain, long-chain, and very-long-chain) (4) and a trifunctional enzyme catalyzing the 3-hydroxyacyl-CoA dehydrogenation, 2-enoyl-CoA hydration, and 3-oxoacyl-CoA thiolysis of long-chain acyl-CoA esters (5, 6). In addition, there are a short-chain-specific enoyl-CoA hydratase, a short-chain 3-hydroxyacyl-CoA dehydrogenase, and two 3-oxoacyl-CoA thiolases (acetoacetyl-CoA-specific and general 3-oxoacyl-CoA thiolase) (7).

In recent years an increasing number of patients with inherited disorders of mitochondrial fatty acid oxidation have been described. In many of these disorders the underlying defect becomes clinically apparent only during periods of fasting, illness, or other metabolic stresses. The clinical features in these patients include hypoketotic hypoglycemic coma, encephalopathy, cardiomyopathy, myopathy, Reye-like episodes, and sudden infant death syndrome. These disorders include abnormalities of all acyl-CoA dehydrogenases (8–10), electron transfer flavoprotein, electron transfer flavoprotein:ubiquinone oxidoreductase (11), trifunctional enzyme (12, 13), CPT I, CPT II (14–16), carnitine–acylcarnitine translocase (17), and primary carnitine deficiency (18). The overall incidence of fatty acid oxidation disorders is unknown but initial studies suggest they are one of the most frequent groups of inborn errors of metabolism. The incidence of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency alone is estimated to be 1 in 6000 to 1 in 20 000 in the UK (19, 20). Since most patients can be treated by diet alone, the accurate and early diagnosis of fatty acid oxidation disorders is of major importance, and a rapid and reliable diagnostic technique applicable to neonatal screening is highly desirable.

Several diagnostic methods for β-oxidation defects are available but many are extremely time consuming and may not detect all possible defects. The pathological accumulation of acylcarnitine and acyl-CoA esters generated in vitro by isolated mitochondria is potentially valuable in the investigation of β-oxidation defects (21). We have shown that mitochondria isolated from a variety of tissues from patients with β-oxidation defects show a specific pattern of intermediates that is pathognomonic for the site of enzyme deficiency (10, 13, 22). The drawback of this method, however, is that it requires either culture of large amounts of fibroblasts or a tissue biopsy. Here we describe a method involving a crude whole-cell preparation as the enzyme source.
which eliminates the need for biopsy or isolation of mitochondria and also minimizes the amount of tissue required.

Materials and Methods

Chemicals

Fatty acids, fatty acid chlorides, acetic anhydride, butyric anhydride, thionyl chloride, and oxalyl chloride were obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK). trans-2,3-Hexadecenoic acid and trans-2,3-tetradecenoic acid were supplied by ICM Biomedicals (Thame, Oxfordshire, UK). trans-2,3-Decenoic, -octenoic, and -hexenoic acids were supplied by Lancaster Synthesis (Morecambe, Lancashire, UK). Acyl-CoA oxidase and ATP were purchased from Boehringer Mannheim (Mannheim, Germany). CoA (lithium salt), L-carnitine hydrochloride, cytochrome c, histone type II-AS, digitonin, saponin, and bovine serum albumin (fatty acid-free) were supplied by Sigma Chemical Co. (Poole, Dorset, UK). DEAE-Sepharcl was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). p-Bromophenacyl-8 was obtained from Pierce (Rockford, IL). Dowex (50W-X8, 200-400 mesh) was purchased from Bio-Rad Lab. (Richmond, CA) and converted to pyridinium form by treating with 500 mL/L pyridine in water. HPLC solvents were from Rathburn Chemicals (Walkerton, UK). Culture media were all obtained from Gibco (Life Technology, Paisley, UK). All other chemicals utilized were of the highest purity available.

[U-14C]Hexadecanoic acid (31.0 TBq/mol) was obtained from Amersham International (Bucks, UK).

Synthesis of Acyl-CoA Esters

Acetyl-CoA and butyryl-CoA were prepared by using their respective acid anhydrides as the acylating reagent (23). Acyl-CoA esters with even-numbered carbon chains (C₆-C₁₆) and heptadecanoyl-CoA were prepared from their corresponding acid chlorides (24). trans-2-Enoyl-CoA esters (C₆-C₁₆) were prepared enzymically from saturated acyl-CoA by using acyl-CoA oxidase. 3-Hydroxyacyl-CoA esters (C₆-C₁₆) were also synthesized enzymically by treating the 2-enzyme-CoA esters with crotonase and purifying by semipreparative HPLC (25). 2-Ynoic acids (C₆-C₁₆), synthesized by the method of Wood and Lee (26), were used to prepare the corresponding CoA esters from the mixed anhydrides (27). These were converted to 3-oxoacyl-CoA esters with crotonase (28).

