MEDIUM-CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY

Diagnosis by Stable-Isotope Dilution Measurement of Urinary n-Hexanoylglycine and 3-Phenylpropionylglycine

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Abstract Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, one of the most common inherited metabolic disorders, is often mistaken for the sudden infant death syndrome or Reye's syndrome. Diagnosing it has been difficult because of a lack of fast and reliable diagnostic methods. We developed a stable-isotope dilution method to measure urinary n-hexanoylglycine, 3-phenylpropionylglycine, and suberylglycine, and we retrospectively tested its accuracy in diagnosing MCAD deficiency.

We measured the concentrations of these three acylglycines in 54 urine samples from 21 patients with confirmed MCAD deficiency during the acute and asymptomatic phases of the illness and compared the results with the concentrations in 98 samples from healthy controls and patient controls with various diseases. The levels of urinary hexanoylglycine and phenylpropionylglycine were significantly increased in all samples from the patients with MCAD deficiency, clearly distinguishing them from both groups of controls. Although urinary suberylglycine was increased in the patients, the range of values in the normal controls who were receiving formula containing medium-chain triglycerides was very wide, overlapping somewhat with the values in the patients with asymptomatic MCAD deficiency.

These results indicate that the measurement of urinary hexanoylglycine and phenylpropionylglycine by our method is highly specific for the diagnosis of MCAD deficiency. The method is fast and can be applied to random urine specimens, without any pretreatment of patients. (N Engl J Med 1988; 319:1308-13.)

MEDIUM-CHAIN acyl-CoA dehydrogenase (MCAD) deficiency is an inborn error of fatty acid metabolism that has recently drawn considerable attention because of its high incidence, upredictable clinical presentation, and high mortality and because of the difficulty of diagnosing it.[1] Since it was first described in 1982 by Kølvraa et al.,[2] approximately 100 cases have been reported in the literature[3] and at least 65 cases have been confirmed by enzymatic assay.[1] Furthermore, Bennett et al.[4] recently reported that the incidence of pathologic dicarboxylic aciduria was 1 in 5000 live births, in a survey conducted in the Sheffield area of England. Although the cause of the dicarboxylic aciduria in these patients was not identified and more accurate epidemiologic studies are necessary, the available evidence is sufficient to suggest that the incidence of MCAD deficiency is one of the highest among the incidences of inborn errors of metabolism.

The typical symptoms of MCAD deficiency are intolerance to fasting, episodic vomiting, lethargy, and coma, accompanied by hypoketotic hypoglycemia and medium-chain dicarboxylic aciduria.[5] An episode may occur abruptly in apparently healthy children. The mortality rate within the first two years of life is 59 percent with the first episode.[1] However, the clinical symptoms of MCAD deficiency are variable. A number of patients were initially considered to have Reye's syndrome.[6-8] Also, family[9,10] and autopsy[11] studies have indicated that in some MCAD-deficient patients the disorder was misdiagnosed as sudden infant death syndrome.

The methods for diagnosis of MCAD deficiency should be sensitive enough to identify asymptomatic patients. However, most current methods either do not have such sensitivity or have not been rigorously tested for this qualification with the use of appropriate controls. Previously, medium-chain dicarboxylic aciduria[12] or the observation of decreased levels of free carnitine and increased levels of acylcarnitines in body fluids and tissues was considered to be diagnostic,[5,13] but recent experience in many laboratories indicates that these findings are not diagnostic. More recently, the identification of octanoylcarnitine and other medium-chain acylcarnitines or of suberylglycine has been proposed to be diagnostic.[14-17] However, the usefulness of these approaches in the diagnosis of MCAD deficiency in asymptomatic patients has not been adequately evaluated. Currently, the only reliable method is enzymatic assay of MCAD activity in patients' cells. All current methods for assaying MCAD are cumbersome, taking several weeks to produce results, and only a few laboratories provide such service worldwide. Thus, the development of a rapid and accurate method for the diagnosis of MCAD deficiency has been urgently advocated.[18]

In addition to suberylglycine, hexanoylglycine has been identified in urine from a number of patients with MCAD deficiency,[3,10,15,19-21] but the excretion of this compound has not been systematically examined. These two acylglycines are abnormal byproducts of fatty acid oxidation. Phenylpropionylglycine has also been detected in several MCAD-deficient patients, with and without loading with phenylpropionic acid.[10,21] In normal subjects, phenylpropionic acid is produced from phenylalanine by intestinal bacteria, further metabolized to benzoate by the action of MCAD,[22] and excreted as hippurate in urine.

