confirmed these mutations at the genomic level. Familial analysis showed that the 9-bp duplication was inherited from the mother and c.1304C > A (T435N) from the father. The 9-bp duplication was a novel mutation and T435N was previously reported in Japanese patients (Fukao et al. 2004).

Transient expression analysis of the c.658-666dupAACGT GATT p.N220_I222dup cDNA showed no residual activity (Fig. 1a), and the N220_I222dup SCOT protein was not detected in immunoblot analysis (Fig. 1b). Since SCOT protein is a homodimer and the T435N mutant retains significant residual SCOT activity, as previously reported (Fukao et al. 2004), we investigated possible interallelic influence between T435N and N220_I222dup by cotransfection of these two mutant cDNAs. However, no apparent interallelic complementation or dominant negative effect were observed.

The T435N mutation was previously identified in two Japanese SCOT-deficient families from the Amami islands (Fukao et al. 2004). There was no apparent consanguinity or relationship between these two families, and the patients were homozygotes of T435N. Since the Amami islands have a population of about 120,000, this mutation might be prevalent in that region. However, the father of GS21, who carries the T435N mutation, has no relation to the islands as far as he knows.

Recently, the crystal structure of human SCOT was determined (PDB entry 3DLX). The SCOT protein is a homodimer; both T435 and N220_I222 lie close to the SCOT dimerization interface and are not directly connected to the active site. Their overall localization in the context of the dimer is shown in Fig. 2a. T435 lies in a loop, with the side chain pointing inward to a rather hydrophilic environment (Fig. 2b); the hydroxyl group makes a hydrogen bond with a backbone carbonyl group from V394. Thus, it is expected that a T435N substitution will perturb the SCOT structure only a little, being able to make hydrogen bond interactions. This is also reflected in the fact that the T435N mutation is associated with significant residual SCOT activity. NVI220-222 is a short beta strand from a beta sandwich domain involved in SCOT dimerization (Fig. 2c). This small domain also contains other previously identified SCOT point mutations, namely, G219E, V221M, R224K, and R268H (Fukao et al. 2000, 2007; Yamada et al. 2007), making it a hot-spot for SCOT mutations. The small beta sandwich is tightly folded, and

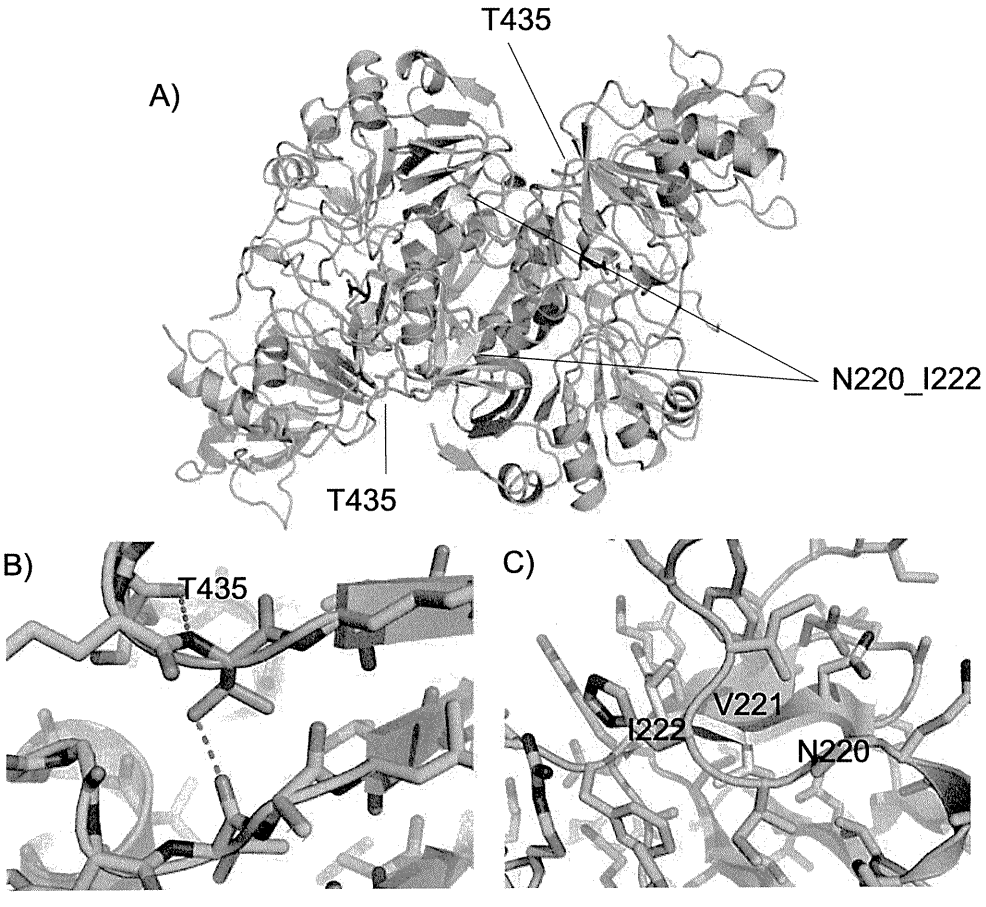

Fig. 2 Tertiary structure around mutations. **a** A human succinyl-CoA:3-ketoacid CoA transferase (SCOT) dimer, with E344 (highlighting the active site of SCOT) in *blue*, N220_I222 in *yellow*, and T435 in *orange*. The two monomers are shown in *green and light blue*. **b** Detailed surroundings of T435 (*orange*). Note the side chain is buried in a hydrophilic environment, making a hydrogen bond with a backbone oxygen. There are also additional potential hydrogen bonding partners in the vicinity. **c** The N220_I222 (in *yellow*) correspond to a short buried beta strand in a tightly packed environment. The dimer interface is at the *top* in this view

one can expect a duplication of three residues within the beta strand to disrupt its folding. This might affect the overall folding of the SCOT monomer and/or the formation of functional SCOT dimers.

Clinical issues

GS21 developed his first ketoacidotic crisis at the age of 2 days, although one of his mutated alleles retained residual SCOT activity. Almost half of the patients with SCOT deficiency develop their first ketoacidotic crisis at the age of 2–4 days (Mitchell and Fukao 2001). We summarized the mutations and their clinical phenotypes for several SCOT-deficient patients, including GS21, in Table 2. GS10, a homozygote of the R268H mutation, which retained residual activity, also developed his first ketoacidotic crisis at the age of 2 days, whereas his sibling (GS10s) with the same mutation developed her first crisis at the age of 6 months (Fukao et al. 2007). On the other hand, GK15, a homozygote of the null mutation, R217X, developed her first ketoacidotic crisis at the age of 8 months (Longo et al. 2004). Neonatal onset, hence, does not appear to be related to residual SCOT activity.

Permanent ketosis, or ketonuria, is a pathognomonic feature of SCOT deficiency (Mitchell and Fukao 2001). We previously reported that patients (GS08, GS09, and GS09b) who are homozygous for T435N did not show permanent ketosis or ketonuria (Fukao et al. 2004). In the case of GS21, the blood levels of FFA and TKB at 3 h after feeding were measured at the ages of 3 weeks, 3 monthsm and 4 months (Table 1). The level of TKB at the age of 3 weeks (0.15 mmol/L) was much less than those at the ages of 3 months and 4 months (2.54, 1.59 mmol/L, respectively). The FFA/TKB ratio at the ages of 3 and 4 months was 0.21 and 0.26, respectively, but was nearly 1.0 at the age of 3 weeks. In the cases of SCOT-deficient patients, this ratio was reported to be <0.3 early in a fasting test (Bonnefont et al. 1990). Even at 6 h after feeding at the age of 3 weeks, the level of TKB was 0.81 and the ratio was 0.57. These facts may mean that a hyperketotic status is not apparent during a nonepisodic condition in the neonatal period. The blood levels of FFA and TKB were not available during the age of 2–3 years, but urinary ketone bodies varied from negative to 3+ without any symptoms in GS21 and were always positive in GS02 and GS02s, whose mutations do not retain residual SCOT activity (Sakazaki et al. 1995). Hence, GS21 has no permanent ketonuria. It is very important to state that SCOT deficiency is the most probable diagnosis if permanent ketosis/ketonuria is present but that SCOT deficiency is not excluded even if permanent ketosis/ketonuria is absent.

Table 2 Succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patients

Number	Country	Sex	Mutations	Res. activity[a]	Onset	First ketoacidotic crisis				PK[b]	No. of episodes	Prognosis	References
						pH	HCO3	TKB	glucose				
GS02	Japan	M	V133E/C456F	–	6mo	7.08	5.1	12.2	6.7	+	3	Good	Sakazaki et al. 1995
GS02s	Japan	F	V133E/C456F	–	prenatal diagn.					+	1	Good	Fukao et al. 1996
GS10	South Africa	M	R268H/R268H	+	2d	6.94	5	14.2	4.4	–	>5	Good	Pretorius et al. 1996
GS10s	South Africa	F	R268H/R268H	+	6mo	7.12	8	5.1	6.6	–	5	Good	Pretorius et al. 1996
GS15	USA	F	R217X/R217X	–	8mo	6.98	<5			+	1	Good	Longo et al. 2004
GS08	Japan	M	T435N/T435N	+	1y5m	7.12	3.7	18.5	6.0	–	3	Good	Fukao et al. 2004
GS09	Japan	M	T435N/T435N	+	10mo	7.00	5.8			–	>5	Good	Fukao et al. 2004
GS09b	Japan	M	T435N/T435N	+	10mo	7.09	5.4		7.1	–	4	Good	Fukao et al. 2004
GS21	Japan	M	T435N/N220_I222dup	+	2d	7.02	5.8		3	–	3	Good	This study

[a] Residual SCOT activity in transient expression analysis of mutant complementary DNAs, [b] permanent ketosis

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Carnitine Palmitoyltransferase 2 Deficiency: The Time-Course of Blood and Urinary Acylcarnitine Levels during Initial L-Carnitine Supplementation

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Carnitine palmitoyltransferase 2 (CPT2) deficiency is one of the most common mitochondrial beta-oxidation defects. A female patient with an infantile form of CPT2 deficiency first presented as having a Reye-like syndrome with hypoglycemic convulsions. Oral L-carnitine supplementation was administered since serum free carnitine level was very low (less than 10 μmol/L), indicating secondary carnitine deficiency. Her serum and urinary acylcarnitine profiles were analyzed successively to evaluate time-course effects of L-carnitine supplementation. After the first two days of L-carnitine supplementation, the serum level of free carnitine was elevated; however, the serum levels of acylcarnitines and the urinary excretion of both free carnitine and acylcarnitines remained low. A peak of the serum free carnitine level was detected on day 5, followed by a peak of acetylcarnitine on day 7, and peaks of long-chain acylcarnitines, such as C16, C18, C18:1 and C18:2 carnitines, on day 9. Thereafter free carnitine became predominant again. These peaks of the serum levels corresponded to urinary excretion peaks of free carnitine, acetylcarnitine, and medium-chain dicarboxylic carnitines, respectively. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had accumulated within the tissues. The excretion of medium-chain dicarboxylic carnitines dramatically decreased on day 13, suggesting improvement of tissue acylcarnitine accumulation. These time-course changes in blood and urinary acylcarnitine levels after L-carnitine supplementation support the effectiveness of L-carnitine supplementation to CPT2-deficient patients.

