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Role of short-chain hydroxyacyl CoA dehydrogenases in SCHAD deficiency

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Abstract

Short-chain hydroxyacyl CoA dehydrogenase deficiency is an ill-defined, severe pediatric disorder of mitochondrial fatty acid β-oxidation of short-chain hydroxyacyl CoAs. To understand the relative contributions of the two known short-chain hydroxyacyl CoA dehydrogenases (HADH) tissue biopsies of six distinct family individuals were analyzed and kinetic parameters were compared. Steady-state kinetic constants for HADH 1 and HADH 2 suggest that type 1 is the major enzyme involved in mitochondrial β-oxidation of short-chain hydroxyacyl-CoAs. Two patients are heterozygous carriers of a HADH 1 polymorphism, whereas no mutation is detected in the HADH 2 gene of all patients. The data suggest that protein interactions rather than HADH mutations are responsible for the disease phenotype. Pull-down experiments of recombinant HADH 1 and 2 with human mitochondrial extracts reveal two proteins interacting with HADH 1, one of which was identified as glutamate dehydrogenase. This association provides a possible link between fatty acid metabolism and the hyperinsulinism/hyperammonia syndrome.

Keywords: β-Oxidation; Hydroxyacyl-CoA dehydrogenase deficiency; Sudden infant death syndrome; Hydroxyacyl CoA dehydrogenase; Hyperinsulinism/hyperammonemia syndrome

Mitochondrial fatty acid β-oxidation constitutes the essential physiological response to energy depletion caused by fasting, severe febrile illness or increased muscular activity. This pathway results in production of acetyl-CoA by the sequential oxidation and cleavage of straight-chain fatty acids. Importantly, hepatic β-oxidation provides the source of energy for extrahepatic tissues through ketone body formation upon fasting [1].

The complexity of fatty acid oxidation (FAO) disorders has been highlighted by genetic analysis of patients with symptoms of fasting intolerance such as hypoglycemia and manifestations like encephalopathy, myoglobinuria, rhabdomyolysis, cardiomyopathy or liver steatosis [1–8]. Various genetic or functional defects of fatty acid transport or utilization have been identified contributing to sudden infant death syndrome (SIDS) or fatty liver of pregnancy [1,6].

L-3-Hydroxybutyryl CoA dehydrogenase or SCHAD/HADH (short-chain hydroxyacyl CoA dehydrogenase) catalyzes the penultimate step in β-oxidation. SCHAD deficiency has only recently been described and is characterized by an urinary organic acid profile with elevated

Abbreviations: SIDS, sudden infant death syndrome; FAO, fatty acid oxidation; SCHAD, short-chain hydroxyacyl CoA dehydrogenase.

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medium-chain dicarboxylic and 3-hydroxydicarboxylic metabolites, and by lowered C4 hydroxyacyl CoA dehydrogenase activity in tissue biopsies [1,2]. Family histories suggest a genetic defect with an autosomal recessive transmission in some cases [1,2]. Hyperinsulinemia was observed in several patients in association with hypoglycemia indicating a unique involvement between fatty acid β-oxidation and insulin regulation [4,5,9].

Two different enzymes with L-3-hydroxybutyryl CoA dehydrogenase activity (HADH 1 and HADH 2) and wide, overlapping tissue-expression have been described [10–13]. HADH 1 constitutes the well-characterized L-3-hydroxybutyryl CoA dehydrogenase, isolated from heart and liver [10,12], with the gene located on chromosome 4q22-q26. HADH 2 is a short-chain hydroxyacyl CoA dehydrogenase [11], first isolated from bovine liver [14], and localized in humans to chromosome Xp11.2. This isoform was initially identified as an amyloid β-peptide binding protein, catalyzing the NAD^+-dependent oxidation of straight and branched short-chain hydroxyacyl CoAs [11,15,16]. Mutations in the HADH 2 gene were found to cause a novel inborn error of isoleucine metabolism with a distinct pattern of neurodegenerative symptoms [15,17]. The enzymes belong to distinct protein superfamilies [10,18,19]. Knock-out models for HADH I and II have both been described, defining the metabolic roles of these enzymes. There are symptoms of fasting-induced hypoglycemia in the HADH I knock-out and development of steatosis in both models [20]. These demonstrated defects at an enzymatic level and the correlation of clinical and animal model phenotypes, prompted us to compare enzymological properties of human HADH I and II as candidate genes for enzymatic SCHAD deficiency.

