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No replenishment of carnitine from trimethyllysine during pivalate-induced carnitine loss in humans

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Pivaloylcarnitine formation from exogenous carnitine and from carnitine precursor 6-N-trimethyllysine was investigated in two groups of pivampicillin treated subjects. In the first group, medication of pivampicillin led to the formation of and urinary excretion of pivaloylcarnitine. Oral L-carnitine supplementation, introduced after the third day of treatment, caused 2 fold urinary excretion of carnitine esters. For this group, the plasma levels and urinary output of butyrobetaine also decreased on the third day of pivampicillin treatment, but it normalized after carnitine administration. In contrast, urinary output of the carnitine precursor, trimethyllysine did not change during the study period. For the second group, oral trimethyllysine supplementation was started on the 4th day of pivampicillin treatment. Administration of trimethyllysine had no effect on the urinary output of carnitine esters, although the urinary excretion of it increased from approximately 25 to 450 μmol/day. Plasma levels and urinary output of butyrobetaine decreased during the pivampicillin treatment and administration of trimethyllysine did not restore the levels. Administration of trimethyllysine produced a large increase in urinary trimethyllysine output, but it did not affect the fast atom bombardment mass spectrometry signal intensity of other carnitine precursors. The urinary metabolite profile shows, that the conversion process of trimethyllysine to hydroxy-trimethyllysine represent an obstacle in butyrobetaine and carnitine biosynthesis in humans in vivo. *Because the administered trimethyllysine was not converted to carnitine or carnitine precursors to any significant extent, its nutritional value with respect to the replenishment of carnitine reserves is questionable.* (J. Nutr. Biochem. 8:147–151, 1997.) © *Elsevier Science Inc. 1997*

Keywords: carnitine; trimethyllysine; pivampicillin; pivaloyl-carnitine

Introduction

The primary biochemical function of carnitine is related to the ester-forming capability of the β-hydroxyl group. In addition to its involvement in β-oxidation of the long-chain fatty acids,[1,2] it can form ester with several medium- and short-chain endogenous or exogenous fatty acids.[2–5]

In mammals, the body stores of carnitine have exogenous and endogenous origin.[1,6,7] Several lines of evidence suggest that carnitine should be considered a vitamin-like compound in humans because carnitine deficiency can develop in healthy individuals treated with certain drugs.[8–12] Pivalate is an apolar component of several widely used drugs (pivampicillin, cefetamet-pivoxil, etc). Pivalate, liberated from pivampicillin (PIVA), forms an acyl ester with the β-hydroxyl group of carnitine in humans;[9–11] the non-metabolizable pivaloylcarnitine is excreted in the urine. During prolonged treatment with the drug, the muscle stores of carnitine can be depleted by an order of magnitude,[11,13] indicating that endogenous synthesis is not adequate to meet the increased needs.

In mammals four enzymes are involved in the synthesis of carnitine from trimethyllysine (TML).[6,7] Although their activities have been detected in several tissues,[6,7,14–16] the relative contribution of the different organs is not clear,

This work was supported in part, by a grant from Hungarian Scientific Foundation (OTKA T-006244, T-014650), and, in part, by a grant from Hungarian Ministry of Welfare (ETT-TKT 03 662/93). Mass spectra were acquired at the MSU-NIH Mass Spectrometry Facility, which is supported, in part, by a grant (RR 00480) from the NIH National Center for Research Resources.

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Received July 25, 1996; accepted December 17, 1996.

