0031-3998/06/6001-0093
PEDIATRIC RESEARCH
Vol. 60, No. 1, 2006
Printed in U.S.A.

Monitoring of Ketogenic Diet for Carnitine Metabolites by Subcutaneous Microdialysis

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ABSTRACT: The ketogenic diet (KD) provides ketones from the degradation of free fatty acids for energy metabolism. It is a therapeutic option for pharmacoresistant epilepsies. Carnitine is the carrier molecule that transports fatty acids across the mitochondrial membrane for degradation into ketones. The integrity of this transport system is a prerequisite for an adequate ketogenic response. For monitoring of tissue metabolism with KD, we used the sampling method of s.c. microdialysis (MD), which permits minimally invasive, frequent, and extensive metabolic monitoring independent of blood tests. By using this new method, we monitored changes in carnitine metabolism induced by KD, particularly in free carnitine (C0), acetylcarnitine (C2), and hydroxybutyrylcarnitine (C4OH). Correlation of microdialysate and tissue concentrations for carnitines *in vitro* was about 85%. Carnitine metabolism was monitored in seven children started on a KD for pharmacoresistant epilepsy after a conventional initial fasting period. Detected metabolic changes consisted of a slight decrease in s.c. C0 and a marked increase in C2/CO and C4OH/CO levels. The levels of s.c. C4OH strongly correlate with β-hydroxybutyrate (β-OHB) levels in plasma providing an additional parameter for the carnitine reserve of the body and reflect an optimal ketogenic energy supply. Subcutaneous MD allows close and extensive monitoring of metabolism with a KD. **(*Pediatr Res* 60: 93–96, 2006)**

A high-fat, low-carbohydrate KD has been proven to be efficient for intractable childhood epilepsies (1). The metabolic effects of the KD are comparable to prolonged fasting. Glucose substrates are replaced by β-OHB, acetoacetate, and free fatty acids. Carnitine plays a major role in the degradation of fatty acids. As a trimethylated amino acid, it facilitates translocation of fatty acids into the mitochondrion and is therefore an essential cofactor in fatty acid oxidation and ketogenesis (2). In mammals, changes in the carnitine pattern in plasma and several tissues have been demonstrated with alterations in nutritional state. Studies in humans have shown a delayed decrease in plasma free carnitine and a rapid increase in long- and particularly short-chain acylcarnitines during fasting or diabetic ketosis (3–5). A study in children demonstrated that the changes in acylcarnitines during fat load (ingestion of sunflower oil) are more or less comparable to those during fasting (6). However, studies on the dynamics of carnitine metabolism, in particular C4OH, during initiation of a KD have not been reported so far.

The technique of MD is a potent tool for the study of tissue metabolism. The method is based on the diffusion of substances through a semipermeable dialysis membrane implanted in the tissue of interest. It allows repeated measurement of the concentrations of tissue molecules that have crossed the membrane. Water-soluble analytes with a molecular weight below the exclusion size of the catheter cross the membrane until their concentrations in the extracellular fluid and the microdialysate are equal (7,8). In clinical practice, MD has been established, especially in neurointensive care for bedside monitoring of glucose, lactate, pyruvate, glycerol, and urea (9,10).

Carnitines measured in the MD fluid reflect unbound carnitine metabolites of the surrounding tissue. C0 and short-chain acylcarnitines like C2 and C4OH predominantly occur in their free form. Acylcarnitines in plasma and interstitium are partially bound to plasma proteins. The rate of protein bound carnitines increases with the length of the bound fatty acid.

So far, MD has not been used for carnitine measurements in human tissues. In our study, we show that MD can be used for the determination of carnitine metabolites in the s.c. tissue. Moreover, this technique permits detailed analysis of changes in the s.c. carnitine pattern with a KD over time.

PATIENTS AND METHODS

The MD device used (CMA/Microdialysis AB, Solna, Sweden) is CE certified for the clinical application on the human brain and s.c. tissue. The study was approved by the local ethics committee, and written consent was obtained from the parents.

Patients. Seven pediatric patients have started on the KD for intractable childhood epilepsy. Patient's median age was 2.5 y (range 0.9–10.6 y).

Received November 30, 2005; accepted February 4, 2006.

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DOI: 10.1203/01.pdr.0000219479.95410.79

Abbreviations: C0, free carnitine; **C2,** acetylcarnitine; **C4OH,** hydroxybutyrylcarnitine; **KD,** ketogenic diet; **MD,** microdialysis; **RR,** relative recovery; **β-OHB,** β-hydroxybutyrate

Table 1. *Relative recovery in vitro for C0, C2, and C4OH*

Carnitine	RR (mean ± SD), %	Tested substance concentrations, range (μmol/L)
C0	86 ± 7	5–174
C2	88 ± 7	7–65
C4OH	83 ± 9	0.09–0.43

The KD consists of long-chain triglycerides at a rate of 4:1 (4 g fat/1 g protein+carbohydrates) and was introduced with an initial fasting period according to a standard protocol (11,12).