Materials and methods

Clinical features of SCHAD deficiency patients. Clinical parameters for patients 1–3 have been previously given [2], all demonstrated ketosis and abnormal medium-chain 3-hydroxydicarboxylic aciduria. In a follow-up of patient 1, a sibling was born in whom metabolic studies performed at birth were non-diagnostic. However, this patient followed a similar clinical course to the index case and also died unexpectedly. Permission for autopsy was not granted and enzymology could not be performed. Case 4 was a male, the first born of non-consanguineous Caucasian parents. Pregnancy was uneventful and the child normal at birth. He died unexpectedly at five days of age. Metabolic studies were not performed. At autopsy, he was noted to be mildly jaundiced and to have moderate to severe fatty infiltration of the liver with macro- and microvesicular steatosis. Measurement of enzyme activities revealed a marked deficiency of C4-hydroxyacyl CoA dehydrogenase activity (Table 1). Case 5 was a female who presented at nine months of age with intussusception, developmental delay and liver cirrhosis. This progressed to liver failure, requiring transplantation from which she did not recover. Metabolic studies suggested LCHAD deficiency, however LCHAD activity was elevated in skeletal muscles samples (Table 1), and interestingly a marked deficiency of C4 HAD activity were found. Activities of long and short-chain keto-acyl thiolases were normal in muscle. Skin fibroblasts were studied enzymatically which revealed normal activities. Case 6 was a 6-week-old male who died suddenly during sleep. The family history was remarkable for two previous sibling deaths attributed to SIDS. This strong family history prompted further enzymatic evaluation in this case. Liver enzymes revealed a modest reduction in the C16 HAD activity and a marked deficiency of C4 HAD.

Table 1
Summary of hydroxyacyl CoA dehydrogenase activity measurements in patients with suspected SCHAD deficiency

Case	Tissue	C16	C4	C4:C16
1	Liver	446	115	0.26
2	Liver	176	30	0.17
3	Liver	220	63	0.29
4	Liver	148	162	1.09
5	Muscle	296	182	0.61
6	Liver	233	104	0.44
Control	Liver	300 ± 14	839 ± 183	2.79 ± 0.48
Control	Muscle	182 ± 10	844 ± 17	4.57 ± 0.31

Activities (C16 palmitoyl CoA, C4 butyryl CoA), dimensions nmol · min^{-1} mg $protein^{-1}$ were measured as described in the Experimental section. Control activities in liver (sample number $n = 50$) and skeletal muscle ($n = 5$) were performed on biopsy samples from apparently healthy subjects.

Metabolite analysis and measurement of SCHAD enzyme activities. Urine or plasma samples were analyzed by tandem mass spectrometry. Enzyme activities for C4 and C16 OH-acyl CoAs were determined in tissue homogenates as described [2].

Sequence analysis of human SCHAD I and SCHAD II genes. Genomic DNA from patient samples was extracted using Genomic DNA Isolation kit (Amersham Pharmacia Biotech). Using the primer sets outlined in Supplementary Table S1, exon sequences with neighbouring intron sequences of SCHAD 1 and SCHAD 2 genes were amplified by PCR using TaqPlus long (Stratagene) or HotStar Taq (Qiagen).

Western blot analysis. Patient samples were analysed by SDS/PAGE (12%) followed by transfer to nitrocellulose membranes. Membranes were incubated with immunopurified anti-SCHAD II rabbit antibodies (against SCHAD II protein, absorbed on membrane). Detection was achieved with the ECL system (Amersham Pharmacia Biotech).

Determination of kinetic constants for recombinant human SCHAD I and SCHAD II. Enzyme activities were measured as NAD(H)-dependent hydroxybutyryl-CoA and acetoacyl-CoA (Sigma) activities by determination of the change of absorbance at 340 nm, using a molar extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. Recordings were carried out with a Cary 300Bio instrument. Reactions were performed in 1.0 ml at 25°C. Kinetic constants were calculated from initial velocity data by linear regression analysis using Hanes or Eadie-Hofstee plots.

Isolation and characterization of SCHAD binding proteins. Human liver mitochondria were isolated from liver as described [21]. Solubilization was carried out using different concentrations of non-ionic detergents (Triton X-100, Tween 20; concentration range 0.5–2%) and varying concentrations and ratios of the ionic detergent sodium taurodeoxycholic acid (TDCA, 0.1–0.5%). Aliquots (60 μl) of these preparations were incubated for 30 min on ice with purified recombinant SCHAD I or SCHAD II (20 μg/ml). As negative control recombinant 3β/17β-hydroxysteroid dehydrogenase was used [18]. His-bind resin (100 μl in equilibration buffer (20 mM MOPS, 0.1% TDCA, pH 7.4) was added, and the mixture was incubated for 10 min. Washing steps with 10 and 60 mM imidazole in equilibration buffer were performed twice, before complexes were disrupted by boiling in SDS sample buffer, followed by SDS/PAGE on 12% gels. Bands were excised and analyzed by tryptic digestion, followed by MALDI-TOF mass spectrometry (Voyager DE Pro, PE Biosystems).

