Acylcarnitine Profiles in Fibroblasts From Patients With Respiratory Chain Defects Can Resemble Those From Patients With Mitochondrial Fatty Acid β-Oxidation Disorders

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Mitochondrial fatty acid β-oxidation (FAO) is coupled to the respiratory chain (RC). Functional defects of one pathway may lead to secondary alteration in flux through the other. We investigated the acylcarnitine profiles in cultured fibroblasts obtained from 14 healthy subjects, 31 patients with 8 different primary enzyme deficiencies of FAO, and 16 patients with primary RC defects including both isolated and multiple enzyme complex defects. Intact cells were incubated in media containing deuterium-labeled hexadecanoic acid and L-carnitine, and the acylcarnitines analysed using an electrospray tandem mass spectrometer. All FAO-deficient cell lines revealed disease-specific acylcarnitine profiles related to the sites of defects. Some cell lines from patients with RC defects showed profiles similar to those of controls, whereas others had abnormal profiles mimicking those found in FAO disorders. The acylcarnitine profiles of patients with RC enzyme defects were not predictable, and in some patients defects caused by mutations in either nuclear-encoded or mitochondrial DNA were associated with acylcarnitine abnormalities. While in vitro acylcarnitine profiling is useful for the diagnosis of FAO deficiencies, abnormal profiles do not exclusively indicate these disorders, and primary defects of the RC remain a possibility. Awareness of this diagnostic pitfall will aid in the selection of subsequent confirmatory tests and therapeutic options.
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MITOCHONDRIAL fatty acid β-oxidation (FAO) and respiratory chain (RC) oxidative phosphorylation play pivotal roles in energy production. Both pathways are linked via the ubiquinone pool at 2 stages,[1] and also via the activity of the citric acid cycle. Inborn errors of mitochondrial fatty acid β-oxidation,[2-4]and the respiratory chain[5,6]are clinically and biochemically heterogenous. Whereas FAO defects usually involve single enzymes, defects in the RC may occur as multiple defects of 2 or more of the 5 enzyme complexes. These 2 groups of disorders have some clinical and biochemical features in common, including muscle weakness, cardiomyopathy, encephalopathy, hepatopathy, metabolic decompensation during catabolic stress, hypoglycemia, and lactic acidosis. Patients with primary RC defects can exhibit organic acidurias mimicking FAO disorders[7-9] Functional enzyme analyses have revealed concomitantly reduced activities of some enzymes in both pathways.[10-13] Histopathological findings[11,14] and in vitro oxidation rate studies[15] may not discriminate between defects of these 2 groups of disorders. It is apparent that impairment of one pathway may lead to secondary alteration in flux through the other.

Incubating cells from patients with documented FAO defects in media enriched with fatty acids and L-carnitine revealed disease-specific acylcarnitine profiles,[16-21] and thus in vitro acylcarnitine profiling is a useful screening tool for the diagnosis of patients suspected of having one of the numerous enzyme defects in this group of disorders. It is not clear whether acylcarnitine profiling is entirely specific for FAO disorders, as fibroblasts from patients with RC defects have not been studied in depth. The current report examines this question.

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MATERIALS AND METHODS

Cell Lines and Cell Culture

Controls. Normal control cultured skin fibroblasts were from 14 healthy laboratory workers.

Mitochondrial FAO-deficient cell lines. These were obtained from 31 patients with 8 different enzyme deficiencies confirmed by specific enzyme assay and/or mutational analysis. The FAO enzyme deficiencies included hepatic carnitine palmitoyltransferase I (CPT IA; n = 1), carnitine palmitoyltransferase II (CPT II; n = 5), carnitine acylcarnitine translocase (CACT; n = 2), very-long-chain acyl-CoA dehydrogenase (VLCAD; n = 3), long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD; n = 5), medium-chain acyl-CoA dehydrogenase (MCAD; n = 9), multiple acyl-CoA dehydrogenase (MAD; n = 5), and short-chain acyl-CoA dehydrogenase (SCAD; n = 1). Table 1 shows the clinical phenotype and gene defects where known.

