Short Communication

2-Methylacetoacetylcarnitine in blood of beta-ketothiolase deficiency and HSD10 disease

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Abstract Together with 2-methyl-3-hydroxybutyrylcarnitine, 2-methylacetoacetylcarnitine has been newly identified in significantly increased amounts in serum and dried blood spot of patients with β-ketothiolase deficiency. In patients with HSD10 disease, however, the amounts of 2-methylacetoacetylcarnitine were considerably low as compared with those in patients with β-ketothiolase deficiency, and the decreased ratio of 2-methylacetoacetylcarnitine to 2-methyl-3-hydroxybutyrylcarnitine in dried blood spot could be an additional index to discriminate HSD10 disease from β-ketothiolase deficiency.

Key words: beta-ketothiolase deficiency, HSD10 disease, hydroxy-pentanoylcarnitine, organic aciduria

Introduction

Mitochondrial 2-methylacetoacetyl-CoA thiolase deficiency, or β-ketothiolase deficiency (KTD), is a rare autosomal recessive disorder of isoleucine and ketone body metabolism. HSD10 disease is a rare X-chromosomal disease caused by a moonlighting protein encoded by the HSD17B10 gene, and this protein catalyzes the 2-methyl-3-hydroxybutyryl-CoA dehydrogenation (MHBD) reaction in isoleucine metabolism[1]. Chemical diagnosis for these disorders is based on the elevated concentrations of metabolites originating from isoleucine breakdown, and urinary organic acid analysis typically shows elevated excretion of 2-methyl-3-hydroxybutyrate (MHBA) and tiglylglycine in both disorders, although increased 2-methylacetoacetate (MAAA) has been detected only in KTD[2]. However, due to broad spectrum in clinical pictures and biochemical abnormalities of these disorders, the chemical diagnosis seems problematic in not a few patients with these disorders, and characteristic metabolites in urine were reportedly missing or obscure in the patients with a milder form of KTD or those with atypical forms of HSD10 disease[2−7]. On the other hand, elevated hydroxy-pentanoylcarnitines (C5-OH) and tiglylcarnitine (C5:1) in dried blood spots were observed in tandem mass spectrometric screening for KTD[8], and 2-methyl-3-hydroxybutyrylcarnitine (MHBC) in serum of KTD patients has been reportd in liquid-chromatography tandem mass spectrometric (LC-MS/MS) analysis[9]. In the present study, we have investigated 2-methylacetoacetylcarnitine (MAAC) and the other metabolites related

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to these disorders in blood by LC-MS/MS in order to clarify how useful these metabolites are as diagnostic markers.

Materials and Methods

Biological samples

Three patients with KTD and two with HSD 10 disease were included in the present study. Patient A with KTD had increased levels of MHBA, TG and MAAA in urine, constantly: serum specimen and dried blood spot (DBS) were collected in stable condition at the age of 19 years. Patient B with KTD had also increased levels of the three metabolites mentioned above in urine during a ketoacidotic episode at the age of 12 months: DBS was collected during this episode. Patient C with KTD had very mildly increased excretion of these metabolites during his first ketoacidotic episode at the age of 18 months: serum specimen was collected during this episode. Patient D with HSD10 disease had typical clinical phenotype and increased levels of MHBA and TG in urine during ketoacidotic events: DBS was collected for newborn screening at the age of 5 days and was stored in freezer before the present analysis. Patient E with HSD10 disease[3] had atypical clinical phenotype and had increased excretion of MHBA and TG during a ketoacidotic event: the serum specimen was collected during this event. The diagnosis for these patients was confirmed by mutation analysis.

Chemicals

NeoSMAAT kit for MS/MS newborn screening, which contains labeled acylcarnitines, including [$^2H_9$]3-hydroxyisovalerylcarnitine and [$^2H_9$]isovalerylcarnitine, was purchased from Sekisui Medical CO. (Tokyo, Japan). 2-Methylacetoacetylcarnitine was synthesized by mixing carnitine hydrochloride and 2-methylacetoacetyl chloride in trifluoroacetic acid, which was formed from ethyl 2-methylacetoacetate (Sigma-Aldrich CO., Tokyo, Japan). Tiglylcarnitine was synthesized from carnitine hydrochloride and tiglyl chloride (Tokyo Chemical Industry Co., Tokyo, Japan).

