Normalization of Short-Chain Acylcoenzyme A Dehydrogenase after Riboflavin Treatment in a Girl with Multiple Acylcoenzyme A Dehydrogenase–deficient Myopathy

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A 12-year-old girl was shown to have carnitine-deficient lipid storage myopathy and organic aciduria compatible with multiple acylcoenzyme A (acyl-CoA) dehydrogenase deficiency. In muscle mitochondria, activities of both short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD) were 35% of normal. Antibodies against purified SCAD, MCAD, and electron-transfer flavoprotein were used for detection of cross-reacting material (CRM) in the patient's mitochondria. Western blot analysis showed absence of SCAD-CRM, reduced amounts of MCAD-CRM, and normal amounts of electron-transfer flavoprotein–CRM. The patient, who was unresponsive to treatment with oral carnitine, improved dramatically with daily administration of 100 mg oral riboflavin. Increase in muscle bulk and strength and resolution of the organic aciduria were associated with normalization of SCAD activity and "reappearance" of SCAD-CRM. In contrast, both MCAD activity and MCAD-CRM remained lower than normal. These results suggest that in some patients with multiple acyl-CoA dehydrogenase deficiency riboflavin supplementation may be effective in restoring the activity of SCAD, and possibly of other mitochondrial flavin-dependent enzymes.

DiDonato S, Gellera C, Peluchetti D, Uziel G, Antonelli A, Lus G, Rimoldi M. Normalization of short-chain acylcoenzyme A dehydrogenase after riboflavin treatment in a girl with multiple acylcoenzyme A dehydrogenase–deficient myopathy. Ann Neurol 1989;25:479–484

The acylcoenzyme A (acyl-CoA) dehydrogenase deficiencies include the inborn defects of the enzymatic system catalyzing the oxidation of straight-chain acyl-CoAs, branched-chain acyl-CoAs, and glutaryl-CoA [1]. Individual genetic defects of long-chain (LCAD), medium-chain (MCAD), and short-chain (SCAD) acyl-CoA dehydrogenases have been reported in humans [2–5]. Most patients have been infants with nonketotic hypoglycemia, organic aciduria, hepatomegaly, and secondary carnitine deficiency. Prognosis is poor. A few late-onset patients have been described; in some of them, the clinical picture was characterized by an isolated carnitine-deficient lipid storage myopathy [6, 7].

Genetic defects of the electron-transfer flavoprotein (ETF) or ETF dehydrogenase (ETF-DH) have been reported in several cases of so-called multiple acyl-CoA dehydrogenase (MAD) deficiency [8, 9]. Glutaric aciduria type II, the laboratory hallmark of the disease, is associated with acute neonatal acidosis and low tissue and plasma carnitine. In most patients, death occurs in infancy. Oxidations of several substrates specifically metabolized by the acyl-CoA dehydrogenases are defective in cultured fibroblasts [8]. A milder, later-onset form of MAD deficiency is characterized by ethylmalonic-adipic aciduria [10].

Clinical improvement of MAD deficiency after riboflavin supplementation has been described [11] in several patients affected by either the infantile [12, 13] or the adult myopathic form [14]. Autosomal inheritance was evident in some of these patients [15]. Among the possible explanations of this riboflavin-responsive MAD deficiency are abnormalities of mitochondrial transport of flavin adenine dinucleotide (FAD) or defects in the binding of FAD to ETF or to ETF-DH [11].

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Received Jun 6, 1988, and in revised form Aug 12 and Oct 27. Accepted for publication Nov 4, 1988.

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In this paper, we describe a case of MAD deficiency in which low SCAD and MCAD activities in muscle were increased by riboflavin supplementation.

Materials and Methods

Case Report

The proposita is a 12-year-old girl born to consanguineous parents (their great-grandparents were cousins). Her brother died at age 8 years of an acute illness characterized by mental confusion, hypotonia, muscle weakness, and hepatomegaly. A 13-year-old sister is healthy.

