Leucine Loading Test is Only Discriminative for 3-Methylglutaconic Aciduria Due to AUH Defect

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Received: 04 September 2013 / Revised: 07 March 2014 / Accepted: 11 March 2014 / Published online: 23 April 2014
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Abstract Currently, six inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature are known. The “Primary 3-methylglutaconic aciduria,” 3-methylglutaconyl-CoA hydratase deficiency or AUH defect, is a disorder of leucine catabolism. For all other subtypes, also denoted “Secondary 3-methylglutaconic acidurias” (TAZ defect or Barth syndrome, SERAC1 defect or MEGDEL syndrome, OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect, “not otherwise specified (NOS) 3-MGA-uria”), the origin of 3-methylglutaconic aciduria remains enigmatic but is hypothesized to be independent from leucine catabolism. Here we show the results of leucine loading test in 21 patients with different inborn errors of metabolism who present with 3-methylglutaconic aciduria. After leucine loading urinary 3-methylglutaconic acid levels increased only in the patients with an AUH defect. This strongly supports the hypothesis that 3-methylglutaconic aciduria is independent from leucine breakdown in other inborn errors of metabolism with 3-methylglutaconic aciduria and also provides a simple test

to discriminate between primary and secondary 3-methylglutaconic aciduria in regular patient care.

Introduction

Currently, six inborn errors of metabolism with 3-methylglutaconic aciduria (3-MGA-uria) as a discriminative feature are known (3-MGA-uria-IEM; for classification and nomenclature, see (Wortmann et al. 2013a). The “Primary 3-MGA-uria” 3-methylglutaconyl-CoA hydratase (3-MGH) deficiency or AUH defect is a disorder of leucine catabolism. For all other subtypes the origin of 3-MGA-uria has not been discovered but is hypothesized to be independent from leucine catabolism (for review (Wortmann et al. 2012)). These three groups of “Secondary 3-methylglutaconic aciduria” encompass disorders of defective phospholipid remodeling (TAZ defect or Barth syndrome, SERAC1 defect, or MEGDEL syndrome) and mitochondrial membrane associated disorders (OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect). The remaining patients with significant and consistent 3-MGA-uria in whom the abovementioned syndromes have been excluded are referred to as “not otherwise specified (NOS) 3-MGA-uria” until elucidation of the underlying pathomechanism enables proper classification.

One can discriminate AUH defect from the other 3-MGA-uria-IEMs based on (1) the level of 3-MGA increase which is higher than in all other subtypes, (2) the elevated urinary 3-hydroxyisovaleric acid (3-HIVA), and (3) the 2:1 ratio for *cis:trans* isoforms of 3-MGA (compared to 1:1 in all other types; (Engelke et al. 2006; Wortmann et al. 2009).

Here we test the hypothesis, that, as opposed to the observation in patients with the AUH defect, other types of 3-MGA-uria-IEM are independent of leucine catabolism by

Communicated by: Jerry Vockley, M.D., Ph.D.

Competing interests: None declared

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presenting leucine loading test in 21 patients with different types of 3-MGA-uria-IEM.

Material and Methods

Patients

Twenty-one patients with different 3-MGA-uria-IEM (three AUH defect, nine TAZ-defect, one OPA3-defect, one SERAC1-defect, seven NOS-3-MGA-uria) were included in this study. In seven patients leucine loading tests were performed at our center, the other patients were reported in literature (Barth et al. 1999; Christodoulou et al. 1994; Duran et al. 1982; Ensenauer et al. 2000; Haan et al. 1987; Kelley et al. 1991; Ruesch et al. 1996).

Leucine Loading Tests

The tests of the patients P12–P18 were performed as part of the individual diagnostic work-up, not as a scientific study. Oral informed consent was obtained from all patients. In our center 100 mg/kg (max. 6 g) leucine powder dissolved in vanilla pudding or lemonade was given orally to all patients. Prior to the loading test, and 1 h after the leucine gift, a urine portion was collected for urinary organic acid analysis. Besides, a venous blood sample was drawn to determine ammonia, lactate and glucose, blood serum amino acids, and blood gas analysis 1 h after leucine loading. In children who were very scared no venous blood sample was taken, only bedside glucose measurements were performed (“finger prick”). After the loading, also a 24 h urine sample was also collected for another urinary organic acid analysis.

