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Metabolic response to carnitine in methylmalonic aciduria

An effective strategy for elimination of propionyl groups

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SUMMARY Patients with methylmalonic aciduria have an excessive intramitochondrial accumulation of acylcoenzyme A compounds that may reduce the availability of free coenzyme A (CoA) for normal metabolic requirements, producing profound metabolic disturbances. Giving carnitine to a patient with methylmalonic aciduria produced an increase in hippurate excretion (an index of intramitochondrial adenosine triphosphate (ATP) and CoA availability), a large increase in short chain urinary acylcarnitines, and a reduction in excretion of methylmalonate and methylcitrate. These acylcarnitines were shown by fast atom bombardment and B/E linked scan mass spectrometry to be propionylcarnitine and acetylcarnitine. Carnitine acts by removing (detoxifying) propionyl groups, thereby releasing CoA and restoring ATP biosynthesis and concentrations towards normal. L-carnitine may play a central role in maintenance of mitochondrial and cellular homoeostasis in methylmalonic aciduria and propionic acidaemia. These principles may provide an approach to the treatment of this and other disorders, inherited and acquired, in which accumulation of acyl CoA metabolites results in sequestration of free CoA, thereby perturbing metabolic homoeostasis.

Recent reports indicate the potential need for supplementary carnitine in patients with several disorders of intermediary metabolism, particularly those associated with the organic acidurias.[1–3] Many of these latter disorders are associated with the accumulation of short chain acylcarnitines (Chalmers, Roe, Hoppel, and Stacey, unpublished data). Carnitine was administered to a child with methylmalonic aciduria because of the clinical benefit seen after giving L-carnitine to another patient with propionic acidaemia. Propionic acidaemia and methylmalonic aciduria have in common the accumulation of propionyl coenzyme A (CoA) and its metabolites, with their untoward metabolic effects. We believe that there is an increased carnitine requirement in methylmalonic aciduria, we detail the beneficial effects on intermediary metabolism after giving carnitine, and identify acylcarnitines excreted in large quantities after carnitine supplementation. This study supports a role for carnitine in the management of patients with this disorder and those with propionic acidaemia.

This study was approved by the ethical committee of Northwick Park Hospital and the Clinical Research Centre, Harrow.

Clinical description

The patient, a girl, was the first child of unrelated caucasian parents. After a normal pregnancy, delivery at term was complicated by breech presentation requiring caesarean section. She was breastfed initially, but on day three of life she became tachypnoeic and hypotonic, and proceeded into coma with profound metabolic acidosis (base deficit, 21 mmol/l). Gasliquid chromatography of her urine and blood plasma followed by mass spectrometry gave a diagnosis of methylmalonic aciduria, and this was confirmed by enzymology on cultured skin fibroblasts. Attempts to treat with

vitamin B_{12} (1 mg daily IM) failed to improve her clinical or metabolic presentation. She was subsequently managed by restriction of natural protein intake (up to 1 g/kg body weight daily) with supplementary amino acids (devoid of isoleucine, valine, threonine, and methonine) at a value equivalent to an additional 1 g protein/kg body weight, daily. Supplementary vitamins and minerals were included, with additional calories provided by carbohydrate polymer and lipid. Her postnatal course was characterised by multiple hospital admissions for metabolic regulation, complicated by anorexia and recurrent vomiting associated with intermittent upper gastrointestinal bleeding. She has generally delayed development with particular delay in speech, and poor muscle tone and power. At the time of the study she was 21 months old, with a developmental age of 11 months, height <3rd centile, weight 3rd–10th centile, and head circumference 50th centile

Methods

Urinary organic acids were quantitatively extracted using DEAE-Sephadex and established procedures,[4] and they were analysed by capillary gas chromatography of their trimethylsilyl (TMS) and TMS-ethoxime derivatives on 25 m fused silica columns coated with a non-polar chemically bonded polymethylsiloxane (CP-SIL-5, Chrompak, UK London). A split injection (70:1) of 2 μl sample at 200°C was temperature programmed at 6°C/min from 70°–250°C. The carrier gas was helium, 1·4 ml/min. Components were identified using retention indices, and quantified using n-tetracosane as a standard. Identifications were subsequently confirmed using capillary gas chromatography mass spectrometry.

Plasma and urinary carnitine and acylcarnitines were determined by radioenzymatic assay utilising [1-^{14}C]-acetyl-CoA and carnitine acetyltransferase, according to the method of Brass and Hoppel.[5] Acylcarnitines in plasma were separated into an acid soluble fraction (short chain acylcarnitines) and an acid insoluble fraction (long chain acylcarnitines). The results are expressed in μmol/l for plasma and nmol/mg creatinine for urine.