The patient was apparently normal until age 9 years, when she started complaining of easy fatigability and muscle weakness. "Hepatitis" was diagnosed because of high levels of transaminases in her serum. Administration of multiple vitamins had no effect on muscle symptoms. A muscle biopsy (first biopsy) taken at age 10 showed lipid storage in muscle. Biochemically, total carnitine content in muscle was markedly low (see Results), and excretion of ethylmalonic acid in the urine was increased (Table 1, Sample 1). Treatment with 2 gm of oral L-carnitine per day was started with no beneficial effects in the following months. At age 11½ years the patient was admitted to the Instituto Neurologico (Milan) because of a general malaise and severe weakness.

Physical examination showed a thin, pale girl in poor general condition, with moderate hepatomegaly, abdominal-type breathing, and generalized muscle weakness. The oropharyngeal and upper trunk muscles were predominantly affected. Some degree of muscle wasting was also present. Deep tendon reflexes were absent in both arms. Serum glutamic oxaloacetic transaminase activity was 380 U (normal, < 60); serum glutamic pyruvic transaminase was 440 U (normal, < 40); lactate dehydrogenase was 3,300 U (normal, < 240); and creatine kinase was 1,100 U (normal, < 120). Blood glucose was occasionally low (61–63 mg/dl); blood lactate and pyruvate were normal. On one occasion, overnight fasting ketones were abnormally high: β-hydroxybutyrate was 1,330 μEq/L (normal range, 20–120 μEq/L); acetoacetate was 487 μEq/L (normal range, 20–100 μEq/L); ratio of β-hydroxybutyrate to acetoacetate was 2.73 (normal ratio < 3). The 24-hour urinary excretion of organic acids had a pattern corresponding to glutaric aciduria type II and compatible with the diagnosis of MAD deficiency (see Table 1, Sample 2). Total plasma carnitine was high-normal, but esterified carnitines were markedly increased (see Results). Because of rapidly progressive clinical deterioration, oral riboflavin (100 mg/day) was added to the carnitine therapy. One week later, only ethylmalonic and methylsuccinic acids were elevated in the urine (see Table 1, Sample 3). During the 3 following weeks, the patient achieved dramatic clinical improvement with marked increase of muscle strength. A second muscle biopsy performed at this time showed that total carnitine in muscle was increased, although it was still low compared with controls (see Results). She left the hospital 40 days after admission in good clinical condition. Laboratory examinations were normal. Six months later, while still taking riboflavin and carnitine, she was in good health, muscle strength was normal, and organic acids were absent in the urine (see Table 1, Sample 4). A third muscle biopsy was taken for biochemical studies (see Results).

Table 1. Urinary Organic Acids[a] in the Patient

	Sample[b]				
Acid	1	2	3	4	Controls
Lactic	128	170	38	23	<90
Pyruvic	42	73	13	11	<20
3-Hydroxybutyric	135	631	139	83	<90
Ethylmalonic	37	65	53	ND	< 5
Succinic	71	47	56	14	<90
Methylsuccinic	13	27	22	ND	< 5
Glutaric	18	542	13	3	< 5
Adipic	10	3,011	18	10	<25
Pimelic	ND	131	5	ND	< 1
Suberic	15	485	10	8	<20
Azelaic	ND	38	ND	ND	< 1
Sebacic	10	146	8	11	<20
2-Hydroxyglutaric	237	841	134	32	<40
2-Hydroxyadipic	49	950	32	5	<25
Isovaleryl-glycine	ND	77	ND	ND	ND
Hexanoyl-glycine	ND	123	15	ND	ND

[a]Values represent μg/mg creatinine.
[b]Sample 1 urine collected before admission; Sample 2 urine collected at admission during a metabolic attack; Sample 3 urine collected after 1 week of riboflavin supplementation; Sample 4 urine collected after 6 months of riboflavin supplementation.

ND = not detectable.

Reagents

Ready-Solv MP scintillation liquid was from Beckman Analytical, Milan, Italy. 1-^{14}C-octanoate (30 mCi/nmol) was from NEN Research Products, Florence, Italy. 1-^{14}C-pyruvate (20 mCi/nmol), 1-^{14}C-palmitate (50 mCi/nmol), and 1-^{14}C-butyrate (50 mCi/nmol) were from Amersham International, Buckinghamshire, UK. Anti-rabbit immunoglobulin-conjugated alkaline phosphatase was from BioRad, Lab SRL, Milan, Italy. Butyryl-CoA, octanoyl-CoA, palmitoyl-CoA, and all the other high-grade reagents were from Sigma Chemicals Co, St Louis, MO.

