Journal of Chromatography B, 877 (2009) 2648–2651

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enzymatic evaluation of glutaric acidemia type 1 by an *in vitro* probe assay of acylcarnitine profiling using fibroblasts and electrospray ionization/tandem mass spectrometry (MS/MS)☆

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A R T I C L E I N F O

Article history:
Received 7 November 2008
Accepted 26 April 2009
Available online 3 May 2009

Keywords:
Glutaric academia type1
GA1
Glutarylcarnitine
C5DC
Acylcarnitine
Electrospray ionization/tandem mass spectrometry
MS/MS
Fibroblasts
In vitro probe assay
Lysine
Aminoadipate
Glutaric acid

A B S T R A C T

Glutaric acidemia type 1 (GA1) is usually diagnosed with an accumulation of glutaric acid (GA) or 3-hydroxyglutaric acid by GC/MS. In some cases, however, excretion of GA is low. We investigated enzymatic evaluation of GA1 using fibroblasts and MS/MS. After loading substrates, lysine, 2-aminoadipate (2AA), or GA, in fibroblasts, and incubating for 96 h, glutarylcarnitine (C5DC) levels in the media were measured. A significant increase of C5DC was observed in GA1 patients, irrespective of substrates added. 2AA showed the largest difference between patients and controls ($p = 0.0004$). Results suggested enzymatic evaluation of GA1 is useful under appropriate culture conditions.

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1. Introduction

Glutaric acidemia type 1 (GA1) is a metabolic disorder of organic acids due to a defect of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7), which acts in the intermediate metabolic steps of lysine, hydroxylysine, and tryptophan (Fig. 1) [1–3]. GA1 shows an autosomal recessive inheritance, and its frequency has been reported in one out of 100,000 people. The GCDH gene, the causative gene for GA1, has been mapped to chromosome 19q13.1. The active enzyme is a homotetramer consisting of 43.3-kDa subunits, localized in the mitochondrial matrix [4,5].

Many disorders of organic acid metabolism induce a rapid development of symptoms after early neonatal onset; however, GA1 shows the slow and gradual development of neuronal regression in most cases. If not diagnosed early and treated, disease onset starts from infancy with extrapyramidal symptoms or neuronal regression, such as myotony and dystonia [6–8]. In patients with GA1, a characteristic appearance of the brain can be seen [9]; namely, marked enlargement of the sylvian fissure, atrophy of the brain cortex, and enlargement of the cerebral ventricle.

Urinary organic acid analysis is useful for the diagnosis of GA1, with the characteristic findings of increases in glutaric acid (GA), 3-hydroxyglutaric acid (3-OH-GA), and glutaconic acid. Furthermore, blood acylcarnitine analysis using electrospray ionization/tandem mass spectrometry (MS/MS) shows an increase of glutarylcarnitine (C5DC). However, it has been reported that GA1 can be classified into two types based on excreted GA levels: a high GA excretion (GA > 100 mmol/mol creatine), and a low one (GA < 100 mmol/mol creatine) [1–2]. In some GA1 cases with low GA excretion, additional examinations are necessary, including measurement of the enzymatic activity or gene analysis.

Schulze-Bergkamen et al. reported enzymatic evaluation for GA1 on peripheral blood mononuclear cells (PBMC) using an *in vitro* probe assay and MS/MS, in which 2-oxoadipic acid was used as a substrate [13]. However, 2-oxoadipic acid is no longer available on a commercial basis, which led us to determine the alterna-

☆ This paper was presented at the 33rd Meeting of the Japanese Society for Biomedical Mass Spectrometry, Tokyo, Japan, 25–26 September 2008.

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doi:10.1016/j.jchromb.2009.04.043

tive substrate. Additionally, to date, little has been done to apply the enzymatic evaluation of using fibroblast and MS/MS to organic acidemia. In the present study, we investigated the efficacy of the *in vitro* probe assay using fibroblasts and MS/MS in enzymatic detection of GA1, with three different substrates.

2. Materials and methods

2.1. Subjects

Human skin fibroblasts obtained from 10 Japanese patients diagnosed as having GA1 based on the characteristic metabolic profiles of urinary organic acids and genetic analysis were studied. Among the 10 patients, 6 were previously described in case reports [9–12]. The 7 control cell lines were also used.

2.2. Cell culture

Skin fibroblasts were cultured and maintained in a minimal essential medium (MEM), containing modified Eagle's essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan), and 2 mmol/l of L-glutamine (Nacalai Tesque) at 37 °C and 5% CO_2 in a humidified atmosphere until confluency.