Acetylcarnitine and propionylcarnitine were synthesised according to the method of Bohmer and Bremer,[6] using the acylchlorides and L-carnitine under reflux conditions followed by multiple recrystallisations. Aqueous eluates from urine samples passed over Dowex-1 colums at neutrality[7] and concentrated by lyophilisation were analysed by fast atom bombardment (FAB) mass spectrometry,[8] using xenon gas and samples in a glycerol matrix with a VG 7070 HS mass spectrometer. Acylcarnitines were further analysed by linked scanning at constant B/E ratios that provided an independent fragmentation pattern for each major ion in the mass spectrum.[9]

At the time of the study, although requiring nasogastric tube feeding, the child was under reasonably good metabolic control as shown by normal plasma glucose, ammonia, electrolyte, acid base status, and by urinary organic acids. D, L-carnitine, 12·4 mmol (200 mg/kg body weight) was given as a single oral dose representing 100 mg/kg of the L-isomer (the isomer measured in the radioenzymatic assay).

Results

Before being given supplementary carnitine the child's plasma showed a total carnitine of 53·2 μmol/l (normal, mean (SD) 46·1 (10·0) μmol/l), free carnitine 35·8 μmol/l (normal, mean (SD) 36·7 (7·6) μmol/l), acid soluble (short chain) acylcarnitines 17·1 μmol/l (normal, mean (SD) 5·7 (3·5) μmol/l), and acid insoluble (long chain) acylcarnitines 0·3 μmol/l (normal, mean (SD) 3·7 (1·5) μmol/l).[10] These results show normal plasma free carnitine values but considerably raised short chain acylcarnitines.

In the urine, free carnitine was 142 nmol/mg creatinine (normal, mean (SD) 51·3 (40·1) nmol/mg creatinine) and combined acylcarnitines were 352 nmol/mg creatinine (normal, mean (SD) 73·7 (39·5) nmol/mg creatinine), giving a total urinary carnitine of 494 nmol/mg creatinine (normal, mean (SD) 125·1 (74·7) nmol/mg creatinine). The urine therefore showed increased excretion of both free carnitine and acylcarnitines.

After giving carnitine, urine samples were collected sequentially over a 24 hour period for organic acid analysis and acylcarnitine measurements. During this period, 476 μmol of carnitine, as free and acylcarnitines, were excreted in the urine. This amount represented 8·1% of the ingested dose. The total carnitines excreted comprised 269 μmol free and 207 μmol acylcarnitines. Urinary free carnitine excretion paralleled acylcarnitine excretion throughout the study period. Most of the excretion—77·2%—occurred in the first 12 hours.

Although there were no notable fluctuations in other metabolites, there were major changes in urinary hippurate, methylmalonate, methylcitrate, and acylcarnitines during the first 12 hours (Fig. 1). There was a rapid 6 fold increase in hippurate concentration at 30 minutes after the dose, with a peak value attained at 3 hours, and the values decreasing thereafter. In contrast, there was a less than twofold increase in acylcarnitine excretion by 30 minutes,

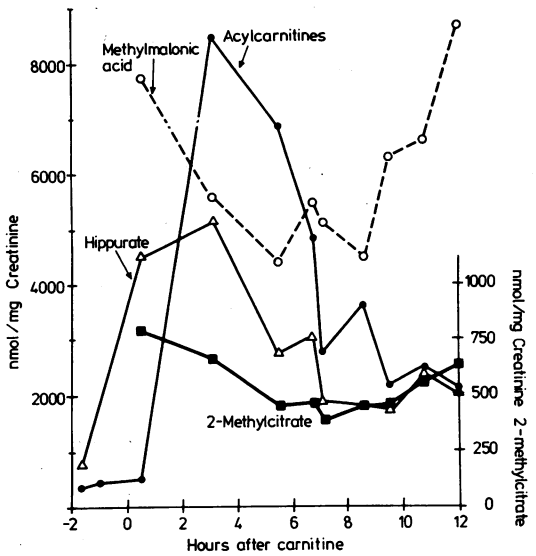

Fig. 1 *Metabolic effects in the urine of a patient with methylmalonic aciduria after a single oral dose of D, L-carnitine 200 mg/kg body weight (given at arrow).*

but then a rapid excretion took place by five hours, representing a 24 fold increase over the baseline value. As the acylcarnitine excretion increased, both methylmalonate and methylcitrate excretion decreased progressively, by 43% and 57% respectively compared with earlier values at 30 minutes. Plasma carnitine analysis at this time showed major changes in short chain acylcarnitine concentrations, from 17·1 μmol/l before carnitine supplementation to 27·4 μmol/l afterwards. Free carnitine increased from 35·8 to 66·1 μmol/l. There was no appreciable change in plasma long chain acylcarnitines. The relation between urinary acylcarnitines and the metabolites, methylmalonate and methylcitrate, persisted for 12 hours, by which time the metabolites had returned to their previous high values, and acylcarnitine excretion had decreased.