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We hypothesized that these three acylglycines might serve as reliable diagnostic markers for this disease. In the present study we measured urinary hexanoylglycine, phenylpropionylglycine, and suberylglycine with a stable-isotope dilution method. A brief preliminary report of this study has been published elsewhere.[23]

Methods

Study Groups

Patients

The numbers of urine specimens from patients with MCAD deficiency and various control groups are shown in Table 1. The diagnosis of MCAD deficiency in all the patients had previously been or was later confirmed by assay of MCAD activity in cultured fibroblasts or leukocytes.[24] Urine specimens from MCAD-deficient patients were requested from the primary physician–investigators by means of a letter accompanied by a questionnaire. The ages of the patients ranged from one week to six years. Of 54 urine specimens from 21 MCAD-deficient patients, 26 (from 13 patients) were sent to us without identification of the patient's diagnosis. Nineteen samples, each marked with a serial number, were sent together with those of the controls and analyzed in a blind fashion. The other seven were samples from patients in whom a disorder had not been diagnosed at the time of analysis but in whom a positive diagnosis according to the method described below was later confirmed by the enzyme assay. The remaining 28 specimens were obtained from eight patients in whom the diagnosis was known at the time of analysis. All samples were categorized as those of asymptomatic patients or patients with acute episodes, according to the information provided in the questionnaire.

Patient Controls

Urine specimens from two patients with long-chain acyl-CoA dehydrogenase deficiency and five with Reye's syndrome (all in Stage IV[25] at the time of urine collection) were received from the Children's Hospital of Philadelphia. Most of the specimens from the other patient controls had been previously received and stored at the Department of Human Genetics, Yale University School of Medicine. A patient with ethylmalonic–adipic aciduria and two of four patients with glutaric aciduria Type II had electron-transfer flavoprotein dehydrogenase deficiency.[26-28] One patient had electron-transfer flayoprotein deficiency. The diagnoses of these patients were confirmed by the respective assays of Drs. S.I. Goodman and F.E. Frerman (University of Colorado). The fifth patient had riboflavin-responsive glutaric aciduria Type II; this diagnosis was also confirmed by enzymatic study.[29] Among the patients with unexplained dicarboxylic aciduria were three patients (six specimens) with heterogeneous clinical symptoms, including failure to thrive and developmental delay. In the cells from all these patient controls, levels of acyl-CoA dehydrogenase activity were confirmed as normal, except for reduced activity of long-chain acyl-CoA dehydrogenase in patients with a deficiency of this enzyme and low levels of short-chain and medium-chain acyl-CoA dehydrogenase activity in the patient with riboflavin-responsive glutaric aciduria Type II. Two patients with a diagnosis of systemic carnitine deficiency were siblings who had developmental delay, heart failure, and low tissue levels of carnitine. The activities of the three acyl-CoA dehydrogenases in fibroblasts from the older sibling were normal.

Normal Controls

Twenty-two urine specimens from 17 normal infants were obtained at Yale–New Haven Hospital, and 17 samples from 8 normal infants were obtained at the Department of Pediatrics, University of Padua, Italy. The ages of the infants ranged from one week to nine months. All were receiving their usual diet at the time of urine collection. The formulas containing medium-chain triglycerides included Pregestimil, Similac 24, and Alprem. Breast-fed infants and those on proprietary cow's-milk formulas (Similac and Enfamil) or soybean formulas (Isomil and Prosobee) were included among the infants placed on regular formulas.