2.3. Substrate loading to cultured fibroblasts

As shown in Fig. 1, to determine an appropriate substrate to add to the culture medium, 3 compounds related to glutaryl-CoA metabolism were used: lysine (Lys; Sigma), L-2-aminoadipate (2AA; Wako, Osaka, Japan), and glutaric acid (GA; Wako). Confluent cells were harvested by trypsinization (0.25%-trypsin/1 mM-EDTA; Nacalai Tesque), then distributed onto 6-well-microplates (35 mm i.d.; Iwaki, Tokyo, Japan), and re-cultured. When they reached confluence, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA), and 1 ml of MEM with containing each substrate plus L-carnitine (0.4 mmol/l; Sigma) was added. After 96 h, the supernatant was collected, and acylcarnitine profiling by MS/MS was performed. The measured blood acylcarnitine levels were corrected by the protein concentration of the cells at the starting point. The protein concentration was calculated based on the previously described method by Lowry et al. [14]. The experiment was repeated at least 3 times for each sample.

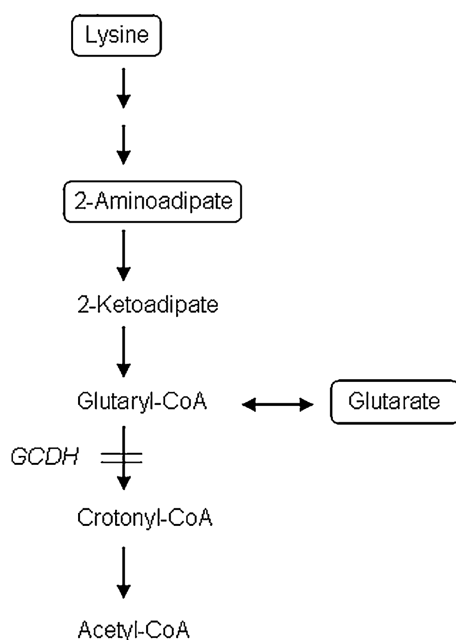

Fig. 1. Flow chart of added substrates and metabolic map of GA1.
The substrates shown in the boxes were added in the present study. GCDH is an abbreviation of glutaryl-CoA dehydrogenase.

2.4. Sample preparation for MS/MS analysis

Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). The contents of an acylcarnitine reference standard kit (NSK-B, Cambridge Isotope Laboratories, Andover, USA): ^{2}[H]$_9$-carnitine, ^{2}[H]$_3$-acetylcarnitine, ^{2}[H]$_3$-propionylcarnitine, ^{2}[H]$_3$-butyrylcarnitine, ^{2}[H]$_9$-isovalerylcarnitine, ^{2}[H]$_3$-octanoylcarnitine, ^{2}[H]$_9$-miristoylcarnitine, and ^{2}[H]$_3$-palmitoylcarnitine, were diluted in methanol and used as an internal standard.

The routine sample preparation method for MS/MS was used for acylcarnitine analysis [15–17]. Briefly, 10 μl of the supernatant was transferred to a 96-well microplate, and 200 μl of the methanol reference standard kit was added to each well. The aliquots were centrifuged at 4000 rpm for 10 min, and then 150 μl of supernatant was obtained. After drying the sample under a gentle stream of nitrogen, 50 μl of 3N *n*-butanol–HCl was added, and butylation was performed at 65 °C for 15 min. After drying up, the sample was reconstituted in 100 μl of 80% acetonitrile:water (4:1, v/v, without formic acid).

2.5. Acylcarnitine analysis by MS/MS

An API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) in combination with a SIL-HTc autosampler (Shimadzu, Kyoto, Japan) was used, with a sample volume of 10 μl. Quantitative analysis was conducted using ChemoView^{TM} software (Applied Biosystems/MDS SCIEX, Toronto, Canada) by comparing the signal intensity of an analyte against the corresponding internal standard (Supplemental "Fig. 3"). The heated gas temperature was 250 °C, and the ion spray voltage was 5500 eV. All acylcarnitines were measured by positive precursor ion scan of m/z 85 (scan range m/z: 200–500, C5DC m/z: 388.2) with declustering potential of 40 V, entrance potential of 10 V, and collision cell exit potential of 5 V.

2.6. Data analysis

The data were expressed as mean plus or minus standard deviation. Statistical analysis was performed using Student's *t*-test in JMP version 5.01a. ^{2}[H]$_3$-octanoylcarnitine was used as an internal standard of glutarylcarnitine (C5DC). The quality assurance of MS/MS analysis was validated by measuring the standard samples including C5DC at the concentration of 0.06, 0.23, 0.39, and 0.72 nmol/ml. The calibration curves of the measurements ($n = 10$) indicated good linearity ($p < 0.01$, $r = 0.99$). Intra- and inter-assay variability was 9.9% and 17.3%, respectively ($n = 6$).