Synthesis of Acylcarnitine Esters

Saturated acylcarnitines with even-numbered carbon chains (C₆-C₁₆) and undecanoylcarnitine were synthesized by treating the corresponding acylchlorides with L-carnitine hydrochloride in trifluoroacetic acid (29). trans-2,3-Enoic acids of chain lengths C₂₂, C₂₄, C₂₆, C₂₈, and C₃₀ were converted to the acid chlorides and were used to prepare the corresponding acylcarnitine esters as described above. 3-Oxohexadecanoic and 3-oxotetradecanoic acids were synthesized as described (30) and were similarly converted to the respective acylcarnitines. 3-Hydroxyhexadecanoylcarnitine and 3-hydroxytetradecanoylcarnitine were prepared from the corresponding 3-oxoacylcarnitines by treatment with sodium borohydride (31).

The identity of all the acyl-CoA and acylcarnitine esters was confirmed by fast-atom bombardment mass spectrometry and their HPLC retention times were determined (Pourfarzam et al., in preparation).

Patients' Cells

Cell lines were obtained from normal subjects (volunteers or patients who had no evidence of metabolic disorders during the course of routine investigations) and patients with deficiencies of CPT I, CPT II, carnitine-acyl carnitine translocase, very-long-chain acyl-CoA dehydrogenase (VLCAD), long-chain 3-hydroxyacyl-CoA dehydrogenase component of trifunctional enzyme, and MCAD. The diagnosis of fatty acid oxidation defect was established by direct enzyme assay (10, 12, 32, and Ogier et al., ms. in preparation) or by the presence of common mutations for MCAD deficiency.

Skin fibroblasts were cultured to confluency in minimum essential medium (MEM) containing Earle's salts, supplemented with 100 mL/L fetal bovine serum, 0.1 g/L streptomycin, 100 kIU/L penicillin, 2 mmol/L glucose, MEM nonessential amino acids, and MEM vitamins. All cell lines were shown to be free of mycoplasma.

Preparation of Fibroblasts

Fibroblasts from two 80-cm² flasks were harvested by trypsinization, washed twice with 10 mL of phosphate-buffered saline, and resuspended in 0.7 mL of medium containing 250 mmol/L sucrose, 3 mmol/L EDTA, and 20 mmol/L HEPES, pH 7.4, in a 1.5-mL microcentrifuge tube. The cell suspension was centrifuged (avg. 12 000g) for 1 min; the pellet was then resuspended in 0.5 mL of incubation medium and recentrifuged (avg. 12 000g, 45 s). Finally the pellet was resuspended in 1.0 mL of incubation medium and used directly for the measurement of β-oxidation flux and intermediates.

Protein concentration and activities of citrate synthase and lactate dehydrogenase were determined according to standard procedures (33–35).

Incubation with [U-14C]Hexadecanoate

Incubations were made in 1 mL of medium containing 110 mmol/L KCl, 5 mmol/L potassium phosphate, 1 mmol/L (ethylenbis(oxyethylenenitrilo)tetraacetic acid (EGTA), 5 mmol/L ATP, 5 mmol/L MgCl₂, 0.2 g/L cytochrome c, 0.1 mmol/L CoA, 1 mmol/L L-carnitine, 10 mmol/L HEPES (pH 7.4), and fibroblasts from two 80-cm² flasks at 35°C in a shaking water bath (120 strokes/min). After a 5-min preincubation, the reaction was started by addition of 50 μL of substrate (73 nmol of [U-14C]hexadecanoate, specific radioactivity 50 Ci/mol) complexed to albumin in a 5:1 molar ratio. For the flux measurement, 25-μL aliquots of the incubation mixture were removed after 0, 20, 40, and 60 min of incubation and quenched with 25 μL of acetic acid. The acid-soluble
Radioactivity was then measured as described previously (36). For determination of intermediates, the remainder of the incubation mixture was quenched after 60 min by the addition of 100 μL of 1 mol/L H₂SO₄.