Respiratory chain defects. Cultured skin fibroblasts were available from 16 patients with RC defects confirmed by measurement of the activities of the enzyme complexes in one or more tissues including skeletal muscle, heart muscle, liver, and cultured skin fibroblasts, with DNA mutation analysis performed in some cases. The defects included isolated complex I (n = 5); complex II (n = 1); complex IV (n = 3); combined deficiencies of complexes I, III, and IV (n = 5); and combined complexes I, II + III, III, and IV deficiencies (n = 2). Cells between passage 4 and 13 were used for the assays, except for one, R10, at passage 19. A summary of clinical phenotype and gene defects (if identified) for each patient are presented in Table 2.

Skin fibroblasts were grown in Ham F10 nutrient mixture supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 1% penicillin, streptomycin, and fungizone in a 25-cm^2 culture flask until 100% confluent. The cells were checked for microbial contamination using the Hoechst 33258 stain (Hoechst, St Louis, MO) whenever they were trypsinized.

Table 1. Incubation of Fibroblasts From Controls and Patients With Mitochondrial Fatty Acid β-Oxidation Deficiencies With $^2H_5$-Hexadecanoate and L-Carnitine for 72 Hours

Enzyme Deficiencies	Gene Defect	Clinical Phenotype		Acylcarnitine (nmol/mg protein) in Media at 72 h								
				C_{4}-	C_{5}-	C_{6}-	C_{8}-	C_{10}-	C_{12}-	C_{14}-	C_{16}-	C_{16}-OH
CPT IA (n = 1)	NK	Liver dysfunction	Average	0.3	0.9	<0.1	<0.1	<0.1	<0.1	<0.1	0.3	<0.1
CPT II early (n = 2)	NK	Neonates, died before 2 m	Average	<0.1	0.9	0.1	0.1	0.1	0.6	0.2	**13.4**	<0.1
			Limits§	<0.1	0.5-1.8	<0.2	<0.2	<0.2	0.1-1.9	0.1-0.3	**6.7-22.6**	<0.1
CPT II late (n = 3)	NK	Childhood and adult onset; rhabdomyolysis	Average	1.2	0.9	0.5	0.6	0.8	0.2	0.1	**3.3**	<0.1
			Limits§	0.6-2.4	0.6-1.0	0.3-0.9	0.3-0.8	0.3-1.3	0.1-0.3	<0.2	**2.3-4.9**	<0.1
CACT (n = 2)	NK	1 neonatal death, 1 with mild symptoms	Average	0.3	1.5	0.3	0.3	0.3	0.7	0.1	**10.2**	<0.1
			Limits§	0.1-0.6	1.4-1.6	0.1-0.4	0.3-0.4	0.2-0.5	0.2-1.3	<0.2	**9.2-11.2**	<0.1
MAD: profile 1 (n = 3)	NK	All neonatal deaths	Average	0.1	1.8	0.2	0.2	0.5	0.6	**1.5**	**10.5**	<0.1
			Limits§	<0.3	0.9-5.6	0.1-0.6	0.1-0.8	0.1-1.3	0.1-1.2	**0.3-2.1**	**5.5-19.8**	<0.1
MAD: profile 2 (n = 2)	NK	Proximal muscle weakness	Average	0.7	1.2	0.9	1.5	**2.3**	**2.7**	**2.3**	3.1	<0.1
			Limits§	0.2-1.3	0.9-1.7	0.5-1.1	1.0-1.8	**1.8-3.1**	**1.8-3.8**	**1.9-2.8**	2.1-4.9	<0.1
VLCAD (n = 3)	(1) R429W/R429W	(1) Fatal neonatal onset	Average	1.4	0.9	0.2	0.3	0.5	**1.0**	**2.4**	4.0	<0.1
	(2) G869A/G881A	(2) Reye-like at 2 yr	Limits§	0.4-5.2	0.5-1.7	0.1-0.3	0.2-0.5	0.3-0.8	**0.4-2.2**	**0.5-4.9**	0.9-8.1	<0.1
	(3) NK	(3) Asymptomatic¶										
LCHAD (n = 5)	G1528C/G1528C (n = 3)	All died before 6 mo of age	Average	0.7	1.3	0.3	0.4	0.6	**0.9**	**0.6**	**5.7**	**1.7**
	G1528C/NK (n = 2)	Both are well on low-fat diet	Limits§	0.2-2.1	0.7-2.2	0.1-0.6	0.2-0.8	0.2-1.5	**0.4-2.1**	**0.2-1.0**	**3.0-10.4**	**0.9-2.4**
MCAD (n = 9)	G985A/G985A (n = 4)	Included 4 clinical and 5	Average	1.4	1.0	2.4	**6.5**	1.4	0.2	0.1	1.2	<0.1
	G985A/Others (n = 5)	asymptomatic¶ patients	Limits§	0.4-3.9	0.4-2.9	1.0-5.5	**2.5-12.3**	0.4-5.4	0.1-0.4	<0.2	0.4-4.0	<0.1
SCAD (n = 1)	A57V/Q374X	Asymptomatic¶	Average	**10.2**	0.8	0.3	0.5	0.6	0.2	0.1	0.8	<0.1
Controls (n = 14)			Mean of 72 observations	0.9*	1.1	0.5*	0.6	0.8	0.2*	0.1*	0.9*	<0.1†
			Reference range‡	0.3-2.2*	0.5-1.7	0.2-1.4*	0.1-1.1	0.1-1.6	<0.4*	<0.2*	0.4-2.2*	0.1†

