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On the differences between urinary metabolite excretion and odd-numbered fatty acid production in propionic and methylmalonic acidaemias

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Summary: In five subjects with methylmalonic acidaemia (MMA) and five with propionic acidaemia (PA) both the level of odd-numbered fatty acids (OLCFA) in erythrocyte lipids and the excretion of propionate-derived metabolites in urine were longitudinally analysed. At a given intake of amino acid precursors of propionyl-CoA and otherwise stable metabolic conditions, subjects with MMA excreted considerably more propionate-derived metabolites, and accumulated less OLCFA in erythrocyte lipids than subjects with PA. We suggest that renal metabolite excretion indicates the efflux of organic acids from cells and might be a measure of the individually determined intracellular relief from toxic acyl-CoA esters. It does not necessarily reflect the total amount of acyl-CoA intermediates produced in the body. OLCFA levels seem to reflect the continuous burden of propionyl-CoA toxicity within the cells and thereby might serve as a reliable tool for evaluating the quality of long-term metabolic control in these disorders.

Patients with inborn errors of propionate metabolism such as propionic (PA, McKusick 232000) and methylmalonic (MMA, McKusick 251000) acidaemias share some characteristic features, most probably due to intramitochondrial accumulation of potentially toxic acyl-CoA intermediates. Both propionyl-CoA and methylmalonyl-CoA cause a complex disturbance of mitochondrial metabolism (Martin-Requero et al 1983; Brass and Bayerinck 1988; Corkey and Deeney 1990; Matsuishi et al 1990; Fenton and Rosenberg 1995). The mainstay of therapy in these disorders is the dietary restriction of the amino acid precursors of propionyl-CoA: L-valine, L-isoleucine, L-methionine and L-threonine (Ney et al 1985; Fenton and Rosenberg 1995). For optimal treatment, intake of these precursor amino acids in the form of natural protein must be sufficiently high to

allow for optimal growth and nutrition but must not go beyond an individual limit in order to minimize the possibility of accumulation of acyl-CoA intermediates to potentially toxic levels within the mitochondria. This therapeutic approach should result in an adequately low excretion of the main propionate-derived metabolites in urine such as 3-hydroxypropionate and methylcitrate in PA, and methylmalonate in MMA (Thompson et al 1989).

The amount of odd-numbered long-chain fatty acids (OLCFA) in lipids has been shown to be elevated in patients with PA and MMA (Wendel 1989). Since propionyl-CoA acts as a primer in the biosynthesis of these fatty acids (Lynen et al 1962), a quantitative relationship of OLCFA synthesis and the amount of propionyl-CoA formed has been postulated (Wendel 1989).

Here we demonstrate for both disorders an inverse relationship between the amount of metabolites excreted in the urine and the amount of OLCFA accumulating within the erythrocyte lipids. Our data lend support to the hypothesis that renal metabolite excretion indicates the relief of the organism from potentially toxic acyl-CoA intermediates, whereas the accumulation of OLCFA reflects the continuous burden of propionyl-CoA that remains within the cells.

METHODS

Materials: Unless otherwise stated, all chemicals were purchased in the highest available purity from Merck (Darmstadt, Germany) or Sigma (Diesenhofen, Germany).

Standards: Methylmalonic acid was purchased from Aldrich (Steinheim, Germany). Methylcitric acid, trisodium salt was prepared from3-oxoglutaric acid diethyl ester, methyl iodide (Aldrich, Steinheim, Germany), and potassium cyanide as detailed by Beach et al (1977). For final purification, however, the original paper chromatography was replaced by an anion exchange chromatographic procedure (von Korff 1965). 3-Hydroxypropionic acid, sodium salt, was prepared by alkaline hydrolysis of 3-hydroxypropionitrile as detailed by Read (1967) and purified by anion exchange chromatography (von Korff 1965). As checked by gas chromatography–mass spectrometry and ^{1}H NMR spectroscopy, methylcitrate was pure (>97%), essentially free from citric acid, and contained minor amounts of dimethyl compounds (<3%). The sodium 3-hydroxypropionate preparation was >96% pure and contained some as yet unidentified carboxylic acid derivatives as impurities.