Laboratory Studies

Plasma and muscle free, short-chain, long-chain, and total carnitines were measured as previously described [16]. Organic acids in 24-hour urinary samples were analyzed by capillary gas-liquid chromatography as described [17]. Fibroblasts were cultured in modified Eagle minimal essential medium supplemented with 10% fetal calf serum. Substrate oxidation rates were measured in cells between the sixth and fifteenth passages [18].

Muscle biopsy specimens (2 gm each) were obtained from the quadriceps femoris. Approximately 1 gm of each specimen was used to isolate mitochondria [19]. The remaining portion was used for morphological studies, carnitine determination, and oxidation rate studies on muscle homogenates [17].

Antibodies Against Pig Liver ETF, SCAD, and MCAD

Highly purified ETF, SCAD, and MCAD were obtained from pig liver mitochondria essentially as described by Hu-

Table 2. Acyl-CoA Dehydrogenase Activities[a] in Muscle Post-Mitochondrial Extract

Biopsy[b]	SCAD	SCAD[c]/CS	MCAD	MCAD[c]/CS	LCAD	LCAD[c]/CS
Second	4.0	3.3	13.2	10.9	13.5	11.1
Third	7.1	9.9	10.7	15.0	9.7	13.6
Controls	9.0 ± 2.8	8.9 ± 2.8	40.1 ± 10.7	31.7 ± 10.1	16.5 ± 9.3	12.7 ± 6.4

[a]Values are expressed as nmol/min/mg protein.
[b]Second biopsy taken after 3 weeks of riboflavin supplementation. Third biopsy taken after 6 months of riboflavin supplementation.
[c]Flavoprotein dehydrogenase activity/citrate synthetase activity × 1,000.

Acyl-CoA = acylcoenzyme A; SCAD = short-chain acyl-CoA dehydrogenase; CS = citrate synthetase; MCAD = medium-chain acyl-CoA dehydrogenase; LCAD = long-chain acyl-CoA dehydrogenase.

sain and Steenkamp (for ETF) [20] and by Ikeda and colleagues (for MCAD and SCAD) [21]. Antibodies against each enzyme protein were raised in rabbits by intradermal injection of 400 μg of purified MCAD, SCAD, or ETF mixed with complete Freund's adjuvant. A booster injection of 400 μg protein in incomplete Freund's adjuvant was given after 3 weeks and again after 6 weeks. A week later the animals were bled, and antisera were stored frozen in small aliquots. Both the monospecificity of each antiserum and its good cross-reactivity against the corresponding human antigen were demonstrated by Western blot and inhibition curve analysis, using either pig or human liver mitochondria.

Soluble proteins of the matrix were obtained by ultracentrifugation of sonicated mitochondria at 100,000 *g* for 1 hour. Protein aliquots corresponding to 50 mU citrate synthetase activity (approximately 50–100 μg protein) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [22] and blotted onto nitrocellulose filters as described [23]. The filters were incubated for 30 minutes at room temperature with a solution containing 5% nonfat dry milk in 50 mM TRIS-HCl, 100 mM NaCl, pH 7.5 (buffer A). The sheets were then incubated for 1 hour at room temperature with anti-SCAD, anti-MCAD, or anti-ETF antibodies diluted in buffer A. Dilutions were 1:50, 1:200, and 1:200, respectively. After two 5-minute washings with TRIS-buffered saline solution, 0.1% Tween-20 (buffer B), the sheets were incubated for 30 minutes at room temperature with alkaline-phosphatase goat anti-rabbit immunoglobulin G diluted 1:3,000 in buffer A. After two washings as above, the staining solution was added, containing 0.6 mg/ml tetrazolium *p*-nitro blue and 0.3 mg/ml 5-bromo-4-chloro-3-indolyl phosphate-toluidine in 0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8. The reaction was allowed to proceed for 15 minutes and was stopped by washing the sheets repeatedly with distilled water.