Keywords: carnitine palmitoyltransferase 2; CPT2; L-carnitine; acylcarnitine profile; carnitine administration
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Carnitine palmitoyltransferase 2 (CPT2) deficiency (EC 2.3.1.21, OMIM 600650) is one of the most common disorders of mitochondrial fatty acid oxidation. CPT2 deficiency has several clinical presentations (Bonnefont et al. 1999). The adult form is characterized by episodes of rhabdomyolysis triggered by prolonged exercise. The infantile form presents as severe attacks of hypoketotic hypoglycemia, occasionally associated with sudden infant death or a Reye-like syndrome (Demaugre et al. 1991; Hug et al. 1991). The most severe kind, the neonatal form, is almost always lethal during the first month of life.

Secondary carnitine deficiency, characterized by low levels of total and free carnitines associated with an increase in the long-chain acylcarnitine fraction, is observed in the infantile form of CPT2-deficient patients (Bonnefont et al. 2004; Longo et al. 2006). Hence, L-carnitine supply might be useful in severe CPT2 deficiencies (Bonnefont et al. 2004), although supplementation with L-carnitine in patients with beta-oxidation defects of long-chain acyl-CoA has long been a matter of controversy (Costa et al. 1998;

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Liebig et al. 2006; Primassin et al. 2008).

In this report, we describe a CPT2-deficient patient who presented as having a Reye-like syndrome with secondary carnitine deficiency. We focused on time-dependent changes in the serum and urinary acylcarnitine profiles after intial L-carnitine supplementation.

Clinical Report

The patient, a female, was born to nonconsanguineous Japanese parents. She had been well until 15 months of age when she suddenly had tonic-clonic convulsions at 3:00 a.m. for about 30 minutes and became unconscious. Ten days before the convulsions, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. When she arrived at another hospital, she had hypoglycemia (blood glucose 1.1 mmol/L), hepatic dysfunction (AST 85 IU/L, ALT 55 IU/L, LDH 402 IU/L), and mild hyperammoniemia (NH3 84 μmol/L). Urinary ketones were not detected. Brain MRI and cerebrospinal fluid were normal. She was suspected of being affected by a Reye-like syndrome and transferred to Gifu University Hospital.

On admission, her height was 72 cm (−1.5S.D.) and her weight was 10 kg (+0.73S.D.). She had a fever (38.3°C) and exhibited lethargy. Physical examination revealed mild hepatomegaly. A laboratory test showed AST 382 IU/L, ALT 441 IU/L, LDH 557 IU/L, PT 31%, NH3 84 μmol/L, and blood glucose 4.7 mmol/L.

We tentatively diagnosed her as having a Reye-like syndrome and treated her with intravenous glucose. Her consciousness level became clear on the 4th hospital day and she started oral intake of food. An abdominal CT scan still showed hepatomegaly and a fatty liver (20HU) on the 6th hospital day. The finding of cardiac ultrasonography was normal. Urinary organic acid analysis during the hypoglycemic condition showed hypoketotic dicarboxylic aciduria. The initial measurements of serum free carnitine and acyl-

Table 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS.

	Day	- 1	3	5	7	9	13
Serum (μmol/L)	range						
C0	10 - 55	2.98	12.70	40.75	24.31	18.49	58.22
C2	4 - 60	2.25	3.85	14.87	20.15	8.37	14.8
C8	- 1.0	0.035	0.024	0.088	0.058	0.073	0.10
C8DC	- 0.25	0.035	0.046	0.12	0.89	0.97	0.063
C10	- 0.8	0.055	0.062	0.25	0.12	0.17	0.21
C10DC	- 0.1	0.063	0.12	0.24	0.33	0.53	0.19
C12:1	- 0.2	0.038	0.038	0.18	0.15	0.15	0.091
C12DC	- 0.05	0.053	0.064	0.19	0.14	0.27	0.054
C14:1	- 0.1	0.075	0.16	0.47	0.58	0.68	0.18
C16	- 0.5	1.01	1.29	2.99	4.45	8.07	2.56
C18	- 0.3	0.49	0.65	1.46	1.67	3.07	0.99
C18:1	- 0.46	1.50	1.84	4.21	6.09	10.03	3.62
C18:2	- 0.3	0.46	0.67	1.47	1.43	2.05	0.98
(C16+C18:1)/C2	- 0.36	1.12	0.81	0.48	0.52	2.16	0.42
C total		12.35	26.74	86.07	84.99	67.46	85.52
Urine (μmol/mmol Cr)	range*						
C0	5.67 - 56.09	0.61	1.31	82.33	37.85	45.95	329.15
C2	6.87 - 60.48	0.56	0.02	25.44	128.00	41.83	53.58
C4	0.07 - 0.74	0.31	0.47	0.92	0.47	1.38	2.32
C6	0.04 - 0.48	0.18	0.09	0.21	0.22	0.61	0.23
C6DC		1.25	1.34	1.63	15.69	83.33	2.93
C8	0.05 - 0.39	0.00	0.02	0.33	0.98	1.33	0.62
C8DC		0.25	0.52	0.83	23.90	122.99	1.11
C10	0.03 - 0.36	0.05	0.06	0.11	2.66	1.76	0.12
C10DC		0.11	0.02	0.10	0.75	4.03	0.08
C12DC		0.00	0.02	0.01	0.23	1.52	0.01
C16	0.05 - 1.55	0.04	0.02	0.02	0.18	0.63	0.08
C total		4.75	6.86	122.16	226.51	344.91	408.34

* Reference values for urine acylcarnitines were obtained from data reported by Mueller et al. (2003) (10th - 90th percentile)

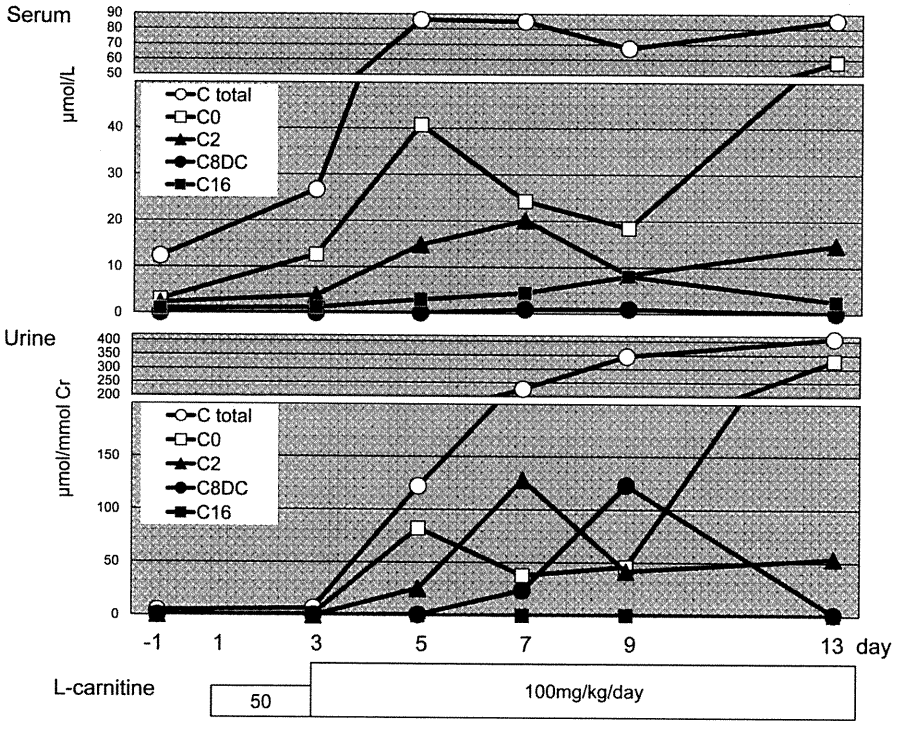

Fig. 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS. The levels of representative acylcarnitines are shown. The first day of L-carnitine supplementation is designated as day 1. Urinary carnitines were assayed using the first urine in the morning.

carnitine fractions by the enzymatic cycling method were 9.5 and 5.9 μmol/L, respectively. The initial serum acylcarnitine profile (Table 1) showed a very low free carnitine level and relatively high long-chain acylcarnitine levels. This profile was compatible with the secondary carnitine deficiency due to CPT2 or translocase deficiency.

After confirmation of the carnitine deficiency, we supplied her with L-carnitine orally from the 15th hospital day (day 1 in the Table 1 and Fig. 1) at a dose of 50 mg/kg/day for the first two days and 100 mg/kg/day from day 3. Blood and urinary samples were obtained before plus 3, 5, 7, 9 and 13 days after L-carnitine supplementation. During carnitine supplementation, the patient had continuous intravenous glucose infusion of 2.5 mg/kg/min until day 11. We analyzed the serum and urinary acylcarnitines by tandem mass analysis, as previously reported (Mueller et al. 2003; Kobayashi et al. 2007a,b). Table 1 shows details of the analyses. Fig. 1 shows the changing patterns of free carnitine (C0), acetyl-carnitine (C2), C8DC representing medium-chain dicarboxylic acylcarnitines, and C16 representing long-chain acylcarnitines in the serum and urine. Urinary excretion of C0 and acylcarnitines remained at very low levels on day 3. Sequential peaks of free carnitine (day 5), acetylcarnitine (day 7), and long-chain acylcarnitines (day 9) were found in the serum, which corresponded to peaks of free carnitine, acetylcarnitine, and dicarboxylic medium-chain acylcarnitines in the urine.