Radio-HPLC Analysis of Acyl-CoA Esters

Acyl-CoA esters were extracted from the fibroblast incubations and analyzed by radio-HPLC as described previously (25) with the following modifications: Hep-tadecanoyl-CoA was used as internal standard, HPLC analysis was performed by using a Hypersil 5-μm RPC₁₈ column [250 × 4.6 mm (i.d.); Shandon HPLC, Cheshire, UK], and acyl-CoA esters were resolved by a binary gradient of acetonitrile in 50 mmol/L phosphate (pH 5.3) as follows: isocratic 50 mL/L acetonitrile (5 min); isocratic 100 mL/L acetonitrile (0.1 min); linear gradient to 300 mL/L acetonitrile (9.9 min); linear gradient to 500 mL/L acetonitrile (30 min); isocratic 500 mL/L acetonitrile (5 min); and linear gradient to 50 mL/L acetonitrile (5 min). The flow rate was 1.7 mL/min and the total run time 55 min.

Radio-HPLC Analysis of Acylcarnitine Esters

Acylcarnitines were extracted as described previously (25) except that undecanoylcarnitine was used as internal standard. The acylcarnitines were analyzed by radio-HPLC, as their 4-bromophenacyl derivatives, by using a Hypersil 5-μm RPC₁₈ column [250 × 4.6 mm (i.d.)]. The following ternary gradient of acetonitrile (A), water (B), and 150 mmol/L triethylamine phosphate, pH 5.6 (C), was used for separation: isocratic 600 mL/L A, 380 mL/L B, 20 mL/L C (3 min); linear gradient to 800 mL/L A, 180 mL/L B, 20 mL/L C (9 min); linear gradient to 920 mL/L A, 80 mL/L C (18 min); isocratic 920 mL/L A, 80 mL/L C (10 min); linear gradient to starting condition (5 min). The flow rate was 1.3 mL/min and the total run time 45 min. Radioactivity associated with eluted compounds was detected on-line as described (25, 37) except that the scintillation fluid was methanol:Ecosint A (National Diagnostics, Atlanta, GA) (1:2 by vol).

The identity of each acyl-CoA and acylcarnitine ester generated during the fibroblast incubations was determined by its relative retention time compared with those of standard compounds. Quantification was based on the integrated radioactive peaks (37) after correction for recovery with appropriate internal standard.

Results

Preparation of Permeabilized Fibroblasts

In preliminary experiments we investigated whether intact cultured human skin fibroblasts were suitable for studies on mitochondrial fatty acid oxidation. Although intact cells were able to oxidize [U-¹⁴C]hexadecanoate, as measured by the release of ¹⁴CO₂ or acid-soluble radioactivity, no intermediates could be detected when these incubations were analyzed for U-¹⁴C-labeled acyl-CoA or acylcarnitine esters by radio-HPLC. This was the case for cell lines from both normal individuals and patients with proven defects of mitochondrial fatty acid oxidation. It was, therefore, necessary to permeabilize the plasma membrane to allow greater access to substrate and L-carnitine. This can promote the formation of acylcarnitine esters and allow distribution of these intermediates throughout the whole incubation medium. Among the methods evaluated to selectively permeabilize the plasma membrane while maintaining intact mitochondria were hand homogenization, centrifugation, and the use of permeabilizing reagents such as digitonin (38), saponin (39), and histone II-AS. The degree of permeabilization was assessed by monitoring the release of marker enzymes from the cytosol (lactate dehydrogenase) and mitochondrial matrix (citrate synthase). Each cell preparation was also incubated with [U-¹⁴C]hexadecanoate and acyl-CoA, and the acylcarnitine ester intermediates generated were analyzed to assess which preparation produced the same pattern of intermediates as seen in isolated intact mitochondria from fibroblasts (22). Two preparations proved suitable: histone-treated fibroblasts (1.5 g/L histone II-AS for 1 min), and those disrupted by mechanical forces during centrifugation (12 000g for 60 s, followed by 12 000g for 45 s). All subsequent experiments were carried out in duplicate with the above two preparations. No differences were observed between the two sets of experiments, and the data on fibroblasts prepared by using the latter procedure are presented here because of the simplicity of the preparation. The percentage recovery of citrate synthase measured in four different preparations ranged from 94% to 98%, indicating that most of the mitochondria remain intact, whereas 60% to 65% of lactate dehydrogenase was released into the supernate.