Methods

One punch (1/8 inch of diameter) of DBS was extracted with 100 μL of methanol solution of NeoSMAAT kit (0.075 nmol/ml [$^2H_9$]3-hydroxyisovalerylcarnitine and [$^2H_9$]isovalerylcarnitine) of by the routine manner for newborn screening, and the extract was dried under the nitrogen stream and was re-dissolved in 2% formic acid solution. The mixture of 5 μL of serum specimen and 167 μL of NeoSMMAT kit solution was centrifuged at 10,000 rpm, and the supernatant was collected. The samples (10 μL) were introduced into the LC mobile phase flow of 0.4 mL/min using 150 mm×3.0 mm Scherzo SS-C18 column (Imtakt, Portland, USA). Gradient elution of the analyte was achieved using a program with mobile phase A (aqueous 0.5% formic acid) and mobile phase B ((0.5 M ammonium formate/0.5 M NH_4OH=9:1)/methanol=1 : 9) as follows: 10% B for 1 min, 10% B to 40% B in 4 min, 40% B to 70% B in 8 min, 70% B to 100% B in 0.1 min plus an additional 7 min at 100% B, then back to 10% B in 0.1 min and re-equilibration for 5 min.

For the measurement of short-chain acylcarnitines in DBS and serum specimen by electrospray-ionization LC-MS/MS, a model API 4000 triple-stage mass spectrometer (AB Sciex, Tokyo, Japan) equipped with a model LC10Avp HPLC system and a model SIL-20AC auto-injector (Shimadzu, Kyoto, Japan) was used[10]. The positive ion MS/MS analysis was performed in multiple-reaction monitoring (MRM) mode with the following transitions; m/z 260＞85 for MAAC, m/z 271＞85 for [$^2H_9$]3-hydroxyisovalerylcarnitine, m/z 251＞85 for [$^2H_9$]isovalerylcarnitine, and m/z 244＞85 for tiglylcarnitine. Suitable measurement conditions for the designated transitions were identified with the automatic tune function of the Analyst software. In addition, the positive ion MS/MS was performed in product-ion scan mode, in order to get the product ion spectra of any peak using variable collision-energy settings. The condition for the transition m/z 262＞103 for MHBC was determined based on the data of product-ion scan measurement of MHBC peak in DBS of patient A.

The data were recorded for 13 min after every sample injection. For quantification, the recorded peak areas of the designated MRM ion set were used.

Results

In Fig. 1, MRM chromatogram for serum acylcarnitines of a patient with KTD was shown. Three peaks of MHBC, a single peak of MAAC, and a large peak of [$^2H_9$]isovalerylcarnitine, were observed from 7.8 to 8.8 min of retention time. As shown in Fig. 2, product ion mass spectra for 3 peaks of MHBC agreed with the reported mass spectra [8]. Since MHBC could not be quantitated separately from 3-hydroxyisovalerylcarnitine (HIVC), using transition of m/z 262＞85, as shown in the lowest column of Fig. 3, the

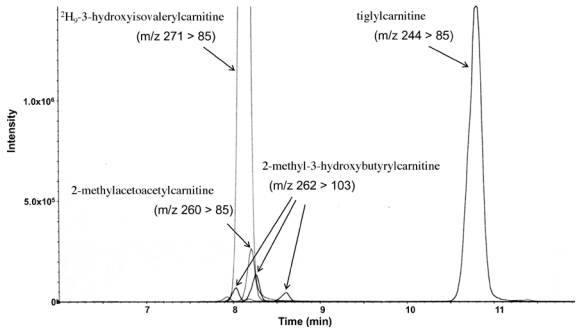

Fig. 1. MRM chromatogram for serum acylcarnitines of a patient with β-ketothiolase deficiency.
Every MRM transition is shown in parenthesis: see text.

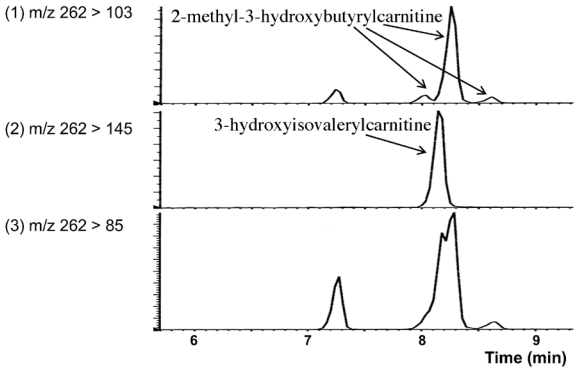

Fig. 3. MRM chromatograms using transition of m/z 262>103 (1), m/z 262>145 (2), and m/z 262/85 (3).