Urinary Organic Acid Analysis

Briefly, a urine sample, in volume equivalent to 4 μ mol of creatinine, is acidified to pH 2, after which the organic acids are extracted by ethylacetate twice, derivatized with trimethylsilyl (TMS), and analyzed on an Agilent 7890A gas chromatograph (GC), coupled to a flame ionization detector (FID) and an Agilent 5975C inert XL MSD mass spectrometer. Quantification of organic acids was done by peak area. The concentration of 3-MGA was estimated by calculating the peak area of 3-MGA, which was compared to the calibration curve obtained with a 3-MGA standard. For this purpose the sum of both isoforms (*cis-trans*) was taken.

Leucine Loading Tests in Patients Described in the Literature

These were performed essentially as described above. Fasting hours before the challenge and time points for

sampling after leucine loading slightly differed between studies. Loading was performed with 100 mg/kg leucine in nine patients, one patient received 580 mg, one 1,200 mg leucine; two patients received 4 g/kg protein (comparable with 200 mg/kg leucine); one patient received 100 mg/kg leucine followed by 4 g/kg protein. Leucine, isoleucine, or protein was administered orally in 12 and intravenously in three patients (for details see Table 1).

Results

The results are summarized in Table 1. Serum ammonia and lactate levels, blood gas analysis, and glucose levels 1 h after leucine loading in our patients were within normal limits (data not shown). Serum amino acid analysis showed elevated leucine levels proving the intake of leucine powder (see Table 1). The patients felt well during and after the test, no one showed signs or symptoms suggesting hypoglycemia, no one reported side effects. To improve comparability of results between different centers all 3-MGA-uria levels are provided in mmol/mol creatinine (in our laboratory reference <10) and the lowest and highest 3-MGA-uria levels before and after leucine loading per patient are presented, as well as the ratio between these levels.

In AUH defect 3-MGA-uria levels were generally higher than in all other 3-MGA-uria-IEM (range before loading 400–761 mmol/mol creatinine, range after loading 1,600–2,982 mmol/mol creatinine). The mean ratio after/before loading was 4.1 ($N = 3$, range 3.9–4.5).

In TAZ-defect 3-MGA-uria levels ranged between 36 and 202 mmol/mol creatinine. The mean ratio after/before leucine loading was 1.8 ($N = 9$, range 1.1–5.3). In OPA3-defect levels ranged between 26 and 29 mmol/mol creatinine. The ratio after/before leucine loading was 0.9 ($N = 1$). In SERAC1-defect levels ranged between 60 and 75 mmol/mol creatinine. The ratio after/before leucine loading was 1.25 ($N = 1$). In NOS-3-MGA-uria levels ranged between 21 and 410 mmol/mol creatinine. The mean ratio after/before leucine loading was 1.0 ($N = 7$, range 0.5–1.3).

Discussion

The pathomechanism leading to 3-MGA-uria in non-AUH-related defect (“Secondary 3-MGA-uria”) remains unclear. Based upon the finding of elevated serum 3-MGA in a subgroup of patients with Smith Lemli Opitz syndrome a link with cholesterol biosynthesis has been hypothesized (Kelley and Kratz 1995). Two possible shuntways are known, the Popjak shunt linking dimethylallyl-PPi in the cholesterol biosynthesis with 3-methylcrotonyl-CoA in the