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0955-2863/97/$17.00
PII S0955-2863(97)00013-2

Table 1 Plasma levels and urinary output of carnitine, carnitine esters, and butyrobetaine for the PIVA and carnitine-treated group (Group A). Day 0: before the PIVA (3 × 500 mg/day) treatment; day 3: on the third day of PIVA administration; day 6: on the last day of combined PIVA and carnitine (3 × 1 g/day) treatment

	Carnitine				
	Total	Free	Ester	Butyrobetaine	Trimethyllysine
Plasma					
Day 0	41.0 ± 3.16	32.2 ± 2.86	8.77 ± 1.26	6.83 ± 1.86	–
Day 3	24.0 + 2.70*	12.1 + 1.63*	11.9 + 1.32*	1.34 + 0.86*	–
Day 6	39.7 ± 2.07†	22.0 ± 1.84†	17.7 ± 1.38†	5.01 ± 1.23†	–
Urine					
Day 0	0.22 ± 0.05	0.07 ± 0.02	0.15 ± 0.02	4.47 ± 1.86	25.9 ± 9.60
Day 3	2.73 ± 0.37*	0.02 ± 0.01*	2.71 ± 0.21*	2.55 ± 0.79*	23.2 ± 7.91
Day 6	6.23 ± 1.79†	0.97 ± 0.08†	5.25 ± 0.45†	4.18 ± 1.31†	23.1 ± 6.53

Plasma values are μmol/L ± SEM, $n = 5$; urinary carnitines are mmol/day; butyrobetaine and trimethyllysine, μmol/day; *$P < 0.05$ versus day 0, †$P < 0.05$ versus day 3.

especially in humans. In rats TML is released from muscle proteins to the circulation and then, after cellular uptake, must enter the mitochondria of the tissues, where TML hydroxylase is located.[16] In rats, TML is readily converted to carnitine,[17] but not in humans.[18,19] Generation of TML is controlled by protein breakdown, but its use for carnitine synthesis is not necessarily affected by the carnitine status.[20] In this study, exogenous TML was administered to humans with enhanced carnitine demands that had been induced by PIVA treatment. The goal was to study the possible limits and obstacle(s) of TML use with the help of metabolite profiling.

Methods and Materials

Patients, study design

A total of 10 male, pediatric-age patients requiring ampicillin treatment by clinical indication participated in this study at the Department of Pediatrics, University of Pécs. Clinical diagnosis included pharyngeal colonization of ampicillin-sensitive *Staphylococci* or *Streptococci*, ampicillin-sensitive bacteriuria, and mild infection of unknown origin (probably viral). The selection criteria included: good general condition, no history of serious illness or previous hospitalization, no evidence for metabolic disorders or illness affecting major organ functions. The patients were randomly divided into two groups. The total study period was 7 days. After a control day, each group received PIVA (Pondocillin, Leo Pharmaceuticals, Denmark) for 6 consecutive days. The dose was 3 × 500 mg daily (a 500-mg PIVA capsule contains 1.08 mmoles of pivalate) In Group A ($n = 5$; average age, 10.4 ± 2.88 years, range, 6 to 13 years; average body weight 31.8 ± 11.6 kg, range, 18 to 46 kg; values are means ± SD), PIVA treatment was combined with 3 × 1 g carnitine daily (in molar terms, 1 g oral solution contains 6.2 mmoles of carnitine), starting 3 days after beginning PIVA treatment. This carnitine dose was chosen because previous studies showed that equimolar amounts of carnitine increased the amount of pivaloylcarnitine formed, but molar excess is required to quantitatively eliminate the pivalate formed probably due to intestinal degradation of carnitine.[20,21]

In Group B ($n = 5$; average age, 11.7 ± 3.01 years; range, 6 to 14 years; average body weight: 33.3 ± 11.4 kg, range 17–45 kg) the basal PIVA treatment was combined with 3 × 1 mmol 6-N-trimethyl-L-lysine glutamate daily beginning 3 days after initiating PIVA treatment. For both groups, blood samples were taken after an overnight fast, and 24-hr urine samples were obtained for the days shown in the Results section. The study design was approved by

Table 2 Plasma levels and urinary output of carnitine, carnitine esters, butyrobetaine, and trimethyllysine for the PIVA and trimethyllysine-treated group (Group B). PIVA (3 × 500 mg/day) treatment was introduced after day 0; trimethyllysine (3 × 1 mmol) was also administered after the day 3 of PIVA treatment