Reproducibility of Incubations with Permeabilized Fibroblasts

The reproducibility of the incubations with permeabilized fibroblasts was examined by preparing a permeabilized preparation of fibroblasts on three separate occasions from the same cell line. Incubations with [U-¹⁴C]hexadecanoate were performed, the β-oxidation rate was measured, and each acyl-CoA and acylcarnitine ester was quantitated. The rates of flux through β-oxidation, as measured by the production of acid-soluble metabolites, were 33.5, 31.6, and 29.3 for a control cell line and 20.7, 20.0, and 18.8 (μmol/g protein per hour) for a trifunctional enzyme-deficient cell line in three experiments. The analysis of acyl-CoA and acylcarnitine intermediates from these incubations revealed a CV of 10–20% for each ester. We also measured the contribution of peroxisomal β-oxidation in these incubations. The data presented in Fig. 1 show the time course of oxidation of [U-¹⁴C]hexadecanoate by permeabilized fibroblasts as measured by the production of acid-soluble radioactivity. The oxidation of substrate was carnitine dependent and was inhibited by respiratory-chain poisons and by etomoxir [R,S-2-(6-[4-chlorophenoxyl]-hexyl)oxirane-2-carboxylate], an inhibitor of CPT I. The contribution of peroxisomes to the oxidation of [U-¹⁴C]hexadecanoate under our experimental conditions is minimal, since peroxisomal activity is both carnitine independent and independent of the respiratory chain. The observed pattern of intermediates, therefore, represents only the mitochondrial pathway.
This is in agreement with our previous findings with mitochondria isolated from control fibroblasts (22) and human skeletal muscle mitochondria (10, 13), in which only saturated acyl-CoA and acylcarnitine ester intermediates were detected. To determine whether the observed pattern of intermediates was consistent during the course of β-oxidation, while the substrate concentration was not rate limiting, we carried out a time-course incubation and analyzed the acyl-CoA and acylcarnitine esters generated. These results are illustrated in Fig. 4 for acylcarnitines and show a consistent pattern of intermediates during 2 h of incubation. Similar results were obtained for acyl-CoA esters (results not shown). Thus, the characteristic pattern of intermediates is achieved rapidly and is maintained for up to 2 h under experimental conditions described here. An incubation time of 60 min was chosen and used throughout the rest of the study.

Flux and Intermediates in Fibroblasts from Patients with Fatty Acid Oxidation Disorders

Trifunctional enzyme deficiency. In fibroblasts from patients with trifunctional enzyme deficiency the rate of fatty acid oxidation was 48% of the mean control values (Table 1). The analysis of acyl-CoA intermediates showed a profile very different from control fibroblasts. There is accumulation of hexadec-2-enoyl-CoA and 3-hydroxyhexadecanoyl-CoA, which are intermediates not seen in incubations with normal fibroblasts. The concentrations of tetradecanoyl-CoA and dodecanoyl-CoA are decreased, and no decanoyl-CoA or octanoyl-CoA was detected (Fig. 2D). Analysis of the acylcarnitine esters showed a similar but more pronounced pattern, with the accumulation of 3-hydroxytetradecanoylcarnitine, 3-hydroxyhexadecanoylcarnitine, tetradec-2-enoylcarnitine, and hexadec-2-enoylcarnitine, and decreased concentrations of chain-shortened products, acetyl carnitine, octanoylcarnitine, and decanoylcarnitine (Table 2; Fig. 3D). The pattern and amount of acyl-CoA and acylcarnitine esters generated in the cell lines from six patients with trifunctional enzyme deficiency were very similar (Table 2) despite evidence for biochemical and molecular heterogeneity in these patients (Jackson et al., ms. in preparation).

MCAD deficiency. The production of acid-soluble metabolites from the oxidation of [U-¹⁴C]hexadecanoate in cell lines from patients with MCAD deficiency was 90% of mean control values. This apparently normal rate is due to the overlapping chain-length specificities of the acyl-CoA dehydrogenases and the fact that medium-chain acyl-CoA and acylcarnitine esters are acid soluble. In all cell lines, the concentration of hexadecanoyl-CoA was normal. The concentrations of tetradecanoyl-CoA and dodecanoyl-CoA were decreased, whereas those of decanoyl-CoA and octanoyl-CoA were increased (Fig. 2B). A similar pattern was seen for the acylcarnitine esters: the concentrations of decanoylcarnitine and octanoylcarnitine were highly increased; those of dodecanoylcarnitine, tetradecanoylcarnitine, and hexadecanoylcarnitine were normal; and hexanoylcarnitine and butyrylcarnitine were not detected (Table 2; Fig. 3B). In these fibroblasts we observed slightly

Table 1. Formation of ¹⁴C-labeled acid-soluble products from [U-¹⁴C]hexadecanoate by cultured skin fibroblasts.