NOTE. Each cell line was analyzed in duplicate in 2 to 6 separate assays. For the FAO-deficient cell lines, the average of each analyte was calculated from all cell lines from different patients with the same enzyme defect.

Abbreviations: NK, not known; CPT IA, hepatic carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CACT, carnitine acylcarnitine translocase; VLCAD, very-long-chain acyl-CoA dehydrogenase; LCHAD, long-chain L-3-hydroxyacyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MAD, multiple acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase.

*Logarithmic transformation of data to determine normal distribution.

†Median and upper limit of observed range.

‡Mean ± 1.96 SD.

§Observed lower and upper limits.

¶Detected by newborn screening program.

Table 2. Incubation of Fibroblasts From Patients With Respiratory Chain Defects With $^2H_5$-Hexadecanoate and L-Carnitine

Complex Defects (patient)	Gene Defect	Clinical Phenotype	Acylcarnitine (nmol/mg protein) in Media at 72 h								
			C_4-	C_5-	C_6-	C_8-	C_{10}-	C_{12}-	C_{14}-	C_{16}-	C_{16}-OH
Complex I											
R1	NK	Dysmorphic, LA	2.0	1.5	0.3	0.3	0.4	0.2	0.1	1.6	<0.1
R2	NK	Cardiomyopathy	1.7	1.3	0.6	0.4	0.6	0.2	0.1	1.4	<0.1
R3	mt $tRNA^{Leu(UUR)}$	MELAS	**2.8**	0.8	0.3	0.4	0.5	0.1	0.1	0.9	<0.1
R4	mt $tRNA^{Leu(UUR)}$	Skeletal myopathy	**3.0**	1.1	0.5	0.6	0.7	0.2	0.1	1.1	<0.1
R5	NK	Leigh syndrome	1.3	1.0	0.5	0.5	0.6	0.2	0.1	1.1	<0.1
Complex II											
R6	NK	Hepatic failure	1.5	1.1	**1.9**	**2.8**	**1.9**	0.3	0.1	1.5	<0.1
Complex IV											
R7	NK	Leigh disease	0.9	1.9	0.7	0.8	1.1	0.3	0.1	1.4	<0.1
R8	SURF-1	Dysmorphic, LA	0.9	1.0	1.5	**2.2**	**2.6**	**1.3**	**0.6**	2.0	<0.1
R9	NK	Neonatal LA	1.1	1.0	0.7	0.7	0.6	0.2	0.1	1.9	<0.1
Complex I, III, and IV											
R10	NK	Hepatic failure	0.4	0.9	0.1	0.2	0.2	0.2	0.3	**6.7**	<0.1
R11	NK	LA	1.3	1.3	1.0	1.0	1.3	0.3	0.1	1.4	<0.1
R12	mt $tRNA^{Lys}$	MERRF	0.7	1.2	0.3	0.4	0.8	0.4	0.3	3.1	**0.4**
R13	NK	Hypoglycemia, LA	0.8	0.9	0.2	0.2	0.4	0.1	0.1	1.6	<0.1
R14	NK	Hepatic failure	1.3	1.3	0.3	0.4	0.5	0.5	0.3	**7.5**	<0.1
Complex I, II + III, III, and IV											
R15	NK	LA	**3.1**	1.3	0.5	0.5	0.4	0.2	0.1	**4.8**	<0.1
R16	NK	LA	2.2	1.1	0.6	0.7	1.1	0.2	0.1	1.2	<0.1
Controls (N = 14)		Mean of 72 observations	0.9*	1.1	0.5*	0.6	0.8	0.2*	0.1*	0.9*	<0.1†
		Reference range‡	0.3-2.2*	0.5-1.7	0.2-1.4*	0.1-1.1	0.1-1.6	<0.4*	<0.2*	0.4-2.2*	0.1†

