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Original article

Effect of heat stress and bezafibrate on mitochondrial β-oxidation: Comparison between cultured cells from normal and mitochondrial fatty acid oxidation disorder children using in vitro probe acylcarnitine profiling assay

Hong Li [a,b], Seiji Fukuda [a], Yuki Hasegawa [a], Hironori Kobayashi [a], Jamiyan Purevsuren [a] Yuichi Mushimoto [a], Seiji Yamaguchi [a,*]

[a] *Department of Pediatrics, Shimane University School of Medicine, 89-1 En-ya, Izumo, Shimane 693-8501, Japan*
[b] *Department of Pediatrics, The Affiliated Hospital of Ningxia Medical University, 804 Sheng-li-jie, Yinchuan, Ningxia 750004, China*

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Abstract

Hyperpyrexia occasionally triggers acute life-threatening encephalopathy-like illnesses, including influenza-associated encephalopathy (IAE) in childhood, and can be responsible for impaired fatty acid β-oxidation (FAO). In this regard, patients with impaired FAO may be more susceptible to febrile episodes. The effects of heat stress and a hypolipidemic drug, bezafibrate, on mitochondrial FAO were investigated using cultured cells from children with FAO disorders and from normal controls, using an in vitro probe acylcarnitine (AC) profiling assay. Fibroblasts were incubated in medium loaded with unlabelled palmitic acid for 96 h at 37 and 41 °C, with or without bezafibrate. AC profiles in culture medium were analyzed by electrospray ionization tandem mass spectrometry. Heat stress, introduced by 41 °C, significantly increased acetylcarnitine (C2) but slightly decreased the other acylcarnitines (ACs) in controls and medium-chain acyl-CoA dehydrogenase (MCAD)-deficient cells. On the other hand, in very long-chain acyl-CoA dehydrogenase (VLCAD)-deficient cells, accumulation of long-chain ACs were enhanced at 41 °C, compared with that at 37 °C. In contrast, bezafibrate decreased long-chain ACs with significant increase of C2 in both control and VLCAD-deficient cells at 37 °C. These data suggest that heat stress specifically inhibits long-chain FAO, whereas bezafibrate recovers the impaired FAO. Our approach is a simple and promising strategy to evaluate the effects of heat stress or therapeutic drugs on mitochondrial FAO.

Keywords: Heat stress; Bezafibrate; Mitochondrial fatty acid β-oxidation disorder; In vitro; Acylcarnitine profiling

1. Introduction

Hyperpyrexia occasionally triggers acute life-threatening encephalopathy-like illnesses, including influenza-associated encephalopathy (IAE) in childhood [1,2], and it has been reported that heat stress can be responsible for impaired fatty acid β-oxidation (FAO) in IAE [3–5]. In this regard, patients with impaired FAO may be more susceptible to febrile episodes. The mitochondrial FAO is a central energy generating process particularly during long fasting, infection or acute metabolic stress, such as hyperpyrexia [6,7]. Patients with inherited mitochondrial FAO disorders occasionally present acute life-threatening symptoms, such as

* Corresponding author. Tel.: +81 853 20 2216; fax: +81 853 20 2215.
E-mail address: seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

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encephalopathy or cardio-myopathy, due to energy crisis of metabolic decompensation [8,9], which are often remediable [10,11]. In addition, heat stress was considered as a model of thermal injury to the central nervous system (CNS) in a number of research reports [3,4,12].

In recent decades, in vitro probe acylcarnitine (AC) profiling assay was developed to evaluate FAO disorders [13–16]. AC profiles in culture medium after incubating with various fatty acids as substrates were determined by electrospray ionization tandem mass spectrometry (MS/MS). In order to investigate the consequence of heat stress on impaired FAO, we compared the quantitative AC profiles at 37 and 41 °C in cultured fibroblasts from Japanese children with mitochondrial FAO disorders [15,16] as well as from normal controls. Furthermore, we utilized the same approach to assess the effects of bezafibrate, a common hypolipidemic drug, which acts as activator of peroxisome proliferators activated nuclear receptors (PPARs) and up-regulates the expression of genes encoding mitochondrial enzymes [17], on mitochondrial FAO in the same groups. We report here the effects of heat stress and bezafibrate on mitochondrial FAO with the findings of the in vitro probe AC profiling assay.

2. Materials and methods

2.1. Skin fibroblasts

We cultured fibroblasts from 6 controls (healthy volunteers, passages 3–16) and 9 Japanese VLCAD-deficient children (passages 3–14). VLCAD deficiency is clinically divided into three subgroups [15]: (1) a severe form, with early onset (infancy), high mortality, and high incidence of cardio-myopathy; (2) an intermediate form, with onset from infancy to early childhood, low mortality, and high incidence of hypo-glycemia; and (3) a myopathic form (milder form), with frequent onset in later childhood, adolescence or after, and isolated skeletal muscle involvement triggered by exercise, long fasting or other metabolic stresses. In this study, cell lines from VLCAD deficiency, 2 cases of the severe; 3 of the intermediate; and 4 of the myopathic forms, were examined. We also used 3 cell lines from medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (passages 4–9) for reference in the study.

2.2. In vitro probe assay of AC profiling

Fibroblasts were cultured in 75-cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Nacalai Tesque) at 37 °C in a humidified 5% CO_2/95% air incubator.

Confluent cells were harvested by trypsinization (0.25%-Trypsin/1 mM-EDTA; Nacalai Tesque) and seeded onto 6-well microplates (35 mm i.d.; Iwaki) with fresh above medium (2 mL/per well) until they reached confluence again. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate A, MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, or substrate B, medium of substrate A added bezafibrate (0.4 mmol/L; Sigma). The start and the end points of the 96-h incubation are expressed as T_0 and T_{96}, respectively. AC profiles in the culture medium were analyzed at T_{96}.

Cultured cells were incubated with substrate A at 37 or 41 °C for 96 h to determine the effects of heat stress on mitochondrial FAO. Cells were also incubated in substrate B, medium containing bezafibrate and palmitic acid, at 37, and 41 °C to determine the effects of bezafibrate. In a replicate experiment, the supernatants were collected at 24, 48, 72 and 96 h to determine their time course effects on the FAO.

2.3. Quantitative acylcarnitines analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium, for 30 min. Portions were centrifuged at 1000*g* for 10 min, and then 150 μL of the supernatant was dried under a nitrogen stream, and butylated with 50 μL of 3 N *n*-butanol–HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v) and then the ACs in 10 μL of the aliquots were determined using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

2.4. Protein concentration, cell count and cell viability

Protein concentrations were measured at T_0 and T_{96}, by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [18]. Cells were enumerated in a model Z1 Coulter Counter (Beckman Coulter Electronics, Luton, UK). The ratio of viable cells at 24, 48, 72 and 96 h of incubation were determined using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [19].

2.5. Data and statistical analysis

The results are expressed as mean ± SD from at least two independent experiments. The AC concentrations

are expressed as nmol/mg protein. Data were statistically analyzed by the one-way analysis of variance (ANOVA) and post hoc LSD test for multiple group comparisons, and Independent-Samples T test for comparisons of two groups using SPSS version 11.5 software for Windows.

3. Results

3.1. Clinical characters of VLCAD-deficient patients

The clinical characteristics of the patients with VLCAD deficiency are summarized in Table 1. Patients 1 and 2 with the severe form both died in infancy. The patients (from No. 3 to 9) with the intermediate or myopathic forms remain alive, although patients classified into the intermediate form had episodes of cardio-myopathy, acute encephalopathy, hypo-glycemia or hyperammonemia.

3.2. AC profiles in VLCAD-deficient cells under heat stress

As shown in Fig. 1, the levels of C2 in all forms of VLCAD deficiency were lower than that of normal controls at 37 °C, and the levels of long-chain ACs, such as C12, C14, C14:1 and C16, were higher in VLCAD deficiency. In particular, the amount of C14 was remarkably elevated in the severe form, while C12 was significantly increased in all forms of VLCAD deficiency.