Table 1. Numbers of Subjects and Urine Samples Analyzed, According to Study Group.

Group	No. of Subjects	No. of Samples		
		Acute Phase	Asymptomatic Phase	Total
Patients with MCAD deficiency	21	16	38	54
Patient controls				
Glutaric aciduria Type II	4	4	6	10
Ethylmalonic–adipic aciduria	1	14	9	23
Long-chain acyl-CoA dehydrogenase deficiency	2	0	4	4
Systemic carnitine deficiency	2	3	0	3
Reye's syndrome	5	5	0	5
Nonspecific ketosis	8	8	0	8
Unexplained dicarboxylic aciduria	3	6	0	6
Normal controls				
Receiving medium-chain–triglyceride formula	13	NA*	24	24
Receiving regular formula	12	NA*	15	15
Total	*71*	*56*	*96*	*152*

*Not applicable.

Radiolabeling and Assay Procedures

n-Hexanoyl-[1,2-^{13}C]glycine, 3-phenylpropionyl-[2-^{13}C,^{15}N]glycine, suberyl-[2-^{13}C,^{15}N]glycine, and their unlabeled counterparts were synthesized in our laboratory according to methods previously described.[17,30]

Urine containing 0.03 to 1.0 mg of creatinine was first added to 5 μg each of the labeled acylglycines. It was then extracted and methylated according to a method previously described.[31] The final methylated samples were concentrated, injected into the capillary column in a gas chromatograph–mass spectrometer computer, and analyzed in the chemical ionization-selected ion-monitoring mode. The measurements of hexanoylglycine, phenylpropionylglycine, and suberylglycine were highly reproducible. Interassay coefficients of variation of each acylglycine at different concentrations were 0.5 to 0.7 percent, 1.1 to 1.2 percent, and 0.3 to 3.3 percent, respectively.

Statistical Analysis

Logarithmic conversion was used to give the values of the study groups a normal distribution. The transformed values were compared with the use of Student's t-test.

Results

The urinary excretion of n-hexanoylglycine, 3-phenylpropionylglycine, and suberylglycine is shown in the figures cited below. The concentrations of these acylglycines in some of the patients with MCAD deficiency were two to three orders of magnitude greater than the corresponding values in the control groups. Therefore, the values are shown on a logarithmic scale so that the wide variation in the levels of a metabolite can be presented in a single figure. The range of excretion in MCAD-deficient patients and various controls is summarized in Table 2.

Urinary Hexanoylglycine

The urinary concentration of hexanoylglycine in all 54 samples from the 21 MCAD-deficient patients was greater than the highest normal value (Table 2 and

Table 2. Range of Urinary Acylglycine Excretion, According to Study Group.

Group	Hexanoyl-glycine	Phenyl-propionyl-glycine	Suberyl-glycine
	micrograms per milligram of creatinine		
Patients with MCAD deficiency			
Acute episode	23–653	2.5–180	109–4553
Remission	3.1–171	1.2–111	13–1007
Patient controls			
Glutaric aciduria Type II			
Acute episode	20–103	0.25–2.7	2.7–105
Remission	2.5–16	0.12–0.63	0.10–6.6
Ethylmalonic–adipic aciduria			
Acute episode	61–152	0.35–0.65	66–262
Remission	6.0–75	0.21–0.42	9.7–64
Long-chain acyl-CoA dehydrogenase deficiency	0.36–2.1	0–0.27	0.1–3.5
Systemic carnitine deficiency	0.95–1.7	0.16–1.8	1.1–19
Reye's syndrome	0.47–3.2	0.31–1.5	0–7.1
Nonspecific ketosis	0.60–3.1	0.11–0.72	2.0–21
Unexplained dicarboxylic aciduria	0.29–1.55	0.23–0.67	0.10–33.4
Normal controls (mean ±SD)	0.21–1.9 (0.88±0.40)	0–1.1 (0.44±0.24)	0–95 (4.2*)

*The median value is shown for both the controls receiving regular formula and those receiving formula containing medium-chain triglycerides, since the values for the latter controls did not follow a gaussian distribution. The mean ±SD in the controls receiving regular formula was 4.0±3.6.