NOTE. Patient results are the average of 4 to 8 observations obtained in 2 to 4 separate assays.

Abbreviations: NK, not known; LA, lactic acidosis; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy ragged red fiber syndrome.

*Logarithmic transformation of data to determine normal distribution.

†Median and upper limit of observed range.

‡Mean ± 1.96 SD.

Culture Media and Chemicals

All cell culture media, antibiotics, trypsin-EDTA were from Gibco BRL (Life Technologies, Grand Island, NY). Stable-isotope labeled palmitate, [15,15,16,16,16]-$^2H_5$ hexadecanoic acid was obtained from C/D/N Isotopes (Quebec, Point-Claire, Canada). Bovine serum albumin (essentially fatty acid free), L-carnitine, L-isovalerylcarnitine (C_5-), DL-hexanoylcarnitine (C_6-), DL-octanoylcarnitine (C_8-), DL-decanoylcarnitine (C_{10}-), and L-palmitoylcarnitine (C_{16}-) were obtained from Sigma (St Louis, MO). Internal standards (IS) containing a mixture of $^2H_3$-labeled butyrylcarnitine (C_4-), C_8-, C_{16}-acylcarnitine, and $^2H_9$-labeled C_5- and tetradecanoylcarnitine (C_{14}-) were purchased from NeoGen (Cambridge Isotope Laboratory, Andover, MA).

The In Vitro Acylcarnitine Assay in Intact Fibroblasts

The in vitro acylcarnitine assay in intact skin fibroblasts was performed as described[19] with some modifications. At 100% confluency, the fibroblasts were detached with trypsin, the cells counted, 10^5 cells (50 to 95 μg protein) subcultured into a well of a 24-well culture dish (Costa Products, Corning, NY), and settled for 24 hours to form a cell monolayer. Duplicate wells were set up for every cell line in each batch. The culture media was then replaced with 0.5 mL of reaction media containing $^2H_5$-labeled hexadecanoic acid 0.11 mmol/L complexed to bovine serum albumin 0.5 mg/mL, L-carnitine 0.4 mmol/L, and fetal calf serum 10% in Ham F10 nutrient mixture, prepared just before use. After incubation at 37°C in a water jacketed incubator for 72 hours, the reaction media in each well was transferred to a tube for acylcarnitine analysis, and the cell monolayer retained for protein quantitation.

Quantitative Profiling of Acylcarnitine Using Electrospray Tandem Mass Spectrometer

IS prepared in ethanol were added to each tube of reaction media, mixed, and centrifuged at 28,000 × *g* min to remove the precipitate. The supernatant was evaporated to dryness under a stream of air, derivatized with 300 μL butanolic-hydrochloric acid 10% (vol/vol) with heating at 60°C for 15 minutes, and dried again. The dried butylated sample was reconstituted with 100 μL of 50% acetonitrile: water (vol/vol) and transferred to a 96-well plate for automated injection.