	Carnitine				
	Total	Free	Ester	Butyrobetaine	Trimethyllysine
Plasma					
Day 0	42.8 ± 4.05	32.8 ± 4.34	10.0 ± 2.37	5.60 ± 0.57	–
Day 3	29.5 ± 5.31*	13.6 ± 3.08*	15.9 ± 3.34*	0.97 ± 0.19*	–
Day 6	20.3 ± 4.61*	12.7 ± 2.61*	7.57 ± 2.59*	0.49 ± 0.17*	–
Urine					
Day 0	0.21 ± 0.06	0.09 ± 0.01	0.12 ± 0.04	4.88 ± 1.79	25.0 ± 12.0
Day 1	2.97 ± 0.99*	0.05 ± 0.01*	2.92 ± 0.99*	4.94 ± 2.12	26.1 ± 9.34
Day 2	2.57 ± 0.72*	0.01 ± 0.01*	2.56 ± 0.72*	3.61 ± 1.52	20.7 ± 12.3
Day 3	2.56 ± 0.67*	0.01 ± 0.01*	2.55 ± 0.66*	2.11 ± 0.45*	27.9 ± 9.83
Day 4	2.55 ± 0.61*	0.01 ± 0.01*	2.54 ± 0.61*	1.06 ± 0.32*	387.7 ± 98.4*
Day 5	2.54 ± 0.92*	0.01 ± 0.01*	2.44 ± 0.92*	1.24 ± 0.60*	535.9 ± 86.9*
Day 6	2.45 ± 0.89*	0.01 ± 0.01*	2.44 ± 0.89*	0.59 ± 0.25*	441.8 ± 158.0*

Plasma values are expressed as μmol/L; urinary carnitine output as mmol/day; urinary butyrobetaine and trimethyllysine as μmol/day; *$P < 0.05$ versus day 0.

the local Ethical Committee, and informed consent was obtained from the parents of all participants in the study.

Analytical methods, procedures

Plasma and urinary free and acid-soluble short-chain carnitine esters were measured radiochemically as described.[21] Urinary TML was determined by pre-column derivatization with orthophthalaldehyde after cation exchanger purification of samples (as described) using a Beckman System Gold HPLC.[22] Butyrobetaine was determined by conversion of butyrobetaine to carnitine by partially purified butyrobetaine hydroxylase.[23] Approximately 10 mL urine were lyophilized for NMR analyses,[21] NMR analyses were performed at the Department of Chemistry, UTD, Richardson, with a GN 500 equipment (General Electric, Fremont, CA USA). FAB-MS analysis was carried out on a JEOL HX-110 (JEOL USA, Peabody, MA USA) double-focusing instrument at the MSU Mass Spectrometry Facility. The acceleration voltage was 10 kV and xenon was used as the FAB gas with a collison energy of 6 KeV. For analysis of urinary quaternary nitrogen compounds, two different purification methods were used. Homocarnitine (5-N-trimethyl 2-hydroxy pentanoic acid, MW = 175 for the inner salt) was used as the internal standard for both methods. For one, urine samples were passed through a Dowex 1 × 8 anion exchanger (200 to 400 mesh) acetate form, similar to the method used for carnitine assays.[21] For the other, quaternary nitrogen compounds were purified from urine using a cation exchanger resin.[23–26] To a 2 mL column of Dowex 50 W × 8 (200 to 400 mesh), H^+ form, 1 mL urine was applied. After washing with two column volumes of water, the quaternary amines were eluted with 1 M NH_4OH, and the effluents were evaporated to dryness under nitrogen and were dissolved in 20 μL water. In separate experiments the acids were converted to the isopropyl esters to increase the sensitivity and to facilitate more accurate mass spectral quantitation.[25,26]

For statistical analysis the Student's *t*-test for paired samples was used.