The reciprocal relation and temporal sequence between the metabolites, methylmalonate and methylcitrate, and urinary acylcarnitines suggested the possibility of removal of a short chain acyl CoA precursor to the organic acid metabolites. Fast atom bombardment (FAB) mass spectrometry, an ionisation technique particularly useful for highly polar solids, was used for investigation of the chemical nature of the acylcarnitines. The acylcarnitines exhibit intense parent cations of the type $(CH_3)_3$ N CH_2CH $(OR)CH_2CO_2H$ (R=acetyl, propionyl, etc) with prominent fragment ions at m/z 58, 85, 100, and 144. The characteristic parent ions for acetylcarnitine and propionylcarnitine have m/z values of 204 and 218, respectively. These compounds characteristically produce a major fragment corresponding to M-59. The FAB spectrum of a urine sample obtained at the peak of acylcarnitine excretion (5 hours) showed prominent ions at m/z 218, 204, and 162 (Fig 2(a)). The other fragment ions such as m/z 58, 85 etc, are also seen in this mass spectrum. Accurate mass measurements of the parent ions m/z 218, 204, and 162 by peak matching at a resolution of 6000 (10% valley)

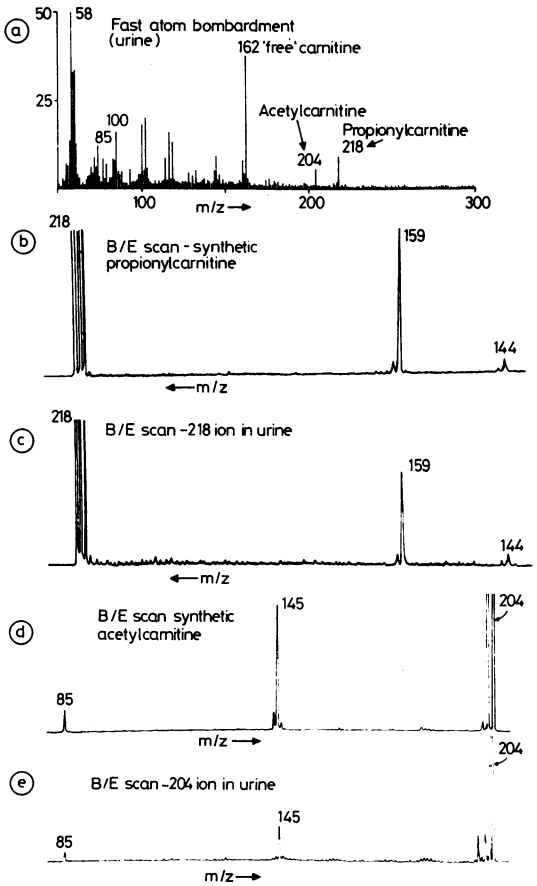

Fig. 2 *(a) Fast atom bombardment (FAB) mass spectrum of urinary acylcarnitines from a sample obtained 5 hours after carnitine was given to the patient with methylmalonic aciduria.*
(b)–(e) B/E linked scans of synthetic and urinary propionylcarnitine and acetylcarnitine.

using a glycerol matrix ion (m/z 185.1025) for reference gave the following values: (a) 218.1390 consistent with the calculated mass of 218.1392 for propionylcarnitine ($C_{10}H_{20}O_4N$), (b) 204.1231 consistent with the calculated mass of 204.1236 for acetylcarnitine ($C_9H_{18}O_4N$), and (c) 162.1114 consistent with the calculated mass of 162.1130 for free carnitine ($C_7H_{16}O_3N$).

Further evidence for the presence of propionylcarnitine and acetylcarnitine was provided by linked scanning at a constant B/E ratio[9] that produces an independent fragmentation pattern for each major ion in a mass spectrum. The constant B/E linked scan spectra for the m/z 218 ions from synthetic propionylcarnitine and the urine extract are compared in Fig. 2(b) and (c), respectively. Similarly, comparison of the linked scan spectra of synthetic acetylcarnitine, m/z 204, and that ion in the urine are shown in Fig. 2(d) and (e), respectively. The similarities in these linked scans between the synthesised acylcarnitines and those excreted by the patient seem identical. In each case the characteristic loss of 59 daltons corresponding to $(CH_3)_3N$ is seen. The m/z 218 produces the m/z 159 from propionylcarnitine while the m/z 204 yields the m/z 145 from acetylcarnitine.