The fatty liver and hepatomegaly improved as judged by an abdominal CT scan on the 26th hospital day (day 13). Informed consent for a skin biopsy, enzyme assay, and DNA was obtained from the parents. CPT2 activity in the patient's fibroblasts was 0.18 nmol/min/mg of protein (3 controls; 0.82, 1.27, and 1.26 nmol/min/mg of protein), confirming the diagnosis of CPT2 deficiency.

Now the patient is 4 years of age. After carnitine supplementation, she did not experience hypoglycemia at all. She is being treated with 1,000 mg L-carnitine/day (current body weight 19.8 kg). Her growth and development are within normal ranges. She had some rhabdomyolysis attacks (the highest CK recorded was 16,769 IU/L) during a febrile illness even after L-carnitine supplementation.

Discussion

The diagnosis of CPT2 deficiency was first suspected by the data on urinary organic acid analysis and acylcarnitine analysis and was confirmed by enzyme assay using fibroblasts. Our patient is a compound heterozygote of a previously reported E174K mutation from the father and an unknown mutation from the mother which was not detected by exon sequencing. According to an in vitro expression analysis of mutant CPT2 cDNAs carrying E174K, the mutant E174K protein was present as much as a wild type protein and retained 10% residual CPT2 activity (Wataya et al. 1998). This "mild" mutation from the father, together with possible null mutation from the mother, may result in an infantile form of CPT2 deficiency.

Initially, she developed secondary carnitine deficiency. Chronic administration of pivalate-conjugated antibiotics is

a major cause of secondary carnitine deficiency even in healthy children (Stanley 2004). Ten days before the onset of the Reye-like syndrome, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. The initial serum acylcarnitine profile showed no elevation of hydroxy-C5 carnitine, nor of pivaloylcarnitine. While the antibiotic might have contributed to secondary carnitine deficiency in part, the acute attack with fasting was more likely the course of the low carnitine in the patient at presentation.

The time-course changes in the serum and urinary acylcarnitine levels after L-carnitine supplementation were studied. These changing profiles suggest that accumulated and potentially toxic long-chain acylcarnitines in the mitochondria were eliminated from the body by day 13. The majority of accumulated long-chain acylcarnitines in the mitochondria may be eliminated by the following steps: 1) a large amount of accumulated long-chain acylcarnitines should be transferred from the mitochondrial matrix by carnitine acylcarnitine translocase if there is a sufficient amount of free carnitine outside of the mitochondrial matrix; 2) then peroxisomal beta-oxidation reduces the chain length of such accumulated fatty acids; 3) the resultant medium-chain fatty acids can be catalyzed in the mitochondria, or further ω-oxidized into dicarboxylic acids in the microsomes; 4) these medium-chain DC and their carnitine conjugates can be excreted into the urine efficiently. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted and it took several days for them to be replenished. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had probably accumulated within the tissues.

It is noteworthy that the acetylcarnitine in both the serum and the urine was a predominant acylcarnitine on day 7 (Fig. 1). Elevation of acetylcarnitine in the serum and urine indicates the presence of enough acetyl-CoA in the mitochondria and the availability of acetyl-CoA for carnitine acyltransferase reactions in the cells, and might account for the increased beta-oxidation rates upon L-carnitine therapy (Fontaine et al. 1996). In general, acetylcarnitine is a major acylcarnitine in healthy controls and is regarded as a marker of undisturbed beta-oxidation (Costa et al. 1998). Since CPT2-deficient patients have beta-oxidation restrictions of long-chain acyl-CoA, L-carnitine supplementation may increase beta-oxidation of medium-chain acyl-CoAs, which could be supplied via peroxisomal beta-oxidation of long-chain acyl-CoA.

Carnitine supplementation in the treatment of long-chain beta-oxidation defects is still controversial. In patients with a defect in the mitochondrial beta-oxidation spiral, when a preceding L-carnitine deficiency is normalized, and transport into the mitochondria of long-chain fatty acids is also normalized, acyl-CoAs accumulate instead of being oxidized by the defective reaction and, consequently, in such cases, free CoA is depleted in the mitochondria (Yoshino et al. 2003). This may be true in beta-oxidation defects such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and trifunctional protein deficiency. Studies on VLCAD-deficient mice suggested carnitine supplementation results in the induction of acylcarnitine production in various tissues and significant accumulation of potentially toxic intermediate acylcarnitines in tissues (Liebig et al. 2006; Primassin et al. 2008). However, blockage of the CPT2 step causes the accumulation of long-chain acylcarnitines but does not primarily cause the accumulation of intermediate CoA esters in beta-oxidation.

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In vitro probe acylcarnitine profiling assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry predicts severity of patients with glutaric aciduria type 2☆

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ABSTRACT

Glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency, MAD) is a multiple defect of mitochondrial acyl-CoA dehydrogenases due to a deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase. The clinical spectrum are relatively wide from the neonatal onset, severe form (MAD-S) to the late-onset, milder form (MAD-M). In the present study, we determined whether the in vitro probe acylcarnitine assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry (MS/MS) can evaluate their clinical severity or not. Incubation of cells from MAD-S patients with palmitic acid showed large increase in palmitoylcarnitine (C16), whereas the downstream acylcarnitines; C14, C12, C10 or C8 as well as C2, were extremely low. In contrast, accumulation of C16 was smaller while the amount of downstream metabolites was higher in fibroblasts from MAD-M compared to MAD-S. The ratio of C16/C14, C16/C12, or C16/C10, in the culture medium was significantly higher in MAD-S compared with that in MAD-M. Loading octanoic acid or myristic acid led to a significant elevation in C8 or C12, respectively in MAD-S, while their effects were less pronounced in MAD-M. In conclusion, it is possible to distinguish MAD-S and MAD-M by in vitro probe acylcarnitine profiling assay with various fatty acids as substrates. This strategy may be applicable for other metabolic disorders.

1. Introduction

Fatty acid oxidation disorders (FAODs) potentially cause energy crises that are triggered by stress brought on by starvation or infection, and manifest themselves through nonketotic hypoglycemia, acute encephalopathy, or symptoms similar to those of Reye's syndrome. Among FAODs, medium-chain acyl-CoA dehydrogenase deficiency (MCAD-def) is most common among Caucasian [1], whereas very long-chain acyl-CoA dehydrogenase deficiency (VLCAD-def) and carnitine palmitoyl-CoA transferase 2 deficiency (CPT2-def) are common in Japanese, followed by glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency: MAD) [2].

In MAD, multiple mitochondrial FAD-dependent dehydrogenases are impaired due to a defect in α- or β-subunits of electron transfer flavoprotein (ETF-α and ETF-β; OMIM 608053 and 130410 respectively) or ETF dehydrogenase (ETF-DH; OMIM 231675) [3,4]. The clinical forms of MAD include the neonatal-onset form (severe form: MAD-S) and the late-onset form (milder form: MAD-M). MAD-S occurs during the neonatal period, and is fatal. MAD-M often becomes symptomatic after infancy, and has episodic symptoms of hypotonia, tachypnea, skeletal muscle symptoms such as myalgia or rhabdomyolysis, and biochemical abnormalities including liver dysfunction, hypoglycemia, or hyperammonemia [5]. These 2 clinical forms can be sharply separated. Biochemical diagnosis is made by blood acylcarnitine analysis using electrospray ionization tandem mass spectrometry (MS/MS) that measures increases in C4 to C18 acylcarnitine, or through urinary organic acid analysis using gas chromatography mass spectrometry (GC/MS) that detects an increase of ethylmalonic acid, glutaric acid, isovalerylglycine, or dicarboxylic acids. However, it is not always feasible to make a definitive diagnosis of MAD by GC/MS or MS/MS, especially during the stable phase of MAD-M [6]. While genetic diagnosis may represent alternative strategy to make accurate diagnosis of MAD, the genotype/phenotype correlation is not clear.

The usefulness of in vitro probe acylcarnitine assay using cultured fibroblasts and MS/MS for the diagnosis of many FAODs has recently been reported [7]. Other reports showed that severity are associated with acylcarnitine prolife in deficiencies of MCAD, VLCAD, CPT2 and long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) [8–12]. Subsequent reports also demonstrated that it

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is feasible to distinguish deficiency with carnitine–acylcarnitine translocase (CACT) from CPT2 as well as LCHAD from MTP deficiency [13,14]. Herein, we investigated if the severity of MAD can be determined by in vitro probe acylcarnitine assay.

2. Materials and methods

2.1. Patients with MAD

The 14 patients with MAD were studied. MAD was biochemically diagnosed using GC/MS and/or MS/MS, and further conclusively diagnosed using gene analysis and immunoblotting. Their clinical features were previously described [15]. They included four cases with ETF-α deficiency, two subjects with ETF-β deficiency, and eight patients with ETF-DH deficiency. The clinical phenotype included 3 cases with MAD-S, 10 subjects with MAD-M, and 1 asymptomatic child who was detected before disease onset by the neonatal screening. No obvious correlation between clinical severity and the specific defective enzyme was seen. All 3 cases with MAD-S died during the neonatal period. Seven of the 10 cases of MAD-M developed the disease during infancy with nonketotic hypoglycemia, acute encephalopathy, or Reye-like syndrome. The remaining 3 cases with MAD-M showed muscle symptoms such as myalgia and rhabdomyolysis or occasional general fatigue in later childhood or later.

2.2. Cultured fibroblasts

Fibroblasts from 14 Japanese patients with MAD were used. We also analyzed cultured fibroblasts from 4 healthy controls, one case each of MCAD-def, VLCAD-def, CPT2-def, or mitochondrial trifunctional protein deficiency (MTP-def) and primary carnitine deficiency (PCD) to validate the specificity of our in vitro probe acylcarnitine assay using cultured fibroblasts with MS/MS and to compare with MAD samples. Cells were cultured in modified eagle medium (MEM; Nissui) with 2 mM L-glutamine, 10% BSA (Sigma) and 1% penicillin/streptomycin until achieving confluency at 37 °C and 5% of CO_2.