<table>
<thead>
<tr>
<th>Cell-line deficiencies</th>
<th>n</th>
<th>Per g of protein</th>
<th>Per kU of citrate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>37.7 ± 8.0</td>
<td>1080.6 ± 230.0</td>
</tr>
<tr>
<td>TFE</td>
<td>6</td>
<td>18.1 ± 4.8</td>
<td>489.0 ± 144.0</td>
</tr>
<tr>
<td>MCAD</td>
<td>4</td>
<td>34.0 ± 11.0</td>
<td>1058.0 ± 415.0</td>
</tr>
<tr>
<td>VLCAD</td>
<td>3</td>
<td>6.91 ± 4.2</td>
<td>204.5 ± 109.0</td>
</tr>
<tr>
<td>CPT II</td>
<td>2</td>
<td>3.49</td>
<td>891.5</td>
</tr>
<tr>
<td>CPT I</td>
<td>1</td>
<td>19.9</td>
<td>469.7</td>
</tr>
<tr>
<td>Translocase</td>
<td>1</td>
<td>6.67</td>
<td>182.0</td>
</tr>
</tbody>
</table>

TFE, trifunctional enzyme.
higher concentrations of C₁₀ intermediates than C₈-intermediates, whereas the predominant abnormal metabolites in patients with MCAD deficiency are C₈ products. The reason for this difference is not known, although possible explanations include further chain-shortening of C₁₀ fatty acids in peroxisomes or incorporation into lipids.

**VLCAD deficiency.** The rate of [U-¹⁴C]hexadecanoate oxidation in fibroblasts from patients with VLCAD deficiency was ~18% of mean control values (Table 1). The pattern of acyl-CoA and acylcarnitine intermediates was also different from that of controls. The only intermediates generated were hexadecanoyl-CoA, tetradecanoyl-CoA,
Fig. 3. Radio-HPLC chromatogram of acylcarnitine esters generated from the incubation of fibroblasts with [U-14C]hexadecanoate. A, control; B, MCAD deficiency; C, VLCAD deficiency; D, trifunctional enzyme deficiency; E, CPT II deficiency; F, CPT I deficiency. Conditions of incubation and peak identification are as indicated in Fig. 2. Only clearly identified peaks with appropriate retention times (Rₜ) for acylcarnitine esters are marked.

and their corresponding carnitine esters. No chain-shortened acyl-CoA or acylcarnitine esters shorter than C₁₄ were detected, and the generation of acetylcarnitine was significantly reduced (Table 2; Figs. 2C and 3C).

CPT II deficiency. Acid-soluble metabolites formed during the oxidation of [U-14C]hexadecanoate in the two cell lines with CPT II deficiency were surprisingly normal under the conditions used (Table 1). This apparently normal rate is presumably because the sum of the accumulated acid-soluble chain-shortened intermediates (i.e., C₂–C₁₀) is similar to that of controls (Table 2; Fig. 3E). In both cell lines hexadecanoyl-CoA was the only detectable acyl-CoA ester and its concentration was normal (patients, 2.1 and 2.6 µmol/g protein per hour; controls, 2.64 ± 0.64, mean ± SD, n = 6; Fig. 2E). The concentration of hexadecanoylcarnitine is considerably increased, and tetradecanoylcarnitine, dodecanoylcarnitine, and decanoylcarnitine are not detected, whereas the concentrations of short-chain acylcarnitines (i.e., C₄–C₈) and particularly acetylcarnitine are increased (Table 2, Fig. 3E). The finding of high concentrations of acetylcarnitine in the incubations of these cell lines is surprising and presumably results from inhibition of the further oxidation of acetylcarnitine.

CPT I deficiency. Flux through β-oxidation was im-
paired, and the rate of formation of acid-soluble metabolites from [U-14C]hexadecanoate was 53% of mean control values (Table 1). The analysis of intermediates from this cell line showed a characteristic pattern: Hexadecanoyl-CoA was the only acyl-CoA detected and its concentration was highly increased (patient, 7.6 μmol/g protein per hour; controls, 2.64 ± 0.64 mean ± SD, n = 6; Fig. 2F), whereas hexadecanoylcarnitine was present in very small amounts (20% of mean control values; Table 2). Acylcarnitine production was reduced and tetradecanoylcarnitine and dodecanoylcarnitine were not detectable, whereas acylcarnitine esters with chain-lengths C12–C10 were present (Table 2; Fig. 3F).