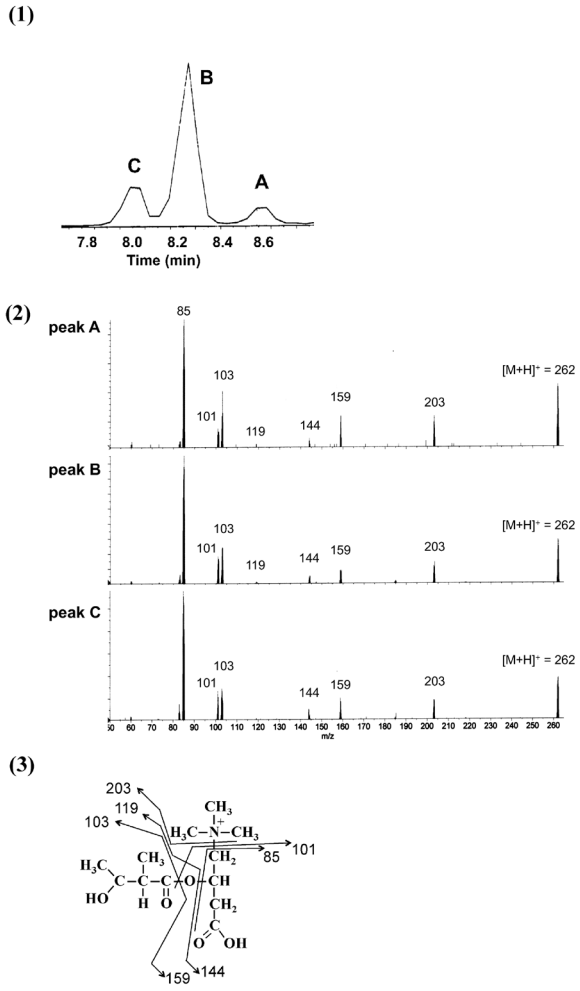

Fig. 2. Three peaks in MRM chromatogram using transition of m/z 262>103 and product ion mass spectra in the measurement of 2-methyl-3-hydroxybutyryl-carnitine.
(1) MRM chromatogram using transition of m/z 262> 103, (2) Product ion mass spectra of precursor ion (m/z 262) for three peaks, (3) supposed fragment ions from $[M+H]^+$ of 2-methyl-3-hydroxybutyrylcarnitine.

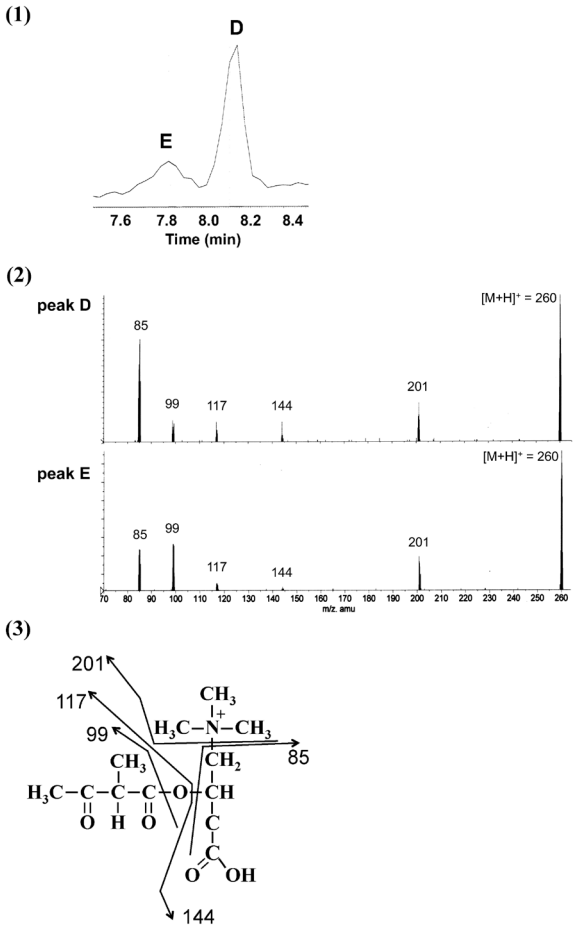

Fig. 4. Two peaks in MRM chromatogram using transition of m/z 260>85 and product ion mass spectra in the measurment of 2-methylacetoacetylcarnitine.
(1) MRM chromatogram using transition of m/z 260> 85, (2) Product ion mass spectra of precursor ion (m/z 260) for two peaks, (3) supposed fragment ions from $[M+H]^+$ of 2-methylacetoacetylcarnitine.

