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Complex changes in the liver mitochondrial proteome of short chain acyl-CoA dehydrogenase deficient mice

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ABSTRACT

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency is an autosomal recessive inborn error of metabolism that leads to the impaired mitochondrial fatty acid β-oxidation of short chain fatty acids. It is heterogeneous in clinical presentation including asymptomatic in most patients identified by newborn screening. Multiple mutations have been identified in patients; however, neither clear genotype–phenotype relationships nor a good correlation between genotype and current biochemical markers for diagnosis has been identified. The definition and pathophysiology of this deficiency remain unclear. To better understand this disorder at a global level, quantitative alterations in the mitochondrial proteome in SCAD deficient mice were examined using a combined proteomics approach: two-dimensional gel difference electrophoresis (2DIGE) followed by protein identification with MALDI-TOF/TOF and iTRAQ labeling followed by nano-LC/MALDI-TOF/TOF. We found broad mitochondrial dysfunction in SCAD deficiency. Changes in the levels of multiple energy metabolism related proteins were identified indicating that a more complex mechanism for development of symptoms may exist. Affected pathways converge on disorders with neurologic symptoms, suggesting that even asymptomatic individuals with SCAD deficiency may be at risk to develop more severe disease. Our results also identified a pattern associated with hepatotoxicity implicated in mitochondrial dysfunction, fatty acid metabolism, decrease of depolarization of mitochondria and mitochondrial membranes, and swelling of mitochondria, demonstrating that SCAD deficiency relates more directly to mitochondrial dysfunction and alteration of fatty acid metabolism. We propose several candidate molecules that may serve as markers for recognition of clinical risk associated with this disorder.

1. Introduction

Short chain acyl-CoA dehydrogenase (SCAD) is a member of the acyl-CoA dehydrogenase (ACAD) family of enzymes that catalyze the initial reaction of the mitochondrial fatty acid oxidation (FAO) spiral leading to sequential cleavage of two carbon units. FAO serves as an important energy source for the body during times of fasting and metabolic stress. Electrons from reduced SCAD are passed to the electron transfer flavoprotein and then directed into the electron transport chain via electron transfer flavoprotein-ubiquinone oxidoreductase [1]. SCAD is a homotetrameric enzyme encoded in the nuclear genome that functions in mitochondria. It shares structural and functional similarity with other ACADs, each of which has a unique pattern of substrate utilization [2,3]. The human *ACADS* gene is located on chromosome 12q22-qter and spans approximately 13 kb, consisting of 10 exons [4]. The mouse *ACADS* gene maps to chromosome 5 at the *Bed-1* locus, and is a compact, single-copy gene approximately 5 kb in size also containing 10 exons [5]. An SCAD deficient Balb/cByJ mouse has been described and is due to a 278-bp deletion at the 3′ end of *ACADS* gene leading to reduced steady-state levels of SCAD mRNA [6].

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is an inborn error of metabolism that leads to impaired mitochondrial β-oxidation of fatty acids with a carbon backbone length of 4 or 6 carbon units. SCADD results in the accumulation of butyryl-CoA, which in turn leads to the excess production of butyrylcarnitine, butyrylglycine, ethylmalonic acid (EMA), and methylsuccinic acid in blood, urine, and

Abbreviations: EMA, ethylmalonic acid; ETC., electron transport chain; FAO, fatty acid oxidation; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; MALDI-TOF/TOF, matrix assisted laser desorption ionization time of flight/time of flight; MS, mass spectrometry; OTC, ornithine transcarbamylase; OXPHOS, oxidative phosphorylation; SCAD, short-chain acyl-CoA dehydrogenase; SCADD, short-chain acyl-CoA dehydrogenase deficiency; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels; TCA, tricarboxylic acid; 2DIGE, two-dimensional gel difference electrophoresis.

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cells [7]. It was originally identified in symptomatic children and adults with heterogeneous symptoms including metabolic acidosis, ketotic hypoglycemia, epilepsy, myopathy, hypotonia, developmental delay and behavioral changes [8,9]. This deficiency was originally thought to affect approximately 1 in 40,000–100,000 newborns, but with the widespread implementation of expanded newborn screening programs through tandem mass spectrometry measurement of acylcarntines in blood spots; additionally, mostly asymptomatic SCAD deficient infants have been identified [10]. The clinical heterogeneity seen in SCADD raises a fundamental question about what (if any) pathophysiologic changes are induced by deficiency of this enzyme. It is clear that biomarkers currently characterized in SCADD patients, including increased urinary EMA and butyrylglycine, increased plasma butyrylcarnitine, and decreased SCAD activity, are not sufficient to predict the development of clinical symptoms and disease severity. Thus, additional biomarkers to categorize effects of SCADD would be of great value.

Approximately 70 genetic variations have been identified in the *ACADS* gene in symptomatic and asymptomatic individuals [10]. No clear genotype–phenotype relationships have been seen in either clinical patients or those identified through newborn screening. Nor is there a good correlation between genotype and the biochemical characteristics of EMA and butyrylglycine excretion [11]. Decreased SCAD activity in skin fibroblasts or muscle is not a good predictor of clinical symptom. In vitro studies suggest an increase in oxidative stress and protein misfolding in SCADD cells, but in total, the clinical relevance of the biochemical defect and the need to treat affected individuals remain unclear [12–16] Most of the biochemical findings in SCADD mice parallel those seen in the human disease, including organic aciduria with excretion of EMA, methylsuccinic acids and N-butyrylglycine, and development of a fatty liver upon fasting or dietary fat challenge [17]. In contrast to the complicated genetic situation in humans with SCADD mutations, the SCAD deficient mouse provides an excellent model to study pathogenesis of this disease in vivo in a homogenous genetic background.