2.3. In vitro probe acylcarnitine assay of fibroblasts using MS/MS

An in vitro probe assay was performed as described by J.G. Okun et al. with some minor modification [8]. Briefly, the cultured fibroblasts were seeded into a 6-well plate, and washed twice with PBS when they reached confluent. Cells were subsequently cultured in MEM loaded with 0.2 mM palmitic acid. In some experiments, palmitic acid was replaced with either octanoic acid or myristic acid. After incubating for 96 h, the culture medium was collected to analyze acylcarnitines by MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Statistic analysis was performed using Mann–Whitney's *U*-test.

3. Results

3.1. Acylcarnitine in cultured fibroblasts with MAD-S and MAD-M shows distinct profile

In the pilot experiments, we confirmed that our in vitro acylcarnitine probe assay demonstrates specific metabolic profile for

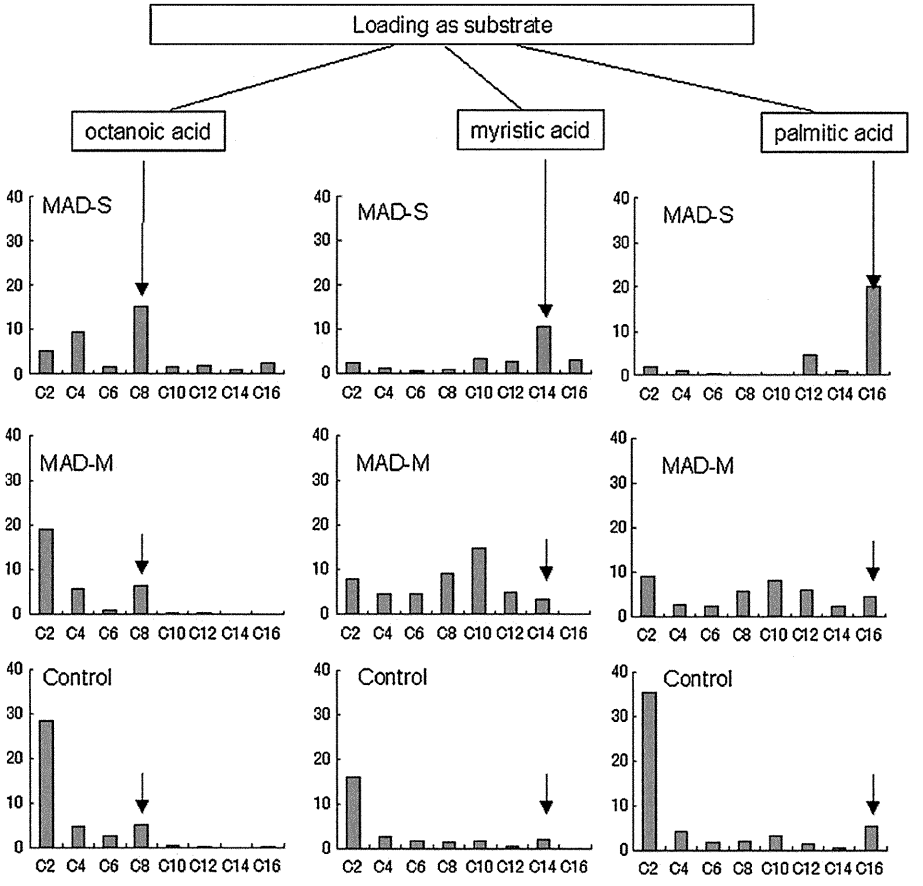

Fig. 1. The acylcarnitine profiles of fibroblasts from MAD-S and MAD-M. Arrows indicate loaded fatty acids; octanoic acid, myristic acid or palmitic acid. The *Y* axis represents values of acylcarnitines expressed as nmol/mg protein/96 h.

Table 1
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled palmitic acid for 96 h.

Clinical type	Acylcarnitine ratio; mean ± SEM				
	Palmitic acid				
	C16/C14	C16/C12	C16/C10	C16/C8	C16/C2
MAD-S (n = 3)	30.1 ± 16.9*,† (4.08–61.8)	6.02 ± 1.08*,† (3.85–7.12)	59.0 ± 24.6*,† (9.85–84.0)	69.1 ± 17.9*,† (33.5–89.5)	5.28 ± 1.75*,† (1.81–7.48)
MAD-M (n = 10)	4.33 ± 0.83 (1.52–10.0)	0.97 ± 0.17 (0.31–2.11)	1.05 ± 0.58 (0.22–6.20)	1.19 ± 0.70 (0.00–7.38)	0.73 ± 0.43** (0.08–4.57)
Asymptomatic (n = 1)	1.63	0.93	0.79	1.06	1.55
Control (n = 4)	7.89 ± 1.10 (5.89–10.8)	1.95 ± 0.64 (0.78–3.73)	0.89 ± 0.33 (0.53–1.89)	1.02 ± 0.16 (0.68–1.46)	0.13 ± 0.03 (0.05–0.23)

Abbreviations: MAD-S and MAD-M: severe and milder forms of MAD, respectively; asymptomatic: a case detected in the neonatal screening.
* P = 0.01 compared MAD-S with MAD-M.
** P = 0.04 compared MAD-M with control.
† P < 0.05 compared MAD-S with control.

Table 2
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled octanoic acid or myristic acid for 96 h.

Clinical type	Acylcarnitine ratio: mean ± SEM					
	Octanoic acid			Myristic acid		
	C8/C6	C8/C4	C8/C2	C14/C12	C14/C10	C14/C2
MAD-S (n = 3)	6.67 ± 3.63 (2.06–13.8)	1.20 ± 0.24 (0.81–1.63)	3.01 ± 1.92* (1.02–6.87)	3.66	3.03	4.42
MAD-M (n = 10)	2.73 ± 0.42 (0.88–4.56)	0.93 ± 0.14 (0.31–1.85)	0.73 ± 0.15** (0.14–1.38)	0.72	0.24	0.45
Asymptomatic (n = 1)	4.58	1.46	2.15	–	–	–
Control (n = 4)	2.10 ± 0.91 (0.69–4.71)	0.80 ± 0.25 (0.40–1.51)	0.15 ± 0.04 (0.08–0.24)	2.55	1.08	0.14

* P < 0.05 compared MAD-S with control.
** P < 0.05 compared MAD-M with control.

different FAOD (data not shown). Next, we determined if the acylcarnitine profiles can differentiate the clinical severity of MAD. When palmitic acid was loaded, a substantial accumulation of C16 was observed in the culture medium of cells with MAD-S, whereas the downstream C14, C12, C10, C8 or C2 did not show any increase. In contrast, C14, C12, C10 or C8 were elevated but C16 was lower in MAD-M compared to MAD-S (Fig. 1, right column). Therefore, the ratios of C16/C14, C16/C12, C16/C10, or C16/C8 were significantly higher in MAD-S than in MAD-M (Table 1). These findings suggest that oxidation of palmitic acid is inhibited in MAD-S, resulting in severely impaired production of acetyl-CoA.

Incubation with octanoic acid led to higher accumulation of C8-acylcarnitine in MAD-S cells compared to MAD-M (Fig. 1, left column). On the other hand, C2 was lower in MAD-S cells than that of MAD-M, as observed by palmitic acid. The ratio of C8/C2 showed significant increase in MAD-S compared to MAD-M (Table 2). Similarly, C8/C6 was higher in MAD-S (6.67 ± 3.63) than MAD-M (2.73 ± 0.42), though the difference was insignificant. Loading myristic acid to the MAD-S cells led to elevation of C14 acylcarnitine compared to control and MAD-M (Fig. 1, middle column). The ratios of C14/C12 and C14/C10 were also higher compared with those of MAD-M, as seen by palmitic or octanoic acid (Table 2). No difference was observed in the profile between infant and later childhood onset cases of MAD-M. The acylcarnitine profile was not associated with specific enzyme defect or gene; i.e. ETF-α, ETF-β and ETF-DH.

4. Discussion

MAD is a target disease of the neonatal screening using MS/MS. However, abnormalities on blood filter papers may not be detected in the stable condition of MAD-M or the presymptomatic stage, while the biochemical abnormalities are obviously observed in MAD-S and in the acute stage of MAD-M by GC/MS or MS/MS analyses. Actually, accurate biochemical diagnosis of MAD in presymptomatic stage is often difficult. Although genetic mutations of patients with MAD have been reported in various ethnic groups, almost all mutations do not seem to be associated with particular phenotype with a few exceptions [15], making it difficult to predict severity of the patients. The purpose of this study was to determine if the clinical severity in MAD can be evaluated using the in vitro probe acylcarnitine assay. Our data indicates that the in vitro probe acylcarnitine assay can clearly distinguish MAD-S from MAD-M.

An increase in C16 was observed exclusively in cells with MAD-S by loading palmitic acid, as opposed to a reduction in C14, C12, C10, C8 as well as C2. Loading octanoic acid or myristic acid also resulted in specific elevation of C8 or C14, respectively, in MAD-S. In contrast to MAD-S, the increase of C16 by palmitic acid was trivial in cells with MAD-M, whereas elevation of the downstream acylcarnitines C14, C12, C10 or C8 was larger compared to MAD-S. Similar to palmitic acid, such specific increase in C8 or C14 was barely detectable in cells with MAD-M upon incubation with octanotic or myristic acid, respectively. These results suggest that the milder enzyme deficiency in MAD-M allows the exogenous fatty acid substrates to process to some degree, resulting in elevation of downstream metabolites originated from loaded fatty acids. On the contrary, severe enzyme deficiency in MAD-S hampers to metabolize the loaded fatty acids to a shorter product, leading to a dramatic accumulation of the fatty acid corresponding to the substrates added. These hypotheses are consistent with significantly higher ratios between C16 and downstream acylcarnitines; i.e. C16/C14, C16/C12, C16/C12 or C16/C8, in cells with MAD-S compared to

MAD-M (Table 1). These findings strongly suggest that severity of MAD can be evaluated by quantitating the ratio between fatty acids loaded and the downstream metabolites.