Results

Lipid storage in muscle fibers was a prominent feature in the first muscle biopsy (performed at age 9 years), and it was still present in the second biopsy, performed at age 11 years, 3 weeks after the beginning of treatment with riboflavin. However, after 6 months of oral riboflavin administration, light and electron microscopy examinations of the third biopsy specimens showed the virtual normalization of muscle morphological features (data not shown).

The total carnitine content in muscle increased from 2.7 nmol/mg noncollagen protein (NCP) (normal, 22.0 ± 8.9 [SD]) in the first biopsy, performed before carnitine supplementation, to 14.6 nmol/mg NCP in the second biopsy. It declined to 8.5 nmol/mg NCP in the third biopsy specimen. Carnitine esters were normal in all three biopsy specimens.

Under oral carnitine supplementation, total plasma carnitine was 77.6 nmol/ml, and carnitine esters were as high as 48.3 nmol/ml (normal, 53.0 ± 12.9 [SD] and 7.8 ± 4.9, respectively).

Since low muscle carnitine can be due to MAD deficiency, we investigated the ability of the patient's muscle to oxidize fatty acids in vitro. In the second biopsy specimen, oxidation rates for butyrate and octanoate were 16 pmol CO_2 produced/min/mg NCP (normal, 39 ± 12 [SD]) and 13 pmol/min/mg NCP (normal, 23 ± 8), respectively. In the third biopsy specimen, they normalized to 29 pmol/min/mg NCP for butyrate, and 22 pmol/min/mg NCP for octanoate. Oxidation rates for pyruvate and palmitate were low-normal in the second biopsy specimen and did not change significantly in the third. In addition, we measured directly the enzymatic activities of SCAD, MCAD, LCAD, and citrate synthetase in isolated muscle mitochondria. Both SCAD and MCAD were markedly decreased in mitochondria obtained at the second biopsy (Table 2). Accordingly, a low ratio (approximately 35% of the mean value of controls) was obtained between SCAD and MCAD activities versus activity of citrate synthetase, the latter being an index of mitochondrial abundance in our preparations.

In the third biopsy, however, the SCAD–citrate synthetase ratio increased to normal values. MCAD–citrate synthetase ratio, although still low, was also increased to 50% of the control values (see Table 2). LCAD activity was normal in both muscle biopsies. To understand whether the observed defects of SCAD and MCAD activities were associated with decreased amounts of the enzyme proteins, titration of the corresponding cross-reacting materials (CRM) was performed by Western blot analysis. Figures 1 and 2 show the results of Western-blotted mitochondrial proteins from the patient and controls immunostained with our anti-SCAD, anti-MCAD, and anti-ETF monospecific