Fasting was started at 1900 h of the day of MD catheter insertion (d 0) and continued for 24 h (d 1). At 1900 h of d 1, patients received the first ketogenic meal, consisting of one third of their final caloric requirement. On d 2, the amount of calories was increased to two thirds and divided into four meals. From d 3 on, patients received the full amount of calories in four ketogenic meals per day.

As the risk of metabolic decompensation is especially high during initiation of the KD, we closely monitored the patients, determining glucose, lactate, and pyruvate by s.c. MD. Additionally, every morning on an empty stomach and then every 4 h β-OHB, glucose, and gas checks were obtained by capillary blood tests.

Microdialysis. The principles of MD have been described in detail previously (13–15). We used a CMA 70 MD catheter (CMA/Microdialysis AB) with a dialysis membrane length of 20 mm. The molecular exclusion size of the polyamide membrane was 20 kD. According to the application in neonates and children (16,17), the MD catheters were inserted under sterile conditions upon transdermal local anesthesia (EMLA, Wedel, Germany) into the s.c. tissue of the lateral thigh (younger children) or the forearm (older children). Intravenous plastic cannulas (Vasofix Braunüle, 18 G; Braun Melsungen, Melsungen, Germany) were used as guides.

The catheter was continuously perfused with a sterile isotone solution (NaCl 0.9% Braun Melsungen). The low flow rate of 0.3 μL/min was ensured by a battery-driven pump (CMA 106 MD pump, CMA/Microdialysis AB). The dialysate samples were collected in microvials (CMA/Microdialysis AB) in a vial holder fixed at the end of the catheter outlet tube. MD duration ranged from 4 to 7 d and was performed without any complications. Dialysates were collected every 2 h and were first analyzed for glucose, lactate, and pyruvate at the bedside in the CMA 600 Microdialysis Analyzer (CMA/Microdialysis AB). The residual dialysates were frozen at −21°C for subsequent carnitine determination.

In vitro determination of the relative recovery rate for carnitines. Concentrations of the substances of interest in the dialysate are proportional to the concentrations in the extracellular fluid, depending on membrane length and texture, flow rate and tissue temperature, expressed in the relative recovery rate (RR) of the MD system: RR = concentration (dialysate)/concentration (surrounding medium).

The relative recovery in our system was determined *in vitro* by immersing the catheter in test solutions of diluted or carnitine-supplemented human serum.

By adding 0.9% NaCl or carnitine stock solution to human serum, solutions of six different concentrations were obtained. They contained C0 in the range of 5 to 174 μmol/L, C2 in the range of 7 to 65 μmol/L, and C4OH in the range of 0.09 to 0.43 μmol/L.

To avoid matrix effects of serum proteins and to create tissue fluid conditions, the standard solutions were prepared by ultracentrifugation, using ultracentrifuge tubes (Centrisart®, Sartorius AG Göttingen, Germany) with a molecular exclusion size of 20,000 D.

A CMA 70 MD catheter with a membrane length of 20 mm was then immersed in the ultrafiltrates and stirred at room temperature. MD was performed at the *in vivo* flow rate of 0.3 μL/min and was equilibrated for at least 4 h before dialysate collection. C0 and acylcarnitines in ultrafiltrates and dialysates were quantified, and the ratio of carnitine concentrations in the ultrafiltrates and the dialysates was used to determine the RR of the MD system.

Carnitine determination. C0 and acylcarnitines were quantified in a Perkin Elmer API 365 tandem mass spectrometer (18). Carnitines in microdialysates and ultrafiltrates were directly determined by mass spectrometry.

Statistical analysis. Statistical analysis was performed using the computer program Statistical Package for Social Science version 11.5 (SPSS Inc, Chicago, IL). Statistical significance was tested by analysis of variance for repeated measurements. *Post hoc* tests were performed one sided. Using a Bonferroni correction, the significance level was set at 1.7%. Results are expressed as median (range) or mean (± SD).

RESULTS

RR in vitro. The RR *in vitro* was determined for the CMA 70 MD catheter at a flow rate of 0.3 μL/min. The ratio of carnitine concentrations in the dialysate and in the ultrafiltrate revealed a mean RR of 86% (± 7%) for C0, of 88% (± 7%) for C2, and of 83% (± 9%) for C4OH (Table 1).