Our results demonstrate that elevation of C16 by palmitic acid is one of the markers to characterize MAD-S. However, when compared with other FAODs, elevation of C16 is not specific to MAD-S, since palmitic acid also induced accumulation of C16 acylcarnitine in CPT2 deficiency without augmenting downstream metabolites (data not shown), which make acylcarnitine profile by palmitic acid in MAD-S look alike to CPT2 deficiency. However, a significant increase in C8 or C12 was observed by loading octanoic acid or myristic acid, respectively, in MAD-S, which was not observed in CPT-2 deficiency. This indicates that the enzyme activity for medium-chain fatty acids as well as long chain fatty acids is impaired in MAD-S, allowing MAD-S to be distinguished from CPT-2. However, cells from patients with respiratory chain defects may also show abnormalities similar to FAODs in the in vitro probe acylcarnitine assay [16,17], suggesting that the definitive diagnosis should be made in combination with acylcarnitine profiling and other laboratory tests, including genetic tests and enzymatic analysis.

In conclusion, our study indicates that the in vitro probe acylcarnitine assay using cultured fibroblasts loaded with various fatty acids allows us not just to distinguish MAD from other FAODs, but also clearly identify the severity of MAD. This strategy may be applied to evaluate the severity of the other metabolic diseases.

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Heat stress deteriorates mitochondrial β-oxidation of long-chain fatty acids in cultured fibroblasts with fatty acid β-oxidation disorders☆

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Acylcarnitine profiling

ABSTRACT

Mitochondrial fatty acids β-oxidation disorder (FAOD) has become popular with development of tandem mass spectrometry (MS/MS) and enzymatic evaluation techniques. FAOD occasionally causes acute encephalopathy or even sudden death in children. On the other hand, hyperpyrexia may also trigger severe seizures or encephalopathy, which might be caused by the defects of fatty acid β-oxidation (FAO). We investigated the effect of heat stress on FAO to determine the relationship between serious febrile episodes and defect in β-oxidation of fatty acid in children. Fibroblasts from healthy control and children with various FAODs, were cultured in the medium loaded with unlabelled palmitic acid for 96 h at 37 °C or 41 °C. Acylcarnitine (AC) profiles in the medium were determined by MS/MS, and specific ratios of ACs were calculated. Under heat stress (at 41 °C), long-chain ACs (C12, C14, or C16) were increased, while medium-chain ACs (C6, C8, or C10) were decreased in cells with carnitine palmitoyl transferase II deficiency, very-long-chain acyl-CoA dehydrogenase deficiency and mitochondrial trifunctional protein deficiency, whereas AC species from short-chain (C4) to long-chain (C16) were barely affected in medium-chain acyl-CoA dehydrogenase and control. While long-chain ACs (C12–C16) were significantly elevated, short to medium-chain ACs (C4–C10) were reduced in multiple acyl-CoA dehydrogenase deficiency. These data suggest that patients with long-chain FAODs may be more susceptible to heat stress compared to medium-chain FAOD or healthy control and that serious febrile episodes may deteriorate long-chain FAO in patients with long-chain FAODs.

1. Introduction

Tandem mass spectrometry (MS/MS) has been introduced to newborn screening for inherited metabolic diseases since early 1990s and become popular in diagnosis for mitochondrial fatty acid β-oxidation disorders (FAODs) [1,2]. Fatty acid β-oxidation (FAO) in mitochondria is a key energy generating process particularly under several conditions of metabolic stresses, like long fasting, prolonged exercises, infection or hyperpyrexia [3,4]. FAOD occasionally causes acute encephalopathy or even sudden death in children [5,6]. On the other hand, hyperpyrexia may also trigger some serious neurological symptoms, such as convulsion or acute encephalopathy [7,8]. There is a possibility that serious neurological symptoms related to hyperpyrexia are caused by transient or inherited defects of FAO. Recent reports suggest that long-chain fatty acid β-oxidation is inhibited during hyperpyrexia and that febrile episode may be one of potential reasons for the serious neurological events in influenza-associated encephalopathy (IAE) [9,10].

The β-oxidation of fatty acids is stepwise cycles and each turn of the cycle shortens the chain of fatty acid by two carbon atoms. There are multiple functional enzymes for each of fatty acids oxidative constituent step responsible for the oxidation of specific length chain fatty acids in mitochondria. If the enzymes involved in long-chain FAO such as carnitine palmitoyl transferase II (CPT2), very-long-chain acyl-CoA dehydrogenase (VLCAD), or mitochondrial trifunctional protein (MTP) are defective, long-chain ACs (C12, C14 or C16) will be accumulated [11–13]. If enzymes regulating medium-chain FAO are damaged, like medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, medium-chain ACs (C6, C8 or C10) will be accumulated [14]. In case electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) are impaired, also called multiple acyl-CoA dehydrogenase (MAD) deficiency, a wide range

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Table 1
The values of R_p/R_c at different temperatures.

Groups (n = number of subjects)	Conditions	C4	C6	C8	C10	C12	C14	C16
Control	37 °C	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(n = 6)	41 °C	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MCAD deficiency	37 °C	6.4 ± 1.4	3.2 ± 1.0	15.9 ± 3.4	3.1 ± 1.8	1.5 ± 0.2	1.3 ± 0.6	1.6 ± 0.5
(n = 4)	41 °C	3.4 ± 1.0*	2.9 ± 1.0	13.0 ± 4.0	3.5 ± 0.8	1.0 ± 0.4	1.1 ± 0.4	1.1 ± 0.2
CPT2 deficiency	37 °C	4.5 ± 0.6	2.3 ± 1.1	0.8 ± 0.5	0.5 ± 0.3	3.9 ± 0.4	6.3 ± 2.5	17.0 ± 2.2
(n = 2)	41 °C	5.0 ± 1.0	1.8 ± 0.1	2.0 ± 1.0	1.3 ± 0.1	12.2 ± 0.4*	9.9 ± 0.8	33.0 ± 3.6*
VLCAD deficiency	37 °C	1.0 ± 0.3	1.5 ± 0.5	2.3 ± 0.6	2.1 ± 0.6	6.0 ± 0.7	6.7 ± 2.2	2.6 ± 0.6
(n = 4)	41 °C	1.5 ± 0.5	1.0 ± 0.3	2.2 ± 0.2	2.7 ± 1.2	10.6 ± 1.5*	43.4 ± 5.6*	6.2 ± 0.8*
MTP deficiency	37 °C	1.3 ± 0.0	0.9 ± 0.1	0.9 ± 0.5	0.9 ± 0.4	8.2 ± 2.7	4.1 ± 1.5	8.7 ± 1.0
(n = 2)	41 °C	1.2 ± 0.2	2.1 ± 0.1*	1.2 ± 0.2	0.9 ± 0.3	25.7 ± 5.1*	17.9 ± 1.3*	19.2 ± 3.2*
MAD deficiency	37 °C	6.4 ± 0.8	10.1 ± 0.7	15.3 ± 0.1	15.2 ± 0.3	11.8 ± 1.4	13.8 ± 2.5	3.0 ± 0.3
(n = 2)	41 °C	1.3 ± 0.0*	1.7 ± 0.6*	4.4 ± 1.0*	4.7 ± 2.3*	12.3 ± 2.8	28.0 ± 0.8*	5.3 ± 1.1

Note: R_p represents the ratios of Cn/C2 (Cn: C4, C6, C8, C10, C12, C14, C14:1, C16) in patient cells. R_c represents the ratios of Cn/C2 in controls. C2–C16 represent specific length chain acylcarnitines, as shown in figure. The value of R_p/R_c represents fold change of patients compared to controls.
* $P<0.05$, values showed significant difference at 41 °C compared to 37 °C.

AC species from short to long-chain (C4–C16) will be elevated [15]. Furthermore, production of acetylcarnitine (C2), the final product of FAO cycle, will be suppressed in FAODs compared with that in normal control [16].

In the present study, we evaluated the effect of heat stress on impaired FAO, using MS/MS and cultured fibroblasts from several types of FAODs and healthy controls in order to determine the relationship between febrile episodes and defect in β-oxidation of fatty acid in children.

2. Materials and methods

2.1. Subjects

Human skin fibroblasts from 14 patients (passages 3–15) with various FAODs, which were diagnosed previously based on clinical and biochemical findings, plasma acylcarnitine profiles by MS/MS, as well as enzyme assay, were studied. These include 4 of VLCAD deficiency (def), each two of CPT2 def, MTP def, and MAD def, as well as 4 of MCAD def. Six cells (passages 3–16) from healthy volunteers were used as the control.

2.2. Cell culture

Cells were cultured in modified Eagle's minimal essential medium (MEM; Nissui Pharmaceutical Co., Ltd., 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 until confluency.

2.3. In vitro probe acylcarnitine profiling assay in cultured cells under heat stress

Confluent cells in a 75 cm^2 flask were harvested by trypsinization (0.25%-Trypsin/1 mM-EDTA; Nacalai Tesque), then seeded onto 6-well microplates (35 mm i.d.; Iwaki) and re-cultured. When they reached confluence, 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 MEM with essential fatty acid–free BSA (0.4%; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, at 37 °C or 41 °C. After 96 h, AC profiling in the culture medium were analyzed by MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA).

2.4. MS/MS analysis

Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). The contents of an acylcarnitine (AC) 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 internal standard.

The sample preparation method for MS/MS analysis was described previously [17,18]. Briefly, 10 μL of the supernatant from culture medium was transferred to a 96-well microplate, and 200 μL methanol containing reference standard kit was added to each well. The aliquots 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 3N 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). The ACs in 10 μL of the aliquots were determined using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

2.5. Data statistical analysis

The results were expressed as mean ± SD from at least two independent experiments using the individual sample. The AC concentration was expressed as nmol/mg protein. Data were statistically analyzed by the one-way analysis of variance (ANOVA) and post hoc 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. Acylcarnitine profiling in various FAODs under heat stress

Incubation of cells from controls and patients deficient for MCAD, VLCAD, and MAD at 41 °C, increased C2 compared with 37 °C. The short-chain (C4), medium-chain (C6, C8 and C10), as well as long-chain ACs (C12, C14 or C16) were barely affected at 41 °C in control (Fig. 1A) and MCAD def (Fig. 1B). In contrast to MCAD def or control, long-chain ACs (C16 and/or C14 and C12) were increased at 41 °C in the cells from long-chain FAODs; CPT2 def (Fig. 1C), VLCAD def (Fig. 1D), and MTP def (Fig. 1E). Furthermore, while long-chain ACs (C12–C16) was elevated, short to medium-chain ACs (C4–C10) were significantly reduced at 41 °C in cells from MAD def (Fig. 1F).