Analytical methods. Urinary metabolites: For determination of the major propionate metabolites methylmalonate, 3-hydroxypropionate and methylcitrate (Thompson et al 1989, 1990), organic acids were extracted from acidified urine: 0.5 ml urine was mixed with 0.25 ml of propan-1,2,3-tricarbonic acid (10 mmol/L in water; Aldrich, Steinheim, Germany) as an internal standard; 0.1 ml of this mixture was acidified with 0.03 ml of 6 mol/L HCl and extracted with 0.75 ml of ethyl acetate. The organic extract was dried under a stream of nitrogen. Then the organic acids were converted to their trimethylsilyl (TMS) derivatives by MSTFA (Macherey & Nagel, Düren, Germany) in tetrahydrofuran at ambient temperature for 1 h. The TMS derivatives were separated by capillary column gas–liquid chromatography on a DB-1 column (0.25 mm ID × 30 m, 0.25 μm film; J & W

Scientific, Folsom, CA, USA) using a temperature programme from 95 to 270°C at 40°C/min with a 1-μl sample and a 70:1 split injection. Carrier gas was helium at 1.8 ml/min. A Dani GC 86.10 equipped with a FID was used. Components were identified from gas chromatographic retention data and quantified by reference to the internal standard using relative response factors as obtained with authentic standards (standard solutes in water). Results from spot urine samples were related to creatinine concentrations. Creatinine was measured by the Jaffe reaction (Boehringer, Mannheim) on a Hitachi analyser.

Fatty acids in erythrocyte lipids: Erythrocytes were isolated from EDTA-blood, washed as described (Wendel 1989) and stored at −20°C until analysis. The workup of the samples — extraction of membrane lipids, base-catalysed transesterification of the fatty acids, and analysis of the fatty acid methyl esters by capillary column gas–liquid chromatography — was performed exactly as described previously (Laryea et al 1988). Fatty acids of carbon length 14–22 were identified by comparison with authentic standards.

Subjects: Four children with a severe neonatal form and one child with a vitamin B_{12}-responsive form of MMA (McKusick 251100), and five children with neonatal-onset form of PA were studied over a period of 18 months. Diagnosis had been established on the basis of clinical features, increased urinary excretion of propionate-derived metabolites and, in some cases, *in vitro* enzyme assays. Treatment consisted of restriction of dietary protein intake and supplementation with essential amino acids, free of valine, isoleucine, methionine and threonine, and carnitine (30–100 mg/kg per day). The patients' intake of amino acid precursors of propionyl-CoA in the form of natural protein was adjusted according to clinical condition. In patients with severe MMA, intake of natural protein ranged from 0.75 to 1.4 g/kg body weight per day. Patient Y.M., who responded well to 20 mg/day hydroxycobalamin orally at the age of 2 years had as much as 1.6 g/kg per day of natural protein without amino acid supplement. Patients with PA ingested from 1.0 to 1.4 g/kg per day of natural protein. During the study, the patients were in good and stable conditions except for patient M.K., who was fairly controlled, and for patients K.S. and K.M., who experienced some episodes of intercurrent illness. Important data at the time of the study are compiled in Table 1.

The study was approved by the institutional ethics committee of the University Hospital Düsseldorf and was performed with the informed consent of the parents of each child.

Protocol: Four spot urine samples were obtained from each patient once a month, every morning and evening of two consecutive days. Urine was immediately frozen at −20°C and sent later to Düsseldorf, where it was stored at −70°C until analysed. Venous blood samples (3–5 ml) were collected in EDTA tubes every 3–5 months and sent to Düsseldorf. There, erythrocytes were isolated, washed and stored at −70°C until analysis. We were not able to obtain the complete set of data in each case, since the patients were not hospitalized during the study.

Calculations: The sum of the OLCFA — 15- and 17-carbon saturated and 17-carbon mono-unsaturated fatty acids ($C_{15:0}$, $C_{17:0}$, $C_{17:1}$) — was calculated and expressed as

Table 1 Urinary excretion of propionate-derived metabolites and red cell OLCFA levels in patients with methylmalonic and propionic acidaemias during an 18-month period

Subject (*sex*)	*Age at start of study* (years)	*Intake: natural protein* (g/kg per day)	*N*	*Excreted metabolites*[a] (mol/mol creatinine)	*OLCFA*[b] (%)
Cobalamin responsive methylmalonic acidaemia (cblA)					
Y.M. (m)	2	1.6	4	2.1 (0.7)	1.1 (0.2)
Methylmalonic acidaemias					
K.L. (f)	1	0.8	7	9.2 (2.5)	1.1 (0.3)
K.A. (f)	3	1.2	4	13.4 (2.3)	1.5 (0.2)
W.K. (f)	7	1.0	5	4.1 (1.0)	1.9 (0.3)
M.K. (f)	1	1.4	6	8.6 (3.0)	2.3 (0.5)
Propionic acidaemias					
A.D. (m)	1	1.1	3	0.5 (0.2)	3.2 (0.5)
B.D. (m)	1	1.0	3	1.5 (0.3)	2.3 (0.2)
K.M. (m)[c]	2	1.4	7	0.7 (0.2)	2.5 (0.7)
K.S. (f)[c]	5	1.4	4	0.6 (0.2)	2.6 (0.4)
G.Y. (m)	11	1.0	5	1.3 (0.2)	2.1 (0.4)

