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Measurement of short-chain acyl-CoA dehydrogenase (SCAD) in cultured skin fibroblasts with hexanoyl-CoA as a competitive inhibitor to eliminate the contribution of medium-chain acyl-CoA dehydrogenase

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Abstract

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency has so far been reported in only very few patients. This is due, in part, to the problems involved in measuring the activity of SCAD unequivocally. The main reason for this difficulty is that butyryl-CoA, the substrate preferably used for SCAD activity measurements, is also dehydrogenated by medium-chain acyl-CoA dehydrogenase (MCAD). Elimination of this contribution can be achieved by means of immune precipitation with a specific MCAD antibody. We now describe a relatively straightforward assay based on the use of gas chromatography/mass spectrometry for detection. The contribution of MCAD to overall butyryl-CoA dehydrogenation was eliminated by adding excess hexanoyl-CoA to the assay medium. The validity of the method developed was checked by SCAD-activity measurements in fibroblasts from an established SCAD-deficient patient.

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1. Introduction

In the past few years an increasing number of patients have been identified with an impairment in mitochondrial fatty acid β-oxidation [1,2]. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is most frequent among these disorders. Identification of such patients is relatively straightforward due to a characteristic pattern of urinary organic acids that is frequently, but not invariably, found. Furthermore, methods have become available to measure the activity of MCAD reliably in white blood cells and/or fibroblasts. Finally, the discovery that most MCAD-deficient patients are homozygous for the $A_{985} \rightarrow G$ mutation has greatly helped identification of patients. The clinical and biochemical characteristics of short-chain acyl-CoA dehydrogenase (SCAD, EC 1.3.99.2) deficiency have not been worked out so thoroughly. Indeed, only very few patients with proven SCAD deficiency have been described [3–9]. This is partly due to the fact that the urinary organic acid excretion profile is not very specific and that correct assessment of the activity of SCAD has proved to be quite difficult.

The main reason for the difficulty in assessing SCAD activity is that butyryl-CoA, the substrate preferably used for SCAD activity measurements, is also dehydrogenated by MCAD [10,11]. When the ETF-based assay is used, the two dehydrogenases contribute about equally to the dehydrogenation of butyryl-CoA [10]. As a consequence SCAD-activity can only be measured reliably if MCAD is removed specifically by means of immune precipitation using a specific antibody. This adds a level of complexity to the analysis.

In the present paper we describe a relatively simple, straightforward assay for SCAD using gas chromatography/mass spectrometry. The enzyme assay is an elaboration of previous work [12–14] and is based on measuring the production of 3-OH-butyryl-CoA ester from the substrate, butyryl-CoA. Furthermore, we show that the contribution of MCAD to the total dehydrogenation of butyryl-CoA can be eliminated by adding hexanoyl-CoA to the incubation medium, which is a substrate for medium-chain acyl-CoA dehydrogenase but not for short-chain acyl-CoA dehydrogenase. The method is validated by measurements in fibroblasts from an established SCAD-deficient patient [9].

2. Materials and methods

2.1. Materials

Ham's F10 medium and trypsin were obtained from Flow (UK). Fetal calf serum, L-glutamine, penicillin and streptomycin were purchased from Gibco (UK). Cysteine, phenazine methosulphate, crotonase (EC 4.2.1.17), butyryl-CoA and hexanoyl-CoA were obtained from Sigma Chem Co. (USA). Flavine adenine

dinucleotide (FAD, the sodium salt) is a product of Boehringer Mannheim (Germany).

The standards, 3-OH-butyric acid and [1,2,3,4-^{13}C]3-OH-butyric acid were custom synthesized by Tracer Technologies Inc (USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide was obtained from Pierce (USA). All other chemicals were purchased from Merck (Germany) and were of analytical grade.

