Ethylmalonic and Methylsuccinic Aciduria in Ethylmalonic Encephalopathy Arise From Abnormal Isoleucine Metabolism

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Ethylmalonic encephalopathy (EE), an organic aciduria of unknown etiology characterized by developmental delay, hypotonia, and vascular instability associated with lactic acidemia and urinary excretion of ethylmalonic acid (EMA) and methylsuccinic acid (MSA), has been described in 11 patients. To test the possibility that the underlying biochemical defect involves isoleucine catabolism, we determined the response to oral L-isoleucine (Ile) load (150 mg/kg) in a 5-year-old girl with EE and in three healthy, age- and sex-matched controls. Following Ile load in the patient, there was accumulation of 2-methylbutyrylglycine (2-MBG) and a delayed and lower peak urinary excretion of tiglylglycine (TGL), suggesting a partial defect in 2-methyl-branched chain acylcoenzyme A dehydrogenase (2M-BCAD). In vitro measurements 2M-BCAD activity in cultured skin fibroblasts from patients with EE have been reported to be normal. Our results show that isoleucine is a source for the elevated EMA and MSA in patients with EE, and suggest a functional, possibly secondary, deficiency of activity of 2M-BCAD in vivo.

ETHYLMALONIC ENCEPHALOPATHY (EE), first described by Burlina et al,[1] is characterized by developmental delay, acrocyanosis, petechiae, and chronic diarrhea associated with lactic acidemia and excretion of ethylmalonic acid (EMA) and methylsuccinic acid (MSA) in the urine. The underlying metabolic defect is unknown. Two enzymes whose deficiencies could theoretically produce the biochemical findings observed in EE are short-chain acylcoenzyme A dehydrogenase[1] and 2-methyl–branched chain acylcoenzyme A dehydrogenase (2M-BCAD).[2] However, the in vitro activities of these two dehydrogenases in skin fibroblasts from patients with EE have been reported to be normal.[1,2] Sixteen patients with EE have been reported in the literature,[1-7] with anecdotal reports of five others (R. Duran and F. Treftz, personal communication, December 1995).

2M-BCAD is involved in the *S* isoleucine catabolic pathway, where it catalyzes the conversion of 2-methylbutyrylglycine (2-MBG) to tiglylglycine (TGL) (Fig 1). In the *R* catabolic pathway, 2M-BCAD catalyzes the conversion of *R* 2-methylbutyrylCoA to 2-ethylmalonyl semialdehyde,[7] leading to the production of EMA and MSA as final products (Fig 1). 2M-BCAD activity toward *R* 2-methylbutyrylCoA is 22% that of its affinity toward the *S* entantiomer.[8] A defect of 2M-BCAD would result in production of EMA and MSA because of activation of the *R* catabolic pathway. If that hypothesis were true, an L-isoleucine (Ile) load in a patient with EE would result in elevation of plasma L-alloleucine (Allo) via the transamination of the *R* 2oxo3methylvalerylCoA formed by the ketotautomerisation of the *S* metabolite, and in increase of excretion of EMA and MSA. We investigated the effect of an oral Ile load on 2M-BCAD in a patient with EE by measuring the serum levels of Ile and Allo and the amounts of metabolites of the Ile catabolic pathway excreted in the urine.

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CASE REPORT

Clinical History

The clinical features of the patient are reported elsewhere.[9] Based on clinical and biochemical findings, this girl was diagnosed with EE at the age of 5 years. She had a similarly affected younger brother. Results of biochemical testing are listed in Table 1. Results of fatty acid oxidation assays in cultured skin fibroblasts, according to a previously published method,[10] are listed in Table 2, and those of mitochondrial electron transport chain assays on cultured skin fibroblasts and fresh muscle are listed in Table 3.

Urinary S-Sulfocysteine

A random urine samples was analyzed for the presence of S-sulfocysteine according to a previously published method.[11] The level in the patient was 20.6 mmol of S-sulfocysteine per mole creatinine.