[a]In methylmalonic acidaemias, methylmalonate; in propionic acidaemias, sum of 3-hydroxypropionate and methylcitrate. Data are mean (SD); *N*=number of data pairs

[b]Control values for OLCFA in erythrocytes: 0.70%±0.12% of the total $C_{14}-C_{22}$ fatty acids in the sample

[c]K.M. and K.S. experienced four episodes of intercurrent illness

percentage of the total $C_{14}-C_{22}$ fatty acids in the sample. The monthly values of propionate metabolites excreted in urine were expressed as the means of four consecutive spot urine samples standardized to creatinine excretion. In order to get suitable pairs of data, the mean of three monthly values of metabolite excretion obtained in the period around blood sampling for measuring OLCFAs was attached to this OLCFA value.

RESULTS

The four patients with a severe form of MMA excreted large amounts of methylmalonic acid with mean values ranging from 4.1 to 13.4 mol/mol creatinine (Table 1). At the same time, the mean OLCFA levels in erythrocyte lipids ranged from 1.1% to 2.3% (normal controls 0.70%±0.12%). In contrast, patients with PA excreted much smaller amounts of propionate-derived metabolites, with means ranging from 0.5 to 1.5 mol/mol creatinine, and showed at the same time, with levels of 2.1% to 3.2%, clearly higher erythrocyte OLCFA levels. As can be seen in Table 1, natural protein intake and clinical status were comparable in both patient groups. Obviously, the mean values of metabolite excretion were not strictly related to individual protein intake.

When the metabolite excretion was plotted against the simultaneously measured OLCFA values (Figure 1), the data points for each MMA patient were spread over an apparently limited and individually situated area. The data set for the PA patients was clearly separated from that of MMA patients without any overlap. Within the PA group no individual areas of data points could be distinguished.

The single patient with the cobalamin-responsive form of MMA excreted only small amounts of methylmalonic acid in the urine and also showed low OLCFA levels. This was

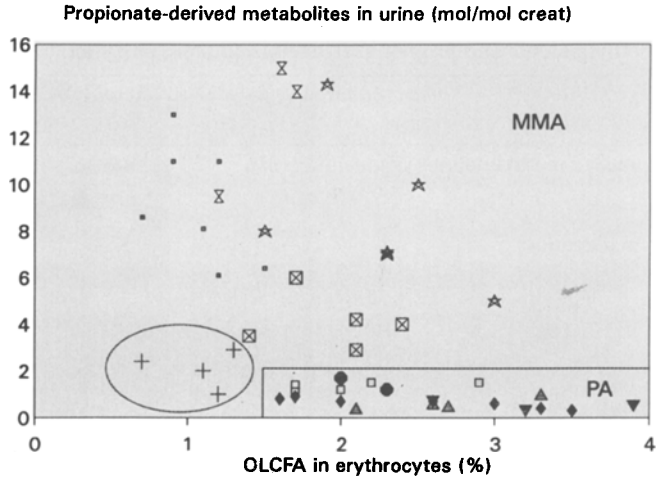

Figure 1 Plot of metabolite excretion in urine versus the OLCFA values in erythrocytes of patients with MMA and PA. Open symbols: patients with MMA, solid symbols: patients with PA. The samples were obtained simultaneously in the course of an 18-month study period. The data of the patients with cobalamin-responsive MMA are encircled

despite a much higher intake of natural protein than in patients with the severe form of MMA.

DISCUSSION

Subjects with severe forms of MMA excreted large but individually variable amounts of methylmalonic acid in the urine. In order to explain the fact that the amount of excreted methylmalonic acid varied from patient to patient, and obviously was not strictly related to the amount of ingested amino acid precursors of propionyl-CoA, two points have to be considered. (1) Even when the intake of precursor amino acids is exactly known, the net influx into the intracellular pool of acyl-CoA intermediates remains obscure. In MMA, the acyl-CoA pool comprises propionyl-CoA and D- and L-methylmalonyl-CoA and is maintained from different sources, among which catabolism of precursor amino acids is the most important but not the only one (Walter et al 1989; Thompson et al 1990). Other sources are propionate production by gut bacteria and oxidation of OLCFA during lipolysis. (2) With D-methylmalonyl-CoA hydrolase (EC 3.1.2.17) an active deacylating system exists in liver tissue which might be responsible for the release of large amounts of methylmalonic acid from mitochondria, which is then cleared into the urine (Kovachy et al 1983). This enzyme is active on the D-isomer but not on the L-isomer of methylmalonyl-CoA and almost inactive with propionyl-CoA (Kovachy et al 1983).