Carnitine–acylcarnitine translocase deficiency. Flux through β-oxidation was impaired and the rate of formation of acid-soluble metabolites from [U-14C]hexadecanoate was 17% of mean control values (Table 1). The concentration of hexadecanoyl-CoA was slightly reduced in the incubation of this cell line (patient, 2.02 μmol/g protein per hour; controls, 3.04 ± 0.64, mean ± SD, n = 6) and no acyl-CoA ester shorter than C18 was detected. The concentration of hexadecanoylcarnitine, however, was grossly increased, whereas those of tetradecanoylcarnitine and dodecanoylcarnitine were reduced. No acylcarnitine shorter than C10 was detectable, and acetylcarnitine concentration was low (Table 2). However, small amounts of 3-hexadecenoylcarnitine and 3-hydroxyhexadecanoylcarnitine were present, although the site of formation of these intermediates is uncertain.

Discussion

The diagnosis of disorders of mitochondrial fatty acid oxidation remains difficult. In fibroblasts, assessment of the pathway by measuring the release of CO2 or acid-soluble radioactivity from U-14C-labeled fatty acids (40–42) or detritiation of 9,10-3H-labeled fatty acids (43, 44) may detect the presence or absence of a defect, but does not discriminate between abnormalities of the different enzymes. The identification of abnormal organic acids, acylcarnitines, and acylglycines in blood, urine, or tissue samples is diagnostic but these substances are often detected only when the patients are metabolically stressed (45). Recent developments in mass-spectrometric techniques have enabled the detection and quantitative analysis of individual acylcarnitines with very low detection limits (46 and literature cited therein), and this technique appears to be an effective means for whole-population neonatal screening. However, the analysis of body-fluid acylcarnitines is applicable only to disorders that are characterized by the consistent presence of pathognomonic metabolites, and its validity to differentiate between all the known disorders of mitochondrial β-oxidation is not documented. Furthermore, even very accurate and sensitive analysis of acylcarnitines in body fluids may not always detect a defect of fatty acid oxidation if the patient is well (47). Because of the intermittent presence of abnormal metabolites, provocation tests such as fasting (21), medium-chain triglyceride loading (48), and phenylpropionic acid loading tests (49) have been proposed to precipitate the excretion of characteristic metabolites, but they are potentially dangerous and the medium-chain triglyceride-loading test is particularly hazardous in patients with MCAD deficiency. More recently, DNA studies have been used in the diagnosis of MCAD deficiency (50), and one can use this method for the diagnosis of defects in which the site of the molecular lesion is known. However, this technique is currently limited to MCAD deficiency.

An inborn error of metabolism can be identified unambiguously by direct measurement of the appropriate enzyme in cultured skin fibroblasts or tissue biopsy. If, as is frequently the case for inherited disorders of mitochondrial β-oxidation, there is not sufficient clinical or biochemical information to suggest which enzyme is to be measured, then in principle all enzymes could be assayed. However, this is not an acceptable solution because the enzymes of mitochondrial β-oxidation are technically difficult to measure. Thus, measurement of all the enzymes, even for a highly selected group of patients, is a major undertaking and is clearly completely unrealistic as a screening strategy. Alternatively, if the pathway is stressed in vitro by administration of exogenous substrate, the intermediates proximal to the block will accumulate and, since the defects involve different enzymes with different substrate and chain-length specificities, the pattern of intermediates is characteristic for each enzyme defect.

We have pursued this approach and developed the analytical techniques required (25, 37, 51). This method has proven effective and appears to provide diagnosis by a single test (10, 13, 22). The major disadvantage of this method previously, however, was that it required respiring mitochondria isolated from cultured fibroblasts or other tissues such as skeletal muscle or liver. The latter tissues are not readily available, and preparation of mitochondria from fibroblasts requires culturing large number of cells, which is both expensive and laborious. Here, we have shown that a simple preparation of permeabilized fibroblasts can be used for the investigation of fatty acid oxidation defects. This system has several advantages: It is simple and rapid, does not require detergent, and since no isolation of mitochondria is involved, provides optimal recovery of mitochondria so that sufficient information can be obtained from a small number of cells. The permeabilization is brought about by a defined centrifugation procedure. The centrifugal forces imposed on cells during this procedure are sufficient to disrupt the plasma membrane without damaging the mitochondrial structure. The reproducibility of the method is demonstrated by preparing permeabilized cells on different occasions from the same cell line and analyzing 14C-labeled acyl-CoA and acylcarnitine intermediates generated during the oxidation of [14C]hexadecanoate. These results, together with a narrow normal range for both flux through β-oxidation and intermediates, indicate that this system is suitable for β-oxidation studies.