Control cells exposed to heat stress (41 °C) showed a significant elevation of C2 ($p = 0.012$), but slightly decreased other species from short-chain to medium-chain ACs (Fig. 1B). Similarly, C2 was significantly elevated in the intermediate and myopathic forms of VLCAD-deficient cells incubated at 41 °C compared to 37 °C ($p < 0.01$). Most importantly, accumulation of C16 was significantly enhanced in all the clinical forms of VLCAD deficiency ($p < 0.01$) (Fig. 1B).

Since overall accumulation of long-chain ACs represents the impairment of long-chain FAO rather than individual long-chain ACs, we calculated the ΣLC, the sum of long-chain AC productions (C12 + C14 + C14:1 + C16), and the ratio of ΣLC/C2, which may be more useful to evaluate the capacity of long-chain FAO. Regardless of temperature, the ΣLC and the ratio of ΣLC/C2 values in VLCAD-deficient cells were significantly higher than those of controls ($p < 0.01$), as shown in Fig. 2. The ΣLC was significantly higher at 41 °C than that at 37 °C in VLCAD-deficient cells ($p = 0.043$), among which myopathic form showed the highest ΣLC, suggesting that the long-chain FAO was inhibited by heat stress and the myopathic form is most sensitive to heat stress ($p < 0.01$).

We compared the effects of heat stress between VLCAD-deficient cells (mild form, $n = 4$) and MCAD-deficient cells ($n = 3$) using the same strategy. As shown in Fig. 3, C2 was significantly increased at 41 °C in all VLCAD-, MCAD-deficient cells as well as normal controls. In VLCAD deficiency (Fig. 3A), medium-chain ACs (C6, C8 and C10) decreased, whereas long-chain ACs (C12, C14, C14:1 and C16) elevated at 41 °C. On the other hand, in MCAD deficiency (Fig. 3B), medium-chain ACs (C6 and C8) were also decreased at 41 °C but long-chain ACs did not change. Namely, while medium-chain ACs were down-regulated by heat stress in control, MCAD-, and VLCAD-deficient cells, long-chain ACs were accumulated exclusively in VLCAD deficiency.

3.3. Effect of bezafibrate on FAO disorder

Compared with the basic condition (Fig. 4A), bezafibrate treatment significantly increased the amount of C2 in VLCAD-deficient cells ($p = 0.014$) as well as control cells, while the accumulation of long-chain ACs remarkably decreased ($p < 0.01$) at 37 °C (Fig. 4B). Although the mean values of ΣLC ($p = 0.029$) and the ratio of ΣLC/C2 ($p = 0.015$) remained higher in VLCAD deficiency patients than that in controls (Fig. 5), bezafibrate significantly reduced ΣLC in all the clinical forms to a different extent at 37 °C (Fig. 5A) compared with that without bezafibrate as shown in Fig. 2A. The reduction of

Table 1
Clinical and biochemical characteristics of patients with VLCAD deficiency.

Patient no.	Age at onset	Clinical subgroup	Outcome	Clinical and biochemical findings					
				Cardio-myopathy	Acute encephalopathy	Myalgia or rhabdomyolysis	Hypo-glycemia	Hyper-ammonemia	Hepato-megaly
1	2 m	Severe	Dead (8 m)	+	+	−	+	+	−
2	4 m	Severe	Dead (5 m)	+	+	−	−	+	+
3	1 y4 m	Intermediate	Alive	−	+	−	+	+	+
4	1 y5 m	Intermediate	Alive	−	+	+	−	−	−
5	4 y9 m	Intermediate	Alive	+	−	+	−	−	−
6	1 y	Myopathic	Alive	−	−	+	−	−	−
7	2 y	Myopathic	Alive	−	−	+	−	−	−
8	2 y10 m	Myopathic	Alive	−	−	+	−	−	−
9	8 y	Myopathic	Alive	−	−	+	−	−	−

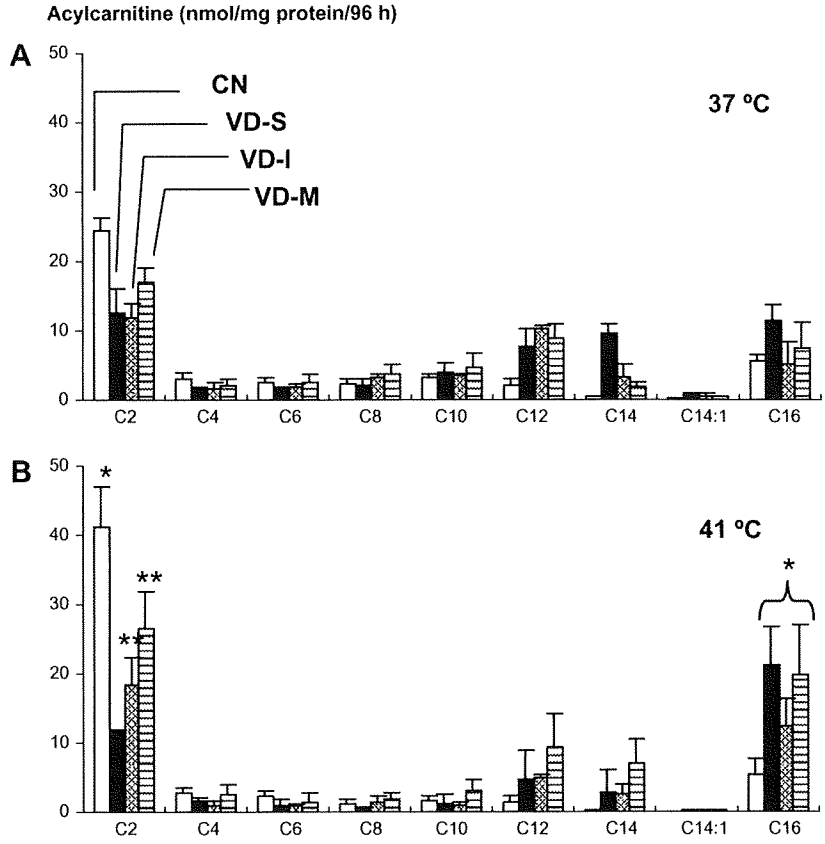

Fig. 1. AC profiles in medium loaded with palmitic acid from VLCAD deficiency with different clinical forms under heat stress. (A) 37 °C; (B) 41 °C. □, Control (CN); ■, severe VLCAD deficiency (VD-S); ▨, intermediate form (VD-I); ▤, myopathic form (VD-M). Data are expressed as mean ± SD (nmol/mg protein/96 h). Significant differences between 37 and 41 °C are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

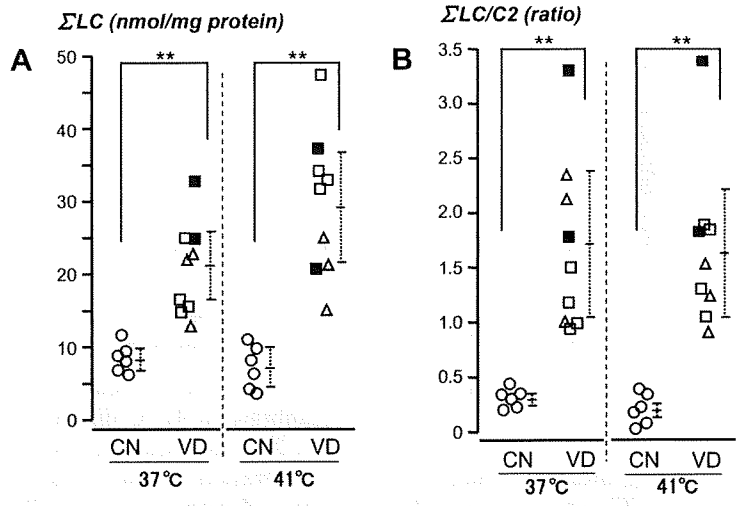

Fig. 2. Comparison of ΣLC and ΣLC/C2 of VLCAD deficiency under heat stress. (A) ΣLC, C12 + C14 + C14:1 + C16; (B) ratio of ΣLC/C2. CN, control; VD, VLCAD deficiency. ○, Control; ■, severe VLCAD deficiency; △, intermediate form; □, myopathic form. Significant differences between different groups are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

long-chain ACs tended to be greater in the intermediate (58 ± 24% reduction) and myopathic forms (54 ± 24% reduction) than in the severe form (35 ± 20% reduction). On the other hand, bezafibrate treatment at 41 °C, curiously, reduced all species of AC including C2 in both control and VLCAD-deficient cells (Fig. 4C).

