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Research Article

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Identification of 3-Methylglutarylcarnitine

A New Diagnostic Metabolite of 3-Hydroxy-3-methylglutaryl-Coenzyme A Lyase Deficiency

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Abstract

Deficiency of 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) lyase affects the metabolism of leucine as well as ketogenesis. This disorder is one of an increasing list of inborn errors of metabolism that presents clinically like Reye's Syndrome or nonketotic hypoglycemia.

Four patients with proven 3-hydroxy-3-methylglutaryl-CoA lyase deficiency were shown to excrete a new diagnostically specific metabolite. The technique of fast atom bombardment and tandem mass spectrometry revealed that only 3-methylglutaryl-CoA is a substrate for acylcarnitine formation. Neither 3-methylglutaconyl-CoA nor 3-hydroxy-3-methylglutaryl-CoA are excreted as acylcarnitines.

The excretion of 3-methylglutarylcarnitine may explain, in part, the apparent secondary carnitine deficiency in this disorder. Carnitine supplementation with moderate dietary restrictions may be a useful treatment strategy for this disorder.

Introduction

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A lyase (HMG-CoA lyase)[1] (EC 4.1.3.4) serves both the degradation of L-leucine and the production of acetoacetate from fatty acid metabolism. A deficiency of this enzyme results in a clinical presentation resembling Reye's syndrome with nonketotic hypoglycemia, metabolic acidosis, encephalopathy, hyperammonemia, and a characteristic organic aciduria (1).

Several patients with this disorder have been reported to have very high levels of urinary acylcarnitines associated with depressed levels of free carnitine, which suggests a need for carnitine supplementation in addition to dietary restriction (2). There has been increasing evidence that specific acylcarnitines are excreted in the organic acidurias that have been studied (3–7).

Here is presented the identification of a new metabolite, 3-methylglutarylcarnitine, which characterizes the HMG-CoA lyase deficiency and suggests a role for carnitine therapy in this organic aciduria.

Methods

Organic acid profiles were obtained on urine samples (2 ml) by derivatization and gas chromatography-mass spectrometry (GC-MS) analysis as described previously (6). The acids were identified by matching retention times and mass spectra with those of standards.

Plasma- and urine-free carnitine and short-chain acylcarnitine and plasma long-chain acylcarnitine concentrations were determined by radioenzymatic assay (8). 3-Methylglutarylcarnitine was synthesized by condensation of the mono-chloride of 3-methylglutaric acid (Fluka, Hauppauge, NY), which was formed in situ from thionyl chloride, with excess L-carnitine essentially as described previously (9). The product that was obtained after repeated precipitation from acetone-methanol-ether was characterized by fast atom bombardment (FAB)-mass spectrometry (MS) (10), which showed the expected protonated molecular ion at a mass/charge ratio (m/z) of 290 with some contamination from L-carnitine (m/z 162).

The patients' urine (25 μl) or standard solution of 3-methylglutarylcarnitine (~1 g/liter, 25 μl) were desiccated over $NaOH/H_2SO_4$, and then methylated (25 μl of 3 M HCl/MeOH, 80°C, 15 min). FAB-MS was also performed on these samples (1–2 μl), and the signal corresponding to the molecular cation of 3-methylglutarylcarnitine (m/z 318) was structurally analyzed by tandem mass spectrometry (MS/MS) (11) on a VG 7070 E-Q tandem mass spectrometer (VG Analytical, Ltd., Manchester, United Kingdom). The collision gas used to induce fragmentation was air and the collision energy was 100 eV.

Another aliquot of urine from one of the patients (1 ml) was partially purified to remove organic acids by elution through an ionic exchange column (Dowex 1(Cl^-); Bio-Rad Laboratories, Richmond, CA), and then hydrolyzed in base (0.2 N KOH, 37°C, 1 h). After acidification, the liberated acids were extracted into ether (2 × 1 ml) and the combined extracts dried, derivatized (50 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA), 80°C, 15 min), and then analyzed by GC-MS.

Results

Four patients with HMG-lyase deficiency were identified by urinary organic acid analysis by capillary GC-MS and enzyme assay from cultured fibroblasts. Large amounts of 3-hydroxyisovalerate, 3-methylglutarate, 3-methylglutaconate, and 3-hydroxy-3-methylglutarate characterized their urinary organic acid profiles, which is consistent with earlier findings (1). The clinical course of two of these patients has been reported (12).

The carnitine distribution in urine from these patients revealed a markedly increased ratio of acylcarnitines to free carnitine ranging from 15–28 (normal ≤ 4). This high ratio indicated an increased production of acyl-CoA compounds acting as substrate for carnitine acyltransferase(s).

