Accumulation of Free 3-Hydroxy Fatty Acids in the Culture Media of Fibroblasts from Patients Deficient in Long-Chain L-3-Hydroxyacyl-CoA Dehydrogenase: A Useful Diagnostic Aid

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Background: The diagnosis of long-chain l-3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) deficiency frequently requires the study of cultured fibroblasts. We developed such a test that does not require disruption and loss of the cells.

Methods: We measured free 3-hydroxy fatty acids (3-OHFAs) in media of skin fibroblasts cultures from 11 patients with a genetic deficiency of LCHAD and the associated disorder of mitochondrial trifunctional protein (MTFP). Fibroblasts were cultured for 24 h with 100 µmol/L nonisotopic palmitate added. 3-OHFAs were measured by selected-ion monitoring, stable-isotope dilution gas chromatography–mass spectrometry with [13C]-labeled internal standards.

Results: 3-OH-hexadecanoic and 3-OH-tetradecanoic FAs were increased 14- and 11-fold, respectively, in all patients with LCHAD or MTFP deficiency when compared with control fibroblast cell lines after overnight incubation with palmitate. 3-OH-dodecanoic FA demonstrated a modest, fivefold increase in LCHAD-deficient cells. The concentrations of all 3-OHFAs were similar whether or not the medium samples were hydrolyzed to release conjugated species such as acylcarnitines, suggesting that 3-OHFAs accumulate in the media as free FAs.

Conclusions: Measurement of 3-OHFA excretion from LCHAD- or MTFP-deficient cell lines can be used as a diagnostic tool. Free FAs are the predominant form of these abnormal metabolic intermediates in culture media.

Mitochondrial fatty acid β-oxidation (FAO) is a spiraling pathway involving four enzymatic steps that serially remove two carbon units from FAs in the mitochondria (1, 2). The four enzymes making up this pathway, in the order that they are utilized, are: (a) an acyl-CoA dehydrogenase, (b) a 2,3-enoyl-CoA hydratase, (c) an l-3-hydroxyacyl-CoA dehydrogenase, and (d) a 3-ketoacyl-CoA thiolase. Each of these enzymes has multiple forms, which are chain-length specific. For example, there are very-long-chain, long-chain, medium-chain (MCAD), and short-chain acyl-CoA dehydrogenases; long-chain and short-chain hydratases; long-chain 3-hydroxyacyl-CoA dehydrogenases (LCHADs) and medium/short-chain 3-hydroxyacyl-CoA dehydrogenases; and long-chain, medium-chain, and short-chain 3-ketoacyl-CoA thiolases. The long-chain forms of hydratase, LCHAD, and the 3-ketoacyl-CoA thiolase exist as a multienzyme complex located in the inner mitochondrial membrane that is called the mitochondrial trifunctional protein (MTFP) (3, 4). Deficiencies have been identified in each of these

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Received January 26, 2001; accepted April 9, 2001.

*Nonstandard abbreviations: FAO, fatty acid β-oxidation; MCAD, medium-chain acyl-CoA dehydrogenase; LCHAD, long-chain l-3-hydroxyacyl-CoA dehydrogenase; MTFP, mitochondrial trifunctional protein; 3-OHFA, 3-hydroxy fatty acid; FBS, fetal bovine serum; 3-OH-C14, 3-OH-tetradecanoic; and 3-OH-C16, 3-OH-hexadecanoic.
FAO enzyme forms with the exception of long-chain acyl-CoA dehydrogenase (5). Depending on the specific enzyme defect and the degree of enzyme deficiency involved, the clinical course can run from relatively mild and eminently treatable to rapidly fatal (6–8).

Diagnosis of FAO disorders is often problematic. In general, FAO defects are initially investigated with the use of urine organic acid profiles and blood acylcarnitine profiles taken when the individual is ill (9, 10). Recently, assays for the measurement of serum FAs and 3-hydroxy-FAs (3-OHFAs) have been introduced to specifically investigate LCHAD defects (11, 12). After the initial work-up on urine and serum, it is frequently necessary to perform confirmatory studies on cultured skin fibroblasts. Typically, fibroblasts will be used for metabolic flux, enzyme, and finally molecular analysis for those disorders in which the gene is known (13, 14). Although fibroblast FAO studies are often used to determine flux through the FAO pathway and decide whether enzyme analysis is necessary, these studies are sometimes inconclusive, possibly because of the number of different enzyme forms involved in this pathway. Recent studies using fibroblasts have been published to help with this problem (15, 16). Fibroblast studies that measure FAO metabolites include (a) preincubation of the fibroblasts with carnitine and free FAs of various chain lengths (17), (b) preincubation with carnitine and stable-isotope FAs (18–20), or (c) preincubation with carnitine and radioactive FAs (21, 22) for up to 96 h. All of these studies require carnitine and measure acylcarnitine species. Our current study demonstrates a rapid test for specific FAO defects that can be performed on fibroblasts in culture, without the need for disruption of the cells and loss of the culture. The assay uses nonisotopic palmitate, requires no addition of carnitine, and takes 24 h. This test clearly separates fibroblasts with defects in the LCHAD and MTFP enzymes from those without these defects.

