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Differentiation of long-chain fatty acid oxidation disorders using alternative precursors and acylcarnitine profiling in fibroblasts

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Abstract

The differentiation of carnitine-acylcarnitine translocase deficiency (CACT) from carnitine palmitoyltransferase type II deficiency (CPT-II) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency from mitochondrial trifunctional protein deficiency (MTP) continues to be ambiguous using current acylcarnitine profiling techniques either from plasma or blood spots, or in the intact cell system (fibroblasts/amniocytes). Currently, enzyme assays are required to unequivocally differentiate CACT from CPT-II, and LCHAD from MTP. Over the years we have studied the responses of numerous FOD deficient cell lines to both even and odd numbered fatty acids of various chain lengths as well as branched-chain amino acids. In doing so, we discovered diagnostic elevations of *unlabeled* butyrylcarnitine detected only in CACT deficient cell lines when incubated with a shorter chain fatty acid, [7-$^{2}H_{3}$]heptanoate plus L-carnitine compared to the routinely used long-chain fatty acid, [16-$^{2}H_{3}$]palmitate. In monitoring the unlabeled C4/C5 acylcarnitine ratio, further differentiation from ETF/ETF-DH is also achieved. Similarly, incubating LCHAD and MTP deficient cell lines with the long-chain branched fatty acid, pristanic acid, and monitoring the C11/C9 acylcarnitine ratio has allowed differentiation between these disorders. These methods may be considered useful alternatives to specific enzyme assays for differentiation between these long-chain fatty acid oxidation disorders, as well as provide insight into new treatment strategies.
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Introduction

Over the last 10 years blood spot and/or plasma acylcarnitine profiles have allowed clinicians to distinguish between the various long-chain mitochondrial beta oxidation defects, namely, very long-chain acyl-CoA dehydrogenase deficiency (VLCAD), long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD) and mitochondrial trifunctional protein deficiency (MTP), carnitine palmitoyltransferase deficiency type II (CPT-II), and carnitine-acylcarnitine translocase deficiency (CACT). The presence of particular acylcarnitine elevations are indicative of long-chain fatty acid oxidation defects (FOD) [1]. For example, C14:1 for VLCAD; C16-OH, and C18:1-OH for LCHAD or MTP, and C16 and C18:1 for CPT-II or CACT. Of these disorders only VLCAD can be diagnosed specifically on the basis of its observed acylcarnitine profile (ACP) in blood and the clinician must realize that the ACP does not help to distinguish, reliably, the severe (neonatal/infantile) CPT-2 from CACT and similarly, LCHAD from MTP. Up until now, the established mitochondrial beta oxidation probe technique using [16-$^{2}H_{3}$]palmitic acid followed by acylcarnitine profiling by tandem mass spectrometry (either ESI or LSIMS) [2] could not reliably distinguish CACT from CPT-II or LCHAD from MTP and therefore, required specific enzyme assays and or mutation analysis for positive confirmation.

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In the process of screening cell lines from patients with confirmed FOD deficiencies expressing interest and/or admission into a dietary investigation with triheptanoin [3], the unique observation was made that CACT deficient cell lines probed with [$^2H_3$]heptanoic acid consistently produced abnormal levels of *unlabeled* C4 acylcarnitine, when compared to normal, CPT-II or other fatty acid oxidation defects. In a similar scenario, incubating LCHAD and MTP cell lines with pristanic acid led to observations which have allowed differentiation between these disorders. When compared to the "gold-standard" enzyme activity results, these observations have proven to be reliable. In laboratories equipped with tissue culture facilities and tandem mass spectrometry this technique should be considered as a further differential tool or as an alternative to direct enzyme assay. As newborn screening becomes more routine and widespread the need for quick and dependable diagnostic assays becomes relevant [4,5]. These observations have also broadened our knowledge and curiosity of the overlap of chain length specificities and interactions amongst different cell pathways and organelles in the face of a deficiency, which may lead to further diagnostic assays as well as investigations into beneficial treatment strategies.