Results

In Group A, plasma free and total carnitine were decreased on day three of PIVA administration (*Table 1*) and short-chain acid soluble carnitine esters were elevated, likely due to pivaloylcarnitine in the circulation.[10] Output of urinary carnitine esters was elevated and free carnitine output was diminished. On the last day of carnitine administration (day 6), urinary carnitine ester output was still increasing, but the urinary output of free carnitine were greater than on day 0. The plasma level and the urinary output of butyrobetaine decreased on day 3 of PIVA administration (*Table 1*). After the administration of carnitine, both plasma and urine butyrobetaine levels returned to normal. Contrary to the butyrobetaine, the TML output did not change during the study period.

For Group B, 24-hr urine collections were obtained for each day of the study. Administration of PIVA caused a large increase in the urinary carnitine ester output (*Table 2*), which was accompanied by reduced free carnitine excretion, similar to results for Group A. Plasma levels of free and total carnitine also decreased; the urinary output of carnitine esters after introduction of the TML treatment was constant and free carnitine levels remained depressed. Administration of TML did not change the plasma free and total carnitine levels. Plasma butyrobetaine decreased on day 3 of PIVA treatment, and it remained depressed during the study. Trimethyllysine administration did not increase urinary output of butyrobetaine after the treatment, but it increased TML output approximately 20 fold (*Table 2*).

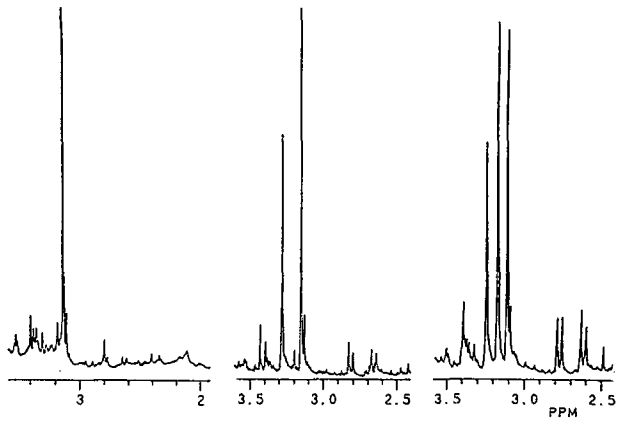

Figure 1 Representative ^1H NMR spectra of urine samples. First spectrum from left: untreated control; resonance at 3.11 ppm represents the creatinine and creatine phosphate. Second spectrum: a new peak appeared at 3.28 ppm in the urine of the PIVA-treated patients; the peak corresponded to the signal of the three methyl groups of the pivaloylcarnitine. Third spectrum: in the TML-treated subjects (Group B), one further resonance appeared at 3.17 ppm during the TML administration; the authentic TML gave the same resonance.

A peak at 3.11 ppm was observed with ^1H NMR in the pretreatment urine samples, which likely corresponded to creatinine and creatine phosphate (*Figure 1*). After treatment with PIVA, an additional resonance appeared at 3.28 ppm for both groups, corresponding to the three methyl groups of authentic pivaloylcarnitine. After administration of TML to Group B, one new peak was detected at 3.17 ppm; this corresponded to the three methyl group of authentic TML.

With FAB-MS analysis, a relatively low signal intensity for all of the intermediates of carnitine biosynthesis was detected in the urine of both groups before treatment (*Figure 2*, panel 1). After treatment with PIVA pivaloylcarnitine was positively identified in the urine samples for both groups of patients at the 246 m/z ratio (*Figure 2*, panel 2). Pivampicillin treatment did not affect the signal intensity of any intermediates of carnitine synthesis. After treatment with TML, the intensity of TML greatly increased; no other increases were detected (*Figure 2*, panel 3).