Proteomics takes a broad, comprehensive and systematic approach to understanding biology, bypassing several inherent limitations to gene expression profiling and genotyping in understanding disease mechanisms. Several approaches have been developed for quantitatively assessing the proteome and comparing differences between the healthy and diseased states. Two-dimensional gel difference electrophoresis (2DIGE) allows comparison of multiple 2D-gels and samples to be analyzed concurrently on the same gel. Compared with conventional two dimensional gel electrophoresis, DIGE shortens the procedure, minimizes the effect of gel to gel variation, and has a large dynamic range allowing differential analysis of abundant proteins, as well as proteins present at low concentration [18]. Isobaric tag for relative and absolute quantification (iTRAQ) has recently come into a more general use in quantitative proteomics [19]. It employs incorporation of 4 to 8 different mass labels into peptides in different samples that can then be compared in one experiment. Labeling multiple peptides per protein increases confidence in identification and quantitation. In this study, we employed both approaches to generate complementary quantitative information on changes in the mitochondrial proteome in SCAD deficient mice compared to wild type animals, and explore novel potential protein markers of disease pathogenesis and severity. Characterization of such changes has the potential to offer new insights into the disease mechanism and provide novel markers for diagnosis.

2. Materials and methods

2.1. Mice

SCAD deficient and wild type BALB/cByJ mice were originally purchased (Jackson Laboratory) at age of 4–5 weeks, and further propagated in the animal facilities at the UPMC Children's Hospital of Pittsburgh. All mice were maintained in a pathogen-free environment, complying with standard housing procedures. Three or five male offspring mice for each genotype were obtained before tissue harvest. All animals were sacrificed at age 6–8 weeks following standard protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Tissues were removed immediately after rapid euthanasia with CO_2.

2.2. Isolation of mitochondria

Freshly removed liver was finely minced in ice cold isolation buffer A (225 mM mannitol, 75 mM sucrose, 10 mM HEPES free acid, 10 mM EDTA, pH 7.4), then gently homogenized with a glass Dounce homogenizer in isolation buffer B (225 mM mannitol, 75 mM sucrose, 10 mM HEPES free acid, 0.1% BSA fatty acid free, 10 mM EDTA, pH 7.4) with the addition of 10 μl/ml Halt™ protease inhibitor cocktail (Pierce). The homogenate was centrifuged at 1300 ×*g* (10 min). A mitochondria pellet was obtained by centrifugation at 10,000 ×*g* (10 min), and then was resuspended in 12% Percoll solution (Sigma). The mitochondrial band between the 30% and 70% layers was acquired after centrifugation at 62,000 ×*g* (35 min). Purified mitochondria were collected by centrifugation at 10,000 ×*g* for 10 min and pellets were stored at −80 °C for use in iTRAQ labeling. The overall experimental workflow is shown in Fig. 1.

2.3. Preparation of mitochondrial protein for DIGE analysis

Mitochondrial protein was extracted immediately after isolation using M-PER Mammalian Protein Extraction Reagent (Pierce). 10 μl/ml of Halt™ protease inhibitor cocktail (Pierce) and 1 μl/ml phosphatase inhibitor cocktail 2 (Sigma) were added to extraction buffer. After removal of cellular debris, protein samples were treated with the 2D Clean-up Kit (GE Health Care) according to manufacturer's instructions. Protein concentration was measured with 2-D Quant Kit (GE Health Care) according to manufacturer's instructions.

2.4. Cy-dye labeling

Stock solutions of PrCy3-N-hydroxysuccinimide ester and MeCy5-N-hydroxysuccinimide ester (Cy3 and Cy5, respectively) were prepared as described [20]. Equal amounts of protein samples from SCAD deficient and wild type mice were individually labeled in a reciprocal manner with equal volumes of Cy3 or Cy5. An equal amount of sample from each genotype mouse was labeled with cyanine dye 2 (Cy2) (GE Health Care) as an internal standard. The labeling reaction was incubated on ice in the dark for 30 min, and was terminated with 1 μl of 40% aqueous methylamine, pH 8.0 [21].

2.5. Difference gel electrophoresis (DIGE)

Each labeled protein sample was dissolved in sample buffer (7 M urea, 2 M thiourea, 2% amidosulfobetaine-14, 10 mg/ml DTT and 1% pharmalytes 3–11). Paired protein samples from deficient and wild type mice were mixed together for subsequent isoelectric focusing on Immobiline DryStrips (24 cm, pH 3–11 nonlinear 3–11 NL) (GE Healthcare). After active rehydration for 10 h at 30 V, IEF was performed on an Ettan IPGphor ΠI system (GE Healthcare) at 300 V for 30 min, 500 V for 30 min, 1000 V for 1 h and up to 80,000 Vh, and then maintained at 30 v as needed. Each strip was equilibrated in 50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and 100 mg DTT for 10–15 min. After additional alkylation with 250 mg iodoacetamide, strips were placed on freshly made 12.5% SDS-polyacrylamide gels (SDS-PAGE). Resolved proteins were separated with a vertical Ettan DALTsix electrophoresis system (GE Healthcare) in a running buffer of 1× TGS (Tris–glycine–SDS) (Bio-Rad).

Gels were visualized and evaluated on a Typhoon 9400 Variable Imager (GE Healthcare). Cy3 images were scanned with a 532 nm laser and