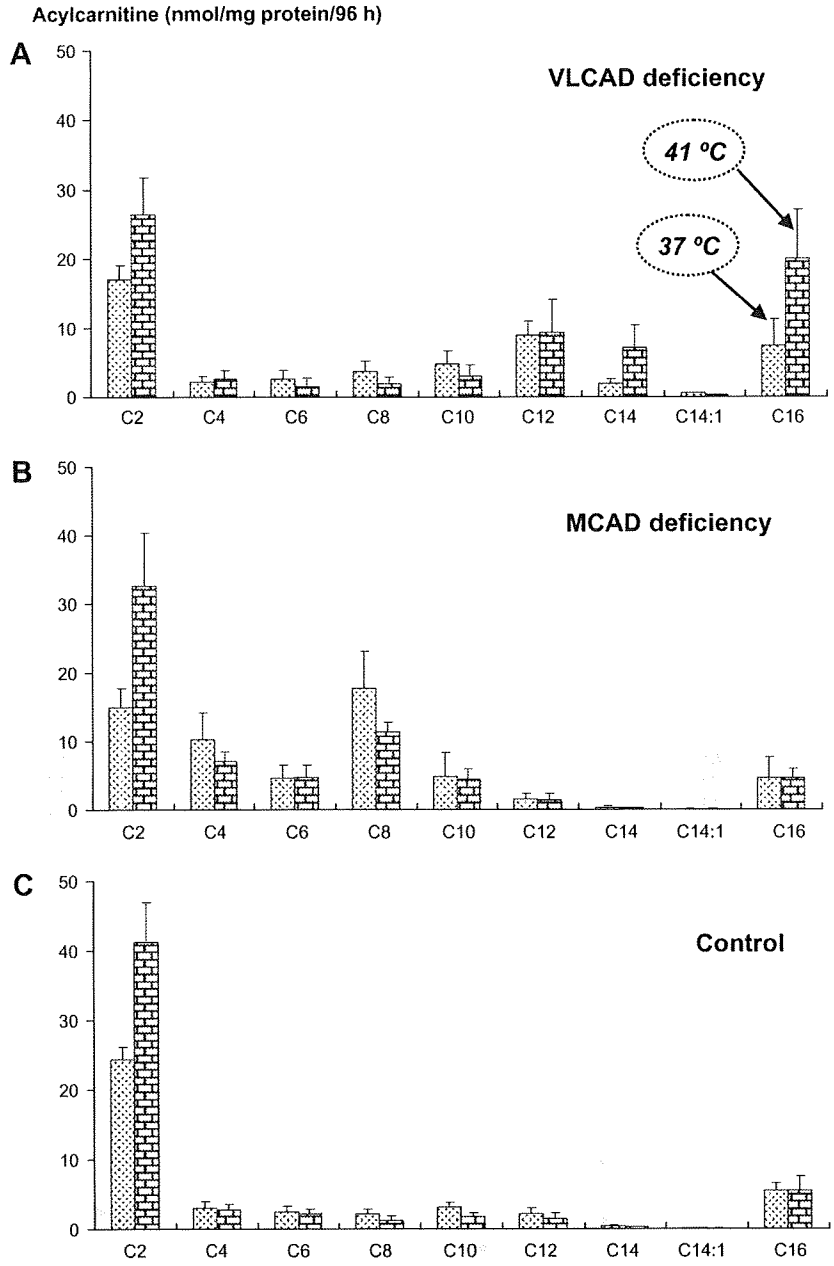

Fig. 3. Comparison of AC profiles in the medium loaded with palmitic acid between VLCAD deficiency and MCAD deficiency under heat stress. (A) VLCAD deficiency; (B) MCAD deficiency; (C) Control. ▨, 37 °C; ▩, 41 °C. Data are expressed as mean ± SD (nmol/mg protein/96 h).

3.4. MTT assay and the protein concentration of cultured cells under different conditions

Since all species of ACs were significantly reduced in the presence of bezafibrate at 41 °C, we measured cell viability using the MTT assay, and protein concentration in lysates to exclude variations in cell number or viability that could otherwise affect ACs in cells cultured in fatty acid-free BSA for up to 96 h under various conditions. The ratio (%) of viable cells (Fig. 6) and the protein concentration (Fig. 7) declined over time in all groups and under all culture conditions. Incubation with bezafibrate at 41 °C diminished the viable cell number after 24 h as shown in Fig. 6 and the average protein concentration at 96-h (T_{96}) was decreased by >50% compared with start point (T_0) as shown in Fig. 7.

4. Discussion

The primary purpose of our study was to investigate the susceptibility of hyperpyrexia, and a hypolipidemic drug, bezafibrate, on mitochondrial FAO capacity using