The patients were given a single oral dose of L-carnitine (100

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1. *Abbreviations used in this paper:* CoA, coenzyme A; FAB, fast atom bombardment; HMG, 3-hydroxy-3-methylglutaryl; GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

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mg/kg), and urine collected during the first 6–12 h was used to determine the nature of the acylcarnitine species excreted in this disorder.

The MS studies on methylated urine from all four patients gave virtually identical information. Fig. 1 *a* illustrates the characteristic FAB mass spectrum which includes the molecular cations of the esters of free carnitine (m/z 176), acetylcarnitine (m/z 218), smaller signals for C_3 (m/z 232), C_4 (m/z 246), and C_5 (m/z 260) acylcarnitines, and a predominant ion at m/z 318. Analysis of this ion by MS/MS (Fig. 1 *b*) confirmed the loss of 59 D characteristic of acylcarnitines, in this case leaving a fragment at m/z 259. The MS/MS spectrum of the same ion from synthetic 3-methylglutarylcarnitine was identical (Fig. 1 *c*). The other acylcarnitine signals were confirmed similarly; m/z 276 and 294 were thus shown not to be acylcarnitines. In many cases, the FAB mass spectrum of underivatized new urine exhibited the protonated molecular ions of the acylcarnitines, including that of 3-methylglutarylcarnitine (m/z 290). High-resolution accurate mass determination of this ion revealed a mass of 290.1627, which is consistent with the elemental composition of $C_{13}H_{24}O_6N$ (required mass, 290.1604). These data are consistent with a six-carbon dicarboxylic monoacylcarnitine which could have been either adipylcarnitine or 3-methylglutarylcarnitine in this metabolic disorder.

GC-MS analysis of the trimethylsilyl derivatives of the acids liberated by mild alkaline hydrolysis of the acylcarnitine fraction from the patients' urines revealed a peak with the appropriate retention time (Fig. 2 *a*) and mass spectrum for 3-methylglutarate

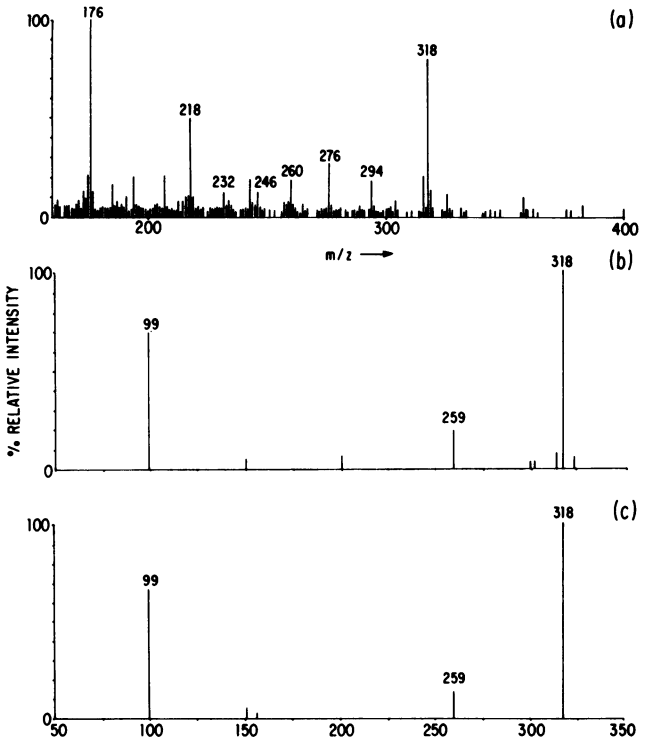

Figure 1. Typical fast atom bombardment mass spectrum from urine of a patient with HMG-lyase deficiency after methylation (*a*). The prominent ion at m/z 318 exhibited an identical daughter-ion spectrum by tandem mass spectrometry (*b*) as did the parent cation of synthetic 3-methylglutarylcarnitine (*c*). Free carnitine (m/z 176), acetylcarnitine (218), and small amounts of C_4 (m/z 246) and C_5 (m/z 260) acylcarnitine methyl esters were also detected in this sample.

