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Chapter 6

Exercise testing in patients with short-chain acyl-CoA dehydrogenase deficiency: biochemical responses and effects of riboflavin therapy

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Abstract

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is a mitochondrial fatty acid oxidation disorder, most frequently associated with neurologic symptoms and/or hypoglycemia. SCADD is biochemically characterized by increased C4-carnitine (C4-C) in blood and ethylmalonic acid (EMA) in urine and caused by rare mutations and/or common gene variants in the SCAD-encoding gene. Previous studies showed that EMA increases and C4-C remains stable during fasting. In addition, EMA and C4-C decreased in response to riboflavin therapy in a subgroup of SCADD patients with a mutation/variant genotype.

Our aim was to examine the biochemical response to exercise in SCADD patients and to assess whether high-dose riboflavin therapy exerts any effects on the observed response. Exercise tests were performed in 3 SCADD patients, all with mutation/variant genotypes and symptoms of exercise intolerance or fatigue. One patient, who responded clinically to riboflavin therapy, was retested during, and 1, and 2 years after riboflavin therapy.

C4-C concentrations in plasma increased in response to exercise, while EMA excretion remained stable. In the patient who was retested during riboflavin therapy, no exercise induced increase of plasma C4-C was seen. However, this C4-C increase was present again 2 years after cessation of riboflavin therapy, though she was still without clinical symptoms. As C4-C increases in SCADD patients during exercise and EMA increases during fasting, preferred tissue-specific pathways might exist. In addition, high-dose riboflavin therapy may prevent C4-C increase during exercise, but appears not to be related to any clinical effect in this particular patient.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD, EC 1.3.99.2) deficiency (SCADD, OMIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid ß-oxidation. SCADD is relatively common, and is most frequently diagnosed as a result of investigations for developmental delay, epilepsy, behavioral disorders, hypoglycemia and hypotonia. Although SCADD appears to go without any clinical significance in many individuals, SCADD is included in newborn screening programs in most US states. In order to obtain insight into the pathophysiological consequences of SCADD, we previously studied the biochemical effects of fasting and fat-loading in SCADD patients. In order to study the efficacy of potential treatments, we assessed the effects of high-dose riboflavin therapy on the biochemical SCADD characteristics and clinical status in SCADD patients. Studies on the biochemical effects of exercise and the effect of riboflavin on the biochemical effects during exercise in SCADD patients have not yet been performed.

The SCAD enzyme catalyzes the dehydrogenation of butyryl-CoA (C4-CoA) and is the first enzyme involved in the short-chain fatty acid ß-oxidation spiral. When SCAD activity is impaired, its substrate C4-CoA accumulates and is subsequently converted into different metabolites, including the corresponding carnitine-ester (butyrylcarnitine, C4-C), butyrate, and ethylmalonic acid (EMA) (Figure 1). C4-C can be measured in blood and EMA can be measured in urine. In our previous study we demonstrated that EMA increased significantly during fasting, while C4-C remained stable.

The SCAD enzyme is a flavoprotein consisting of 4 subunits, each of which contains one molecule of its co-factor flavin adenine dinucleotide (FAD). Riboflavin, vitamin B2 (7,8-dimethyl-10-ribityl-isoalloxazine) is the precursor of FAD and is predominantly

![Figure 1. Metabolic Fate of Butyryl-CoA in SCADD](image-url)
ingested through the consumption of milk and dairy products. FAD binding is important not only for the catalytic activity of flavoproteins, but also for folding, assembly, and/or stability. Treatment with riboflavin could therefore be a potentially effective therapy in SCADD. Indeed, we previously demonstrated a biochemical effect of high-dose riboflavin supplementation in a subgroup of SCADD patients.

The diagnosis of SCADD is based on the presence of inactivating mutations and/or common variants of the SCAD-encoding gene (ACADS). The majority of SCADD patients are homozygous or compound heterozygous for either 1 or 2 common ACADS variants or for ACADS variants in combination with an ACADS mutation. The ACADS variants have been found in the general population with a remarkably high prevalence of homozygosity, with frequencies of approximately 0.3% for the c.511C>T and 5.5% for the c.625G>A variant. These variants are thought to play modifying roles in the pathogenesis of SCADD by conferring susceptibility for clinical disease. Those SCADD patients showing at least biochemical benefit from riboflavin therapy all carry a mutation on one ACADS allele and a variant (c.625G>A) on the other. This points to a potential role of the c.625G>A variant leading to functional SCADD caused by decreased FAD affinity and/or SCAD protein instability.