Analysis of SCAD Gene Polymorphism G625A

Single-strand conformation polymorphism (SSCP) analysis of the patient's 141-bp genomic DNA segment consisting of position 625 showed that the patient had two copies of the normal allele.[12]

METHODS

Ile Loading

Oral Ile was given at a dose of 150 mg/kg. Urine was collected at the beginning of the load (time 0) and at timed intervals (2, 4, 6, 9, 12, 18, and 24 hours) for quantitative organic acid and acylglycine analysis. Quantitative plasma amino acids analysis at 2, 6, 9, 12 and 24 hours was performed with the use of an automated ion-exchange amino acid analyzer. The patient and three age-, weight-, and sex-matched controls were on a low-protein diet for the duration of the study (24 hours). The study was approved by the Research Ethics Board of the Hospital for Sick Children. Informed consent was obtained from the parents; all study controls assented to participate.

Gas Chromatography/Mass Spectroscopy

Urine samples (volume equivalent of 0.05 mg creatinine, to a maximum of 1.0 mL) were acidified to a final pH less than 1.0 by addition of 2N HCl. Internal standard (500 nmol of pentadecanoic acid) was added and the organic acids were extracted with diethyl ether (3 mL) and ethyl acetate (3 mL). External standard (50 mg of C-24) was added to the combined extracted organic phases, which were evaporated

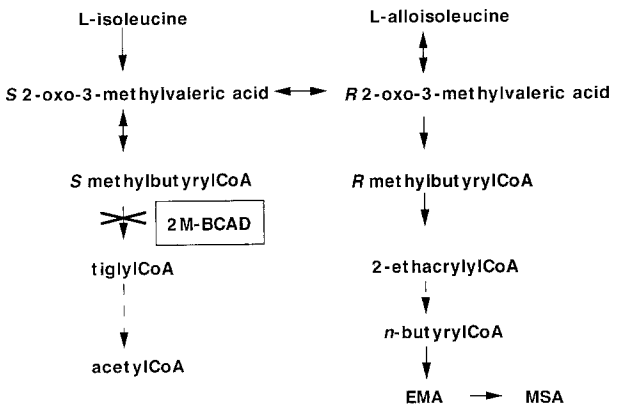

Fig 1. Isoleucine catabolism showing the relationship between the *R* and *S* pathways.

to dryness under N_2. Trimethylsilyl derivatives were prepared by the addition of 0.1 mL of bistrimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane (Pierce Chemical, Rockford, IL) and heating at 60°C for 10 minutes. The samples were then diluted to 1.0 mL by addition of 0.9 mL *n*-hexane. Quantitative analysis of the derivatized organic acids was performed with the aid of a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph, equipped with an autosampler, and an HP-5971 Mass Selective Detector. Samples were injected onto a wall-coated, open tubular glass capillary column (SPB-1, 30 m, 0.25 mm internal diameter, 0.25-mm coating; Supelco, Bellfonte, PA). The carrier was helium, at a flow rate of 1 mL/min operated isothermally at 60°C. Metabolites in the urine were identified by comparison with known spectra listed in the Hospital for Sick Children mass spectra library. Quantitation of EMA, MSA, and lactate was performed by comparison with internal standard (500 nmol of pentadecanoic acid), performed with the aid of Hewlett-Packard MS Chemstation version G1034C software.

Identification and Quantitation of Urinary Acylglycines

Urinary acylglycines were measured using a stable-isotope dilution method according to a previously described method.[13] Quantitation and analysis of methylated samples by capillary gas chromatography/mass spectrometry were performed using a Hewlett-Packard (Palo Alto, CA) gas chromatograph, model 5890 Series II equipped with an HP-5–fused silica column (25 m in length, 0.20 mm internal diameter) interfaced to a quadrupole mass spectrometer VG TRIO-2A (VG Masslab; Fisons Instruments, Broadheath, Cheshire, UK) equipped with the LAB-BASE data system. The stationary phase was cross-linked 5% phenylmethylsilicone gum, 0.33 μm film thickness. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. One microliter of sample or standard was injected in the splitless mode at 250°C. The column temperature was programmed to produce an initial oven temperature of 100°C, held for 1 minute, then increased at 8.0°C/min to 240°C, followed by a second gradient of 20.0°C/min, to a final temperature of 270°C.