2.2. Patient

The patient, whose cultured skin fibroblasts were studied in order to validate the method described in this paper, was the first child of healthy consanguineous Turkish parents [9]. Hospital admission was because of pneumonia, fever, tachypnoea and expiratory stridor at 15 months of age. The main features in the infant were increased urinary excretion of ethylmalonate and methylsuccinate and progressive skeletal muscle weakness (see Ref. [9] for detailed information).

2.3. Fibroblast cultures

Human skin fibroblasts were cultured in Ham's F10 medium supplemented with 8% (v/v) fetal calf serum plus 1 mmol/l L-glutamine, 90 units/ml penicillin and 90 g/ml streptomycin.

Fibroblasts were grown in 75 cm^2 culture flasks and media were changed twice a week. Cultured skin fibroblasts were harvested by trypsinization 10 days after the last sub-culture. Subsequently, harvested cells were washed three times with potassium phosphate buffer (100 mmol/l, pH 7.5) and frozen at −80°C for subsequent assay of acyl-CoA dehydrogenase activities.

2.4. Enzyme activity measurement

We used the assay conditions described previously, using butyryl-CoA at a concentration of 0.35 mmol/l [13,14]. Incubations were carried out at 37°C in a final reaction mixture (0.7 ml) per assay containing the following components: 20 μmol potassium hydrogen phosphate (pH 7.5), 0.25 μmol cysteine, 0.05 μmol FAD, 3.5 μmol phenazinemethosulphate, 50 μl crotonase (= 1.2 μg protein), 0.25 μmol butyryl-CoA and about 0.2–0.4 mg fibroblast protein. The reaction was terminated by adding 500 μl 0.1 mol/l sodium hydroxide. The internal standard, 25 μl [1,2,3,4-^{13}C]3-OH-butyric acid (0.40 mmol/l), was added prior to stopping the reaction with sodium hydroxide.

Incubations were carried out in the presence or absence of the substrate hexanoyl-CoA (concentration 0.8 mmol/l). Enzyme activities are expressed in μmol 3-OH-butyryl-CoA produced/min per g protein.

The 3-OH-butyryl-CoA ester was hydrolysed at 60°C for 30 min. Extraction of the OH-acids from the NaOH-stopped reaction mixture took place after acidifying with HCl [15]. To 0.6 ml of NaOH-stopped reaction mixture, 4 ml of ether/ethyl acetate (1:1, v/v) were added. The organic phase was evaporated to dryness under a stream of nitrogen at 50°C.

tert-Butyldimethylsilyl (TBDMS) derivatives were prepared by heating the dried

residues with 200 μl of pyridine/*N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide for 30 min at 80°C. A di-TBDMS derivative was formed.

GC/MS SIM measurements were performed with a Hewlett Packard 5890 gas chromatograph directly coupled to a 70–250 S mass spectrometer (VG Instruments, Manchester, UK). The combination was operated under the following conditions: carrier gas (helium) flow-rate 1.0 ml/min; injector temperature 250°C; splitless mode; oven temperature program 100°C, 15°C/min to 190°C; 30°C/min to 280°C; interface, ion source and analyzer temperatures, 250°C; and ionization energy, 70 eV.

The capillary gas chromatography column was directly inserted into the ion source of the GC/MS system. Separation was achieved on a fused silica column CP Sil 5 CB (25 m × 0.2 mm) from Chrompack Int BV (The Netherlands). Injection volume of the derivatized extracts was 3 μl.

3-OH-Butyric acid and [1,2,3,4-^{13}C]3-OH-butyric acid TBDMS esters were monitored at *m/z* 275 ($[M - 57]^+$) and *m/z* 279 ($[M - 57]^+$), respectively. The concentration of 3-OH-butyric acid formed was calculated from the GC/MS calibration graph prepared from a mixture of 3-OH-butyric acid and [1,2,3,4-^{13}C]3-OH-butyric acid. The calibration graph ranged from 0 to 0.5 mmol/l 3-OH-butyric acid. The measured ratios were corrected for the natural abundance.