The mitochondrial oxidation of fatty acids plays an important role in energy production, not only during periods of prolonged fasting, but also during moderately intense exercise. Different pathophysiological mechanisms that underlie the inborn errors of fatty acid oxidation (FAO) can be distinguished including the following: 1) inadequate supply of energy, 2) sequestration or loss of vital components of intermediary metabolism, and 3) accumulation of toxic metabolites. The results of our previous study, showing EMA increase during fasting as the only biochemical abnormality in SCADD patients, led us to suspect that, with respect to SCADD, the latter mechanism is the most likely one.

The aims of the current study were: a) to examine the effects of moderately intense exercise on biochemical profiles in SCADD patients and b) to assess whether high-dose riboflavin therapy exerts any effects on these profiles during exercise.

**Methods**

**Patients**

Three patients, 1 male and 2 females, aged 6, 8, and 13 years respectively, who all suffered from exercise intolerance or fatigue, were included in the study group. All patients were diagnosed with SCADD on the basis of increased C4-C in plasma and/or increased EMA in urine under non-stressed conditions on at least two occasions, and the presence of a mutation and/or the c.511C>T or c.625G>A variants on each ACADS allele. Genetic changes indicating SCADD were established by sequence analysis of all exons and flanking intronic sequences. The genotypes and clinical phenotypes of the
participating patients are shown in the table. The selected patients were part of the Dutch SCADD cohort described previously.1

Tests
All tests were performed between January 2002 and January 2008. Written informed consent was obtained from the parents and/or legal representatives of all patients participating in this study. The study was reviewed and approved by the Medical Ethics Committee of the Academic Medical Center.

Exercise protocol
The exercise test consisted of 60 minutes of cycling on a magnetic braked cycle ergometer (E5R, Tunturi Oy Ltd, Finland). Heart rates were monitored continuously and each patient exercised at 60% of his or her predicted maximum heart rate. The value for maximum heart rate was calculated as follows: 60% of maximum heart rate (beats per minute) = (208 – 0.7 x age) x 0.6.28 Blood samples were collected immediately prior to cycling (at t=0), during cycling (at t = 15, 30, 45, and 60 minutes), and in the case of patient 3, 1 hour after the exercise (at t = 120 minutes). Urine samples were collected prior to and after the exercise.

Riboflavin treatment and assessment in patient 3
Riboflavin was administered to patient 3 in a total dose of 150 mg per day divided in 3 doses, ingested during meals. This patient was studied on 4 separate occasions. A first exercise test was performed because of fatigue. As she appeared to respond clinically to riboflavin therapy, a second exercise test was performed after 4 months of therapy and a third and fourth test after riboflavin therapy had been stopped for 1 and 2 years respectively.

Blood and urine analysis
Blood samples were analyzed for glucose, nonesterified fatty acids (NEFA), creatine kinase (CK), alanine-aminotransferase (ALAT), and aspartate-aminotransferase (ASAT). In addition biochemical analyses of blood samples were performed to explore lactate,
pyruvate, ketone bodies (KB), and acylcarnitine profiles. Acylcarnitine profiles were determined using electrospray tandem mass spectrometry. Blood samples for lactate, pyruvate, and KB were immediately deproteinized with perchloric acid and stored on ice, followed by quantitative determination of metabolites using standard spectrophotometric or fluorimetric methods. All samples were delivered to the laboratory within 10 minutes of collection. Urine samples were analyzed for organic acids by gas chromatography/mass spectrometry, and stored at -20°C until analysis.

Interpretation of test results
EMA was considered to be increased if patients had >15 μmol/mmol creatinine for children younger than 2 years and >8 μmol/mmol creatinine for children aged 2 years and older. The upper reference range for C4-C was 0.58 μmol/L.

Results
Exercise tests
None of the patients showed any clinical signs of symptoms during or after the 60 minutes of exercise and remained asymptomatic during and after the test. Glucose, CK, FFA, ALAT, and ASAT remained normal and stable during and after the test. Free carnitine levels remained stable in patients 2 and 3 (data not shown, not measured in patient 1). C4-C values showed a clear increase in patients 2 and 3 (Figure 2A, not measured in patient 1). The variation in urinary EMA excretion was comparable to the at random EMA fluctuations reported previously (Figure 2B).

