学 位 論 文 の 要 旨

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学 位 論 文 名	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
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論 文 内 容 の 要 旨

INTRODUCTION

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 might be more susceptible to febrile episodes. The mitochondrial FAO is a central energy generating process particularly under several conditions of metabolic stress, like long fasting, infection or hyperpyrexia. Patients with inherited mitochondrial FAO disorders occasionally present acute life-threatening symptoms, such as encephalopathy, cardiomyopathy or even sudden death, due to energy crisis of metabolic decompensation, which are often remediable.

In recent decades, in vitro probe acylcarnitine (AC) profiling assay was developed to evaluate FAO disorders. 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℃ in cultured fibroblasts from Japanese children with mitochondrial FAO disorders 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, on mitochondrial FAO. We reported the

effects of heat stress and bezafibrate on mitochondrial FAO using the in vitro probe AC profiling assay.

MATERIALS AND METHODS

We cultured fibroblasts from 6 controls (healthy volunteers) and 9 Japanese children with very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, including three clinical subgroups: 2 cases of the severe; 3 of the intermediate; and 4 of the myopathic forms. Three cell lines from medium-chain acyl-CoA dehydrogenase (MCAD) deficiency were also examined for reference in the study.

Fibroblasts were cultured in modified Eagle's minimal essential medium (MEM) supplemented with 2 mmol/L of L-glutamine, 10% FBS and 1% penicillin /streptomycin at 37℃ in a humidified 5% CO_2/95% air incubator. Confluent cells were harvested by trypsinization and seeded onto 6-well microplates 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 and cultured for 96 h in 1 mL of experimental substrate A, MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA), L-carnitine (0.4 mmol/L), unlabelled palmitic acid (0.2 mmol/L) and 1% penicillin/streptomycin without L-glutamine, or substrate B, medium of substrate A added bezafibrate (0.4 mmol/L). The initiation and the end points of the 96-hour incubation are expressed as T_0 and T_{96}, respectively. ACs in the culture medium were determined at point T_{96}. In a replicate experiment, the supernatants were collected at 24, 48, 72 and 96 hours to determine their time course effects on the FAO.

ACs in culture medium supernatants were analyzed using MS/MS (API 3000). Briefly, methanol (200 μL) including stable isotope-labeled internal standards was added to 10 μL of the supernatant from culture medium, for 30 minutes. Portions were centrifuged at 1000 × g for 10 minutes, 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℃ for 15 minutes. 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.

Protein concentration was measured at T_0 and T_{96}, by a modification of the Bradford method using the Bio-Rad protein assay. Cells were enumerated in a model Z1 Coulter Counter. The viable cells at 24, 48, 72 and 96 hours of incubation were determined using the modified 3-(4, 5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay.

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.

RESULTS AND DISCUSSION

The purpose of our study was to investigate the susceptibility to hyperpyrexia, and to a hypolipidemic drug, bezafibrate, on mitochondrial FAO, using in vitro probe AC profiling assay. We evaluated the FAO capacity based on the accumulation of specific length-chain of ACs. Acetylcarnitine (C2), derived from acetyl-CoA, the final product of FAO cycles, is considered to be the important marker of the whole FAO flux, and the long-chain ACs specifically represent the long-chain FAO flux. We measured cell viability using 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 hours under various conditions.

Under heat stress (introduced by 41℃), a significant elevation of C2 ($p = 0.012$), but slightly decrease of other species from short-chain to medium-chain ACs was seen in controls. Similarly, C2 was significantly elevated in the intermediate and myopathic forms of VLCAD-deficient cells incubated at 41℃ compared to 37℃ ($p < 0.01$). Most importantly, accumulation of C16 was significantly enhanced in all the clinical forms of VLCAD deficiency ($p < 0.01$).

∑LC, the sum of long-chain ACs (C12+C14+C14:1+C16), and the ratio of ∑LC/C2, were also used to evaluate the capacity of long-chain FAO. The ∑LC was significantly higher at 41℃ than that at 37℃ in VLCAD-deficient cells ($p = 0.043$), among which myopathic form showed the highest ∑LC, suggesting that the long-chain FAO was inhibited by heat stress and the myopathic form is most sensitive to heat stress ($p < 0.01$).

In contrast, exposure of MCAD-deficient cells to 41℃ increased C2, decreased medium-chain ACs but did not affect long-chain ACs. 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, which has distinguishable state in long-chain FAO.

Bezafibrate treatment significantly increased C2 in VLCAD-deficient cells ($p = 0.014$) as well as control cells, while the accumulation of long-chain ACs also remarkably decreased ($p < 0.01$) at 37℃. Although the mean values of ∑LC ($p = 0.029$) and the ratio of ∑LC/C2 ($p = 0.015$) remained higher in VLCAD deficiency patients than that in controls, bezafibrate significantly reduced ∑LC in all the clinical forms to a different extent at 37℃ compared with that without bezafibrate. The reduction of 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). These findings suggest that bezafibrate may represent a potential treatment strategy for VLCAD deficiency, specifically for the clinically milder forms.

CONCLUSION

In vitro acylcarnitine profiling assay using unlabeled palmitic acid as substrate is a simple and promising strategy to determine the effects of heat stress or drugs on mitochondrial FAO. It was indicated that heat stress inhibits long-chain FAO in VLCAD deficiency, and that bezafibrate improves impaired long-chain FAO.