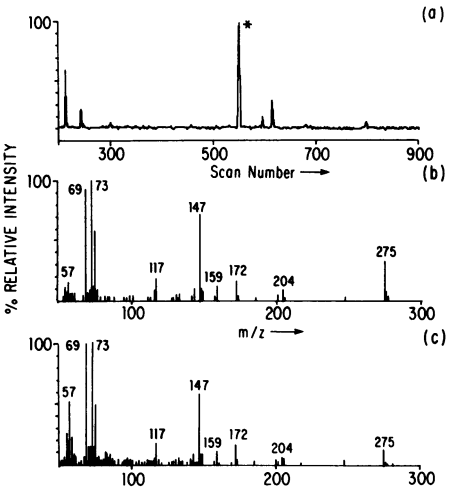

Figure 2. Identification of 3-methylglutaric acid released from the acylcarnitine by mild alkaline hydrolysis of a urine sample from the same patient. The partial chromatogram (*a*) shows a peak coincident in retention time to the standard, 3-methylglutarylcarnitine-di-trimethylsilyl ester (*). The mass spectrum of this peak (*b*) is identical with that of the standard (*c*).

(Fig. 2 *b*). The characteristic ions in the mass spectrum are at m/z 275 (M-15), and at m/z 204, 172, and 159 consistent with the mass spectrum of authentic 3-methylglutarate-di-trimethylsilyl ester (Fig. 2 *c*).

These studies failed to detect signals corresponding to carnitine esters of 3-hydroxyisovaleric, 3-methylglutaconic, or 3-hydroxy-3-methylglutaric acids, despite the high concentration of the free acids in the urine.

Two of these patients are being treated with dietary protein restriction alone and have required additional hospitalizations (12). The other patients have been treated with oral carnitine supplementation (50 mg/kg/d in four divided doses) and starch supplement (2 g/kg in cold water) at bedtime with modest protein restriction (1.5–2.0 g/kg/d). These patients have remained asymptomatic.

Discussion

Identification of acylcarnitine species in several organic acidurias indicates that carnitine represents a significant alternate pathway for the elimination of toxic intermediates which accumulate due to the enzyme deficiency. These acylcarnitine profiles are also of significant diagnostic value. To date, propionylcarnitine is characteristic of both propionic acidemia (3) and methylmalonic aciduria (4); isovalerylcarnitine is the dominant species in isovaleric acidemia (5); medium-chain length acylcarnitines, predominantly octanoylcarnitine, reflect the medium-chain acyl CoA dehydrogenase deficiency (6); *n*-butyrylcarnitine is the major species in ethylmalonic adipic aciduria, which is thought to be a mild form of glutaric aciduria Type II (unpublished results).

Several of the disorders whose acylcarnitine excretion has now been characterized share the clinical features of hypoglycemia, similarity to Reye's syndrome, and potentially affected siblings. Although organic acid analysis by GC-MS clearly identifies patients with HMG-lyase deficiency, other patients presenting with a Reye-like syndrome or with recurrent hypoglycemia need not be subjected to diagnostic fasting. Administration of L-carnitine (100 mg/kg) as a single dose and acylcarnitine

species identification by FAB-MS appears to be an excellent alternative without clinical risk and has recently proven useful in detecting presymptomatic siblings with medium-chain acyl-CoA dehydrogenase deficiency before metabolic deterioration (13).

In four patients with HMG-lyase deficiency, 3-methylglutarylcarnitine has now been identified as the characteristic acylcarnitine species. This represents the first conclusive identification of the intact molecule of a dicarboxylic monoacylcarnitine from humans. Of particular interest is the absence of acylcarnitine species derived from 3-hydroxyisovaleryl-CoA, 3-methylcrotonyl-CoA, 3-methylglutaconyl-CoA, or the major metabolite of this disorder, HMG-CoA. These are all intermediates in leucine degradation (Fig. 3). In HMG-lyase deficiency, both leucine degradation and ketogenesis are impaired. However, fatty acid oxidation is not impaired until after acetoacetyl-CoA is formed. Therefore, it is not surprising that acetylcarnitine is prominent in the acylcarnitine profile.

3-Methylglutarate is believed to originate from reduction of 3-methylglutaconyl-CoA (14), and therefore, serves as an alternate pathway for that compound and its precursors toward acylcarnitine formation. The existence of 3-methylglutarylcarnitine indicates that formation of 3-methylglutaric occurs after reduction of 3-methylglutaconyl-CoA to 3-methylglutaryl-CoA. In the leucine pathway, therefore, only isovaleryl-CoA and 3-methylglutaryl-CoA appear to act as substrates for carnitine acyltransferase(s). 2-Oxoisocaproate, which is not a CoA thio-ester, cannot serve as substrate and is not seen as an acylcarnitine. The remaining intermediates either have unsaturation between carbons 2 and 3 or a hydroxyl attached to carbon 3. Compounds with either a ketone or hydroxyl substituent at carbon 3 (acetoacetate, 2-methylacetoacetate, 3-hydroxypropionate, 3-hydroxybutyrate, 3-hydroxy-2-methylbutyrate, etc.) have not been detected in the urine of patients with propionic acidemia, methylmalonic aciduria, or ketosis. These observations suggest that these functional groups may preclude the formation of acylcarnitines from these intermediates.