Table 1. Characteristic acylcarnitines in HSD10 disease and β-ketothiolase deficiency

	Tiglylcarnitine	MHBC	MAAC	MAAC/MHBC
Pt-A: KTD (serum)	0.874	0.634	0.1471	0.232
Pt-A: KTD (DBS)	0.615	3.610	0.0590	0.016
Pt-B: KTD (DBS)	0.485	4.844	0.1360	0.028
Pt-C: KTD (serum)	0.036	0.048	0.0014	0.029
Pt-D: HSD10 (DBS)	0.247	0.550	0.0005	0.001
Pt-E: HSD10 (serum)	0.440	0.135	0.0016	0.012
Controls DBS (*n*=15) mean±*SD* (range)	0.003±0.001 (0.002−0.004)	0.014±0.004 (0.008−0.018)	(<0.0004)	

Unit of acylcarnitines is nmol/mL.
The internal standards were [$^2H_9$] isovalerylcarnitine for tiglylcarnitine and [$^2H_9$]3-hydroxyisovalerylcarnitine for 2-methyl-3-hydroxybutyrylcarnitine and 2-methylacetoacetylcarnitine, respectively; see text for details.
MHBC; 2-methyl-3-hydroxybutyrylcarnitine, MAAC; 2-methylacetoacetylcarnitine,
KTD; β-ketothiolase deficiency, DBS; dried blood spot

transition of m/z 262>103 for MHBC was used, and the sum of the areas of three peaks was used for calculation. Because authentic MHBC was not available, the peak areas measured by the transition m/z 262>103 were compared to those by m/z 262>85 in DBS of patient B, who had a markedly large amount of MHBC, but a very low amount of HIVC; the mean ratio of the former areas to the latter were 0.32 (*n*=7).

As shown in Fig. 4, the product ion mass spectrum for Peak D was identical to that of synthesized MAAC. The product ion mass spectrum for Peak E, which was observed constantly in both of control subjects and the patients, was similar to that of MAAC.

The concentrations of MHBC and MAAC, which were calculated using [$^2H_9$]3-hydroxyisovalerylcarnitine as an internal standard, and tiglylcarnitine, which were calculated using [$^2H_9$]isovalerylcarnitine, were shown in Table 1. HSD 10 patients had large amounts of tiglylcarnitine and MHBC, but very low amounts of MAAC, while KTD patients had large amounts of tiglylcarnitine, MHBC together with increased amounts of MAAC, which were more clearly shown by the ratio of MAAC/MHBC. In addition, KTD patient 3 had higher concentrations of MHBC in DBS than in serum, while those of MAAC in DBS were lower than in serum.

Discussion

In the present study, accumulated MAAC has been first identified in blood of KTD patients. Increased MAAA in urine of KTD patients suggested the accumulated 2-methylacetoacetyl-CoA, which is a substrate of mitochondrial β-ketothiolase. Therefore, it is speculated that MAAC can be produced from accumulated 2-methylacetoacetyl-CoA by carnitine acetyltransferase[11]. In our experience, however, MAAA seems unstable in body fluids, and sometime could not be detected in the urine of the patients with KTD, especially after long-time storage. Since MAAA was not commercially available unfortunately, the stability of MAAA could not be tested and LC-MS/MS measurement of MAAA has not been performed, yet. Although MAAC may not be stable enough, it could be detected in considerable amounts in blood samples of the patients with KTD, which were stored in freezer for up to 3 years. Blood sample, instead of urine, was suggested to be a useful substitute for chemical diagnosis of KTD.

Increased MHBC, which was assumed to be produced from accumulated 2-methyl-3-hydroxybutyryl-CoA, has been reported in patients with KTD[8]. In the reported LC-MS/MS measurement, there were three isomer peaks of MHBC, which possesses two chiral carbons and exists in four isomers. It is thought that the second peak of 2-methyl-3-hydroxybutyrylcarnitine in Fig. 2 may contain two of the four isomers and three peaks of 2-methyl-3-hydroxybutyrylcarnitine appeared.