Fig. 1). Since it is difficult to distinguish between the lowest values of the asymptomatic patients with MCAD deficiency and the highest values of the normal controls represented in Figure 1, because of the use of a logarithmic scale, these values are also shown in Table 3. The lowest hexanoylglycine concentrations in the MCAD-deficient group were found in five urine specimens from three asymptomatic patients

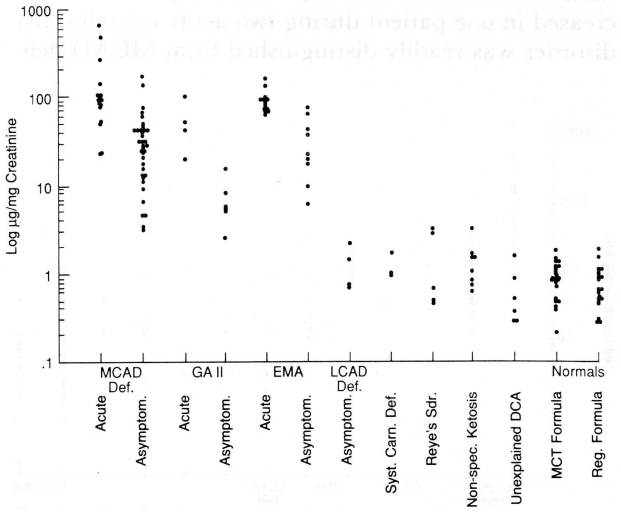

Figure 1. Urinary Excretion of n-Hexanoylglycine in Patients with MCAD Deficiency and Various Controls.

Values are expressed on a logarithmic scale. DCA denotes dicarboxylic aciduria, EMA ethylmalonic–adipic aciduria, GA II glutaric aciduria Type II, LCAD long-chain acyl-CoA dehydrogenase deficiency, MCT medium-chain–triglyceride, and Syst. Carn. Def. systemic carnitine deficiency.

who were being treated with a low-fat diet and supplementation with L-carnitine. The values for the 33 other urine samples from asymptomatic MCAD-deficient patients were 10 to 200 times higher than the mean of normal values. The values for specimens of both the asymptomatic-phase and acute-phase groups were significantly higher than those of the normal controls ($P<0.001$ for each comparison).

The concentration of hexanoylglycine in urine specimens from the patient controls with glutaric aciduria Type II and ethylmalonic–adipic aciduria was increased both during remission ($P<0.001$ for each comparison) and during acute episodes ($P<0.001$ for each comparison), as in the patients previously described.[26,27] The levels in patients with Reye's syndrome (Stage IV) and nonspecific ketosis were essentially within the normal range and much lower than the values observed in patients with MCAD deficiency in the acute phase ($P<0.001$ for each comparison) (Fig. 1), even during the most acute stages of these other disorders. The values observed in four urine specimens from asymptomatic patients with a deficiency of long-chain acyl-CoA dehydrogenase were similar to normal values.

Urinary Phenylpropionylglycine

The concentrations in most of the urine specimens from the acute-phase and asymptomatic-phase groups of MCAD-deficient patients were greatly elevated above the normal range ($P<0.001$ for each comparison). The lowest amount of urinary phenylpropionylglycine in the patients was more than 2 SD greater than the mean of normal values (Fig. 2). It is interesting to note that urinary phenylpropionylglycine excretion in the patients with glutaric aciduria Type II and ethylmalonic–adipic aciduria was usually within the normal range and was significantly lower than that in the patients with MCAD deficiency ($P<0.001$ for each comparison). Only two samples had moderately elevated values; both had been obtained from a 19-year-old woman with a mild deficiency of electron-transfer flavoprotein dehydrogenase, who had had two successive episodes of hypoglycemic coma one month after an abortion occurred at 12 weeks of gestation.[27] The values in the patients with long-chain acyl-CoA dehydrogenase deficiency or nonspecific ketosis were within the normal range.