Table 1. Biochemical Investigations

Variable	Patient	Normal
Plasma lactate (mmol/L)	6.06, 4.30	<2.50
Plasma pyruvate (mmol/L)	0.14, 0.108	0.08-0.15
Plasma lactate/pyruvate ratio	43, 39	<10
Total/free carnitine (μmol/L)	51.8/45.6	48-72/35-50
Urinary acylglycines (μmol/mol creatinine)		
Hexanoylglycine	2.8	<1.7
Isobutyrylglycine	17.4	<8.6
Butyrylglycine	2.1	<1.65
TGL	1.1	<3.85
2-MBG	ND*	<5.4
Isovalerylglycine	ND*	<10.2
Urinary organic acids (μmol/mol creatinine)		
EMA	94.4	8.4 ± 5.7
MSA	8.64	1.3 ± 0.8
Lactate	57.6	25 ± 21
Urinary sulfites	Positive	ND

Abbreviation: ND, not detectable.
*Less than 0.2 mmol/mol creatinine.

Table 2. Results of Fatty Acid Oxidation Assays

Assay	Patient	Controls
1-[^{14}C]butyrate (pmol/mg protein/min)	69, 70, 79, 77, 80	91, 84, 89*; 45, 33, 38; 174, 167, 214; 154, 165, 158; 98, 96, 127
[9,10-^{3}H]myristic acid (nmol/mg protein/h)	3.2, 2.9	3.4, 3.4
[9,10-^{3}H]palmitic acid (nmol/mg protein/h)	5.3, 5.7	5.7, 5.6

*Control results are triplicate determinations on 5 separate cell lines.

Statistical Analysis

A modified *t* test for comparison of two means was used to compare the results for the patient and the mean of the three controls. The values reported were statistically significant at $P \leq .05$.

RESULTS

Plasma levels of Ile and Allo following an oral Ile load are shown in Fig 2. Plasma Ile levels increased in the patient and in controls within 2 hours of loading, and they returned to preload levels within 24 hours. Elevation of plasma Allo was observed in both the patient and the controls. In the patient, Allo returned to preload levels within 24 hours, while in controls it was not detectable by 12 to 18 hours (Fig 2). Within 2 hours after the Ile load, there was a significant increase in the excretion of EMA in the patient, with a corresponding increase in the excretion of MSA, and both remained elevated for the duration of the study

Table 3. Results of Electron Transport Chain Assays (nmol/min/mg mitochondrial protein)

Variable	Patient	Controls
Muscle		
NADH-cytochrome C reductase	84.79	94.6 ± 9.5
Succinate cytochrome C reductase	127.186	102 ± 6.9
Cytochrome oxidase	103.86	119 ± 11.3
Citrate synthase	230.68	
Cultured skin fibroblasts		
Pyruvate dehydrognease (native)	1.07 ± 0.17	0.89 ± 0.25
Pyruvate dehydrogenase (dichloroacetate-activated)	1.34 ± 0.21	1.14 ± 0.11
Pyruvate carboxylase	0.33	0.51
Cytochrome oxidase	5.15 ± 0.45	4.47 ± 0.61
Succinate cytochrome c reductase	5.13 ± 1.15	6.64 ± 0.66
Cellular lactate/pyruvate ratio	22.3 ± 3.0	15.4 ± 1.7