Results

Establishment of short-chain hydroxyacyl CoA dehydrogenase deficiency

Skeletal muscle and liver samples from deceased patients with possible short-chain dehydrogenase deficiency were

analyzed with proper ethical permits for long-chain (hydroxy-palmitoyl CoA, C16) and short-chain (hydroxy-butyryl-CoA, C4) hydroxyacyl CoA dehydrogenase activities (Table 1). Activities for long-chain OH-acyl CoA dehydrogenase measured were slightly lower (patients 2, 3, 4, 6) than control values, or in two subjects (patients 1 and 5) even elevated. In all cases a substantial reduction in short-chain hydroxyacyl CoA dehydrogenase activity was noted, ranging from 30 to 182 nmol/min mg protein (control values 839 ± 183 and 844 ± 17 for liver and skeletal muscle, respectively), accompanied by a concomitant decrease in the ratio of C4/C16 activities, ranging from 0.17 to 1.09 (control values 2.79 ± 0.48 and 4.57 ± 0.31 for liver and muscle, respectively). Taken together, the data provide compelling evidence for an underlying defect in short-chain hydroxyacyl CoA dehydrogenase activity.

Immunoblot analysis

Expression of SCHAD I was described earlier in patients 1–3, but experiments carried out in the Dallas laboratory also confirmed expression in patients 4–6 (unpublished data; M. Bennett, A. Strauss, L. O'Brien). Using affinity purified anti-SCHAD II antibodies, specific signals, and SCHAD II expression were detected in patients 1–4 (Fig. 1). Lack of signal for patients 5 and 6 was probably due to proteolytic degradation, observed in SDS/PAGE for these samples. Based on these data SCHAD I and II proteins are both expressed in liver and skeletal muscle tissues of the SCHAD deficient patients analyzed.

Kinetic analysis of recombinant human short-chain hydroxyacyl CoA dehydrogenases

The two isoforms of short-chain hydroxyacyl CoA dehydrogenases were expressed in *Escherichia coli*, purified

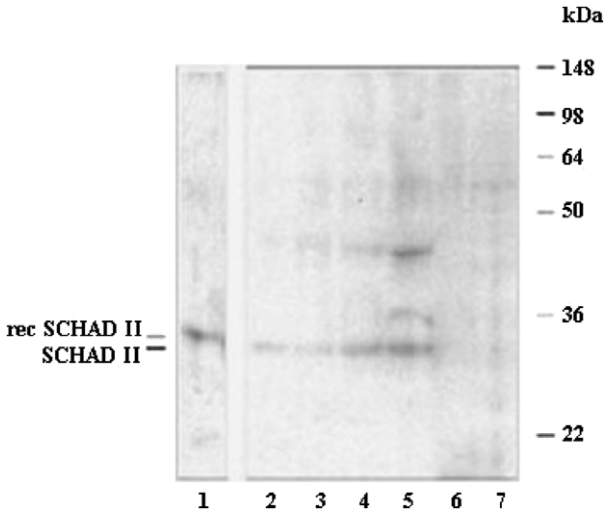

Fig. 1. Immunoblot analysis of tissue lysates resolved by SDS/PAGE and probed with immunopurified anti-SCHADII antibodies, revealing specific signals in patients 1–4. Proteolytic degradation observed in samples 5–6 resulted in lack of immunoreactivity. Recombinant SCHADII displays slightly lower electrophoretic mobility due to the His6-tag present.

to homogeneity (Supplementary Fig. S1), and the kinetic parameters K_M, k_{cat} and k_{cat}/K_M for the substrates acetoacetyl CoA, β-hydroxybutyryl CoA, NADH and NAD^+ were determined (Table 2). Kinetic constants were determined at two different pH values, at the enzyme-specific pH optimum (SCHAD I pH 7.0 for reduction and pH 8.5 for oxidation; SCHAD II pH 7.0 for reduction and pH 9.3 for oxidation) and at pH 8.0, the condition found within the mitochondrial matrix. Both enzymes efficiently catalyze the reversible reaction with short-chain hydroxy/keto-acyl CoAs with k_{cat}/K_M values for acetoacetyl CoA reduction of 32.8 ± 5.9 $10^6\,s^{-1}\,M^{-1}$ for SCHAD I and 0.677 ± 0.119 for SCHAD II, and values for hydroxybutyryl CoA dehydrogenation of 1.19 ± 0.2 and 0.09 ± 0.009 for SCHAD I and II, respectively, at pH 8.0 (Table 2). These values are in the same order of magnitude as the values reported from other laboratories for isozymes isolated from brain or heart of different species. These *in vitro* data clearly indicate a 40- to 50-fold higher catalytic efficiency of the SCHAD I isozyme for β-oxidation of short-chain hydroxy/keto acyl CoAs.