Exercise test results in response to riboflavin therapy in patient 3
On riboflavin therapy, C4-C levels during exercise decreased compared to baseline levels (Figure 3). One year after stopping riboflavin treatment, C4-C levels had increased again but were still lower when compared to the levels of the baseline test (Figure 3). After 2 years without riboflavin therapy, and still without any clinical signs or symptoms, a clear C4-C increase in response to exercise was again detected (Figure 3). EMA excretion was similar during the 3 consecutive tests (data not shown).

Discussion
The present study is the first to describe the biochemical response to exercise and the effects of riboflavin treatment on this response in SCADD patients. In our previous study, an increase in EMA excretion was observed during fasting, while C4-C remained stable. The current study demonstrates the opposite: C4-C increases during exercise in both
patients tested, while EMA excretion remained stable. The increase in EMA excretion, as observed during fasting is likely due to the accumulation of C4-CoA, the substrate of SCAD, which is converted into ethylmalonyl-CoA by propionyl-CoA carboxylase and subsequently into EMA by one of the mitochondrial acyl-CoA hydrolases. The increase in C4-C, as observed during exercise, is most likely caused by the increased production of C4-C from C4-CoA by the mitochondrial enzyme carnitine acetyltransferase. Even though our study only included a limited number of patients, the observed profiles might suggest the existence of a preferred pathway towards EMA during fasting and towards C4-C during exercise. This might be based on higher intramitochondrial carnitine concentrations in muscle compared to liver, resulting in a higher proportion of C4-CoA being converted into C4-C.31

**Figure 2.** Biochemical characteristics of short-chain acyl-CoA dehydrogenase deficiency (SCADD) during exercise in SCADD Patients. The profiles of C4-Carnitine (C4-C) in 2 SCADD patients and ethylmalonic acid (EMA) in 3 SCADD patients during exercise are shown. A, C4-C. The dashed line represents the upper limit of normal levels (0.58 μmol/L). B, EMA. The dashed line represents the upper limit of normal levels (8 μmol/mmol creatinine).

**Figure 3.** C4-Carnitine (C4-C) during exercise before, during, and 1, and 2 years after riboflavin therapy in patient 3.
No studies on the toxicity of C4-C have been performed. As discussed previously the accumulation of potentially toxic metabolites is the most likely mechanism to be involved in the pathophysiology of SCADD.\textsuperscript{1} However, previous studies showed that patients with the highest levels of EMA and C4-C (the mutation/mutation genotype group) were not more severely affected with respect to ketogenesis and clinical symptoms when compared with patients with lower EMA and C4-C levels (the mutation/variant or variant/variant genotype groups).\textsuperscript{1,15} Therefore, no conclusions can yet be made regarding a potential toxic effect from C4-C and/or other metabolites derived from butyryl-CoA.

A clear biochemical response to riboflavin therapy was demonstrated by the prevention of increased C4-C values during exercise in patient 3. As exercise stimulates FAO, repeating these tests during riboflavin treatment offered the potential to assess its functional efficacy in SCADD patients. The observation that C4-C levels in response to exercise clearly increased again 2 years after cessation of riboflavin therapy but without any changes in clinical signs and symptoms, points to 2 different aspects. First of all, discontinuation of riboflavin therapy only showed its effects 2 years later. This can be explained by the fact that there is only little destruction of riboflavin associated with its function in metabolism,\textsuperscript{32} together with the observation that riboflavin intake was high enough to provide for normal FAD levels preceding riboflavin therapy in this patient. Secondly, it supports the hypothesis that the observed responses of SCADD patients to riboflavin are of a biochemical nature and are not related to any improvement in clinical disease.\textsuperscript{16}

In summary, we have demonstrated that C4-C increases during exercise in SCADD patients, whereas during fasting the urinary excretion of EMA increased. We hypothesize that preferred pathways might exist towards EMA during fasting and towards C4-C during exercise. This could be due to a higher intramitochondrial carnitine concentration in muscle as compared to liver. In addition we showed that high-dose riboflavin therapy fully prevented C4-C increase during exercise. However, as the C4-C increase during exercise was present again 2 years after cessation of therapy but was not accompanied by any deterioration in clinical symptoms, the biochemical effect of riboflavin does not seem to be related to any clinical effect in the patient tested.

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