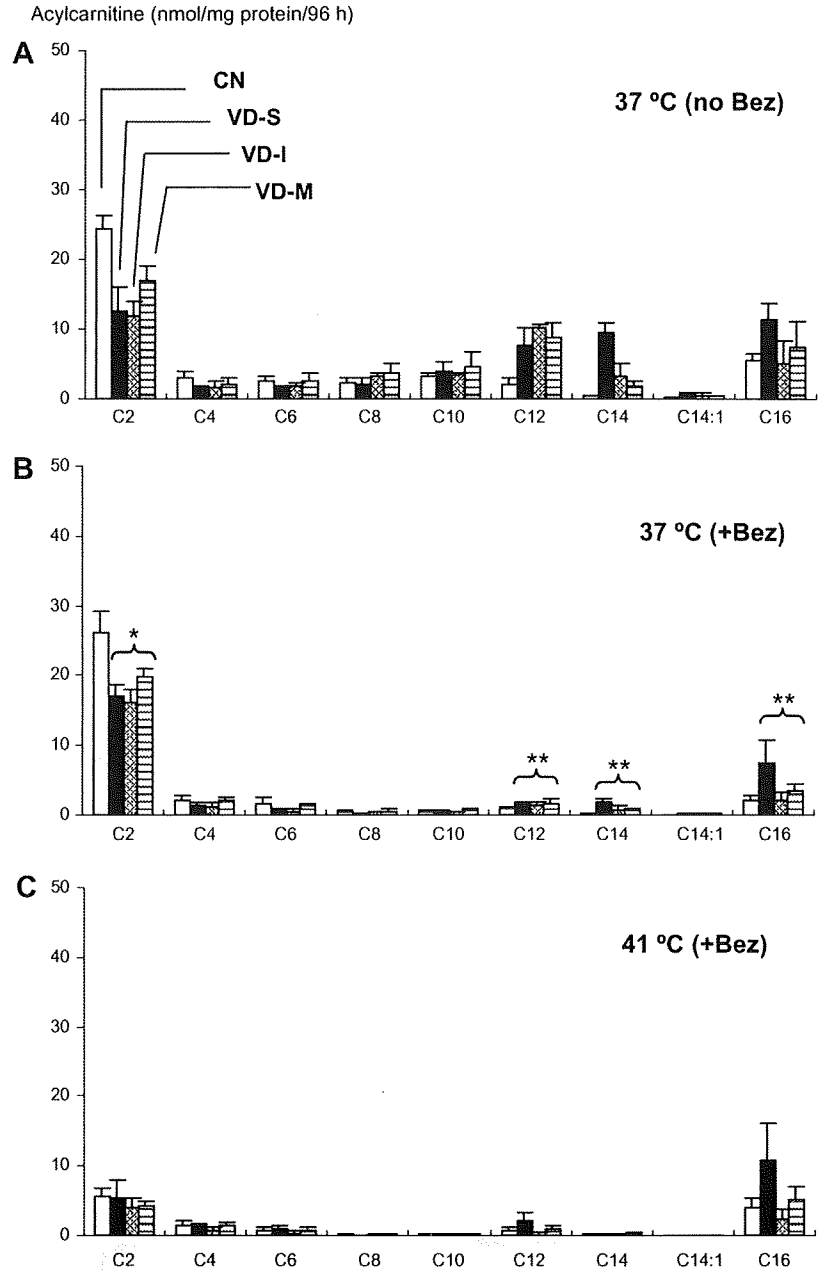

Fig. 4. AC profiles in medium loaded with palmitic acid from VLCAD deficiency with different clinical forms after bezafibrate treatment. (A) 37 °C without Bez; (B) 37 °C + Bez; (C) 41 °C + Bez. □, Control (CN); ■, severe VLCAD deficiency (VD-S); ▨, intermediate form (VD-I); ▤, myopathic form (VD-M). Data are expressed as mean ± SD (nmol/mg protein/96 h). Significant differences between cells treated with or without bezafibrate at 37 °C are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

in vitro probe AC profiling assay. Although hyperpyrexia may be responsible for deterioration of various metabolic disorders in childhood and occasionally is associated with life-threatening encephalopathy such as IAE [1–5], it is difficult to confirm the susceptibility to hyperpyrexia in vivo.

We used fibroblasts from patients with VLCAD deficiency, in which longer chain FAO is impaired, as well as normal controls, to determine the effect of heat stress and bezafibrate on mitochondrial FAO. While mitochondrial acyl-CoA dehydrogenases (ACDHs) share the same basic chemical mechanism, they differ markedly in their specificity towards the 'length' of their acyl-CoA substrates [20]. In this context, we evaluated the FAO capacity based on the accumulation of specific length-chain of ACs. Acetylcarnitine (C2), derived from

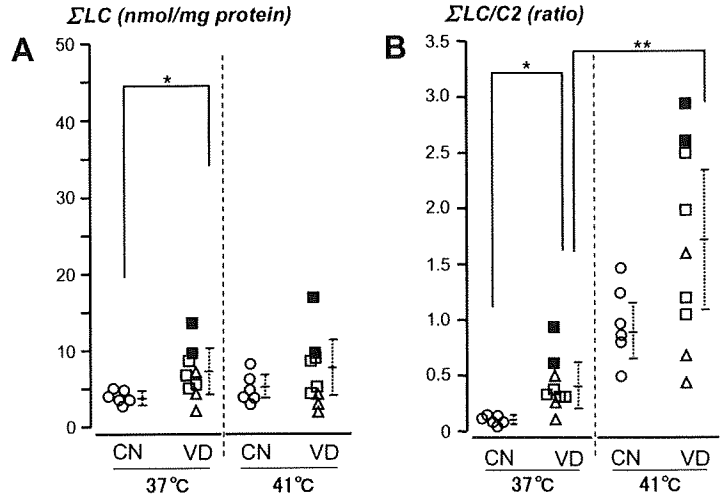

Fig. 5. Comparison of ΣLC and ΣLC/C2 of VLCAD deficiency after bezafibrate treatment. (A) ΣLC, C12 + C14 + C14:1 + C16; (B) ratio of ΣLC/C2. CN, control; VD, VLCAD deficiency; ○, Control; ■, severe VLCAD deficiency; △, intermediate form; □, myopathic form. Significant differences between different groups are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

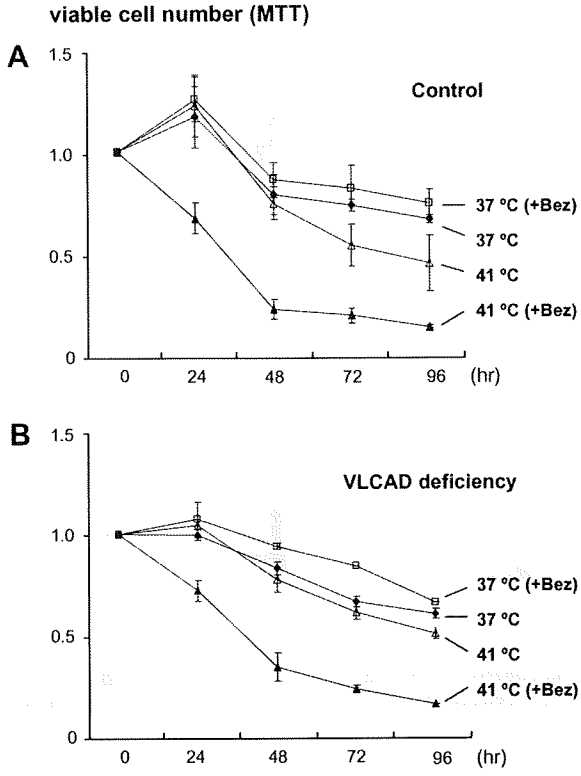

Fig. 6. Cell viability measured by MTT assay at various conditions. (A) Control; (B) VLCAD deficiency. ◆, 37 °C; △, 41 °C; □, 37 °C with bezafibrate; ▲, 41 °C with bezafibrate. Data are expressed as mean ± SD.

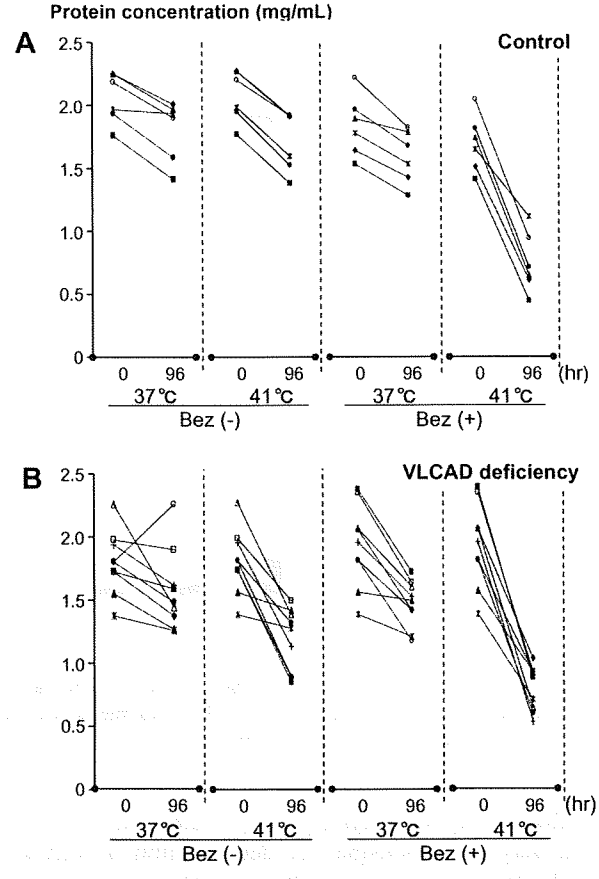

Fig. 7. Protein concentration at T_0 and T_{96} at various conditions. (A) Control; (B) VLCAD deficiency. Each line represents individual cells.

acetyl-CoA, the final product of FAO cycles, is considered to be the most important marker of the whole FAO flux, and the long-chain ACs specifically represent the long-chain FAO flux.

