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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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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).

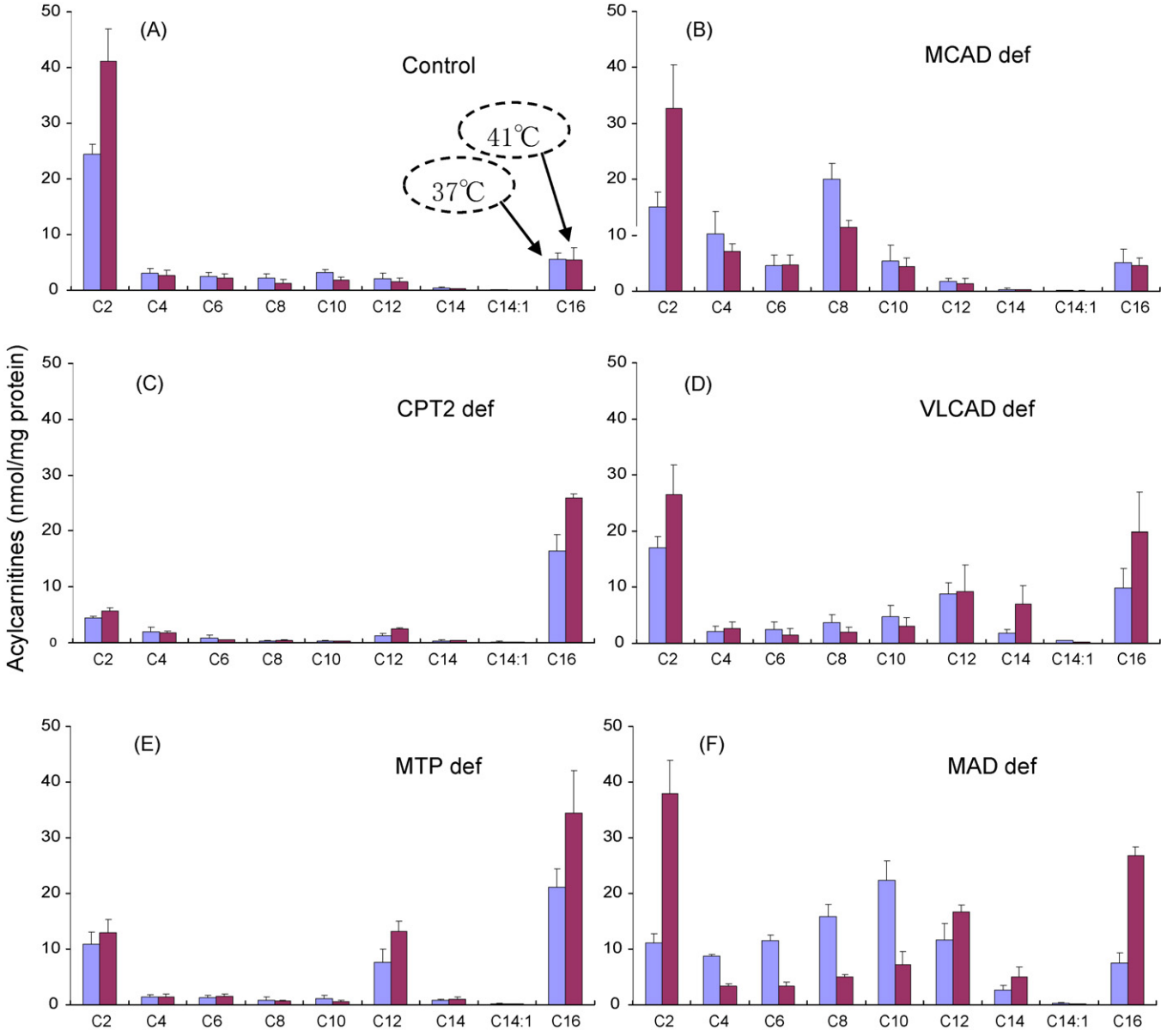

Fig. 1. AC profiles in supernatant of cells cultured with palmitate in various FAODs at 37 or 41 °C. : 37 °C; : 41 °C. A, Control; B, MCAD def (medium-chain acyl-CoA dehydrogenase deficiency); C, CPT2 def (carnitine palmitoyl transferase II deficiency); D, VLCAD def (very-long-chain acyl-CoA dehydrogenase 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 C*n*/C2 (C*n*: 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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