Sequence analysis of SCHAD I and II genes

Genomic DNA was extracted from SCHAD patients, and the complete exon sequences with adjacent intron boundaries were determined for SCHAD I and II genes. Whereas complete identity of SCHAD II to the wild-type allele was established in all 6 patients, a C257T (position in precursor cDNA) polymorphism in exon 2 in patients 2 and 5 was noted for SCHAD I. Both patients are heterozygous carriers with a wild-type allele and a polymorphic form at this position, as indicated by sequence signals for both alleles (Supplementary Fig. S2). Conceptual translation results in an amino acid exchange of Pro86 to Leu, the first residue located in helix α3 of the mature protein. This polymorphic allele is found with a frequency of 5–7% in a normal population (M. Bennett A. Strauss and L. O'Brien, unpublished results). These data indicate that the observed biochemical deficiency is not due to patient-specific mutations in type I or II SCHAD genes.

Protein–protein interactions of SCHAD proteins in liver mitochondria

Protein components in human liver mitochondria interacting with SCHAD proteins were determined in His-tag pull-down experiments *in vitro*. Complexes formed were resolved by SDS/PAGE and visualized by silver-staining (Fig. 2). Whereas no specific binding interactions with SCHAD II or the control were observed, SCHAD I bound specifically five distinct protein components present in human liver mitochondria. For three proteins the masses range between 32 and 37 kDa, whereas two display apparent masses of 60 and 70 kDa, respectively. The 32–37 kDa proteins were identified as proteolytic fragments of SCHAD I, whereas the 60 kDa component was identified

Table 2
Apparent steady-state kinetic constants for dehydrogenase and reductase activities of SCHAD I and SCHAD II

Substrate	Acetoacetyl-CoA			NADH			β-Hydroxybutyryl-CoA			NAD^+		
	K_M	k_{cat}	k_{cat}/K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M
SCHAD I pH optimum	16.6 ± 1.5	77.2 ± 5.9	77.5 ± 13.0	10.6 ± 1.3	21.5 ± 1.4	33.8 ± 6.4	22.5 ± 2.4	7.14 ± 0.75	5.29 ± 1.13	109 ± 11	7.76 ± 0.46	1.19 ± 0.20
pH 8.0	22.2 ± 2.6	40.9 ± 4.4	32.8 ± 5.9	Nd			32.0 ± 1.3	5.02 ± 0.18	2.61 ± 0.20	Nd		
SCHAD II pH optimum	25.7 ± 0.9	1.43 ± 0.07	0.927 ± 0.079	30.6 ± 1.5	1.65 ± 0.1	0.90 ± 0.1	85.2 ± 7.2	0.29 ± 0.01	0.058 ± 0.007	42.3 ± 1.5	0.23 ± 0.02	0.09 ± 0.008
pH 8.0	31.5 ± 2.8	1.28 ± 0.1	0.677 ± 0.11	Nd			38.6 ± 2.6	0.13 ± 0.02	0.058 ± 0.01	Nd		

Substrates used were acetoacetyl-CoA and β-hydroxybutyryl-CoA with NADH and NAD^+, respectively. K_M values are given in 10^{-6} M, k_{cat} values in 10^3 min^{-1} and k_{cat}/K_M values in 10^6 s^{-1} M^{-1}.
Values shown are the average of 3–5 experiments and its corresponding standard deviation value. *Abbreviation:* nd, not determined.

as glutamate dehydrogenase type I. No identification was possible at this end for the 70 kDa protein. These data indicate the possibility that SCHAD I interacts specifically with proteins present in mitochondria also *in vivo*.

Discussion

SCHAD deficiency is at present an ill-defined, clinically diverse defect [1], with a common biochemical abnormality at the level of short-chain hydroxyacyl CoA oxidation. However, the heterogeneous levels of activity found in SCHAD patients, as determined in this or other studies [2–4,22–25], suggest different enzymes or specific regulatory factors to be involved.