A correlation coefficient of >0.99 was routinely found. Linear regression was used to calculate the concentration of 3-OH-butyric acid formed during the enzyme assay in the fibroblast extracts after hydrolysis. Protein concentration was measured by the method of Lowry et al. [16].

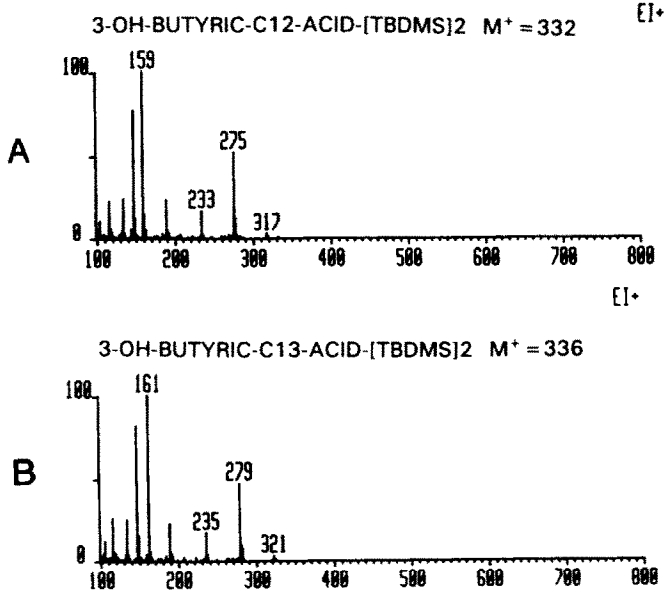

Fig. 1. The GC/MS spectra of hydroxy fatty acid measured in this enzyme assay. (A) 3-OH-butyric acid, standard [^{12}C] used in the GC/MS calibration graph for determining 3-OH-butyric acid produced, after hydrolysis, in the enzyme assay. (B) 3-OH-butyric acid, internal standard [^{13}C] used in the calibration graph. Monitoring of the di-TBDMS derivatives was at *m/z* 275 $([M-57])^+$ (A) and *m/z* 279 $([M-57])^+$ (B), respectively.

3. Results and discussion

Kølvraa et al. [12] were the first to apply GC/MS for the study of acyl-CoA dehydrogenase activity measurements. The method is based on the use of enoyl-CoA hydratase (crotonase), which is commercially available, to convert the enoyl-CoA ester into the corresponding 3-hydroxy acyl-CoA. After alkaline hydrolysis the 3-hydroxy fatty acid can be measured by GC/MS.

We have now used the same principle to measure the activity of SCAD in fibroblasts homogenates. In order to accomplish this we first recorded the spectra of the di-TBDMS derivatives of both [^{12}C] and [^{13}C]3-OH-butyric acids as shown in Fig. 1. The TBDMS derivatives were monitored at *m/z* 275 ($[M - 57]^+$) and *m/z* 279 ($[M - 57]^+$), respectively (molecular ions are 332 and 336).

Fig. 2 shows the results of an experiment in which the formation of 3-hydroxy butyryl-CoA was studied in fibroblast homogenates. A linear relationship was found between the amount of 3-hydroxy butyric acid formed and the amount of fibroblast protein. Furthermore, activity was found to be linear with time up to 100 min. Based