Most probably, in MMA it is the deacylation reaction which functions as an escape valve in order to limit the intramitochondrial accumulation of toxic acyl-CoA esters. Another line of efflux of acyl-CoA esters, by conjugation with carnitine or other compounds, appears of only minor importance (Millington et al 1984; Thompson et al 1989). Methylmalonyl-CoA does not participate in anabolic processes (Jakobs et al 1984). Therefore, the organism strives for immediate elimination of excess methylmalonate

residues by uncoupling from CoA at the level of the D-form of the acyl-CoA ester. Thereupon methylmalonate can cross cellular membranes readily and is readily excreted in the urine — though not without causing damage to the kidney in the long term (D'Angio et al 1991; Molteni et al 1991). It might be the individual deacylating capacity that determines how much of the metabolically produced methylmalonyl-CoA is deacylated and excreted, and how much remains within the cells. In this respect, interindividual differences in the deacylation capacity might, together with the individual propionate oxidation capacity, modify the clinical severity of the disorder (Thompson et al 1989, 1990) that is primarily brought about by the extent of genetic impairment of the methylmalonyl-CoA mutase step.

Patients with severe PA excreted much smaller amounts of propionate-derived metabolites in the urine than patients with MMA, although the amounts of ingested amino acid precursors and the clinical conditions were largely the same in both groups. Hence, in contrast to MMA, urinary metabolite excretion appears to play a minor role in diminishing potentially toxic effects of intracellular excess of acyl-CoA esters. Reportedly, there is no enzyme system for deacylation of propionyl-CoA and only one with relatively low deacylating activity against 3-hydroxypropionyl-CoA, its main secondary metabolite (Fenton and Rosenberg 1995). Lack of a specific propionyl-CoA deacylase is in complete accord with the role which propionyl-CoA can play analogously to acetyl-CoA. It serves as a substrate for the formation of methylcitrate which is then transported into the cytoplasm. From there it may be partly excreted into the urine, or it may be used as a priming molecule for the synthesis of OLCFA.

3-Hydroxypropionyl-CoA formed from propionyl-CoA on a less active pathway (Ando et al 1972) ultimately serves as a substrate for 3-hydroxyisobutyryl-CoA deacylase (EC 3.1.2.4), an enzyme involved in valine oxidation (Rendina and Coon 1957). In PA, the contribution of conjugation of organic acids with carnitine and other compounds (e.g. glycine) to the overall propionate efflux from the cells appears to be limited since relatively small amounts of conjugates are found in urine (Millington et al 1984).

The excretion of propionate-derived metabolites in the urine and the OLCFA content in erythrocyte lipids showed an inverse relation as a general characteristic in subjects with severe MMA and PA. Accordingly, patients with PA presented with higher mean OLCFA values than patients with MMA. This can be attributed to disorder-specific differences in the amount of intracellular propionyl-CoA that can be envisaged from the position of the metabolic block in propionate catabolism, and to the size of efflux of organic acids from the cells. At a given amount of ingested amino acid precursors of propionyl-CoA and otherwise stable metabolic conditions, both factors are in favour of a larger propionyl-CoA pool and thus of higher OLCFA values in patients with PA than in those with MMA.

Our data on the patient with cobalamin-responsive MMA fit into the concept presented. In this subject, the low urinary methylmalonic acid excretion is due to the still high flux of propionyl-CoA through the defective methylmalonyl-CoA mutase step. Only small amounts of acyl-CoA intermediates are produced, which are likely to be almost completely deacylated and excreted and at the same time lead to minimal elevation of OLCFA production.

Our data demonstrate the different mechanisms by which an organism can get rid of potentially toxic acyl-CoA esters. From a teleological point of view the active D-

methylmalonyl-CoA deacylation can be regarded as a preventive measure by which an otherwise healthy organism protects itself against possible harm arising from not infrequently occurring (even mild) cobalamin deficiency, when distinct amounts of D-methymalonyl-CoA are produced (Schneede et al 1994). In case of increased propionyl-CoA production, transportation into the cytosol and participation in synthesis of nontoxic odd-numbered fatty acids apparently signifies a sufficiently active disposal route.

In summary, we hypothesize that the renal excretion of propionate-derived metabolites deals with the efflux of organic acids from the cells and is a measure of the generally possible and individually determined relief from toxic acyl-CoA esters, and does not necessarily reflect the total amount of acyl-CoA intermediates produced in the body. In contrast, OLCFA seem to reflect the continuous burden of propionyl-CoA toxicity that remains within the cells and thereby might serve as a reliable tool for evaluating the quality of long-term metabolic control in these disorders. From our experiences we expect that optimally treated PA subjects with adequately low protein intake show OLCFA levels below 2.2% whereas OLCFA levels in well-treated subjects with MMA are, at 1.5%, somewhat lower.

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