The butyl esters of acylcarnitines were detected using a Micromass Quattro LC electrospray ionization tandem mass spectrometer (EIS-MS/MS, Micromass, Manchester, UK), detecting the precursors of m/z of 85.2 by the multichannel acquisition mode scanning in the range m/z 250 to 500. Following data acquisition, the concentrations of acylcarnitines were processed using Neolynx software (Micromass), measuring the intensities of analyte/internal standard ratio and concentration data from externally calibrated analytes including C_5-, C_6-, C_8-, C_{10}-, and C_{16}-acylcarnitine. Concentrations of C_4-, C_{12}- C_{14}-, and C_{16}-OH-acylcarnitine were calculated using the responses of the nearest counterparts, C_5-, C_{10}-, and C_{16}-acylcarnitine.

Protein Determination

The cell monolayer was washed twice with phosphate-buffered saline, hydrolyzed with 2.0 mol/L sodium hydroxide, and then neutralized with 1.0 mol/L hydrochloric acid. The protein was measured by the Pierce (Rockford, IL) bicinchoninic acid (BCA) method using bovine serum albumin in saline as standard on a Cobas BIO analyzer (Roche Diagnostics, Basel, Switzerland).

Data Analysis

Control cell lines (n = 14) were analyzed repeatedly with increasing passage numbers until a total of 72 observations were obtained. The data distribution of the levels of each $^2H_5$-labeled acylcarnitine species was checked with a test of normality, the Kolmogorov-Smirnov test, after logarithmic transformation of data for some analytes where required (SPSS version 9, SPSS Inc, Chicago, IL). Reference ranges were then derived, based on ±1.96 SD for allanalytes except for C_{16}-OH-acylcarnitine, which was expressed as the median and the upper limit of the observed range.

All patient cell lines were analyzed in duplicate in each batch and batches were repeated 2 to 6 times following successive subculture. For the data obtained from the FAO-deficient cells, the average for each analyte was calculated from all cell lines from different patients with the same enzyme defect, and the lower and upper limit of all these observations recorded (Table 1).

Fibroblast acylcarnitine levels in individual patients with RC defects were calculated as the average of 4 to 8 observations obtained from 2 to 4 separate assays (Table 2).

RESULTS

Butyl esters of $^2H_5$-labeled acylcarnitines derived from the precursor $^2H_5$-palmitate, and unlabeled acylcarnitines originating from the endogenous source of fatty acids and amino acids that had accumulated in the reaction media after 72 hours incubation were detected. Results shown in Tables 1 and 2 are the butyl esters of deuterium labelled acylcarnitines, except for C_5-acylcarnitine, which is the unlabelled species derived from branched-chain amino acids in the fetal calf serum.

The possibility of effect from the passage number (long-term subcultures) was investigated in fibroblasts from an RC affected patient with complex IV deficiency demonstrated in skeletal muscle, transformed lymphoblasts, and fibroblasts, who had homozygous mutation in SURF-1 gene (R8). Retrieved cell line cultured over a period of 6 weeks representing passages 5 to 8 following successive subcultures was analyzed in 4 separate assays. The average (observed lower-upper limit) of $^2H_5$-labeled C_8-, C_{10}-, C_{12}-, C_{14}-acylcarnitine were 2.2 (1.5-3.6), 2.6 (1.9-4.1), 1.3 (0.7-2.3), 0.6 (0.4-1.1) nmol/mg protein, respectively. These analytes were consistently elevated above the reference range of normal controls, and there were no significant changes in pattern or indication of a trend of change in concentration of any analytes with increasing passages.

Table 1 shows the results of quantitative profiling of acylcarnitines generated by fibroblasts from healthy subjects and those from patients with various inherited deficiencies of FAO (data not overlapping with the reference range are in bold type). The control cell lines showed the presence of low concentrations of acylcarnitines with carbon chain lengths corresponding to C_4-, C_5-, C_6-, C_8-, C_{10}-, C_{16}-, even lower levels of C_{12}-, C_{14}-, and near absence of C_{16}-OH-acylcarnitine. CPT IA-deficient cells displayed generally reduced levels of all $^2H_5$-labeled acylcarnitines, but normal level of C_5-acylcarnitine compared to the controls. All cell lines from patients with CPT II and CACT defects showed an elevated concentration of $^2H_5$-C_{16}-acylcarnitine. Two distinct profiles were exhibited by the MAD-deficient cell lines: one with massive accumulation of $^2H_5$-C_{16}-acylcarnitine and variable elevation of C_5-acylcarnitine (n = 3), and another with markedly increased concentrations of a number of medium- and long-chain species, notably $^2H_5$-labeled C_{10}-, C_{12}-, and C_{14}-acylcarnitine (n = 2). Cell lines with VLCAD deficiency were associated with elevation of $^2H_5$-labeled C_{12}-, C_{14}-, and C_{16}-acylcarnitine, with $^2H_5$-C_{14}-acylcarnitine being the predominant species. All of the LCHAD-deficient cell lines revealed accumulation of deuterium-labeled long-chain species and the distinctive C_{16}-OH-acylcarnitine. The MCAD-deficient cell lines were characterized by the predominant elevation of $^2H_5$-C_8-acylcarnitine, and the SCAD deficiency showed increased concentration of $^2H_5$-C_4-acylcarnitine only.