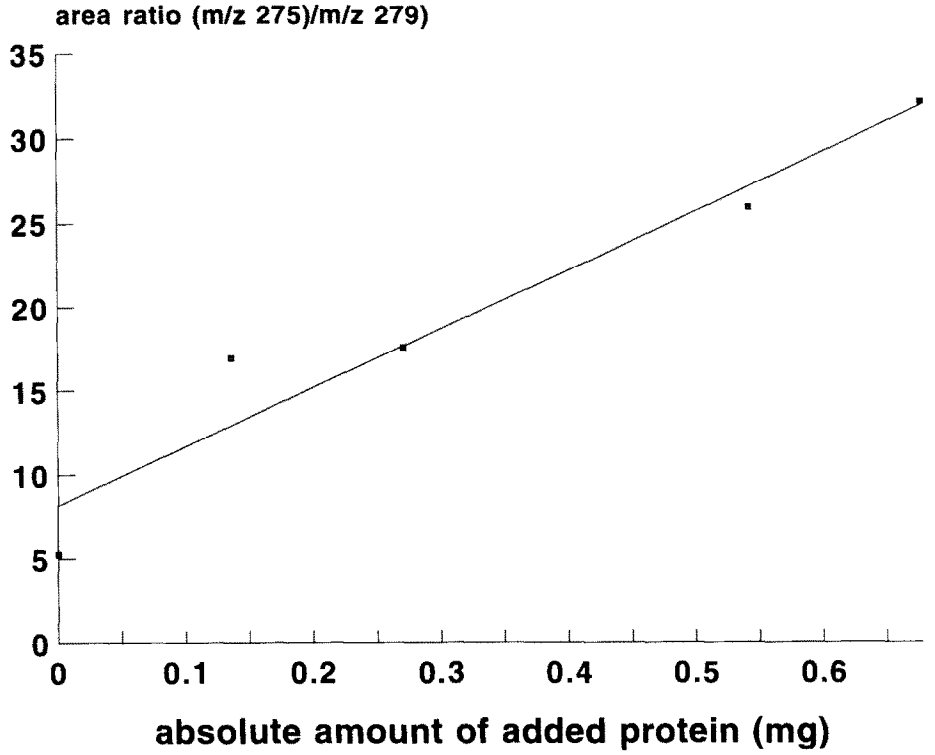

Fig. 2. Butyryl-CoA dehydrogenase activity measurements in homogenates of cultured skin fibroblasts as a function of the amount of protein added. Different amounts of fibroblast protein were incubated in the standard reaction medium for 60 min. Reactions were terminated followed by determination of the 3-hydroxy butyric acid levels using GC/MS (see Materials and methods).