The diagnostic value of this method is demonstrated in the present study of cell lines from patients with proven deficiencies of fatty acid oxidation enzymes. Indeed, three of the patients with trifunctional enzyme
An internal carnitine acyl-CoA esters increased chain showed with ratio deficiency. The accumulation of long-chain 2,3-unsaturated and 3-hydroxylated acyl-CoA and acylcarnitine esters. This pattern is consistent with the site of the defect, a block at the level of 3-hydroxyacyl-CoA dehydrogenase; therefore, 3-hydroxacyl-CoA esters accumulate and are transferred to the mitochondrial matrix as carnitine esters via the carnitine shuttle. Enoyl-CoA esters also accumulate because of the reversibility of 2-enoyl-CoA hydratase. This enzyme activity may also be partially deficient in trifunctional enzyme deficiency, although some activity with the long-chain substrates is preserved because of the overlapping substrate specificity of other 2-enoyl-CoA hydratases.

A finding of slow flux together with the accumulation of large amounts of hexadecanoyl-CoA in the presence of little or no hexadecanoylcarnitine and other long-chain acylcarnitines (i.e., C₁₂ and C₁₄) is diagnostic of CPT I deficiency.

In carnitine–acylcarnitine translocase deficiency, the characteristic features are again slow flux, significantly high concentration of hexadecanoylcarnitine with high ratio of hexadecanoylcarnitine to hexadecanoyl-CoA, and lack of short-chain intermediates.

CPT II deficiency is characterized by high concentration of hexadecanoylcarnitine, absence of chain-shortened long-chain intermediates (i.e., C₁₀–C₁₄), and a high concentration of short-chain acylcarnitines (i.e., C₂–C₆).

In VLCAD deficiency, the pattern of intermediates showed very little chain-shortening, with no detectable intermediates shorter than C₁₄, thus indicating a long-chain enzyme defect.

The intermediates that accumulate in MCAD deficiency are also characteristic, with analysis showing increased concentrations of medium-chain intermediates and no detectable intermediates shorter than C₈. An additional finding in these patients is the presence of normal or marginally higher concentrations of acetyl carnitine, which is different from that in patients with defects at the level of long-chain substrates. This finding suggests the impaired oxidation of acetyl groups, and the most likely explanation is that accumulated medium-chain intermediates prevent further oxidation of acetyl groups by inhibiting either the tricarboxylic acid cycle or the respiratory chain.

The acyl-CoA and acylcarnitine profiles with added internal standards provide quantitative information on individual intermediates. However, absolute quantitation in most cases is not necessary, and a semiquantitative analysis or pattern recognition appears to be sufficient for data interpretation. In addition, although acyl-CoA analysis complements the acylcarnitine profile and extra or confirmatory information can be obtained by analysis of both classes of intermediates, in most cases analysis of acylcarnitines alone provides sufficient information to make a diagnosis. The two mitochondrial acylcarnitine transferases, carnitine acetyltransferase and CPT, have broad chain-length specificities for acyl-CoAs that together include C₂–C₁₈ (52). Enoyl-, 3-hydroxy, and 3-oxoacyl intermediates are also substrates for the carnitine transport system (53). Accumulated acyl-CoA esters thus form acylcarnitines, which then exit the mitochondria. The acylcarnitines can distribute throughout the incubation media and, therefore, accumulate in sufficient amounts to allow detection.

We conclude that the described method allows the diagnosis of fatty acid oxidation disorders with small amounts of cultured skin fibroblasts. This avoids the need for biopsy or large-scale tissue culture for isolation of mitochondria, and the diagnosis can be made in asymptomatic patients. In combination with other sensitive analytical techniques such as tandem mass spectrometry, which reduces the sample preparation and analysis time considerably, this method offers a reliable and fast approach for the detection of defects of fatty acid oxidation and can be adopted for screening populations at risk.

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References