Two candidate mitochondrial isozymes have been isolated from human or other mammalian species [10,11,26,27]. However, the relative contributions of these isozymes in humans have not been reported. The kinetic constants obtained clearly indicate an about 50-fold higher catalytic efficiency of SCHAD I over type II, thus establishing SCHAD I as the major form involved in mitochondrial β-oxidation in humans, in contrast to bovine liver, where both forms apparently contribute to similar extents [27]. Our data agree with the hypothesis that SCHAD II is responsible for the residual levels (about 5% of controls) of activity observed in the patient with a SCHAD I loss-of-function mutation, accompanied by absent SCHAD I expression [4]. Thus, assuming a level of 5% residual hydroxybutyryl CoA activity carried out by SCHAD II, most of our patients examined display more than 5% residual activity, indicating either a strongly attenuated type I contribution to the levels observed in patients, or the presence of a third, yet undefined isozyme. In line with the conclusions from the kinetic data, sequence analysis of SCHAD patients resulted in no detectable mutation within the SCHAD II gene. The fact that patients 2 and 5 are heterozygous carriers of a polymorphic allele for SCHAD I with a Pro/Leu mutation, does not explain the phenotype of at least 4, if not of all patients.

Another hypothesis to explain the observed lowered activity is the existence of regulatory components. Binding proteins for SCHAD I isolated from different species have been reported [14,28,29], but the identity of these interacting partners have not been determined, and in only one case thus far, a regulatory function could be assigned to a 60 kDa protein isolated from rat liver mitochondria [14]. This protein corresponds well in mass to the 60 kDa protein from human liver mitochondria isolated in our study. We identified by mass spectrometry this protein to be type I glutamate dehydrogenase (GDH). This suggests a possible interaction between β-oxidation, and the TCA and urea cycles in mitochondria. Interestingly, gain of function mutations were observed in GDH in the hyperinsulinism/hyperammonemia syndrome which is accompanied by hyperinsulinism [30]. Further studies are warranted to investigate this novel link in hyperinsulinism through FAO/HADH 1 and GDH interactions.

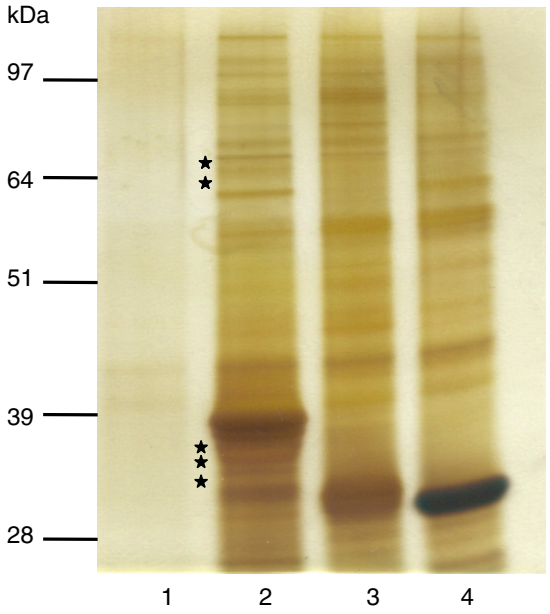

Fig. 2. Identification of binding proteins in human liver mitochondria by SDS/PAGE. Pull-down experiments with His-tagged SCHAD I (lane 2), SCHAD II (lane 3) and 3β/17β-HSD (lane 4) were carried out with solubilized human liver mitochondria as described in the Experimental section. Lane 1: control experiment without added recombinant bait. Molecular masses of a standard mixture are indicated on the left. Specifically interacting proteins with SCHAD I are marked by asterisks, displaying apparent molecular masses of 70, 60 and 37, 35, and 32 kDa.

The 70 kDa component identified by the His-pull-down experiments matches well the size of a SCHAD I interacting protein from pig heart mitochondria [28]. Other components interacting with SCHAD I have been reported, like GLUT 4 [29], but assuming a mitochondrial inner-matrix localization for both SCHAD isozymes, a physiological role of this interaction still has to be proven. We therefore performed binding experiments of recombinant human SCHAD isozymes with solubilized human mitochondrial components derived from healthy donors to verify the data obtained from other species. The results clearly indicate the existence of proteins specifically interacting with SCHAD I, which could be involved in regulating SCHAD activity levels. In summary, we were able to clarify the role of SCHAD/HADH isozymes by kinetic, biochemical and mutational analyses. The data obtained clearly indicate other factors than mutations in the two known SCHAD isozymes responsible for the observed phenotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007. 10.188.

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