Our previous data indicate that in vitro AC profiling assay can identify patients with various FAO disorders [21], which is consistent with the findings of others

[22–26]. Our results showed significant reduction of the amount of C2, and accumulation of long-chain ACs in VLCAD-deficient cells. In particular, the accumulation of C12 and C14 were the most sensitive diagnostic markers to specify the clinical subgroups of VLCAD deficiency.

It has been reported that some of the milder FAO disorders are thermolabile in IAE [3,4] and that bezafibrate improves the residual VLCAD activity in patients with milder form of VLCAD deficiency [17,27]. However, the effect of heat stress or bezafibrate on FAO capacity in VLCAD deficiency by the in vitro probe assay with unlabelled palmitic acid as substrate, has never been characterized. Our approach is simpler than the other methods to determine the effects of metabolic stresses as well as of drugs on FAO disorders in vitro or in vivo.

Under heat stress, C2 significantly increased in cells from controls, intermediate, myopathic forms of VLCAD- and MCAD-deficient patients as well as in normal controls. On the other hand, C2 did not change in the severe form of VLCAD deficiency. The amounts of long-chain ACs, especially C16, were significantly elevated in all forms of VLCAD deficiency, but not in MCAD deficiency at 41 °C, suggesting that long-chain FAO are more susceptible to the heat stress, compared with medium-chain FAO. Although heat stress also increased C2 in the myopathic and intermediate forms as in normal controls, this is most likely a consequence of enhanced short- or medium-chain FAO at higher temperature (Fig. 1B). ΣLC was significantly higher in the myopathic form of VLCAD deficiency compared with that of the other two forms at 41 °C, suggesting that the myopathic form of VLCAD deficiency is most sensitive to heat stress. These results are accordance with the fact that asymptomatic patients with the myopathic form often triggered the symptoms under conditions of metabolic stress [28].

The regulation of mitochondrial FAO by PPARs has been studied extensively by several groups [29]. Bezafibrate, one of the activators of PPARs, represents an alternative therapeutic approach to treat long-chain FAO disorders [17,27,30–32]. In keeping with previous studies, we also explored the effect of bezafibrate on mitochondrial FAO using our in vitro probe assay, and found that bezafibrate (0.4 mM) enhanced the long-chain FAO process. It is hypothesized that the drug diminishes the accumulation of toxic long-chain ACs in cells from VLCAD-deficient patients. We also found that bezafibrate reduced long-chain ACs more effectively in the intermediate and myopathic forms than in the severe form. These findings suggest that bezafibrate may represent a potential treatment strategy for VLCAD deficiency, specifically for the clinically milder forms.

On the other hand, all species of ACs were significantly reduced not only in VLCAD-deficient cells but also in controls, incubated with bezafibrate at 41 °C. We therefore explored the mechanism responsible for this phenomenon. Our data showed that the viable cell number significantly decreased after 24 h in the presence of bezafibrate at 41 °C. The obvious reduction of ACs is probably a consequence of loss of cell viability at this condition. These data may imply a potential toxicity of bezafibrate during fever.

In conclusion, in vitro probe acylcarnitine profiling assay using unlabelled palmitic acid as substrate is a simple and promising strategy to determine the effects of heat stress or drugs on mitochondrial FAO. Heat stress inhibits long-chain FAO specifically in long-chain FAO disorders, and bezafibrate improves impaired long-chain FAO.

Acknowledgments

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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)☆

Yuichi Mushimoto*, Yuki Hasegawa, Hironori Kobayashi, Hong Li, Jamiyan Purevsuren, Isamu Nakamura, Takeshi Taketani, Seiji Fukuda, Seiji Yamaguchi

Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan

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ABSTRACT

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.

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-

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* Corresponding author. Tel.: +81 853 20 2219; fax: +81 853 20 2215.
E-mail address: mushiu1@med.shimane-u.ac.jp (Y. Mushimoto).

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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.

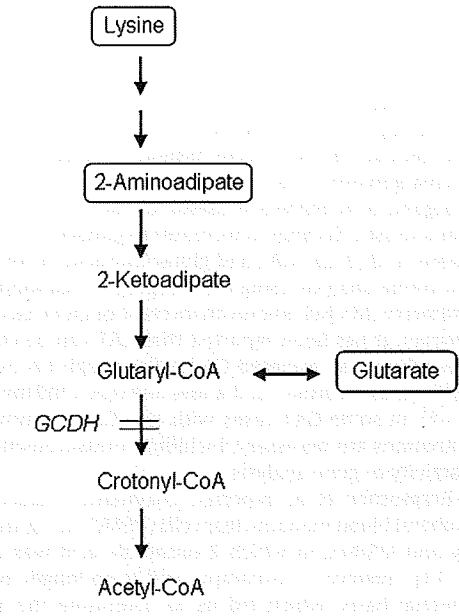

Fig. 1. Flow chart of added substrates and metabolic map of GA1.
The substrates shown in the boxes were added in the present study. GCDH is an abbreviation of glutaryl-CoA dehydrogenase.

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™ 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

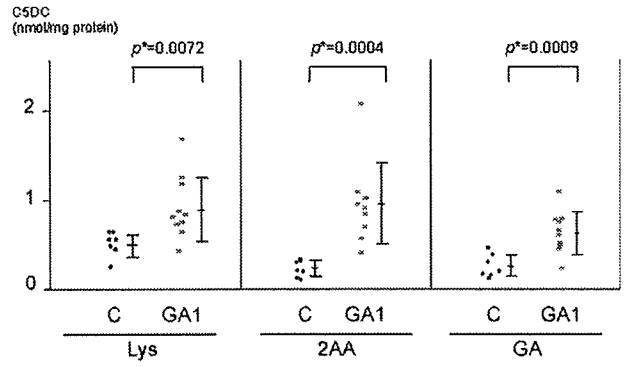

Fig. 2. Amount of C5DC in the medium of GA1 cells and control with different substrates.
As a substrate, 2.0 mM of Lys, 2AA, or GA was added. The amount of C5DC in the medium was compared between patients with GA1 cases and controls. *$p < 0.01$.
Abbreviations: C, control; Lys, lysine; GA, glutaric acid;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

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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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新規 *ETHE1* 遺伝子変異を認めたエチルマロン酸脳症の1例

佐世保市立総合病院小児科[1]，広島大学大学院医歯薬学総合研究科小児科学[2]

大坪　善数[1]　白尾謙一郎[2]　岡田　賢[2]　但馬　剛[2]

佐倉　伸夫[2]　楠本　隆[1]　青木　幹弘[1]　中下　誠郎[1]

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新規 *ETHE1* 遺伝子変異を認めたエチルマロン酸脳症の1例

佐世保市立総合病院小児科[1]，広島大学大学院医歯薬学総合研究科小児科学[2]
大坪　善数[1]　白尾謙一郎[2]　岡田　賢[2]　但馬　剛[2]
佐倉　伸夫[2]　楠本　隆[1]　青木　幹弘[1]　中下　誠郎[1]

要　旨

エチルマロン酸脳症（EE）は，常染色体劣性遺伝形式をとる有機酸代謝異常症である．2004年に *ETHE1* 遺伝子変異が原因であることが判明した．30数例の海外報告例があるが，本邦での報告例はない．

今回，遺伝子解析によりEEの診断が確定した男児例を経験した．生後5か月時に筋緊張低下，発育不良，慢性下痢，四肢のチアノーゼ，点状出血を主訴に当科紹介となり，先天代謝異常症を疑った．本例は確定診断がつかないまま1歳11か月時に死亡したが，EEの典型的な臨床症状を持ち，生前の血中アシルカルニチン分析にてC4の増加，尿中有機酸分析にてエチルマロン酸（EMA），メチルコハク酸，C4-C5アシルグリシンの排泄増加を認め，保存リンパ球を用いた遺伝子解析により *ETHE1* の新規遺伝子変異である112T＞A（exon2：Y38N），154G＞C（exon2：D52H）の複合ヘテロ変異を持つEEの診断に至った．両親，兄弟の遺伝子解析の結果，*ETHE1* 変異遺伝子のヘテロ保因者であることが確認された．