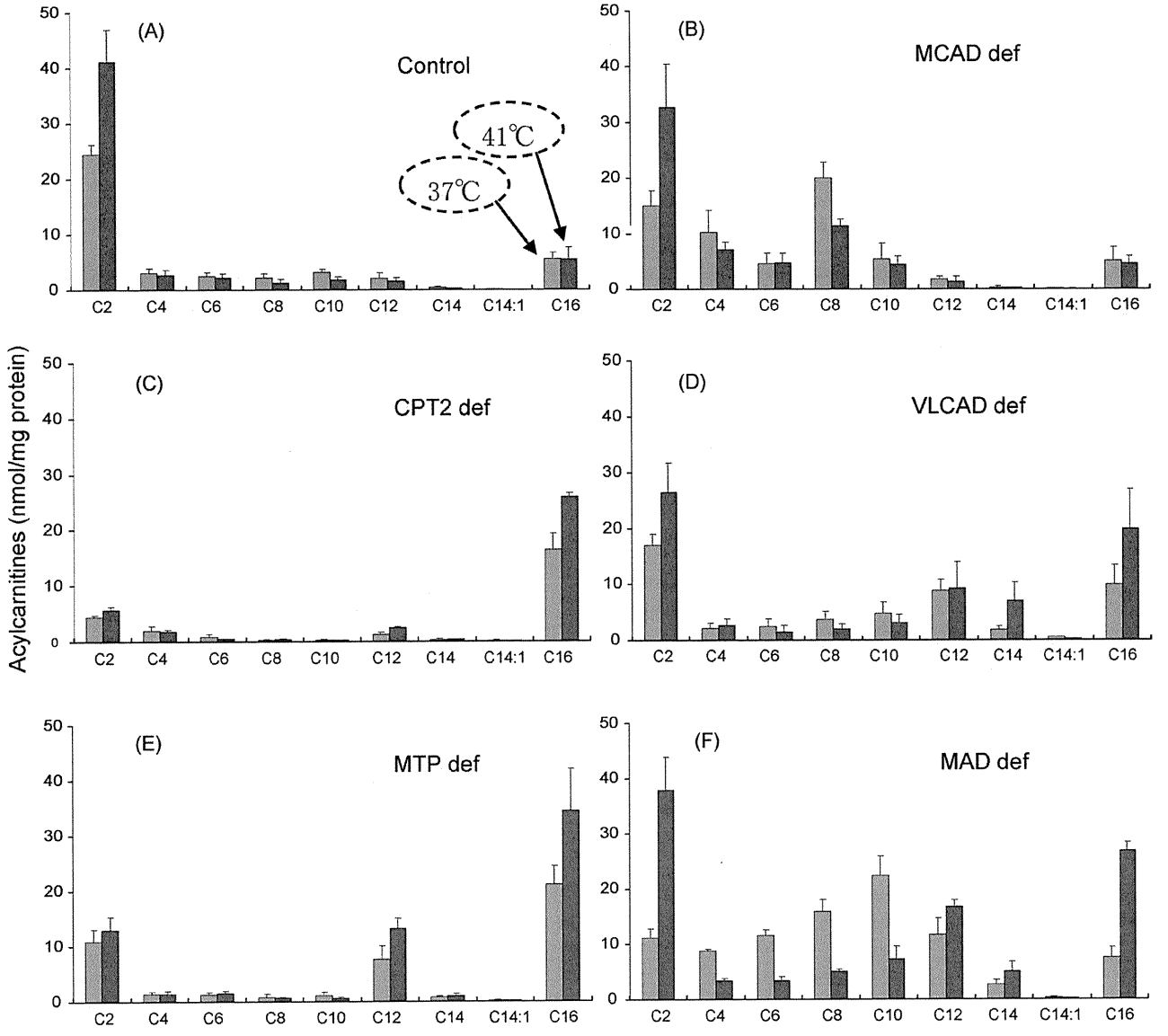

Fig. 1. AC profiles in supernatant of cells cultured with palmitate in various FAODs at 37 or 41 °C. ▒ : 37 °C; ■ : 41 °C. A, Control; B, MCAD def (medium-chain acyl-CoA dehydrogenase deficiency); C, CPT2 def (carnitine palmitoyl transferase II deficiency); D, VLCAD def (very-long-chain acyl-CoA dehydrogenase deficiency); E, MTP def (mitochondrial trifunctional protein deficiency); F, MAD def (multiple acyl-CoA dehydrogenase deficiency). *Abbreviations*: C2, acetylcarnitine; C4, butyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, myristoylcarnitine; C16, palmitoylcarnitine.

3.2. The values of ratios of R_p/R_c at different conditions

We calculated the ratios of R_p/R_c, which represent comparison of Cn/C2 (Cn: C4, C6, C8, C10, C12, C14, C14:1, C16) between patients (R_p) and controls (R_c) at different experimental conditions (Table 1). The value of R_p/R_c did not show any changes in MCAD deficient cells at different temperatures. R_p/R_c in long-chain ACs (C12, C14, C16) showed dramatic increase at 41 °C compared to 37 °C in cells from CPT2, VLCAD, and MTP deficiency. On the other hand, R_p/R_c from short to medium-chain (C4, C6, C8, C10) were lower whereas those from C14 was elevated at 41 °C compared to 37 °C in cells with MAD def.

4. Discussion

The present study evaluated the effect of heat stress, one of the most common metabolic stresses in children, on defective mitochondrial FAO to determine the relationship between febrile episodes and impaired FAO. We previously reported that accumulation of long-chain ACs was significantly enhanced at 41 °C compared with 37 °C in VLCAD-deficient cells [19]. Consistent with this observation, our current study showed that incubation of cells from patient deficient for CPT2, VLCAD or MTP at high temperature deteriorates long-chain FAO compared to physiological temperature. CPT2, VLCAD, and MTP are membrane-bound enzymes, located at inner-membrane of mitochondria and worked together towards β-oxidation of long-chain fatty acids [20]. Our results suggest that long-chain FAODs, such as deficiency for CPT2, VLCAD, as well as MTP, are susceptible to high temperature, which may be associated with metabolic crisis of these patients when they suffer from high fever. In contrast, short or medium-chain FAO was barely affected by heat stress. These data indicates that the effect of heat stress on FAO is different between long-chain ACs and short/medium-chain ACs. Consistent with these findings,

long-chain FAO was impaired whereas short and medium-chain FAO were facilitated by high temperature in patient cells lacking MAD, an enzyme involved in short to long-chain FAO. The data suggest that the electron transfer process by ETF and ETFDH for the flavin-containing dehydrogenases in long-chain FAO may be impaired at higher temperature in MAD deficiency without deteriorating medium-chain FAOs. While enzymes involved in long-chain FAO, such as VLCAD, TFP and CPT2 are bound to inner-membrane of mitochondria, MCAD and SCAD that catalyze medium and short-chain FAO are located in the mitochondrial matrix. These findings suggest that heat stress may selectively impair membrane-bound protein in contrast to those in the matrix. The underlying mechanism responsible for the differential effect of high temperature on ETF or ETFDH activity remains to be determined, but interaction of various dehydrogenases with ETF/ETFDH at differential locations may also partially be responsible for diverse effect on heat lability.

Previous reports suggest that impaired fatty acid β-oxidation may be responsible for influenza-associated encephalopathy (IAE), one of the life-threatening diseases resulting from influenza virus infection in children [9,10]. Other report also showed a decreased thermal stability of CPT2 variants in IAE patients during hyperpyrexia [21]. However, lack of definitive evidence explaining the mechanism responsible for the IAE resulting from deficiency of FAO makes it difficult to prove this association. Our data implies that impairment of mitochondrial FAO as a consequence of hyperpyrexia may be one of the mechanisms responsible for IAE.

In conclusion, our study suggests that patients with long-chain FAODs may be more susceptible to heat stress compared to medium-chain FAODs or healthy controls. Serious febrile episodes may further deteriorate long-chain FAO in FAODs. The underling pathogenic mechanism involved in impaired FAO by various stresses associated with life-threatening neurological episodes should be determined in future studies.

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Simple and rapid analytical method for detection of amino acids in blood using blood spot on filter paper, fast-GC/MS and isotope dilution technique

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ABSTRACT

A simple and rapid method for quantitative analysis of amino acids, including valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met) and phenylalanine (Phe), in whole blood has been developed using GC/MS. In this method, whole blood was collected using a filter paper technique, and a 1/8 in. blood spot punch was used for sample preparation. Amino acids were extracted from the sample, and the extracts were purified using cation-exchange resins. The isotope dilution method using $^{2}H_{8}$-Val, $^{2}H_{3}$-Leu, $^{2}H_{3}$-Met and $^{2}H_{5}$-Phe as internal standards was applied. Following propyl chloroformate derivatization, the derivatives were analyzed using fast-GC/MS. The extraction recoveries using these techniques ranged from 69.8% to 87.9%, and analysis time for each sample was approximately 26 min. Calibration curves at concentrations from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met showed good linearity with regression coefficients = 1. The method detection limits for Val, Leu, Ile, Met and Phe were 24.2, 16.7, 8.7, 1.5 and 12.9 μmol/l, respectively. This method was applied to blood spot samples obtained from patients with phenylketonuria (PKU), maple syrup urine disease (MSUD), hypermethionine and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and the analysis results showed that the concentrations of amino acids that characterize these diseases were increased. These results indicate that this method provides a simple and rapid procedure for precise determination of amino acids in whole blood.

1. Introduction

Free amino acids in whole blood reflect the state of amino acid metabolism; consequently, amino acids in blood or serum are monitored in patients with inborn errors of amino acid metabolism [1–4]. The filter paper technique is widely used for sampling whole blood for analysis of amino acids, acylcarnitines and fatty acids due to its superior features in sampling, transportation and sample retention compared with serum samples [1,5,6].