Discussion

Rats and humans readily form pivaloylcarnitine from the pivalate liberated from pivalic acid containing prodrugs.[8–11] Pivaloylcarnitine excretion increases urinary loss of carnitine that can results in depletion of tissue carnitine after prolonged treatment. Reductions in carnitine levels of an order of magnitude (3–5 to 0.3–0.7 μmol/g tissue) have been reported for human muscle,[11,13] the major storage site for carnitine. The estimated rate of carnitine synthesis is approximately 1.20 $\mu mol \times kg^{-1} \times day^{-1}$ in adults, estimated from the steady-state carnitine excretion by strict vegetarians. For this study, urinary loss of carnitine esters was much greater than 1.20 $\mu mol \times kg^{-1} \times day^{-1}$ for both groups after beginning the PIVA treatment. If decreases of

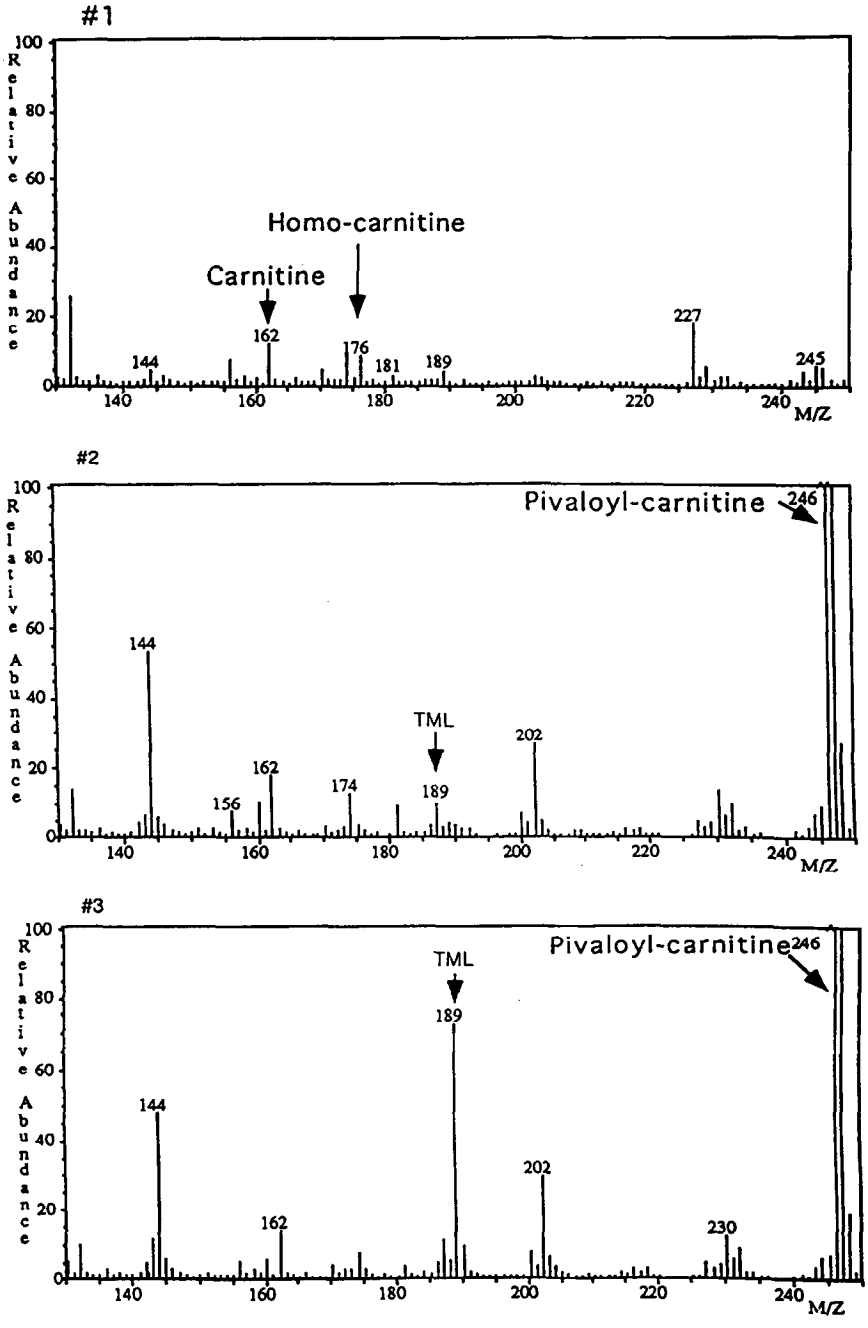

Figure 2 Representative FAB-MS spectra of the urine of the patients. First spectrum (*top*): untreated control sample; the same signal intensity was found in both groups before the treatment. Second spectrum: representative of urine of PIVA-treated patients; the peak at 246 m/z was the pivaloylcarnitine formed. Third spectrum: in the Group B, a further peak appeared at 189, corresponding to the TML administered.

carnitine reserves can affect the synthetic rates of carnitine in humans, the present condition can be used as a model for the synthesis. Note, that carnitine synthesized from its precursors should have been used for pivaloylcarnitine production, similarly to the exogenous carnitine supplement; administration of a molar excess of carnitine enhanced pivaloylcarnitine excretion in Group A, similar to previous results.[10,20,21] If molar excess of carnitine is available as exogenous supplement, detoxification of pivalate prefers the carnitine ester formation in humans.

Pivalate or its derivates do not appear to inhibit carnitine biosynthesis, since no increases were found in the level of any precursors of carnitine biosynthesis in the urine before the TML administration. Carnitine precursors have been detected in the urine of rats[27] and tentatively in humans.[24] For the current study, major increase in precursor levels should have been detected in the urine of the PIVA-treated patients, but urinary levels were unchanged. Thus, the carnitine deficiency in PIVA treatment appears to be caused solely by depleting of the reserves.

The hydroxylation of butyrobetaine to carnitine is not rate-limiting in the biosynthesis of carnitine in humans