本例同様，海外報告例でも乳幼児期死亡例が多く，臨床症状からEEが疑われた場合，血中アシルカルニチン分析，尿中有機酸分析および遺伝子解析による早期診断が重要となる．

キーワード：エチルマロン酸脳症，*ETHE1* 遺伝子，有機酸代謝異常症

はじめに

エチルマロン酸脳症（Ethylmalonic encephalopathy：EE）は，常染色体劣性遺伝形式をとる有機酸代謝異常症であり，1991年にBurlinaらによって初めて報告された[1]．中枢神経症状，血管障害，慢性下痢を主症状とし，生化学的には血中アシルカルニチン分析，尿中有機酸分析で特徴的な所見を認め，頭部MRIで広範に異常信号を呈する[2][3]．2004年に19q13に局在する *ETHE1* 遺伝子変異が原因であることが判明した[4]．非常に稀な疾患ではあるが，特徴的な臨床症状，生化学的所見に加え，遺伝子解析により早期診断が可能となった．現在のところ，発症機序は不明で，確立された治療法は報告されていない．

遺伝子解析により本邦第1例目となるEEの男児例を経験した．これまでの報告例と比較しながら，今回確定診断に辿り着いた経緯について報告する．

症　例

症例：5か月男児

主訴：筋緊張低下，発育不良，慢性下痢，四肢のチアノーゼ，点状出血

家族歴：同胞の兄2人（3歳，1歳）は成長発達に異常なし．両親に血族婚はなく，生来健康．近親に乳幼児期の早期死亡例はみられない．

現病歴：妊娠分娩経過に問題なく，在胎37週5日，出生体重2,790gで出生．出生時，特に異常は認めなかったが，生後数日目より1日に10～15回の水様便を認め，徐々に粘液便となっていった．吸啜は弱く授乳に時間を要し，常に鼻閉感，いびき様呼吸を認めていた．啼泣時には，顔面，四肢のチアノーゼ，点状出血を認め，4か月健診時に体重増加不良，筋緊張低下，頸定の遅れを指摘され，5か月時に当科紹介となった．

初診時現症：身長64cm（－0.9SD），体重6.1kg（－2.0SD），意識清明であったが，表情は乏しく，筋緊張低下を認めた．手掌単一屈曲線，耳介低位が見られ，頸定，寝返りは認めなかった．心音呼吸音に異常認めず，腹部は平坦軟であり，肝脾腫認めず，神経学的には四肢深部腱反射は正常であった．

初診時検査所見：初回血液生化学検査は異常なく，低血糖，代謝性アシドーシス，高アンモニア血症，高乳酸・ピルビン酸血症も認めなかった．また染色体検査は正常核型で（表1），頭部MRI検査では脳萎縮，出血，低酸素性虚血性脳症を示唆する所見は見られなかった．しかし，脳波では，全般にsuppression burst

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表1　初診時検査所見

WBC	13,100 /μl	TSH	3.050 μIU/ml
Hb	12.1 g/dl	F-T4	1.450 ng/dl
Plt	31.6×10^4 /μl	F-T3	3.92 ng/dl
		血清総 IgE	120 IU/ml
T.P	7.0 g/dl	特異的 IgE（牛乳）	0.70 IU/ml
AST	11 IU/L	アンモニア	52 μg/dl
ALT	28 IU/L	乳酸	19.4 mg/dl
LDH	211 IU/L	ピルビン酸	1.0 mg/dl
CPK	150 IU/L		
BUN	8.1 mg/dl	PT（INR）	1.17
Cr	0.2 mg/dl	PT（%）	77.1 %
Ca	10.6 mg/dl	APTT	31.7 sec
CRP	0.6 mg/dl	染色体検査（G 分染法）	46,XY
ESR	14.0 mm/hr		
BS	112 mg/dl	（静脈血液ガス）	
IgG	544 mg/dl	PH	7.326
IgA	36 mg/dl	PCO2	44.1 mmHg
IgM	111 mg/dl	PO2	29.7 mmHg
		$HCO3^-$	22.4 mmol/L
		B.E.	− 3.1 mmol/L

表2　特殊検査所見

血中遊離脂肪酸分析　　(μmol/l)

	7か月	8か月	正常値
C8	18.8	14.8	4.8 ～ 9.2
C10	14.4	8.2	4.2 ～ 7.8
C10：1	5.3	1.8	nd
C14：1	0.6	0	nd

尿中有機酸分析　　(mmol/mol of creatine)

	9か月	1歳9か月	正常値
エチルマロン酸	55.36	119.45	0.00 ～ 6.20
メチルコハク酸	16.02	68.01	0.00 ～ 6.40
2-OH-グルタル酸	6.08	11.22	0.60 ～ 5.90
イソブチリルグリシン	16.53	1.40	0.00 ～ 0.40
イソバレリルグリシン	33.04	9.94	0.00 ～ 0.40
アジピン酸	16.82	35.08	0.50 ～ 5.00

アシルカルニチン分析　　(nmol/ml)

	新生児期濾紙血	血清（8か月時）
遊離カルニチン	31.3（10 ～ 55）	16.0（31 ± 8）
C4 アシルカルニチン	2.33（< 0.25）	1.91（0.25 ± 0.09）

pattern を示し，てんかんと診断した．また眼底検査では網膜血管の蛇行を認めた．

臨床経過：痙攣発作は認めなかったものの，初診時よりカルバマゼピンによる治療を開始した．初診直後より頻回に呼吸器感染症のため入院治療を要するようになり，高熱に伴う痙攣発作の頻度は徐々に増加した．単剤でのてんかん発作のコントロールは困難で，クロナゼパムを併用したところ，比較的良好なコントロールが得られるようになった．また頻回の下痢に対してはRASTの結果よりミルクアレルギーを疑い，アレルギー用ミルク，クロモグリク酸ナトリウム，整腸剤の内服を行ったが，水様下痢，粘液便は持続した．啼泣時のチアノーゼ，点状出血も頻回に出現し，頸定は見られず，筋緊張低下の改善も見られなかった．これら