Materials and Methods

Patients

Fibroblasts included in the study were from 11 patients who were deficient in either the LCHAD enzyme or had complete trifunctional protein deficiency with all MTFP activities reduced. Twelve nondeficient cell lines were used as controls. All deficient fibroblasts came from patients who had diagnoses that were confirmed by enzyme and molecular analysis. The LCHAD-deficient cell lines and their mutations are listed in Table 1.

Materials

Tissue culture media, including low-glucose DMEM (cat. no. 11885-084), no-glucose DMEM (cat. no. 11966–025), and fetal bovine serum (FBS) were obtained from Life Technologies. Palmitate, sodium sulfate, and fatty-acid-free bovine serum albumin were obtained from Sigma. 3-OHFA stable-isotope internal standards were synthesized and used as described previously (12). HPLC-grade ethyl acetate and HCl were from Mallinkrodt. The derivatizing reagent N,O-bis(trimethylsilyl)trifluoroacetamide+trimethylchlorosilane (99:1) was obtained from Supelco.

Substrate Preparation

Nonisotopic palmitate was originally dissolved in absolute ethanol to a concentration of 22 mmol/L, then 0.5 mL was pipetted into 10.5 mL of 100 g/L bovine serum albumin to make a 1 mmol/L palmitate solution, which was used as the stock solution. This stock solution was incubated at 37 °C for 30 min to allow the palmitate to bind to the albumin, and then the stock palmitate was added to the no-glucose DMEM–100 mL/L FBS medium to a final concentration of 0.1 mmol/L. This was the medium used for incubation.

Cell Culture

Cells were cultured in low-glucose DMEM with 100 mL/L FBS. All cells were studied between passages 2 and 15, when cells were at least 90% confluent, generally 5–7 days after subculture. Culture media were removed from the cells, and 5 mL of palmitate-containing medium was added to the T25 flasks. Flasks were incubated 24 h at 37 °C in a 5% CO2–95% air incubator. After 24 h, the palmitate medium was removed and saved for analysis, and the cells were returned to regular culture medium, low-glucose DMEM–100 mL/L FBS. All cell lines were assayed in duplicate.

Extraction of Culture Media

The media were extracted and derivatized as described previously (12) with the following modifications: 4 mL of medium was used for each sample, acidification was accomplished with 250 μL of 6 mol/L HCl, and derivatization was performed using 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide+trimethylchlorosilane. For hydrolysis of media, 400 μL of 10 mol/L NaOH was added before the addition of the 3-OHFA internal standards, and hydrolysis was carried out at 37 °C for 30 min. Acidifica-

| Table 1. LCHAD-deficient cell lines. |
|-----------------------------|-----------------------------|
| Cell line | Mutation | 3-OH-C16 accumulation, nmol/L |
| B918 | Homozygous G1528C | 2288 |
| B1025 | Homozygous G1528C | 1331 |
| B260 | Homozygous G1528C | 1271 |
| B1211 | Homozygous G1528C | 807 |
| B608 | Homozygous A2G splice, exon 7 | 1584 |
| B1298 | Homozygous C181T, exon 4 | 1042 |
| B760 | G1528C/5-bp deletion; crosses splice site after exon 15 | 1211 |
| B762 | G1528C/5-bp deletion | 917 |
| B184 | G1528C/C1678T, exon 16 | 663 |
| B1175 | G1528C/G509A | 597 |
| B858 | G1528C/G2027A | 574 |
tion of the sample was then accomplished by the addition of 1.5 mL of 6 mol/L HCl, instead of 250 μL. Gas chromatographic–mass spectrometric conditions and analyses were as described previously (12), except the gas chromatography–mass spectrometry device used was an Agilent Technologies 6890 gas chromatography system with a 5973 N Series mass spectrometer.

**Statistical Analysis**

The means of the data sets for the 3-OH-tetradecanoic (3-OH-C14) and 3-OH-hexadecanoic (3-OH-C16) FAs were subjected to analysis using the two-tailed Student t-test to determine whether they were statistically different.