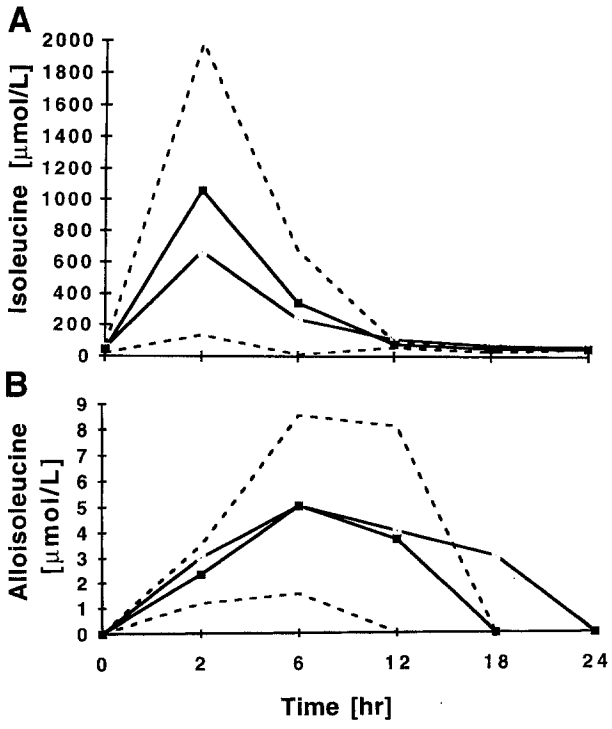

Fig 2. Plasma isoleucine (A) and alloisoleucine (B) concentrations following 150 mg/kg oral dose of Ile. (x) Patient; (■) mean of 3 controls. (-----) Mean control value ± 2SD (3 controls).

($P < .001$) (Fig 3). In the controls' urine samples, these compounds were not detected (data not shown). The patient excretion of 2-MBG surpassed the 2-MBG excretion in the controls within 2 hours following the Ile load ($P < .05$) and reached a peak at 4 hours ($P < .0001$) (Fig 4A). In contrast, TGL excretion in the patient was not detectable before the load ($P < .001$), reached a peak at 6 hours, and at all times remained lower than that of the three controls ($P < .05$) (Fig 4B).

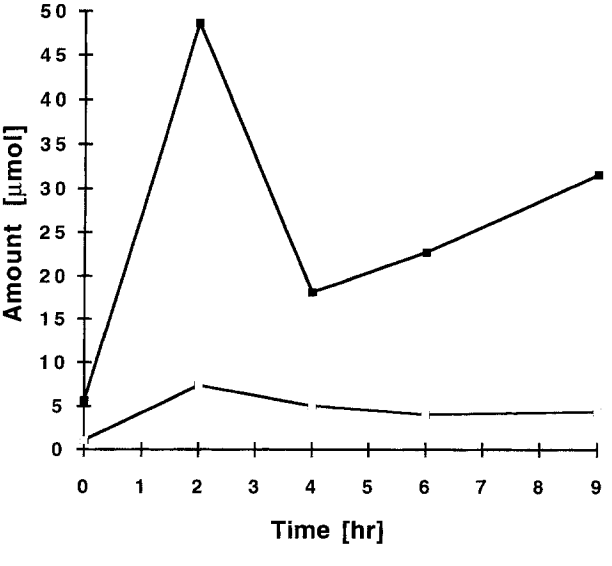

Fig 3. Urinary organic acid excretion following 150 mg/kg oral dose of Ile. (□) MSA; (■) EMA.

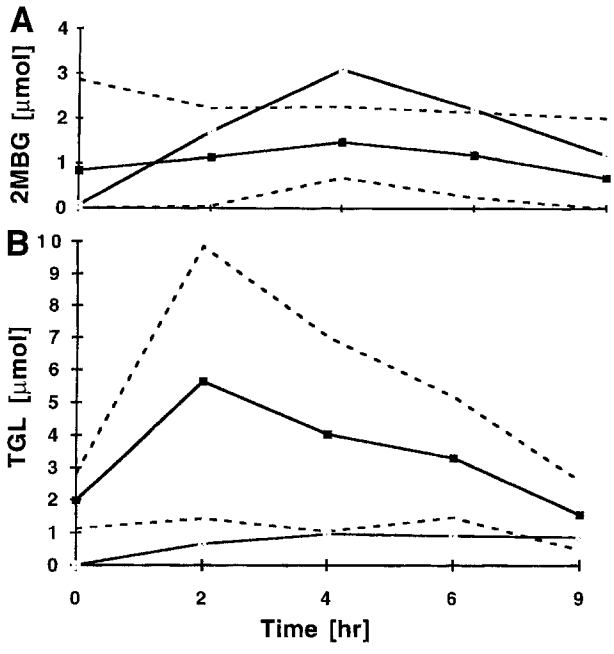

Fig 4. Urinary 2-MBG (A) and TGL (B) excretion following 150 mg/kg oral dose of Ile. (x) Patient; (■) mean of 3 controls. (---) Mean control value ± 2SD (3 controls).