Urinary Suberylglycine

The range of values for urinary suberylglycine excretion in normal infants receiving regular formula was wide, and it was even more so in those receiving medium-chain–triglyceride formula (Fig. 3). Seven specimens from the latter group had high values, the highest being 95 µg per milligram of creatinine. Overall, the values in asymptomatic MCAD-deficient patients were significantly higher than in the normal controls ($P<0.001$), but approximately half these values overlapped with some of the high values in the normal controls receiving medium-chain–triglyceride formula. The values recorded in the patients during

acute episodes of MCAD deficiency were greatly elevated ($P<0.001$ for the difference from normal controls).

Discussion

In this study we have shown that urinary excretion of hexanoylglycine and phenylpropionylglycine, when measured with a stable-isotope dilution assay, was markedly increased in patients with MCAD deficiency, not only during acute episodes but also during remission, and that this elevation was highly diagnostic of this disease. The accuracy of this method was underscored by the finding that all 26 urine specimens from 13 patients with MCAD deficiency were correctly identified although analysis was blinded.

We have shown that the excretion of a very large amount of urinary suberylglycine — more than 500 μg per milligram of creatinine — can support a diagnosis of MCAD deficiency. However, moderately increased values of up to 100 μg per milligram of creatinine, as observed in some samples from asymptomatic MCAD-deficient patients, were also found in the urine of normal infants receiving formula containing medium-chain triglycerides. Therefore, the detection of suberylglycine in concentrations in these ranges is not diagnostic.

The measurement of acylglycines should be performed with a stable-isotope dilution assay. It has previously been observed that the analysis of relatively small amounts of acylglycine according to the ordinary method of screening for organic acids, using gas chromatography or gas chromatography–mass spectrometry, yielded highly variable results.[30,32] In the isotope dilution assay, the labeled internal standard serves as a carrier as well as a quantitative standard. It protects against the loss of small amounts of endogenous acylglycines during the entire procedure and makes possible a high reproducibility of results, with a high sensitivity.

Table 3. Comparison of the Lowest Hexanoylglycine Values in MCAD-Deficient Patients with the Highest Values in Normal Controls.

Group	Hexanoylglycine	Phenylpropionylglycine	Suberylglycine
	micrograms per milligram of creatinine		
Patients*			
A	3.1	2.0	48.5
B	3.3	13.7	12.5
C	4.4	2.2	40.9
D	6.3	3.6	65.6
E	9.0	8.0	33.0
Controls†			
a	1.2	0.22	4.6
b	1.3	0.55	45.8
c	1.3	0.19	24.7
d	1.4	0.73	95.3
e	1.8	0.37	1.7
f	1.9	0.87	10.6

*All were asymptomatic and were receiving long-term therapy with carnitine at the time of urine sampling.

†Controls a through e were receiving medium-chain–triglyceride formula and Control f, regular formula, at the time of sampling.

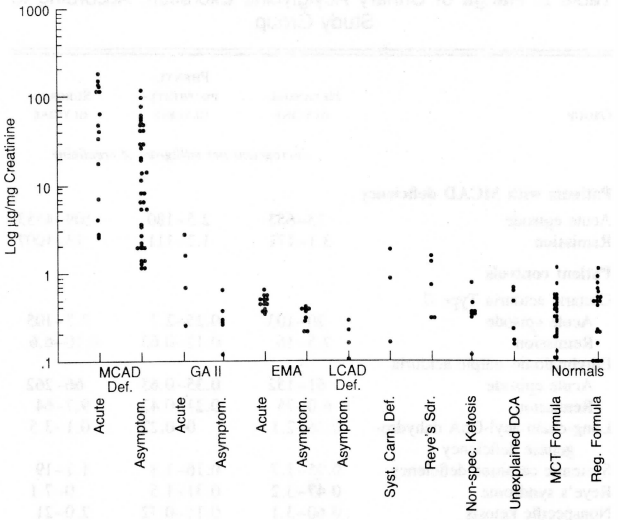

Figure 2. Urinary Excretion of 3-Phenylpropionylglycine in the Study Groups.
For an explanation of abbreviations, see the legend to Figure 1.