Carnitine supplementation would seem to have a therapeutic

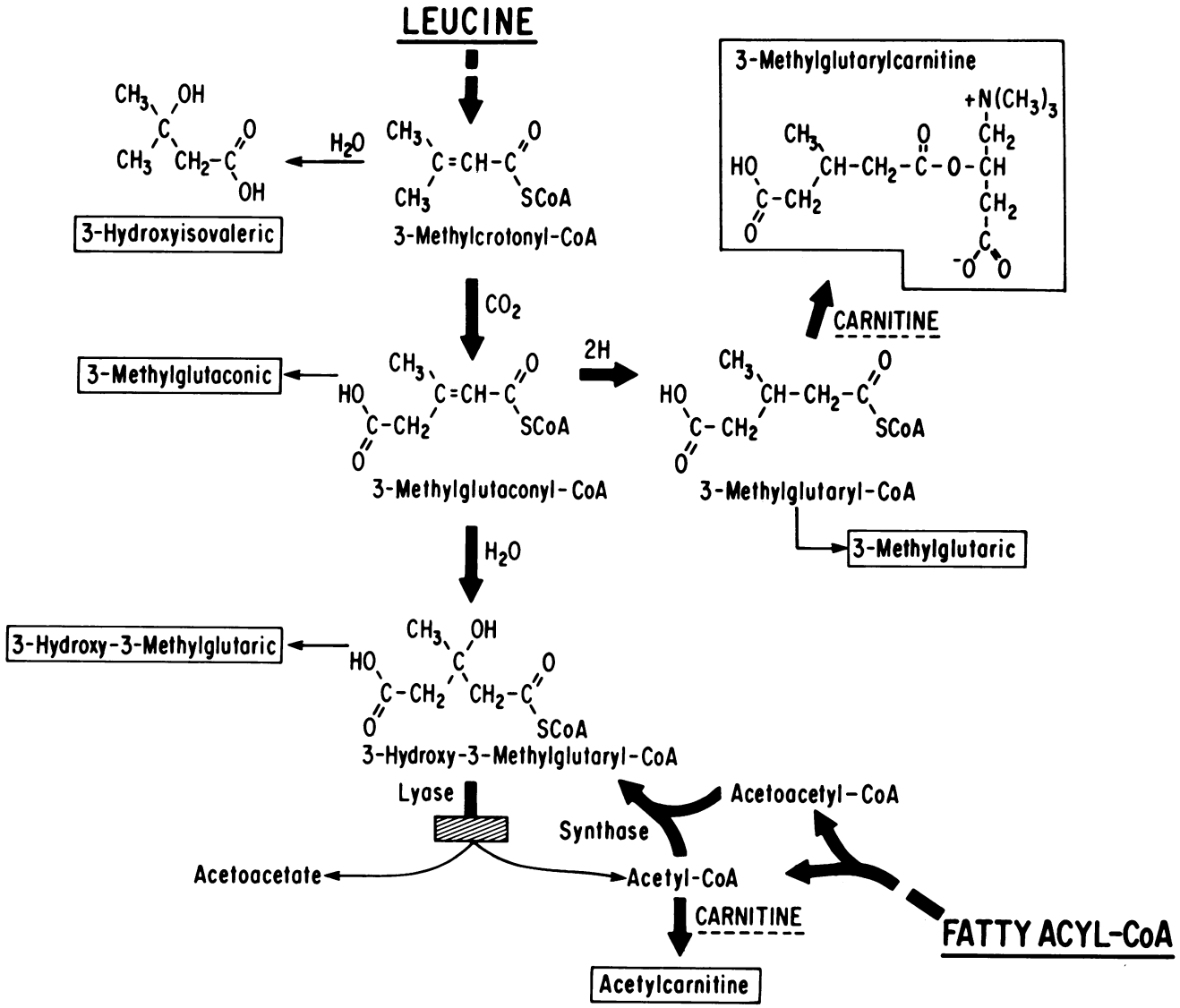

Figure 3. Origins of metabolites in HMG-CoA lyase deficiency. 3-methylglutaryl-CoA is an effective substrate for L-carnitine acylation. Urinary organic acid and acylcarnitine metabolites are enclosed in boxes.

role in this disorder since two of the three dicarboxylic acyl-CoA compounds present can be excreted as 3-methylglutarylcarnitine. After 10 mo and 2 yr of therapy, respectively, the two patients receiving supplementation have not required hospitalization and have remained asymptomatic.

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