On the other hand, MAAC has no chiral carbon and showed a single peak in our LC-MS/MS measurements. Interestingly, one peak with a product ion mass spectrum similar to that of MAAC was observed close to the peak of MAAC. While it appeared in small amounts even in controls and the ingredient of the peak has not been clarified, it

should not be counted as MAAC, accidentally. In our MRM measurements, in addition, very small amounts of MAAC were quantitated even in patients with HSD10, and the peak of MAAC may contain some unknown substance which could influence on the quantitation of MAAC in very low concentration range, using the MRM transition m/z 260 ＞85.

In the report mentioned above[9], MAAC was not observed. It may be partly because the concentrations of MAAC might not be high enough, or simply because the MRM measurement to detect MAAC was not be performed.

In the present study, the accumulation of MHBC was more evident in DBS, as compared with that of tiglylcarnitine, than in serum. The dominant accumulation of 3-hydroxyisovalerylcarnitine in DBS of the infants with biotin deficiency has been reported[12], and MHBC in DBS could be a better marker for KTD and HSD10 than that in serum. As shown in KTD patient C, MAAC was rather low in KTD patient with very mildly increased amounts of tiglylcarnitine and MHBC, while the ratio of MAAC to MHBC seems to be a better marker than MAAC concentration itself to discriminate KTD from HSD10.

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Conflict of Interest

The authors declare that they have no competing interests.

References

1) Vilardo E, Rossmanith W: Molecular insights into HSD10 disease: impact of SDR5C1 mutations on the human mitochondrial RNase P complex. *Nucleic Acids Res* 43: 5112−5119, 2015.
2) Zschocke J: HSD10 disease: clinical consequences of mutations in the HSD17B10 gene. J Inherit Metab Dis. 35: 81−89, 2012.
3) Fukao T, Akiba K, Goto M, Kuwayama N, Morita M, et al: The first case in Asia of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 disease) with atypical presentation. *J Hum Genet* 59: 609−614, 2014.
4) Fukao T, Maruyama S, Ohura T, Hasegawa Y, Toyoshima M, et al: Three Japanese patients with beta-ketothiolase deficiency who share a mutation, c.431A＞C (H144P) in ACAT1: subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis using tandem mass spectrometry. *JIMD Reports* 3: 107−115, 2012.
5) Fukao T, Scriver CR, Kondo N, the T2 Collaborative Working Group: The clinical phenotype and outcome of mitochondrial acetoacetyl-CoA thiolase deficiency (β-ketothiolase or T2 deficiency) in 26 enzymatically proved and mutation-defined patients. *Mol Genet Metab* 72: 109−114, 2001.
6) Sarafoglou K, Matern D, Redlinger-Grosse K, Bentler K, Gaviglio A, et al: Siblings with mitochondrial acetoacetyl-CoA thiolase deficiency not identified by newborn screening. *Pediatrics* 128: e246−e250, 2011.
7) Akagawa S, Fukao T, Akagawa Y, Sasai H, Kohdera U, et al: Japanese male siblings with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 Disease) without neurological regression. *JIMD Rep* 32: 81−85, 2017.
8) Han LS, Ye JW, Qiu WJ, Gao XL, Wang Y, et al: Selective screening for inborn errors of metabolism on clinical patients using tandem mass spectrometry in China: A four-year report. *J Inherit Metab Dis* 30: 507−514, 2007.
9) Penga M, Fangb X, Huanga Y, Caia Y, Lianga C, et al: Separation and identification of underivatized plasma acylcarnitine isomers using liquid chromatography−tandem mass spectrometry for the differential diagnosis of organic acidemias and fatty acid oxidation defects. *J Chromatogr A*, 1319: 97−106, 2013.
10) Shigematsu Y, Hata I, Tajima G: Useful second-tier tests in expanded newborn screening of isovaleric acidemia and methylmalonic aciduria. *J Inherit Metab Dis* 33(Suppl 2); S283−S238, 2010.
11) Violante S, IJlst L, Ruiter J, Koster J, van Lenthe H, et al: Substrate specificity of human carnitine acetyltransferase: Implications for fatty acid and branched-chain amino acid metabolism. *Biochim Biophys Acta* 1832; 773−779, 2013.
12) Tokuriki S, Hayashi H, Okuno T, Yoshioka K, Okazaki S, et al: Biotin and carnitine profiles in preterm infants in Japan. *Pediatr Int* 55(3): 342−345, 2013.