DISCUSSION

We investigated the hypothesis that the biochemical defect in EE involves isoleucine catabolism. We postulated that if a defect existed at the level of 2M-BCAD, it would cause a shift toward the *R* pathway of isoleucine catabolism with resultant increased plasma Allo concentrations and increased excretion of EMA and MSA. A complete block at the level of 2M-BCAD would cause accumulation of the substrate and increased excretion of 2-MBG, and in decreased excretion of the major product of the reaction, TGL.

Elevated concentration of plasma Allo was observed in both the patient and the controls (Fig 2), but there was no statistical difference. However, Allo levels returned to normal earlier in the controls than in the patient (18 *v* 24 hours). We concluded that in normal, healthy controls, appearance of Allo in plasma following Ile oral load does not indicate biochemical pathology.

Analysis of the urinary organic acid excretion showed that within 2 hours following an Ile load, there was a marked increase in the excretion of EMA and a corresponding increase in the excretion of MSA in the patient (Fig 3) consistent with increased *R* pathway flux. In the urine of healthy controls, EMA and MSA were not detected during quantitative urinary organic acid analysis; this was also true following the Ile load. At all times during the study, the patient excreted more 2-MBG than any of the controls ($P < .05$) (Fig 4).

However, the patient also excreted metabolites distal to the step catalyzed by 2M-BCAD (TGL, 2-methyl-3-hydroxybutyrate, 2-methylglutaconate), indicating that some 2M-BCAD activity is present. In the patient, peak TGL excretion occurred at 4 hours after the Ile load, while in the controls, the peak of TGL excretion occurred at 2 hours following Ile ingestion. The amount of TGL excreted by the patient was always lower compared with the controls (Fig 4).

Our observations show that Ile is the source of the elevated EMA and MSA levels in patients with EE and suggest an partial defect of 2M-BCAD activity. Previously reported assays of 2M-BCAD showed normal activity in vitro[2]; our data suggest that 2M-BCAD deficiency in vivo may be secondary to another metabolic defect. Patients with EE manifest a number of biochemical abnormalities whose coexistence has not yet been adequately explained: lactic acidemia, increased lactate/pyruvate ratio, excretion of unrelated acylglycine species, and excretion of EMA and MSA. In addition, Duran et al[14] recently reported increased excretion of inorganic thiosulphate and of two sulfur-containing amino acids, S-sulfocysteine and S-sulfothiocysteine, in four patients with EE; this finding was manifested by our patient as well.

A possible candidate site to unify the biochemical findings in EE could be complex II (succinate conenzyme Q oxidoreductase) of the mitochondrial transport chain, which oxidizes $FADH_2$ to FAD, and transfers electrons to and reduces coenzyme Q. Complex II deficiency, by increasing the intracellular ratio of FADH/FAD, should result in a relative deficiency of FAD and affect the enzymatic reactions dependent on FAD. These include, among others, 2M-BCAD, isovalerylCoA dehydrogenase, and short-chain acylCoA dehydrogenase; their combined deficiencies would result in a urinary excretion pattern similar to that observed in EE, in addition to the observed lactic acidemia and elevated lactate/pyruvate ratio. Complex II includes a number of sulfur-iron clusters. A defect in transfer of sulfur to iron-sulfur clusters of complex II would explain the biochemical findings observed in EE.

Electron transport chain enzyme activities in the patient's tissues measured in vitro were normal. If EE is associated with a defect in complex II with a resultant secondary 2M-BCAD deficiency in vivo, it was not present in the tissues studied. Alternatively, the defect may involve an aspect of complex II activity not demonstrable by the in vitro assays used during routine assays of electron transport chain activities.

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