Tandem mass spectrometry (MS/MS) [1,3], high performance liquid chromatography (HPLC) [2], liquid chromatography mass spectrometry (LC/MS) [7], gas chromatography (GC) [8] and gas chromatograph mass spectrometry (GC/MS) [5,9] methods have been reported for quantitative analysis of amino acids in whole blood using the filter paper technique. In the aforementioned methods, MS/MS is commonly used for screening of inborn errors of metabolism because of the short analysis time [3]. Conversely, HPLC, LC/MS, GC and GC/MS methods have been applied to quantitative analysis due to their performance in chromatographic separation. In particular, the GC/MS method offers exceptional chromatographic separation, detailed mass spectral analysis and low ion suppression.

For these reasons, GC/MS methods for the analysis of amino acids in blood have been developed. In sample preparation process, amino acids are extracted using cation-exchange resins [10–12] after proteins are removed from blood samples using sulphosalicylic acid [10,13,14] and picric acid [12,14] treatments. Extracted amino acids are derivatized for GC/MS analysis. If organic solvent treatment is used for the removal of proteins, the sample goes directly to derivatization following the drying procedure [5,8,15–17]. For the derivatization procedure, trimethylsilylation [4,18,19], tert-butyldimethylsilylation [20,21], esterifcation-acylation [5,16], and alkyl chloroformation [8,9,22–25] have been reported and those methods were summarized by Knapp [26] and Blau and Halket [27]. For quantitative calculation, the absolute calibration method is widely used but the isotope dilution method was used to improve the accuracy [10,17,28].

In this study, we have developed a method for analysis of amino acids in blood using the filter paper technique and GC/MS. For easy and simple sample preparation, a commercially available kit for analysis of amino acids was used; after sample purification

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on cation-exchange resins and propyl chloroformate derivatization were performed, the treated samples were analyzed using the fast-GC/MS method to shorten the analysis time. To improve the accuracy of the results, the isotope dilution technique was also applied for quantitative analysis.

For the validation of this method, phenylalanine, leucine, isoleucine, valine and methionine were chosen as target amino acids in consideration of current Japanese neonatal mass screening, in which phenylalanine, leucine, and methionine are measured for detection of phenylketonuria (PKU), maple syrup urine disease (MSUD), and homocystinuria (HCY), respectively.

This method was successfully applied to the analysis of amino acids in blood spot samples obtained from patients suffering from PKU, MSUD, hypermethioninemia and even neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD).

2. Experimental

2.1. Chemicals

$^2H_8$-valine ($^2H_8$-Val), $^2H_3$-leucine ($^2H_3$-Leu), $^2H_3$-methionine ($^2H_3$-Met) and $^2H_5$-phenylalanine ($^2H_5$-Phe) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) to be used as internal standards. A mixture of the d-labeled amino acids was prepared at a concentration of 1 μmol/l in methanol. The solution mixtures of valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met) and phenylalanine (Phe) were prepared at concentrations of 0.5, 1, 5, 10 and 50 μmol/l by diluting a stock solution of the amino acid mixture (200 μmol/l; EZ:faast™) with 0.05 N HCl solution.

2.2. Preparation

Whole blood samples were absorbed on a filter paper (ADVANTEC PKU, Tokyo, Japan). After drying at room temperature, one 1/8 in. punch from each blood spot was transferred into a 1.5 ml-vial, and 100 μl each of the internal standard mixture and 0.05 N HCl solution was added to the vial. The mixture was vortexed for 10 min, allowed to stand for 40 min and then vortexed a second time for an additional 10 min. The obtained supernatant was prepared following the procedure described in the Phenomenex EZ:faast™ amino acid analysis kit for GC/MS (Phenomenex Inc., CA, US) with the exception of the addition of the internal standard mixture to the solution instead of novaline.

2.3. GC/MS measurement

The blood sample analysis was performed on a gas chromatograph coupled to a quadrupole mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with an automatic injection system (AOC-20i+s) and a split/splitless injection port. The analytical conditions are shown in Table 1. A short capillary column (10 m × 0.25 mm I.D.) was used. The data acquisition interval was set to 0.2 s to collect more than eight data points for each of the observed GC peaks along the GC peaks [15,29].

2.4. Method validation

2.4.1. Recovery of preparation

A control experiment was performed to evaluate the extraction recovery of the amino acids from a blood spot sample; the amounts of amino acids recovered were assumed to be equal to those of the labeled amino acids. Whole blood from healthy control, which contained amino acids within the normal concentration ranges, was spotted onto filter paper, and the blood spot (1/8 in.) was punched. Internal standard (0.1 nmol each of $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe) was added directly to each punch, and they were dried as spiked samples. The punches were treated following the preparation procedure shown in Section 2.2, with the exception that the internal standard mixture was added. For the blank test, five blank samples that did not contain a blood spot punch were prepared in 1.5 ml-vials following the preparation procedure (Section 2.2). The extraction recovery was calculated by dividing the peak areas of spiked samples by those of blank samples for $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe.

Table 1
Analytical conditions for GC/MS.

Gas Chromatography	
Injection volume	1.0 μl
Injection mode	Split (1:15)
Injection temp.	280 °C
Column oven	110 °C→(30 °C/min)→320 °C (0 min)
Carrier gas	He
Flow control mode	70.2 cm/s (linear velocity)
Total Flow	21.8 ml/min
Column Flow	1.18 ml/min
Purge Flow	3.0 ml/min
Mass spectrometry	
Interface temp.	280 °C
Ion box temp.	200 °C
Ionization voltage	70 eV
Emission current	150 μA
Data acquisition rate	0.2 s
Monitor ion *(m/z)*	
1.15–2.09 min	72,74,80,86,89, 116, 124, 130, 133, 158, 166, 172, 175
2.09–2.76 min	61,64, 190, 193
2.76–3.76 min	120, 126, 148, 154, 190, 196

2.4.2. Calibration curve

Blank and standard mixtures of Val, Leu, Ile, Met and Phe (0.5, 1, 5, 10 and 50 nmol/l) were analyzed and used to construct calibration curves according to a least-squares linear regression equation. Because one blood spot punch corresponds to 3 μl of whole blood, the concentrations of the standard mixtures (0.0, 0.5, 1, 5, 10 and 50 nmol/l in analysis) were converted to those of amino acids in whole blood (0.0, 16.7, 33.3, 166.7, 333.3 and 1666.7 μmol/l). The concentrations were varied from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met. A concentration of 33.0 μmol/l of $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe was added as an internal standard for corresponding non-labeled amino acids, and $^2H_3$-Leu was substituted for isotope-labeled Ile.

2.4.3. Method detection limit

The method detection limits were determined by analyzing five blood punches from a healthy control, which contained amino acids within the normal concentration ranges, and multiplying the standard deviation of the mean by the appropriate Student's *t*-value for the 99% confidence level using the appropriate degrees of freedom.

2.5. Healthy control and patient sample analysis

Blood spot samples were obtained from 33 normal controls and analyzed by the methods outlined in Sections 2.2 and 2.3. The mean values and the standard deviations obtained from 33 normal controls were calculated. The cut-off value was defined as the mean plus three standard deviations.

Blood spot samples were obtained from 5 patients with disorders diagnosed by the MS/MS method and clinical symptoms for PKU, MSUD and hypermethionine NICCD. To validate the new method, each patient's sample was analyzed to determine Val, Leu, Ile, Met and Phe concentrations, which were compared with the cut-off values.

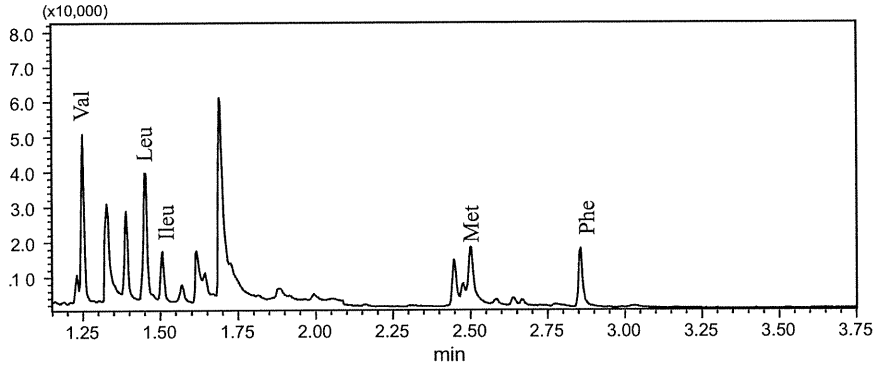

Fig. 1. Total ion chromatogram of a blood spot sample. A total ion chromatogram of a blood spot sample obtained from a healthy control is shown. Val = Valine, Leu = Leucine, Ile = Isoluecine, Met = Methionine and Phe = Phenylalanine.

3. Results

3.1. GC/MS measurement

Fig. 1 shows the total ion chromatogram of a blood spot sample obtained from a healthy control. Retention times of Val, Leu, Ile, Met and Phe were 1.25, 1.46, 1.51, 2.50 and 2.86 min, respectively. All target amino acids were separated by selective ion monitoring (*m/z* 116, 172, 130, 190 and 190 for Val, Leu, Ile, Met and Phe, respectively) without overlapping component peaks (Fig. 2).

3.2. Method validation

3.2.1. Recovery of preparation

The extraction recoveries varied from 69.8% to 87.9%, as shown in Table 2. The repeatabilities for blank samples and control samples were <4.4% and 14.1% (RSD%, $n = 5$), respectively.

Table 2
Recovery and repeatability.

	Blank (n = 5)		Punch of blood (n = 5)		
Compound	Mean[a]	%RSD	Mean[b]	%RSD	Recovery[c]
$^2H_8$-Valine	3575	4.4%	2496	12.3%	69.8%
$^2H_3$-Leucine	6449	2.9%	4515	13.5%	70.0%
$^2H_3$-Methionine	615	2.5%	541	13.4%	87.9%
$^2H_5$-Phenylalanine	2096	3.6%	1655	14.1%	79.0%

[a] The internal standard mixture (0.1 nmol) was added directly to each punch of the blood spot sample obtained from a healthy control. The dried punch was treated following the preparation procedure shown in Section 2.2. The means of the indicated peak areas are shown.