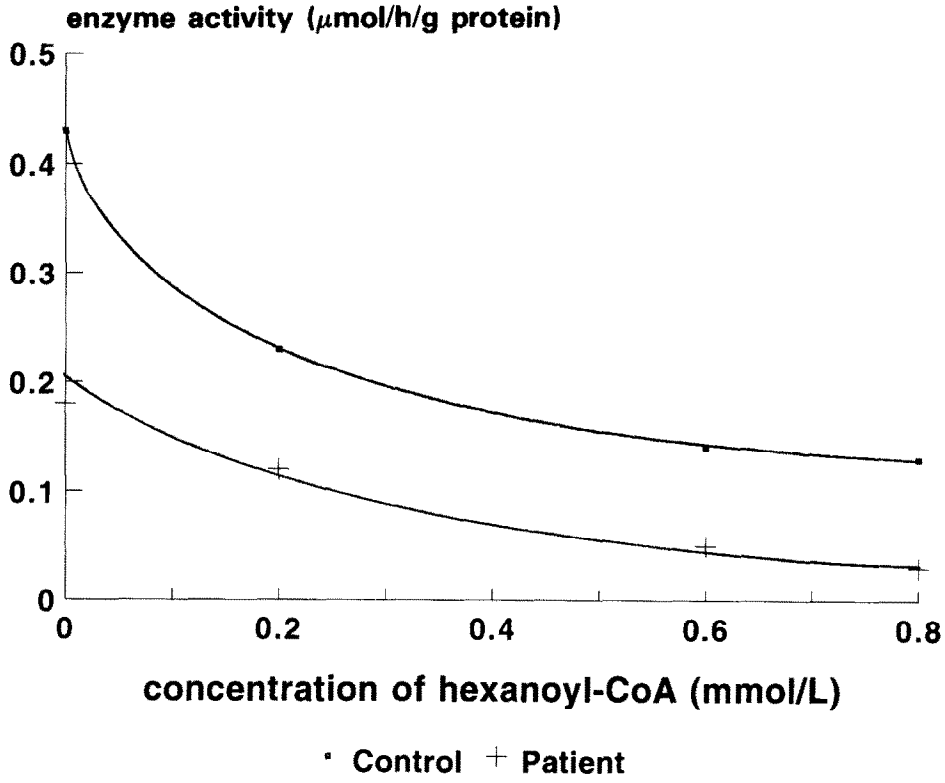

Fig. 3. Effect of addition of increasing amounts of hexanoyl-CoA on the formation of 3-hydroxy butyryl-CoA from butyryl-CoA in control fibroblasts and fibroblasts from a patient with an established SCAD deficiency. Incubations were carried out as described in Materials and methods in the presence of 0.8 mmol/l hexanoyl-CoA (see Materials and methods for details).

on these findings we selected an incubation time of 60 min and a protein concentration of 0.2–0.4 mg at a butyryl-CoA concentration of 0.35 mmol/l.

When the activity of butyryl-CoA dehydrogenase was subsequently measured in fibroblast homogenates from control subjects and a patient with an established defi-

Table 1
Butyryl-CoA dehydrogenase activity measurements in cultured skin fibroblasts from control subjects and an established SCAD-deficient patient

Fibroblasts studied	Butyryl-CoA dehydrogenation activity (μmol/min per g protein)
Control	0.22 ± 0.05 ($n = 5$)
SCAD-deficient	0.03 ($n = 1$)

Enzyme activities were measured as described in Materials and methods in the presence of 0.8 mmol/l hexanoyl-CoA and are expressed as mean ± S.D. with the number of different cell lines tested between parentheses.

ciency of SCAD (see Ref. [9]), it was found that the butyryl-CoA dehydrogenase activity in the SCAD-deficient fibroblasts was only partially deficient with a residual activity of 40% as compared with the activity in control cells (data not shown). This finding compares well with data in the literature in which the ETF-based assay was used [17]. The high residual activity found by us and other investigators is most likely due to the fact that medium chain acyl-CoA dehydrogenase also reacts with butyryl-CoA.

In order to reduce the contribution of medium-chain acyl-CoA dehydrogenase to the total butyryl-CoA dehydrogenating activity in fibroblast homogenates, we repeated the butyryl-CoA dehydrogenase activity measurements in the presence of increasing concentrations of hexanoyl-CoA which is a good substrate for medium-chain acyl-CoA dehydrogenase but not short-chain acyl-CoA dehydrogenase. The results depicted in Fig. 3 show that hexanoyl-CoA inhibits enzyme activity, both in control and SCAD-deficient fibroblasts, with the extent of inhibition increasing with increasing concentrations of hexanoyl-CoA. At the highest concentration of hexanoyl-CoA (0.8 mmol/l), enzyme activity in the SCAD-deficient fibroblasts is very low in contrast to that in control cells.

Table 1 depicts the results of a series of enzyme activity measurements in control and SCAD-deficient fibroblasts using a concentration of 0.8 mmol/l hexanoyl-CoA. The results show much better discrimination between control and the SCAD-deficient fibroblasts under these conditions.

In conclusion, we have developed a simple, straightforward assay for short-chain acyl-CoA dehydrogenase making use of elevated concentrations of hexanoyl-CoA to eliminate the interference by medium-chain acyl-CoA dehydrogenase. Under such conditions butyryl-CoA is expected to react with short-chain acyl-CoA dehydrogenase only, given the K_m values of 71.4 and 8.0 μmol/l for butyryl-CoA and hexanoyl-CoA, respectively, for human medium-chain acyl-CoA dehydrogenase as determined by Finocchiaro et al. [11].

The method described here allows accurate determination of SCAD-activity and avoids the use of ETF (which is not commercially available and must be purified from pig liver [18]) and antibodies against medium-chain acyl-CoA dehydrogenase.

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