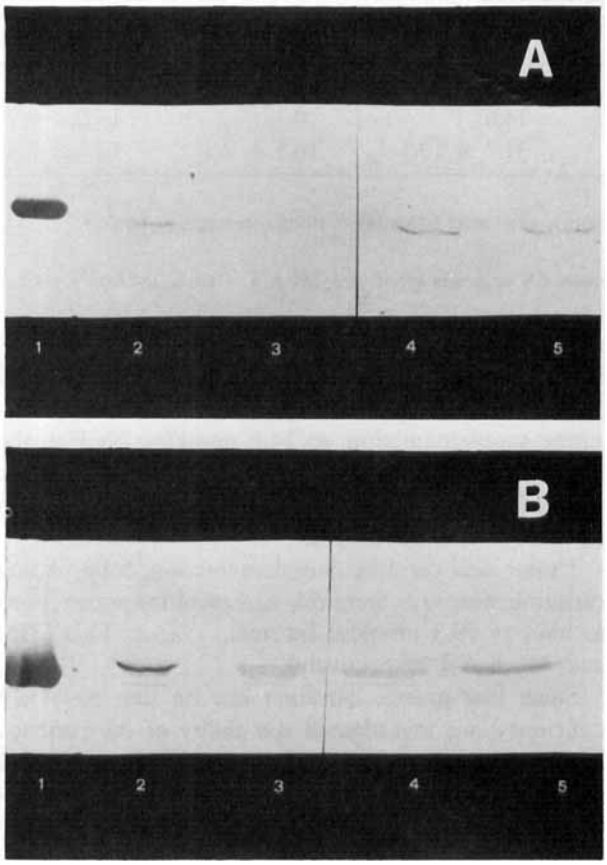

Fig 1. Short-chain (SCAD) (A) and medium-chain (MCAD) (B) acylcoenzyme A dehydrogenase immunoblotting of mitochondrial extracts from patient muscle before and after riboflavin supplementation. Immunoblots were revealed with anti-rabbit alkaline phosphatase–conjugate immunoglobulin G. In each lane the amount of 100,000 g mitochondrial extract seeded was adjusted to citrate synthetase activity (mitochondrial protein seeded = 50 mU of citrate synthetase activity). Lane 1 = purified pig liver SCAD (A) or MCAD (B); lane 2 = Control 1 mitochondria; lane 3 = patient mitochondria (second biopsy); lane 4 = patient mitochondria after 6 months of oral riboflavin supplementation (third biopsy); lane 5 = Control 2 mitochondria.

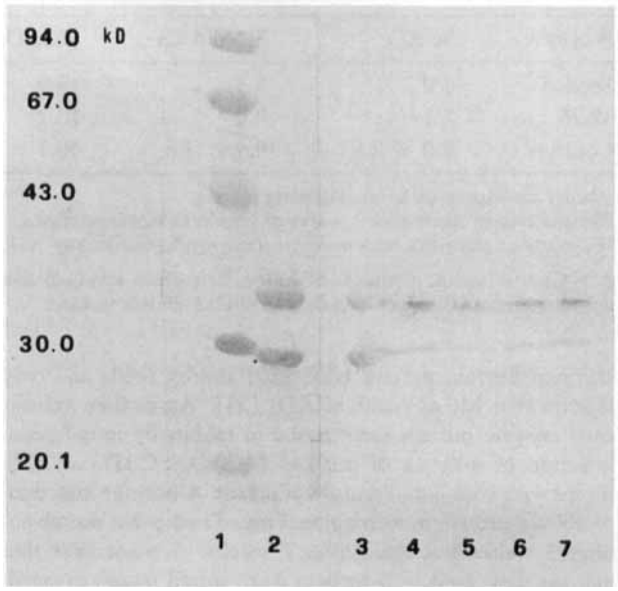

Fig 2. Electron-transfer flavoprotein (ETF) immunoblotting of mitochondrial extracts from patient muscle before and after riboflavin supplementation. Lanes 1 and 2 represent coomassie blue–stained molecular weight markers (lane 1) and purified pig liver ETF (lane 2). In lanes 3 to 7 ETF immunoblots were revealed with anti-rabbit alkaline phosphatase–conjugate immunoglobulin G. In each lane the amount of 100,000 g mitochondrial extract seeded was adjusted to citrate synthetase activity (mitochondrial protein seeded = 50 mU of citrate synthetase activity). Lane 3 = purified pig liver ETF; lane 4 = control 1 mitochondria; lane 5 = patient mitochondria (second biopsy); lane 6 = patient mitochondria after 6 months of oral riboflavin supplementation (third biopsy); lane 7 = Control 2 mitochondria.

antibodies. SCAD-CRM, which was virtually undetectable in mitochondria from the second biopsy specimen, was found in normal amounts in the third biopsy specimen, taken after 6 months of riboflavin treatment (see Fig 1A). On the other hand, in both specimens, MCAD-CRM gave a lower-than-normal reaction (see Fig 1B), and ETF-CRM was in the normal range (see Fig 2).

Finally, oxidation rates on fibroblasts grown in a riboflavin-containing medium (0.1 mg/L) were similar in the patient's and controls' cells. In particular, pyruvate was 106 nmol labeled CO_2/hr/mg cell protein (normal, 72 ± 16 [SD]); butyrate was 4.6 nmol/h/mg (normal, 3.0 ± 0.6); octanoate was 1.4 nmol/h/mg (normal, 1.0 ± 0.4); and palmitate was 5.5 nmol/h/mg (normal, 5.6 ± 1.0).