Fibroblast acylcarnitine profiles in individual patients with RC defects are shown in Table 2. Of the 5 cell lines with isolated complex I deficiency, 3 showed a profile similar to those of controls, while 2 revealed increased concentration of $^2H_5$-C_4-acylcarnitine (R3 and R4), suggestive of SCAD dysfunction. The cell line from a patient with complex II defect (R6) exhibited elevated concentrations of $^2H_5$-labeled C_6-, C_8-, and C_{10}-acylcarnitine, with C_8-acylcarnitine being the dominant species, strongly resembling those with MCAD deficiency. One of the 3 cell lines with isolated complex IV defect (R8) showed accumulation of a number of medium- and long-chain species suggestive of MAD deficiency. Of the 5 cell lines with multiple deficiencies of complexes I, III, and IV, profiles similar to controls (R11 and R13), or mimicking those with CPT II/CACT (R10 and R14), or LCHAD (R12) deficiencies were observed. One of the 2 cell lines with combined deficiencies of complexes I, II + III, III, and IV revealed marked accumulation of $^2H_5$-C_{16}- and $^2H_5$-C_4-acylcarnitine (R15), a profile suggestive of combined dysfunctions of short- and long-chain FAO.

DISCUSSION

In this study, only the acylcarnitines accumulating in the reaction media were analyzed, in contrast to previous studies where cells plus media were used.[21,22] The acylcarnitines are derived from the acyl-CoA esters, catalyzed by the carnitine acyltransferases in the presence of excess L-carnitine. These acylcarnitines leave mitochondria via the reverse action of carnitine acylcarnitine translocase,[23] and exit the cell cytosol possibly with the aid of the plasma membrane carnitine transporter,[24] as indicated by the appearance of acylcarnitines in the blood of patients with FAO defects during metabolic stress. Substantial levels of intracellular acylcarnitines have been found upon in vitro analysis of rat hepatocytes[25] and fibroblasts of normal controls.[26] Analyzing the acylcarnitines in the reaction media without the cells would appear to exclude these intracellular "background levels" of acylcarnitines. Our approach may improve the diagnostic sensitivity of the assay, as indicated by the unique profile of CPT IA deficiency,[27] which has been previously reported to be indistinguishable from controls.[22]

The acylcarnitine profile observed in CPT IA deficiency is different from that of other FAO defects in that all $^2H_5$-labeled acylcarnitines were reduced instead of elevated. All other FAO-deficient cell lines revealed elevation of acylcarnitines related to the site(s) of metabolic block, findings similar to other reports.[16,19-22] The same enzyme defects exhibited disease-specific acylcarnitine profiles (analyte concentrations >1.96 SD of control mean), irrespective of severity of symptoms (CPT II, CACT, VLCAD) or different mutations (LCHAD, VLCAD, MCAD). Hence, in vitro acylcarnitine profiling is a useful test for selected patients suspected with FAO when in vivo metabolite findings are not conclusive.

Of the 16 cell lines from patients with RC defects (Table 2), 8 showed acylcarnitine profiles similar to controls, and the other 8 exhibited abnormal profiles mimicking several enzyme defects in FAO. There was no clear association of a characteristic acylcarnitine profile with a specific respiratory chain defect (R10 to R14). Acylcarnitine abnormalities were seen in some cell lines with defects caused by mutations in the nuclear-encoded DNA (R8) or mitochondrial DNA (R3, R4, and R12).