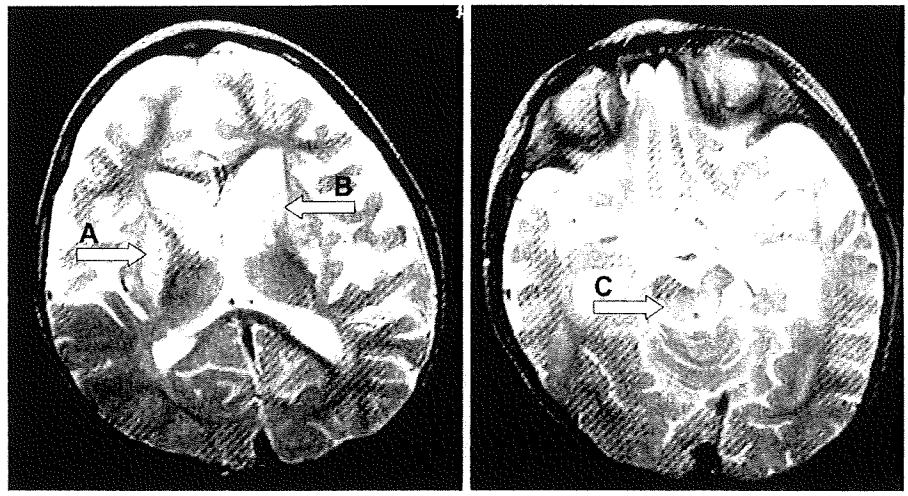

図1　1歳9か月時の頭部MRI単純（T2強調像）所見．前頭葉～側頭葉の萎縮，またレンズ核（A），尾状核頭部（B），中脳背側（C）の高信号域を認める．

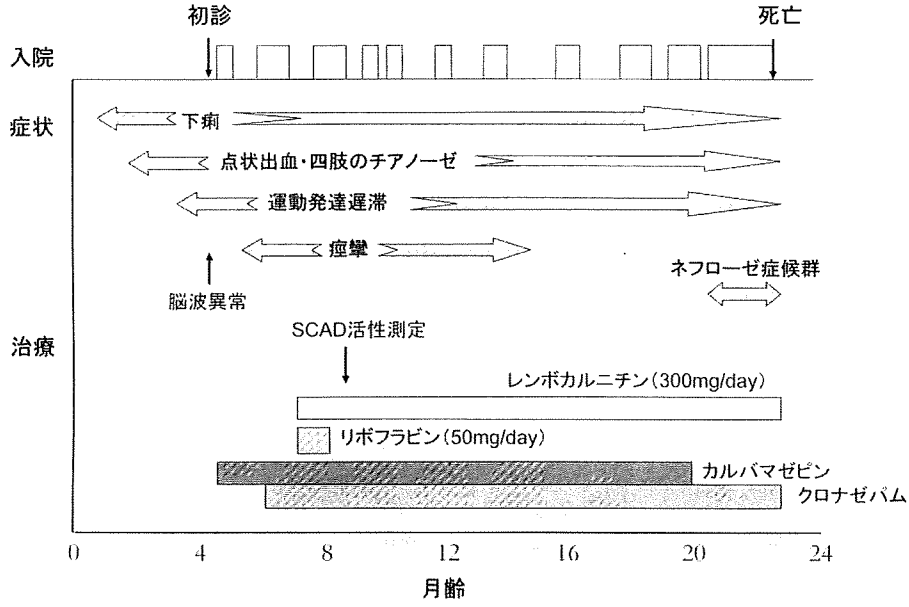

図2　臨床経過

の臨床経過より先天代謝異常症を疑い，血中・尿中アミノ酸分析を行ったが，特定のアミノ酸代謝異常症を示唆する所見は得られず，生後7か月時の血中遊離脂肪酸分析では，C4，C10，C10：1の増加，C14：1の軽度増加を認め，尿中有機酸分析では，短鎖脂肪酸由来であるエチルマロン酸(EMA)，メチルコハク酸の排泄増加に加え，複数のアミノ酸の中間代謝産物である2-OH-グルタル酸（C5），イソブチリルグリシン（C4），イソバレリルグリシン(C5)，中鎖ジカルボン酸であるアジピン酸（C6）の排泄増加も認めた．この時点でグルタル酸尿症II型（GAII）が考えられ，レボカルニチン（300mg/日），リボフラビン（50mg/日）投与を開始した．しかし，8か月時の血中遊離脂肪酸分析ではC14：1の増加は認めず，尿中有機酸分析ではエチルマロン酸増加，また血中アシルカルニチン分析では遊離カルニチン低下，ブチリルカルニチン（C4）増加を認め（表2），短鎖アシルCoA脱水素酵素（SCAD）欠損症の可能性が高いと考えられ，リボフラビンは中止した．確定診断のため，9か月時にリンパ球を用いて行ったSCAD活性測定（Tajimaらが報告したイソ吉草酸血症の酵素診断法に準じる[7]）では酵素活性の低下は認められず，SCAD欠損症は証明できなかった．

臨床経過，生化学的検査から脂肪酸酸化異常症が疑われたが，確定診断がつかないまま，呼吸器感染症による10数回の入退院を繰り返し，1歳9か月時頃よりネフローゼ症候群の合併も認めた．低蛋白血症，感染のコントロールは困難となり，徐々に全身状態は悪化していった．同時期の頭部MRI検査では，前頭葉～側

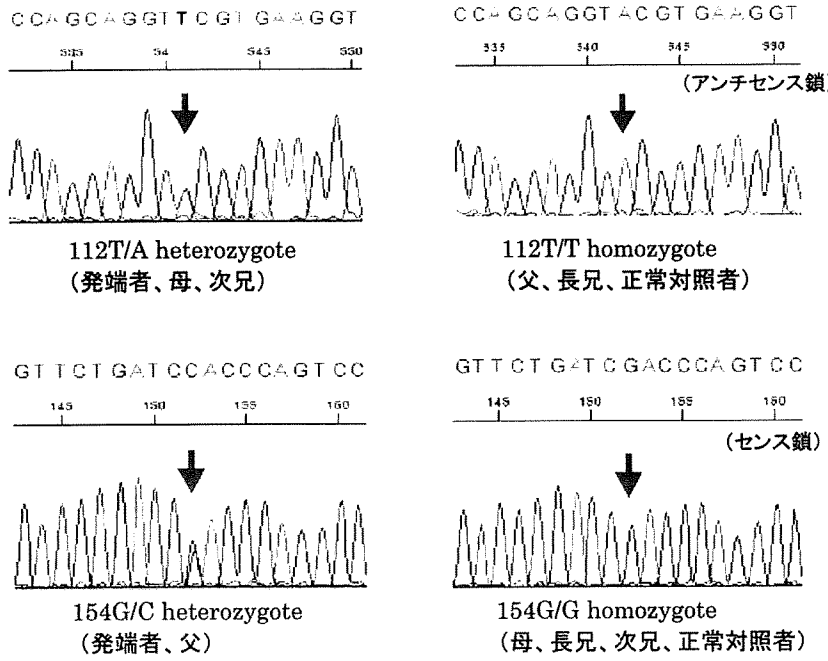

図3　Exon 2 of *ETHE1* gene（112T/154G）

表3　*ETHE1*, *ACADS* 遺伝子解析結果

被験者	*ETHE1* 遺伝子変異	*ACADS* 遺伝子変異
発端者	112T > A（exon2：Y38N） 154G > C（exon2：D52H） (Compound heterozygote)	625G > A（exon6：G209S） 625G > A（exon6：G209S） (Homozygote)
父親	154G > C（exon2：D52H） (Heterozygote)	625G > A（exon6：G209S） (Heterozygote)
母親	112T > A（exon2：Y38N） (Heterozygote)	625G > A（exon6：G209S） (Heterozygote)
長兄（9歳）	No mutation	625G > A（exon6：G209S） (Heterozygote)
次兄（7歳）	112T > A（exon2：Y38N） (Heterozygote)	625G > A（exon6：G209S） 625G > A（exon6：G209S） (Homozygote)

塩基番号はATGコドンのAを＋1，アミノ酸番号は開始メチオニンを＋1として記載した．

頭葉の萎縮，またT2強調像でレンズ核，尾状核頭部，中脳背側の高信号域を認め（図1），てんかん発作も治療抵抗性となっていった．1歳11か月時に敗血症を併発し，人工呼吸器管理，血液透析などの種々集中治療に反応せず死亡した（図2）．

遺伝子診断

本例の臨床像とこれまでのEE報告例の類似性から，遺伝子診断によって病因を解明できることが期待された．そこで，ご両親のインフォームドコンセントを得た上で遺伝子診断を試みた．患児の保存リンパ球からゲノムDNAを抽出後，*ETHE1* の各エクソン（前後のイントロン領域を含む）をPCR法で増幅し，直接シークェンス法で解析した．また，鑑別診断のためにSCAD欠損症の責任遺伝子 *ACADS* についても，同様の方法で解析を行った．その結果，*ETHE1* については112T>A（exon2：Y38N），154G>C（exon2：D52H）の複合ヘテロ変異を検出した．これらの塩基置換はこれまで報告されていないが，同部位はいずれも種を超えて高度に保存されており，正常対照者30検体の解析でも検出されなかったことから，新規遺伝子変異によるEE罹患例と診断した．また母親と次兄は112T>A，父親は154G>Cのヘテロ保因者と判明した（図3）．*ACADS* については，本人と次兄が，625G>A