Materials and methods

Media and reagents

All tissue culture reagents and media were obtained from Gibco/BRL (Gaithersburg, MD) or Atlanta Biologicals (Norcross, GA). Dulbecco's modified Eagle's medium (DMEM), 1×, low glucose (25 mM), with L-glutamine (4 mM) was used as the basic growth media. DMEM, 1×, low glucose, with L-glutamine and devoid of branched-chain amino acids was a custom formulation. Complete medium was prepared by adding 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (v/v) into base medium. Fatty acid free bovine serum albumin (BSA; 60 mg/ml in serum free medium), L-carnitine, [methyl-$^2H_3$]L-carnitine (40 mM stock in serum free media) and [U-^{13}C]valine, [U-^{13}C]isoleucine, and [U-^{13}C]leucine (80 mM stock in water; CIL Isotopes, Andover, MA) were sterilized using a 0.2 μ syringe filter and stored at −20 °C. [16-$^2H_3$]Palmitic acid and [7-$^2H_3$]heptanoic acid (20 mM stock in ethanol) were purchased from CDN Isotopes. Pristanic acid (20 mM stock in ethanol) was a gift from Herman ten Brink (Amsterdam). Unlabeled amino acids and L-carnitine was purchased from Sigma Chemical (St. Louis, MO) and all other chemicals were of analytical grade.

Patients

The fibroblast cell lines used in this study were sent to us on patients who were either suspected of having a mitochondrial β-oxidation defect that was later confirmed or were previously confirmed with a specific FOD defect requesting further investigations. The patient population in this study included seven patients with CACT, of which five were characterized as "severe" presenting with neonatal onset and two as "mild", presenting later in infancy. The CPT-II population consisted of 18 patients, seven presenting in infancy with recurrent hypoglycemia and/or carnitine deficiency and 11 patients presenting in late teens/early adulthood with recurrent muscle weakness or exercise induced rhabdomyolysis or myoglobinuria. Fourteen patients had a confirmed "isolated" deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD; EC 1.1.1.211). Three of the seven patients with confirmed trifunctional protein (MTP) deficiency were found deficient in all three components of the MTP complex: L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase and 2-enoyl-CoA hydratase. The other four patients were assayed for two of these three enzymes, the acyl-CoA dehydrogenase and thiolase, found to be deficient in both and therefore were also classified as MTP deficiency. In some cases, where the suspected diagnosis was CACT vs CPT-II or LCHAD vs MTP, the suspicion was first confirmed by performing the established "in vitro probe" technique using [16-$^2H_3$]palmitic acid in intact fibroblasts and then proven by direct enzyme assay. Additional DNA mutation analysis for the common 1528G > C (E510Q) in LCHAD [6] and the more frequent S113L in CPT-II [7] were performed as requested. Patients reported here to have electron transport flavoprotein deficiency (ETF; $n = 1$) or ETF dehydrogenase deficiency (ETF-DH; $n = 3$) were based on in vitro probe results with [16-^{2} H_3]palmitic acid and ^{13}C-branched-chain amino acids [8] and confirmed by specific enzyme assay of ETF and ETF-QO. Fibroblasts with no obvious defect in fatty acid oxidation as determined by the in vitro probe served as controls. All studies were approved by the Baylor University Medical Center Institutional Review Board and the requesting physicians.

Enzyme activity

Activity was measured predominantly in cultured skin fibroblasts using established methods previously described for CACT [9] and CPT-II [10], LCHAD [11], MTP [12], and ETF-DH/ETF [13].

Fibroblast cultures

Fibroblasts from all patients and controls were maintained in complete DMEM at 37 °C in a humidified 5% CO_2/95% air incubator. Approximately, 1–3 days before initiation of incubation studies, the cells were sub-cultured into duplicate or triplicate T-25 flasks and grown to within 90–95% confluency (∼0.15–0.40 mg protein).

Protein measurement

Protein concentration was determined by a modification of the Bradford method [14] using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with BSA as standard on a COBAS FARA II centrifugal analyzer (Roche, Somerville, NJ).

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