The principal function of FAO is generation of acetyl-CoA, and that of the RC is the production of adenosine triphosphate (ATP). Functional defects in RC should not be associated with acylcarnitine abnormalities theoretically, as acyl-CoA esters are not the intermediate substrates. The observation of abnormal acylcarnitine profiles could be related to the linkages of L-3-hydroxyacyl-CoA dehydrogenases to complex I via NADH, and the acyl-CoA dehydrogenases to ubiquinone via electron transfer flavoprotein (ETF) and ETF dehydrogenase, and the reversible reactions between them. A primary functional defect of the RC resulting in dysfunction of these dehydrogenases in FAO is evident by accumulation of long-chain acyl-CoA esters, their L-3-hydroxyacyl derivatives and acylcarnitines as has been demonstrated in rat heart mitochondria, although high levels were not reached.[28] However, the abnormal metabolites thus accumulated could further impair oxidative phosphorylation,[29] and exacerbate the FAO dysfunction by the inhibitory effects of acylcarnitines on CACT,[30] and L-3-hydroxyacyl-CoA on enoyl-CoA hydratases.[31] The latter reaction could lead to accumulation of 2-enoyl-CoA, which has been shown to inhibit the acyl-CoA dehydrogenases.[32] These and other genetic factors may explain the variable and unpredictable acylcarnitine profiles observed in fibroblasts from patients with primary RC defects.

The profile of the complex IV-deficient cell line (R8) indicating general dysfunction of numerous enzymes which act on medium- and long-chain acyl-CoA is probably not surprising. However, it is not clear how a deficiency in complex I may be related to elevation of $^2H_5$-C_4-acylcarnitine (R3, R4), instead of the hydroxy-acylcarnitines. Complex II performs a key step in the citric acid cycle, in which succinate is dehydrogenated to fumarate. In turn, the citric acid cycle is linked to FAO via acetyl-CoA, the end product of thiolytic cleavage. The predominant accumulation of C_8-acylcarnitine suggesting a specific inhibition of MCAD in the fibroblasts of this complex II-affected patient (R6) is unexpected, especially since the complex II activity was not abnormal in skin fibroblasts, but deficient in liver. There was in fact no association between the expression of the RC defect in skin fibroblasts and a normal or abnormal acylcarnitine profile (data not shown), but our profile results were reproducible. It is possible that these cell lines harbor 2 genetic defects,[33] but the occurrence of abnormal findings in 8 of 16 patients studied seemed too high for this possibility.

Mitochondrial β-oxidation disorders described to date show autosomal-recessive inheritance (nuclear encoded), and may be potentially fatal if undetected. However, in most diagnosed cases the prognosis is generally favorable,[34] even in patients with 2 null mutations.[35] Moreover, treatment is relatively simple, involving avoidance of fasting together with dietary therapy and L-carnitine in some disorders. In contrast, defects in RC can be caused by mutations in nuclear or mitochondrial DNA, and the inheritance patterns may be autosomal (recessive or dominant), or maternal (mitochondrial). Treatment in most individuals has generally been ineffective[34,36] and the prognosis especially in early onset defects is poor, making prenatal diagnosis and genetic counseling particularly important.

In summary, in vitro acylcarnitine profiling in fibroblasts is a useful test for the detection of patients suspected with FAO disorders. However, there are limitations. Neither defects in plasma membrane cellular uptake of long-chain fatty acids[37] nor in ketogenesis not involving acyl-CoA intermediates would be detected. Tissue-specific enzymes that are not expressed in fibroblasts would also be missed. Moreover, abnormal acylcarnitine profiles may also be detected in fibroblasts from patients with RC defects. Whereas the clinical and biochemical abnormalities of FAO and RC defects may be similar, patient management, prognosis and genetic counseling are vastly different. Awareness of this diagnostic pitfall enables the appropriate selection of follow-up confirmatory tests and assists in targeted therapeutic strategies. Integrated evaluation of clinical information, in vivo and in vitro findings, histopathology, enzymology, and molecular studies are required for accurate diagnosis.

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