(G209S) ホモ接合体であり，両親と長兄はヘテロ接合体であった（表3）．

考　察

新規の *ETHE1* 遺伝子変異を伴ったEEの男児例を経験した．我々は当初，尿中有機酸分析および血中遊離脂肪酸分析の結果からGAIIを考えたが，再検査の結果および追加実施した血中アシルカルニチン分析結果からは，GAIIよりもSCAD欠損症の可能性が疑われた．そこでSCAD活性測定を行ったが，酵素活性の低下は証明されなかった．これまでの報告例も，2004年に *ETHE1* 遺伝子が原因であることが判明するまでは，生化学的検査からGAII，SCAD欠損症が鑑別疾患となり，EEに典型的な臨床症状，中枢神経系画像所見を基に診断がなされていた．本例は1歳11か月時に死亡したため，それ以上の検索がなされていなかったが，生前の特徴的な生化学的所見に加え，保存リンパ球を用いた遺伝子解析より *ETHE1* 遺伝子変異が判明し，死亡後4年以上の年月を経て診断が確定した．

EEの責任遺伝子は19番染色体上にあり，約1,000塩基を有し7つのexonから成る[6]．*ETHE1* 蛋白は，ミトコンドリアの恒常性やエネルギー代謝に重要な役割を持つとされるが，詳細な機能は解明されていない．30数例の報告例のうち大半が，イタリア系，アラブ系をはじめとする地中海地域からのものであるが[7]，2001年には3例の韓国人女児例の報告もある[8]．本例は112T>A，154G>Cの複合ヘテロ変異が原因と考えられたが，*ADADS* 遺伝子の625G>A (G209S) ホモ接合体でもあることが判明した．この1塩基置換については，次兄（*ETHE1* の112T>A変異のヘテロ保因者）も625G>Aのホモ接合体であったが，9歳の長兄同様，7歳現在まで成長発達に異常なく，これまでのところ何ら健康面の問題を生じていない．

EEとSCAD欠損症の鑑別に関して，Tirantiらは，臨床的にEEと診断された患者29例と，早期発症の進行性脳症とエチルマロン酸尿を呈するがEEに特異的な他の臨床症状を欠く患者（non-EE EMA）11例を対象とする，*ETHE1* と *ACADS* の遺伝子解析結果を報告した[7]．それによると，EE患者では全例に *ETHE1* 遺伝子変異が確認されたのに対し，non-EE EMA患者では *ETHE1* 遺伝子変異が認められなかった．また *ACADS* 遺伝子では，625G>Aがnon-EE EMA患者全例（22アレル中20アレル）に認められたのに対し，EE患者では29例中15例に見出され，そのうちホモ接合体は3例であった．この結果から，*ETHE1* 遺伝子の変異がEEという均一な臨床像の疾患を惹起するのは明らかであり，*ACADS* 遺伝子の625G>AはEEの病因ではないと考えられるが，彼らのnon-EE EMA患者において病因となっている可能性は否定できない．

この報告からも，発端者の臨床経過は *ETHE1* 変異に起因するEEとしてすべて説明可能と思われ，*ACADS* 625G>Aホモ接合体であることの影響を見て取ることは難しい．しかし，特に次兄（*ETHE1* 112T>Aヘテロ保因者かつ *ACADS* 625G>Aホモ接合体）については，これらの遺伝子変異を有することによる健康への影響を現時点で完全に否定することは難しく，生化学的異常の検索も含めて長期的な経過観察が必要と思われる．

EEの臨床的特徴としては，中枢神経症状，血管障害，慢性下痢などが挙げられる．神経症状は，筋緊張低下，頸定，寝返り，座位保持ができないなどの運動発達遅滞に始まり，錐体路症状，てんかん症状，最終的には脳症症状が出現する．画像的な変化として，前頭葉～側頭葉の低形成や脱随所見，尾状核，被殻そして小脳にまで及ぶ変性所見（MRIのT2強調像で高信号域），梗塞巣などを認める[9]．血管障害による症状としては，点状出血，四肢末端の浮腫・チアノーゼ，網膜血管の蛇行などが見られ，本例にも合併したネフローゼ症候群，また肺うっ血，微小血尿，血性腹水を合併したという報告もある．出血時間，血小板数・機能検査，凝固系検査また免疫学的検査では異常を示さないとされる[2,10]．また生直後より出現する慢性下痢の原因は不明である．

Grossoらは，これら主症状の出現時期はEE患者（2002年までの既報告例20例）のうち25%が新生児期に，75%が2～7か月時としているが，初発時期と臨床経過の進行度には関連がなく，乳幼児期死亡例が多い中，2か月時に発症し，7歳でもなお生存している症例を報告している[9]．本例は乳児期早期に発症し，EEに典型的な症状を有し，頭部MRI検査でも既報告例と合致した所見を呈していた．

EEの特徴的な生化学的所見としては，尿中有機酸分析におけるエチルマロン酸，メチルコハク酸の排泄増加，C4-C5アシルグリシン（n-ブチリルグリシン，イソブチリルグリシン，イソバレリルグリシン，2-メチルブチリルグリシン）の排泄増加，血中アシルカルニチン分析におけるC4，C5-アシルカルニチン増加がある[2,3]．脂肪酸代謝異常症で見られる非ケトン性ジカルボン酸尿症もEEに見られる所見のひとつである．しかし血中・尿中アミノ酸分析では特異的な異常を捉えることは困難である．

治療に関しては，カルニチン，リボフラビン，ビタミンE，グリシン，大量メチルプレドニゾロン投与の報告があるが[2,8]，有効性については様々で確立した治療はない．本例では，GAII，SCAD欠損症を考えていたため，レボカルニチン，リボフラビンの投与を行った

が，病態の進行を止めることはできなかった．

特徴的な臨床症状，生化学的所見を示すEEの診断にあたっては遺伝子解析が有用であり，有機酸代謝異常症を考えた場合，鑑別疾患として念頭におく必要がある．

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The First Japanese Case of Ethylmalonic Encephalopathy with Novel *ETHE1* Gene Mutations

Yoshikazu Otsubo[1], Ken-ichiro Shirao[2], Satoshi Okada[2], Go Tajima[2], Nobuo Sakura[2], Takashi Kusumoto[1], Mikihiro Aoki[1] and Yoshio Nakashita[1]
[1]Department of Pediatrics, Sasebo City General Hospital
[2]Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences

Ethylmalonic encephalopathy (EE) is a rare inborn error of metabolism that is characterized by developmental delay, acrocyanosis, petechiae, chronic diarrhea and early death in infancy. EE also has characteristic abnormalities in brain magnetic resonance imaging, blood acylcarnitine analysis and urinary organic acid tests. It is an autosomal recessive disorder caused by the *ETHE1* gene mutation on chromosome 19q13. While approximately 30 patients have been reported worldwide, no patient has been reported in Japan.

We here report a boy with EE that was genetically proven. He was referred to our hospital at 5 months of age, presenting hypotonia, failure to thrive, chronic diarrhea, repeated cyanosis and petechiae. Although he had been under attentive medical care with suspicion of certain inborn error of metabolism, he died of septicemia with aggravated nephrotic syndrome at 23 months of age.

Having had the typical clinical symptoms and characteristic biochemical and urine abnormalities of EE, his *ETHE1* gene was analyzed after 4 years of his death, making diagnosis of EE with novel compound heterozygous mutations in exon 2. Further investigation revealed his parents and two elder brothers were heterozygotes for either of the *ETHE1* gene mutations, except for the eldest brother who was wild type.

Since most of the previously reported cases as well as the present case died suddenly in early infancy, it is important to perform biochemical and genetic analyses for suspected patients as early as possible for appropriate medical management and genetic counseling.