Discussion

Riboflavin-responsive MAD deficiency was described in infants affected by Reye's-like syndrome and glutaric aciduria type II [11]. In some infants, however, ethylmalonic-adipic aciduria was the most prominent feature, while glutaric acid was present only during metabolic crises (i.e., riboflavin-responsive ethylmalonic aciduria) [24]. A third phenotype, riboflavin-responsive lipid storage myopathy, has been reported in 2 patients [14, 25]. In one of them, β-oxidation was impaired in fibroblasts cultured in the absence of riboflavin [26].

The girl described in this paper had lipid myopathy, low muscle carnitine, decreased β-oxidation in muscle, and normal β-oxidation in cultured fibroblasts and was highly responsive to riboflavin supplementation. Her muscle showed reduced ability to oxidize butyrate and octanoate in vitro. This was associated with markedly reduced activities of both SCAD and MCAD in isolated mitochondria, while LCAD activity was nor-

mal. Low enzymatic activities were associated with the virtual absence of SCAD-CRM and with a less striking, yet evident, decrease in MCAD-CRM in mitochondria. Similar findings are observed in the experimental riboflavin deficiency of the rat [27]. In a detailed study [28], Hoppel and coworkers showed that riboflavin deprivation in the weanling rat was associated with impaired β-oxidation of fatty acids in the liver. The deficiency of β-oxidation was accompanied by decreased activities of acyl-CoA dehydrogenases in mitochondria, SCAD being the most severely affected [28]. The biochemical data from muscle mitochondria from our patient are in agreement with these experimental observations, because after riboflavin supplementation SCAD activity and SCAD-CRM returned to normal. This was associated with a significant increase in MCAD activity.

The multiple organic aciduria found in our patient can be explained either by a combined deficiency of the primary dehydrogenases (LCAD, MCAD, and SCAD) or by a defect of ETF or ETF-DH activity. Although the latter possibility cannot be ruled out (ETF and ETF-DH activities could not be assayed in the patient's mitochondria), we consider it unlikely because, in contrast with what we found for SCAD and MCAD, the mitochondria of our patient contained normal amounts of ETF-CRM.

Most likely, this patient is affected by an abnormality of riboflavin metabolism causing low availability of FAD in mitochondria. Low intramitochondrial FAD content could affect the metabolism of the flavoprotein enzymes either by down-regulating their synthesis or by increasing their breakdown, or both. The different effects of riboflavin deficiency on SCAD, MCAD, and possibly ETF and ETF-DH could be explained by the different affinities of FAD for the flavoprotein apoenzymes [21, 29].

Clinically, our patient was affected by carnitine-deficient lipid storage myopathy. Biochemically, this phenotype can be associated with several defects of β-oxidation. These include generalized and muscle-specific SCAD deficiency [6, 7, 30], MCAD deficiency [31], ETF-DH deficiency [17], and riboflavin-responsive MAD deficiency [14]. Generalized SCAD deficiency is probably genetic in origin, because the defect is present in different tissues as well as in cultured fibroblasts [6]. On the other hand, muscle-specific SCAD deficiency [7, 30] may not be an independent biochemical entity. Supporting this view is the lack of tissue-specific SCAD isoenzymes [21]. Instead, it has recently been suggested that patients affected by myopathy and SCAD deficiency may suffer either from the generalized form of SCAD deficiency or from riboflavin-responsive MAD deficiency. In patients suffering from the latter, expression of the defect would predominantly affect SCAD [6]. We think that this was indeed the case in the 1 patient previously described by us as having muscle-specific SCAD deficiency [30].

Riboflavin-responsive MAD deficiency, however, is likely to be a heterogeneous biochemical entity. We studied a second case with carnitine-deficient lipid storage myopathy, low SCAD and MCAD activities, and low SCAD-CRM and MCAD-CRM in muscle. In contrast to the case presented here, the biochemical data in the latter patient were unchanged after long-term treatment with riboflavin, in spite of dramatic improvement in the clinical picture [32].

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