Changes in the tissue carnitine pattern. During the initial fasting period (24 h) β-OHB levels increased in peripheral blood from 0.13 mmol/L (± 0.14 mmol/L) to 2.80 mmol/L (± 1.91 mmol/L). After 2 d of KD (d 3), β-OHB levels exceeded 5 mmol/L in all patients.

Subcutaneous C2 levels increased significantly ($p = 0.001$) during the fasting period, from 5.13 μmol/L (2.39–6.49 μmol/L) before fasting to 13.13 μmol/L (5.35–21.1 μmol/L) after 24 h of fasting. C2 increased continuously with KD to a concentration of 22.42 μmol/L (9.13–27.24 μmol/L) after 24 h of ketogenic nutrition and then remained stable (Fig. 1*A*).

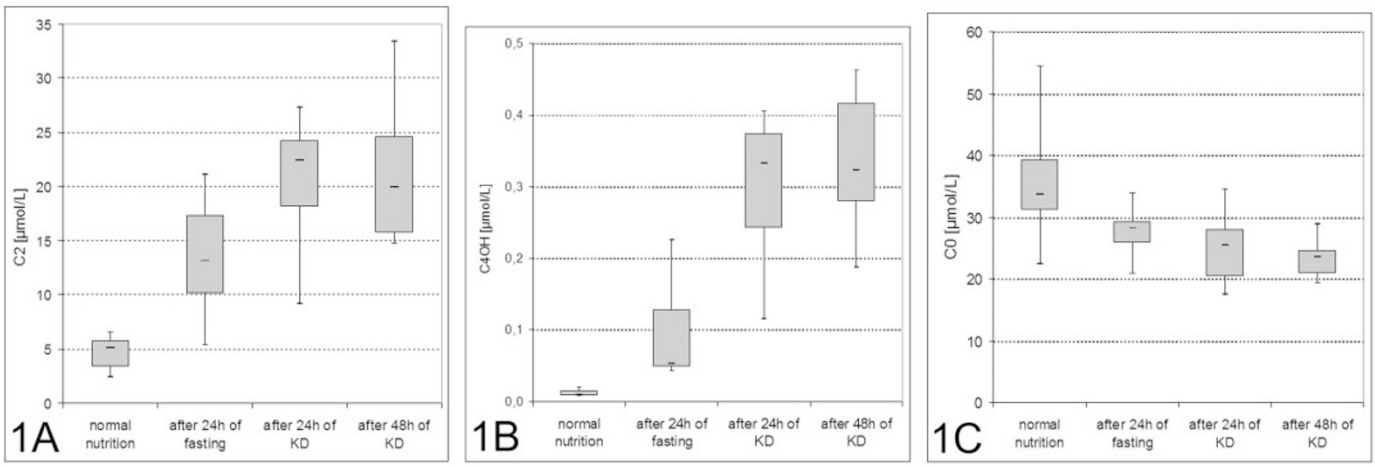

Figure 1. Influence of ketosis on C2 (*A*), C4OH (*B*), and C0 (*C*) in the s.c. tissue. C2 (μmol/L) and C4OH (μmol/L) increased significantly with fasting and ketogenic nutrition ($p_{C2} = 2 \times 10^{-7}$, $p_{C4OH} = 1.4 \times 10^{-5}$). s.c. C0 (μmol/L) decreased slowly with ketosis ($p = 0.001$). Each boxplot is based on 28 values (four values per patient).

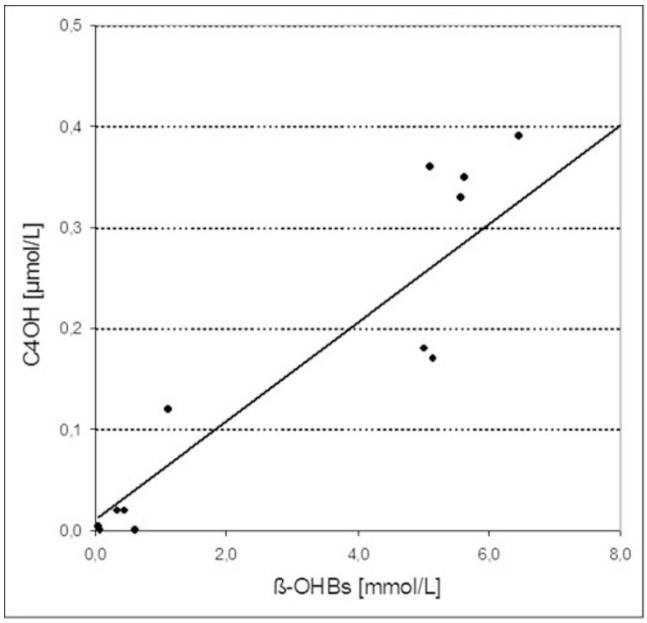

Figure 2. Serum β-OHB correlates strongly with s.c. C4OH. β-OHB is plotted against C4OH in the dialysate ($r = 0.91$, including data for seven patients).