3. Results

3.1. Substrates and concentrations

The effects of different substrates, Lys, 2AA, and GA, on C5DC levels were compared using 5 cell lines from 5 GA1 patients. Each substrate was used at 0.5, 2.0, and 4.0 mM (Supplemental "Fig. 4"). When Lys or GA was added, the C5DC level was significantly higher than in medium alone ($p < 0.01$) at concentrations of 2.0 and 4.0 mM. When 2AA was added, C5DC level was significantly

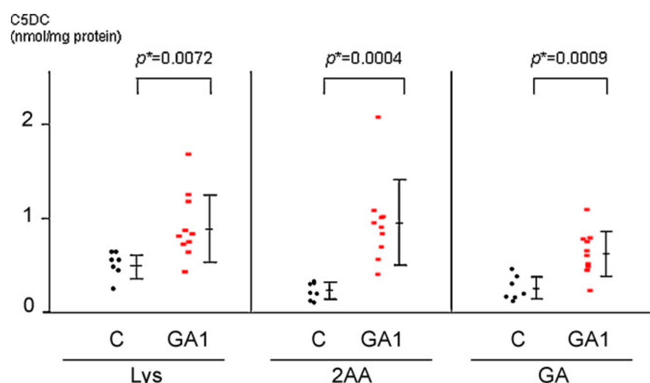

Fig. 2. Amount of C5DC in the medium of GA1 cells and control with different substrates.
As a substrate, 2.0 mM of Lys, 2AA, or GA was added. The amount of C5DC in the medium was compared between patients with GA1 cases and controls. $^*p < 0.01$. Abbreviations: C, control; Lys, lysine; GA, glutaric acid; 2AA, 2-aminoadipate.

increased at a concentration of 2.0 mM ($p < 0.01$). These substrates at 2 mM did not affect the cell viability as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay [18]) (Supplemental "Fig. 5"). Based on these findings, the optimum concentration of substrates was considered to be 2.0 mM in the present study.

3.2. *Amount of C5DC in culture medium in patients with GA1*

The amount of C5DC was compared with control subjects and 10 patients with GA1 by adding substrates (Lys, 2AA, or GA at 2.0 mM) (Fig. 2 and Supplemental "Fig. 6"). In patients with GA1, a significant increase of C5DC was seen in each sample, with the level of significance regarding the difference. The addition of 2AA led to the greatest difference. When cultured without any substrates, no significant difference was seen between patients with GA1 and control subjects.

4. Discussion

Our study verified that enzymatic activity evaluation in patients with GA1 is practical by an *in vitro* probe assay of acylcarnitine profiling using human skin fibroblasts. Since 2-oxoadipic acid used by Schulze-Bergkamen et al. [13] is no longer commercially available, we determined whether the other molecules involved in GCDH represent alternative substrates. Lys and 2AA were used as substrates as they locate upstream of glutaryl-CoA synthesis pathway (Fig. 1). Since administration of GA would increase C5DC in cells with GCDH deficiency as a consequence of accumulation of glutaryl-CoA, to which GA is converted [19], GA was also used as another substrate. The results showed that all substrates increased the amount of C5DC in GA1 cells, compared with normal controls and GA1 cells cultured in medium alone. Our data indicate that 2AA can be used as an alternative substrate to evaluate the enzyme activity of GCDH.

If the concentration of a substrate is higher than 4 mM, the amount of C5DC in GA1 cells reduces in some cases. This tendency was striking when 2AA was added, which is likely due to growth suppression of the cells by the presence of an excessive substrate. However, these substrates at 2.0 mM did not affect the cell viability as determined by the MTT assay (Supplemental "Fig. 5"), making it highly unlikely that the substrate used in our study affected cell proliferation that could influence the amount of cellular protein and the C5DC level. Based on these results, the appropriate substrate concentration was considered to be 2.0 mM in the present study.

The difference of the C5DC between 10 patients with GA1 and 7 control subjects was most remarkable when 2AA was added as a substrate, showing no overlaps. Thus it was considered that 2AA was the most suitable substrate. When Lys or GA was added, there was an overlap in C5DC between GA1 patients and controls.

The *in vitro* probe assay has been reported to be useful for the enzymatic evaluation of β-oxidation disorders [20]. Based on our results, it was indicated that our method was useful for the diagnosis of patients with GA1. In future studies, analysis of C5DC using d6-glutarylcarnitine as internal standard or quantifing the metabolite by a fast liquid chromatography (LC) step or the multiple reactions monitoring (MRM) would make the analysis more quantitative and improve the performance of the results. Although various metabolic disorders of organic acids can be diagnosed through urinary organic acid analysis by GC/MS, some cases require further diagnostic methods for enzymatic evaluation. Our data suggests that the *in vitro* probe assay will be useful for the diagnosis of other organic acidemias, though an appropriate substrate and experimental condition are required. Because PBMC do not allow us to repeat the experiments using the same sample, fibroblasts would provide more accurate and reliable information to determine an appropriate substrate.

Acknowledgements

We are grateful to Yuka Ito, Toyomi Esumi, Midori Furui, Nana Tomita, and Shine Mushimoto for their technical assistance. This study was partly supported by grants from the Ministry of Health, Labour and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.04.043.

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