Table 1 Results of leucine loading tests

	Mutation	Loading	Serum glucose level 1 h after loading (mmol/L, N 4–6 mmol/L)	Serum leucine after loading (μmol/L)	Elevated urinary 3-HIVA	Lowest urinary 3-MGA before loading	Peak urinary 3-MGA after loading	Ratio high/low	Ratio <i>cis:trans</i> isoforms	Mean ratio (range, N patients)
P1 (P1 Duran et al. (1982))	<i>AUH</i>	Protein 4 g/kg p.o.	NA	819 ^a	+	518	2,328	4,5	NA	4,1 (3,9–4,5; N = 3)
P2 (P2 Duran et al. (1982))	<i>AUH</i>	Protein 4 g/kg p.o.	NA	875 ^a	+	761	2,982	3,9	NA	
P3 (Ensenauer et al. (2000))	<i>AUH</i>	100 mg/kg leucine followed by 4 g/kg protein	NA	NA	+	400	1,600	4,0	NA	
P4 (P5 aged 6 years Kelley et al. (1991))	<i>TAZ</i>	Leucine 100 mg/kg p.o.	NA	Three to fourfold elevated	–	61 ^b	75 ^b	1,2	NA	1,8 (1,1–5,3; N = 9)
P5 (P5 aged 7 years Kelley et al. (1991))	<i>TAZ</i>	Leucine 100 mg/kg p.o.	NA	NA	–	36 ^b	38 ^b	1,1	NA	
P6 (P1 Barth et al. (1999))	<i>TAZ</i>	Leucine 100 mg/kg i.v.	NA	NA	NA	69 ^b	159 ^b	2,3	NA	
P7 (P2 Barth et al. (1999))	<i>TAZ</i>	Leucine 100 mg/kg i.v.	NA	NA	NA	38 ^b	202 ^b	5,3	NA	
P8 (P3 Barth et al. (1999))	<i>TAZ</i>	Leucine 100 mg/kg i.v.	NA	NA	NA	106 ^b	150 ^b	1,4	NA	
P9 (P3 Barth et al. (1999))	<i>TAZ</i>	Leucine 100 mg/kg p.o.	NA	NA	NA	123 ^b	192 ^b	1,6	NA	
P10 (JD Christodoulou et al. (1994))	<i>TAZ</i>	Leucine 100 mg/kg p.o.	NA	211 (N 71–175)	–	100	110	1,1	NA	
P11 (ST Christodoulou et al. (1994))	<i>TAZ</i>	Leucine 100 mg/kg p.o.	NA	NA	–	130	140	1,1	NA	
P12	<i>TAZ</i>	Leucine 100 mg/kg p.o.	4.3	NA	–	32	40	1,3	1:1	
P13	<i>OPA3</i>	Leucine 100 mg/kg p.o.	5.2	NA	–	29	26	0,9	1:1	0,9
P14	<i>SERAC1</i>	Leucine 100 mg/kg p.o.	4.5	536 (N 22–276)	–	60	75	1,3	1:1	1,3
P15	<i>NOS</i>	Leucine 100 mg/kg p.o.	5.8	425 (N 22–276)	–	21	23	1,1	1:1	1,0 (0,5–1,1; N = 7)
P16	<i>NOS</i>	Leucine 100 mg/kg p.o.	NA	NA	–	33	16	0,5	1:1	
P17	<i>NOS</i>	Leucine 100 mg/kg p.o.	4.9	310 (N 22–276)	–	52	53	1,0	1:1	
P18	<i>NOS</i>	Leucine 100 mg/kg p.o.	5.3	NA	–	187	234	1,3	1:1	

(continued)

Table 1 (continued)

	Mutation	Loading	Serum glucose level 1 h after loading (mmol/L, N 4–6 mmol/L)	Serum leucine after loading ($\mu\text{mol/L}$)	Elevated urinary 3-HIVA	Lowest urinary 3-MGA before loading	Peak urinary 3-MGA after loading	Ratio high/low	Ratio <i>cis:trans</i> isoforms	Mean ratio (range, N patients)
P19 (TH Ruesch et al. (1996))	NOS	Leucine 580 mg/kg p.o	NA	NA	–	370	410	1,1	NA	
P20 (TH Ruesch et al. (1996))	NOS	Leucine 1,200 mg/kg p.o	NA	NA	–	360	360	1,0	NA	
P21 Haan et al. (1987)	NOS	Leucine 100 mg/kg p.o	NA	NA	NA	100	100	1,0	NA	