Subcutaneous C4OH levels increased 3.6-fold during 24 h of fasting from 0.01 μmol/L (0.01–0.02 μmol/L) to 0.05 μmol/L (0.04–0.22 μmol/L) ($p = 0.0025$). C4OH reached 0.33 μmol/L (0.12–0.41 μmol/L) after 24 h of KD. The level was 23-fold higher than with normal nutrition and remained stable throughout the second day of KD (Fig. 1*B*).

C0 in the s.c. tissue decreased slowly from 33.68 μmol/L (22.48–54.47 μmol/L) to 28.24 μmol/L (20.89–33.94 μmol/L) after fasting (not significant) and reached 23.62 μmol/L (19.31–28.83 μmol/L) after 2 d of KD (significant to the initial value with normal nutrition, $p = 0.0045$) (Fig. 1*C*).

We found a strong positive correlation of β-OHB levels in the blood and C4OH in the s.c. tissue ($r = 0.91$, Fig. 2), and a moderate correlation of β-OHB in the blood and C2 in the s.c. tissue ($r = 0.7$).

In accordance with the carnitine changes in the s.c. tissue, we noted a decrease of the serum C0 as well as an increase in C2 and C4OH with increasing ketosis.

DISCUSSION

We used s.c. MD in children to monitor the KD by changes in the tissue carnitine pattern of C0, C2, and C4OH.

The high relative recovery of the MD system *in vitro* made s.c. MD suitable for monitoring of tissue carnitine concentrations (15).

Ketosis in children was induced by fasting and maintained with ketogenic nutrition, accompanied by characteristic shifts of carnitine metabolites in the s.c. tissue. Thus, C0 slowly decreased, whereas C2 and C4OH rapidly increased with ketosis.

So far, fasting experiments in animals and humans revealed changes in the blood and urine carnitine pattern similar to our findings. In fasting or diabetic ketosis, a delayed decrease in plasma and urine C0 and a rapid increase in long- and particularly short-chain acylcarnitines was found and correlated well with increasing plasma ketone levels (4,5,19). Several animal tissues have also been studied for carnitine metabolites under ketotic conditions, such as liver (20–23), skeletal muscle (20–23), heart (22–24), kidney (20,23), and brain (24).

C0 levels decrease mainly because they are stored in the esterified form. Studies of Hoppel and Genuth (19) revealed an increase in the urinary excretion of acylcarnitines during ketosis, which may lead to a loss of carnitine.

The increase in C2 reflects an increase in acetyl-coenzyme A (CoA), the final product of β-oxidation. Via carnitine acetyltransferase in the mitochondria, the acetyl group is transferred to carnitine dependent on the equilibrium constant of the enzyme. By generating C2 from acetyl-CoA, carnitine may act as an acyl sink to maintain adequate cellular levels of free CoA (25). According to Hoppel and Genuth (19), the C2/carnitine ratio is reflecting the respective CoA ratio and so may reflect the energy level.

Changes in the C4OH pattern, especially in combination with carnitines, have not been studied so far. Our MD study revealed the characteristic changes in the s.c. C4OH pattern, which correlated with increasing levels of β-OHB in the blood. After 24 h of fasting, levels had increased fivefold and further increased with KD to a value 23-fold greater compared with the level before fasting. The s.c. C4OH was highly correlated with the level of ketone bodies in the blood ($r = 0.91$). Depending on its concentration, β-OHB may be linked to carnitine in an unspecific enzymatic reaction, as it is described for acetoacetyl-CoA (25). So C4OH/CO may also be an indirect marker of energy level. C4OH might further be a very sensitive parameter for the extent of the ketotic state as it includes a parameter of the body's carnitine reserve as a prerequisite for the effective metabolism of ketone bodies. Indicating a sufficient level of substituted carnitine, C4OH might be the more accurate parameter for monitoring the state of ketosis than ketone bodies alone.

We conclude that a KD is therapeutic nutrition that causes important changes in patients' metabolism, and close monitoring of patients' energy state is advantageous, especially during initiation of the diet. Subcutaneous MD in combination with spectrometric carnitine determination allows a minimally invasive, close, and extensive monitoring of tissue metabolism.

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