[b] For the blank test, five blank samples that did not contain a blood spot punch were prepared in the 1.5 ml vial were prepared following the preparation procedure (Section 2.2). The means of the indicated peak areas are shown.

[c] Recovery = peak area (punch of blood)/peak area (blank).

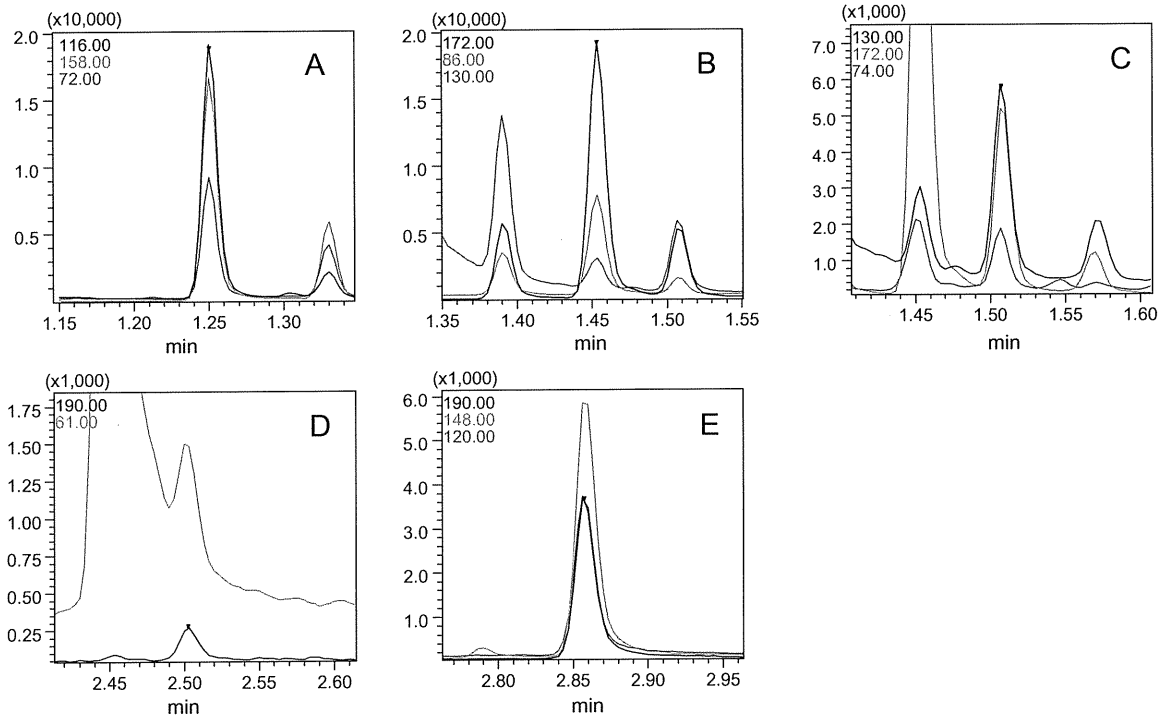

Fig. 2. Selected ion monitoring of a blood spot sample. Selected ion monitoring of amino acids in a blood spot sample obtained from a healthy control is shown. All target amino acids were separated by selected ion monitoring without overlapping of component peaks. A: Valine, *m/z* 116, 129.7 μmol/l; B: Leucine, *m/z* 172, 104.7 μmol/l; C: Isoleucine, *m/z* 130, 41.3 μmol/l; D: Methionine, *m/z* 190, 13.3 μmol/l and E: Phenylalanine, *m/z* 190, 63.0 μmol/l.

Table 3
Calibration curves.

Compound	Equations	R^2 [a]
Valine	$y = 1.41x + 2.60 \times 10^{-2}$	1.000
Leucine	$y = 1.08x + 0.10 \times 10^{-1}$	1.000
Isoluecine	$y = 0.73x + 2.76 \times 10^{-2}$	1.000
Methionine	$y = 0.91x + 4.62 \times 10^{-1}$	0.999
Phenylalanine	$y = 1.12x + 5.17 \times 10^{-2}$	1.000

The concentrations varied from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met. A concentration of 33.0 μmol/l of $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe was added as an internal standard for corresponding non-labeled amino acids; $^2H_3$-Leu was also substituted for isotope labeled Ile. The concentrations of 0.0, 0.5, 1, 5, 10 and 50 nmol/l were converted to 0.0, 16.7, 33.3, 166.7, 333.3 and 1666.7 μmol/l of amino acids in whole blood.

[a] Correlation coefficient.

3.2.2. Calibration curve

The correlation coefficients for Val, Leu, Ile and Phe were 1.000 at concentrations from 0.0 to 1666.7 μmol/l, and the correlation coefficient for Met was 0.999 at concentrations from 0.0 to 333.3 μmol/l as shown in Table 3.

3.2.3. Method detection limit

Results obtained from 5 blood spot samples of a healthy control are shown in Table 4. The repeatabilities for the method (%RSD, $n = 5$) were <5.0% for the evaluated amino acids. The method detection limits ranged from 1.5 (Met) to 24.2 μmol/l (Val).

3.3. Healthy control and patient sample analysis

Table 5 shows the analytical results obtained for blood spot samples from 5 patients suffering from PKU, MSUD and hypermethionine NICCD and from 33 healthy controls. The concentrations of Val, Leu, Ile, Leu + Ile, Met and Phe in the 33 healthy controls were lower than the cut-off values.

4. Discussion

Methods using GC or GC/MS for analysis of amino acids in whole blood, which used blood spot samples on filter papers, have been reported [5,9]. In these previous reports, a sample punch 8 mm in diameter was used; in this study, we used a punch of 1/8 in., which allowed for more sample punches to be taken from the same spot if re-analysis or other biochemical tests are required.

Amino acids were extracted from punches of blood spots with a mixture of methanol, which was used as the solvent for the internal standard, and 0.05 N HCl (1:1, v/v). During the solvent extraction, most of protein could be removed similar as organic solvent treatment [4,5,8,16]. Cation-exchange resins were used to extract amino acids from the solvent. Adsorption efficiency of aliphatic amino acids, such as Phe, on ion exchange resins was not decreased by the non-polar extraction solvent, which was approximately 50% methanol. The extraction process showed excellent recovery and repeatability (79.0% and 14.1% %RSD) for $^2H_5$-Phe. For the other amino acids, the recoveries were more than 69.8%, and the repeatability was <13.5% (%RSD, $n = 5$) without internal standard correction. These results indicate that not only non-aliphatic amino acids, such as Val, Leu, Ile and Met, but also aliphatic amino acids, such as Phe, can be quantitatively extracted using this method.

Amino acids are usually analyzed by GC/MS after derivatization of the amine and carboxylic functional groups. Various derivatization methods were evaluated as described in Section 1. In those methods, residual water in the sample does not interfere with propyl chloroformation and this derivatization method may be highly preferable in biological samples [9,24,25,30]. Additionally, derivatization time was shortened by using propyl chloroformate. Trimethylsilylation, which is commonly used in derivatization for GC/MS analysis, requires solvent dehydration via heating and longer derivatization reaction time (30–60 min). Conversely, propyl chloroformate is unaffected by water, and the reaction is complete within 1 min at room temperature. For these reasons, propyl chroloformation was applied to this study.

Amine and carboxylic functional groups are converted to carboxylicpropyl and propylester, respectively, by the propyl chloroformation derivatization, and the mass number of the molecular ion is increased by 128 u. In Leu, Met and Phe, mass spectra of the derivatives showed molecular ions at m/z 259, 277 and 293, respectively; however, a similar molecular ion was not detected in mass spectra of Ile. In addition, mass spectra of derivatives of Val, Leu, Ile, Met and Phe showed specific ions formed by loss of a C_3H_7COO fragment (87 u) from the molecular ion at m/z 158, 172, 172, 190 and 206, corresponding to the loss of this fragment, i.e. Val: 245 − 87, Leu: 259 − 87, Ile: 259 − 87, Met: 277 − 87 and Phe: 293 − 87 (data not shown). These results demonstrate that the evaluated amino acids were completely derivatized by this method.

A high linear velocity of 70.2 cm/s, which was generated by a short capillary column (10 m × 0.25 mm I.D.), and a fast oven temperature program of 30 °C/min were used to shorten GC/MS analysis time (Table 1). The GC/MS analysis cycle time was approximately 10 min; the Phe had the longest retention time (2.86 min). Under these conditions, all target amino acids were separated and selectively detected (Fig. 2). During 50 sample analyses, interference due to peak overlap did not occur. These results suggest that the solvent extraction and purification on the cation ion-exchange resins were appropriate to selectively separate the amino acids from sample contaminants, and these pre-analysis steps resulted in good chromatographic separation. This method reduces GC/MS analysis time by one-third to one-sixth compared to the conventional method (30–60 min) while retaining good chromatographic separation [9,20,31].

Table 4
Repeatability and method detection limits.

Compound	Concentrations for five blood punches[b] [μmol/l]							
	1	2	3	4	5	Mean	%RSD	MDLs[c]
Valine	129.7	145.7	140.7	135.3	139.3	138.1	4.3%	24.2
Leucine	104.7	114.0	113.7	108.0	113.0	110.7	3.7%	16.7
Isoluecine	41.3	45.3	44.7	40.7	44.7	43.3	5.0%	8.7
Leu + Ile[a]	146.0	159.3	158.3	148.7	157.7	154.0	4.0%	24.9
Methionine	13.3	12.7	12.3	12.7	13.0	12.8	3.0%	1.5
Phenylalanine	63.0	70.7	70.0	66.7	69.7	68.0	4.7%	12.9

[a] Total values of Leu (Leucine) and Ile (Isoluecine).

[b] The method detection limits were determined by analyzing five punches of the same blood spot from a healthy control, which contained amino acids within the normal concentration ranges.

[c] MDLs (Method detection limits) were determined by multiplying the standard deviation of the mean by the appropriate Student's t-value for the 99% confidence level using the appropriate degrees of freedom.