Discussion

Carnitine supplementation in this child with methylmalonic aciduria was considered because of the beneficial clinical and metabolic effects noted after carnitine was given to another patient with propionic acidaemia.[1] Both propionic acidaemia and methylmalonic aciduria are disorders associated with the accumulation of acyl CoA compounds inside the mitochondria. Most metabolites excreted in these disorders share a common precursor, propionyl CoA.[4] This compound and its metabolites are known to produce a variety of metabolic disturbances. These include inhibition of the tricarboxylic acid (TCA) cycle,[11 12] reduced guanosine triphosphate (GTP) synthesis by inhibition of succinate: CoA ligase (GDP) and hence reduced regeneration of ADP from adenosine monophosphate (AMP) via GTP: AMP transphosphorylase,[13] and uncoupling of oxidative phosphorylation by propionate. All of these processes would result in reduced ATP synthesis. Propionyl CoA also inhibits the pyruvate dehydrogenase complex,[14] N-acetylglutamate synthetase,[15] and the glycine cleaveage system.[16] During a catabolic state, which would be associated with increased production of propionyl CoA, these inhibitory actions would have major effects on intermediary metabolism including pyruvate entry into the TCA cycle, activity of the cycle itself, the elimination of ammonia by the urea cycle, and the production of methylene units from glycine cleaveage. These metabolic disturbances lead to the clinical features that may be observed in these patients, associated, with hypoglycaemia, hyperammonaemia, and hyperglycinaemia.[4] Thus the most logical strategic approach to treatment would be the elimination of propionyl groups.

Before being given carnitine the patient had a normal plasma free carnitine concentration, associated with increased short chain acylcarnitines. Her urine contained increased concentrations of both free carnitine and acylcarnitines. These results indicate that she was able to synthesise carnitine and utilise it in the formation of acylcarnitines. However, the excessive excretion of acylcarnitines in the presence of continued excretion of abnormal organic acids suggests she may have insufficient L-carnitine available for her increased metabolic requirements imposed by the intramitochondrial accumulation of propionyl CoA. Supplementation with carnitine resulted in an increase of acylcarnitine excretion to very high values, with a concomitant reduction in urinary methylmalonic acid and methylcitric acid excretion. The major acylcarnitine excreted was shown to be propionylcarnitine, suggesting that removal of propionyl groups is directly responsible for the metabolic changes obserevd.

These results indicate L-carnitine may play a central role in maintenance of mitochondrial and cellular homoeostasis in methylmalonic aciduria and in propionic acidaemia—both disorders in which propionyl groups accumulate as propionyl CoA with sequestration of intramitochondrial CoA. Giving exogenous carnitine and removal of this accumulating propionyl CoA results in release of free CoA with restoration of mitochondrial homoeostasis. This is reflected in the initial and immediate response to supplementary carnitine and the sudden and profound increase in hippurate formation and excretion. Intramitochondrial biosynthesis of hippurate from benzoate is dependent upon availability of both CoA and ATP[17] and this dramatic increase in hippurate excretion indicates that L-carnitine acts by removing propionyl groups, and restoring intramitochondrial CoA and ATP concentrations towards normal.[18] Benzoate, itself an uncoupler of oxidative phosphorylation, must be accumulating in the mitochondria in these disorders because of the inadequate supply of CoA and ATP. Benzoate treatment of hyperammonaemia in patients with disorders of organic acid metabolism, as in the primary hyperammonaemia syndromes,[19 20] would be contraindicated without greatly supplemented L-carnitine cotherapy.

Thus the major problems in methylmalonic aciduria and propionic acidaemia are: (a) lack of availability of CoA, (b) diminished ATP production, and (c) insufficient L-carnitine for the patients' increased metabolic requirements. All of these are caused by the accumulation of propionyl CoA. Since we have shown that propionyl groups can be detoxified and eliminated in the urine as propionylcarnitine, we propose the use of supplementary L-carnitine in the long term management of these diseases. With adequate supplementary L-carnitine, a mechanism is provided whereby flux of propionylcarnitine (formation and excretion) may be made to equal the excess accumulation of propionyl CoA when dietary protein intake is only moderately restricted. These principles may apply to the treatment of a wide group of disorders—some inherited, such as the organic acidurias and some acquired, such as Reye's syndrome[21]—in all of which accumulation of various acyl CoA metabolites results in the sequestration of CoA, thereby perturbing metabolic homoeostasis.

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