3-methylglutaconic acid (3-MGA) levels are in mmol/mol creatinine

^a Reference range not provided

^b Sum of 3-methylglutaric and 3-methylglutaconic acid

leucine catabolism and the HMG Salvage pathway, linking 3-hydroxy-3-methylglutaryl-CoA with 3-methylglutaconyl-CoA. Recently we and others have shown that there is no 3-MGA-uria in a total of 27 (treated and untreated) Smith Lemli Opitz patients as well as in three patients with mevalonate kinase deficiency (Roullet et al. 2012; Wortmann et al. 2013b). This makes significant shunting via the mevalonate shunt as source of 3-MGA-uria unlikely. However, the HMG-salvage pathway has recently been proven to account for the elevated 3-MGA production in a zebrafish model of OPA3 defect. The authors show that simvastatin inhibited mevalonate production from extra-mitochondrial HMG-CoA, which leads to elevated 3-MGA levels (Pei et al. 2010). For the vast majority of patients with 3-MGA-uria mitochondrial dysfunction and in more detail disturbances in the mitochondrial membranes seem to be the common denominator (Wortmann et al. 2013a), but there is no working hypothesis how this membrane imbalance leads to 3-MGA-uria.

Currently six 3-MGA-uria-IEM (Wortmann et al. 2013a) are known. AUH defect is the only “primary 3-MGA-uria,” a disorder of leucine catabolism. For all other types (TAZ defect or Barth syndrome, SERAC1 defect or MEGDEL syndrome, OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect) the origin of 3-MGA-uria remains enigmatic but is hypothesized to be independent from leucine catabolism (for review, see (Wortmann et al. 2012)).

Here, we report on the results of leucine loading tests in 21 patients with different 3-MGA-uria-IEM (Table 1). Urinary 3-MGA-excretion only showed a significant increase (with a factor 4) in patients with AUH defect, but not in all other types (factor 0.9–1.8). The results for patients with TAZ-defect or Barth syndrome (factor 1.8) should be interpreted with care. The ratio suggests that 3-MGA-uria levels nearly double after leucine loading. But, this is predominantly due to the results of one patient (patient seven; patient two in (Barth et al. 1999)) who has a quite low 3-MGA-level of 38 mmol/mol creatinine before and a high level after leucine loading (202 mmol/mol creatinine; ratio high/low 5.3). This is in contrast to the nine other TAZ-patients described. Leaving patient seven out, the ratio for the remaining nine patients is 1.4 which is compatible with the normal fluctuations of urinary organic acid levels as seen in all patients. The authors discuss this issue in their article and argue that in this patient the daily 3-MGA-uria levels in the morning and in the afternoon only differ up to a factor 1.75 which is still lower than the increase after leucine loading. They speculate that the results could be influenced by different fasting times before leucine loading in this patient (Barth et al. 1999).

Our data prove the hypothesis that 3-MGA-uria is independent from leucine breakdown in all secondary

3-MGA-uria-IEM. Neither the patients reported in the literature nor our own patients encountered any side effects of the leucine powder or other medical problems while undergoing the leucine loading test. This test is a simple, inexpensive test to distinguish between AUH defect and all other 3-MGA-uria-IEM. One should keep in mind that also 3-HIVA excretion and the 2:1 ratio between *cis* and *trans* isoforms of 3-MGA is unique to AUH defect patients and can be used for the same purpose.

In conclusion, this article proves the hypothesis that 3-MGA-uria only stems from leucine metabolism in AUH defect, but not in other types of 3-MGA-uria-IEM.

Disclosures

None declared.

Compliance with Ethics Guidelines

Conflict of Interest

Saskia B. Wortmann, Leo A.J. Kluijtmans, Silvia Sequeira, Ron A. Wevers, and Eva Morava declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Oral informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Saskia B. Wortmann and Eva Morava planned the study. Saskia B. Wortmann, Silvia Sequeira, and Eva Morava conducted the study in patients. Leo A.J. Kluijtmans and Ron A. Wevers conducted the biochemical measurements. All authors interpreted and discussed the results. Saskia B. Wortmann wrote the manuscript which was read and corrected by all authors.

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