**Results**

The concentration of palmitate used in the experiments was first optimized by measuring the accumulation of 3-OHFA s in culture media from cells fed with no-glucose DMEM containing increasing amounts of palmitate. The free 3-OH-C16 FAs that accumulated in the culture media at different palmitate concentrations in both LCHAD-deficient and control fibroblasts are demonstrated in Fig. 1. As shown in Fig. 1, LCHAD-deficient cell lines accumulated increasing amounts of 3-OH-C16 intermediates of FAO when the cells were fed increasing amounts of palmitate. Given that the greatest accumulation of intermediates was seen when the cells were fed 100 μmol/L palmitate, this concentration was then used throughout the rest of the study. When the intermediate accumulation for all the carbon chain lengths, 3-OH-hexanoic to 3-OH-C16, was compared between control and LCHAD-deficient fibroblasts, there was no significant difference between the amount of accumulation of the shorter chain lengths, 3-OH-hexanoic to 3-OH-decanoic. 3-OH-dodecanoic showed a moderate fivefold increase in accumulation in the LCHAD-deficient cell lines when compared with control cell lines, with a mean of 40 μmol/L for control cell lines and a mean of 209 μmol/L for LCHAD cell lines. 3-OH-C14 and 3-OH-C16 FAs are the most useful chain-length intermediates for separating the two types of cells. After the cells were fed 100 μmol/L palmitate, there was a >10-fold difference between long-chain metabolites excreted by control and LCHAD-deficient cells. The differences between the means from the control and the LCHAD-deficient cell lines were statistically significant (P <0.0005). The numerical mean, median, and range for the LCHAD-deficient vs the control cell lines for 3-OH-C14 and 3-OH-C16 FAs are given in Table 2. The 3-OH-C14 and 3-OH-C16 metabolite accumulation in control and LCHAD cells from each individual cell line are shown in Fig. 2, demonstrating that there was no overlap in the amount of accumulation between the control cell lines and LCHAD-deficient cell lines. The wide range in the amount of accumulation from the different LCHAD-deficient cell lines is also shown in Fig. 2. Studies performed with medium samples that were hydrolyzed to release conjugated species demonstrated that none of the 3-OHFA intermediates shorter than 3-OH-dodecanoic FA and <5% of the 3-OH-C14 and 3-OH-C16 intermediates were conjugate species.

**Discussion**

Defects of mitochondrial FA oxidation have emerged as significant causes of morbidity and mortality in infants and children. At least 20 genetic defects of FAO are currently known. Some of these disorders occur with relatively high frequency in the population (1–6). MCAD
deficiency occurs commonly enough, ~1 in 14,000 births, and is so treatable that it has been proposed for inclusion in newborn screening programs. Newborn screening with the use of tandem mass spectrometry makes this even more feasible (23, 24). In fact, MCAD testing is already included as part of a routine newborn screen by the New England Newborn Screening Program at the University of Massachusetts Medical School, by Baylor University Medical Center, and by Neo Gen Screening, Inc. MCAD testing is also part of the routine newborn screen in Massachusetts, Maine, North Carolina, and Wisconsin (25–27).

Since the first description of LCHAD deficiency in 1991, LCHAD- or MTFP-deficient individuals have been diagnosed with increasing frequency and with a wide array of symptoms. Molecular genetic analysis is finding more mutations in the LCHAD gene. As seen in Table 1, there appears to be a difference in the accumulation of long-chain species from cell lines with different mutations. Although there is no statistically significant correlation between the type of mutation and the amount of 3-OHFA, there is a definite suggestion that homozygous mutations lead to a greater accumulation of long-chain 3-OH intermediates than do heterozygous mutations. The assay may be a helpful indicator of an individual’s ability to oxidize FAs and may be of assistance in determining treatment options and monitoring therapy.

The concentrations of the 3-OHFA species measured in this study ranged from 200 nmol/L to 2.4 μmol/L. This is in contrast to earlier studies in fibroblast culture, which measured acylcarnitine metabolites after incubation of the cells with carnitine and FAs. These earlier studies reported 100 pmol/L to 10 nmol/L ranges for most acylcarnitine species, often reporting unrecoverable amounts of many of those compounds (17–22). We have found that most of the metabolite species we measured in fibroblast culture media were unconjugated, free 3-OHFA. This is in contrast to our findings in serum, in which 3-OH-C14 and 3-OH-C16 FAs were ~50% conjugated (28). This finding suggests that in systems that assay cell culture media, our assay may be a more useful tool for diagnosing LCHAD deficiency than for measuring acylcarnitines in these cells. Also, the observation that >95% of the 3-OHFA intermediates are nonesterified may have important implications when considering the pathogenesis of LCHAD deficiency. Acylcarnitine species are more water soluble and more likely to be excreted in the urine than the more lipophilic free FA species, which are more likely to be bound to circulating albumin and not excreted.

References