Glutaric aciduria Type II and its milder variant, ethylmalonic–adipic aciduria, were the only other conditions in which the hexanoylglycine concentration was increased. In patients with these two diseases, the oxidation of fatty acids and several amino acids is blocked at the acyl-CoA dehydrogenase step by a deficiency of either electron-transfer flavoprotein or electron-transfer flavoprotein dehydrogenase.[26,28,33] Unexpectedly, the level of urinary phenylpropionylglycine was not increased in these patients during periods of remission. Although this compound was increased in one patient during two acute episodes, her disorder was readily distinguished from MCAD defi-

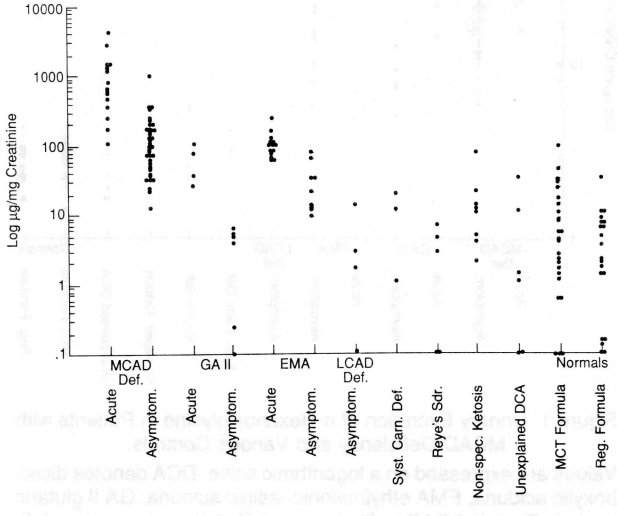

Figure 3. Urinary Excretion of Suberylglycine in the Study Groups.
For an explanation of abbreviations, see the legend to Figure 1.

ciency by the increased urinary excretion of two specific metabolites, glutaric and ethylmalonic acids.

The detection of urinary octanoylcarnitine by fast atom bombardment–mass spectrometry[14,15] or thin-layer chromatography[16] has been described by some investigators as diagnostic of MCAD deficiency. Although the first of these methods[14,15] has provided an accurate diagnosis in a number of MCAD-deficient patients, we found in the course of our study that this technique missed the diagnosis in at least 4 of the 21 patients evaluated.[34] To our knowledge, it also failed to give a correct diagnosis in another patient, whose disorder was readily identified by our method. Since a direct comparison between our method and fast atom bombardment–mass spectrometry was carried out for only a small number of urine samples, the percentage of the cases that might have been missed by testing for octanoylcarnitine may be higher than that given above. Our preliminary comparison suggests that the detection of octanoylcarnitine is not as reliable as the quantitative determination of hexanoylglycine and phenylpropionylglycine. The inconsistency of octanoylcarnitine detection is probably due to the fluctuation of the tissue concentration of free carnitine available for acylation. The diagnostic value of analysis of acylcarnitines by thin-layer chromatography has not been adequately tested. Since thin-layer chromatography is much less sensitive than fast atom bombardment–mass spectrometry, it is reasonable to assume that the accuracy of acylcarnitine detection by thin-layer chromatography is lower.

Since completing this study, we have prospectively identified three additional patients with MCAD deficiency, among them a nine-year-old girl who has a family history of three cases of sudden infant death and who has never had an acute episode of the disorder. This instance illustrates the usefulness of our method in the diagnosis of MCAD deficiency.

The procedure described in our study can be readily performed on a random urine specimen with a low-resolution gas chromatograph–mass spectrometer system, which is available in many major medical centers, and it takes only a few hours. It does not require any preparation of the patient, such as fasting (which is potentially dangerous) or the administration of medium-chain–triglyceride oil, carnitine, or phenylpropionic acid. With this method, it is possible to survey accurately a fairly large population, such as children in families with a history of sudden infant death.

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