because administration of exogenous butyrobetaine in vivo causes massive increase in the carnitine production.[18,19] The plasma level and the urinary output of butyrobetaine decreased in both groups during the PIVA treatment. This likely was because of the increased demand for carnitine caused by pivalate ester formation, which should promote an increase in carnitine biosynthesis from butyrobetaine and a decrease of the butyrobetaine levels. This explanation is supported by the observation that, in Group A, the level and output of butyrobetaine was normalized after carnitine administration.

No changes were detected in the urinary output of TML during the first three days of treatment in any groups. In Group A the urinary output of TML did not change during the study period. Although the exogenous carnitine was a good substrate for pivaloylcarnitine formation, output of carnitine esters was not affected by the administration of exogenous TML in Group B, nor were free carnitine levels in plasma and urinary output of free carnitine affected by exogenous TML. These results show, that the drug induced carnitine depletion was not accompanied by increased use of endogenous TML, and the administered TML was not used for butyrobetaine or carnitine synthesis.

There are several possible explanations for the lack of conversion of TML to butyrobetaine and carnitine; limited or lack of uptake of TML from the circulation; limited or lack of uptake of TML by mitochondria; regulation of TML hydroxylase; as well as low or no functional activity of this enzyme. Further rate-limiting steps in the classic synthetic pathway seems to be unlikely because no increases were found in the excretion of any precursors for carnitine synthesis after administration of TML. The method used would detect any major increases of metabolite levels in the urine, because the increased circulating levels should have been associated with increased filtered load. Thus, the conversion of TML to hydroxy-trimethyllysine, including the uptake and intramitochondrial transport of TML, limited the TML use. Contrary to the situation in rats, this process appears to be regulatory or even not operational in the biosynthesis of carnitine in humans,[28] which does not permit increased flux even if the tissue needs are increased.

Acknowledgments

The authors are grateful to Dr. Carlo Trevisani (Sigma-Tau Foundation, Rome/Pomezia, Italy) for providing carnitine. The 6-N-trimethyllysine glutamate salt was a kind gift from László Kovács (Gedeon Richter Pharmaceuticals, Budapest, Hungary). The skilled technical help of Ilona Szántó and Piroska Sütő is kindly acknowledged.

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