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Circulating tricarboxylic acid cycle metabolite levels in citrin-deficient children with metabolic adaptation, with and without sodium pyruvate treatment

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ABSTRACT

Citrin deficiency causes adult-onset type II citrullinemia (CTLN-2), which later manifests as severe liver steatosis and life-threatening encephalopathy. Long-standing energy deficit of the liver and brain may predispose ones to CTLN-2. Here, we compared the energy-driving tricarboxylic acid (TCA) cycle and fatty acid β-oxidation cycle between 22 citrin-deficient children (age, 3–13 years) with normal liver functions and 37 healthy controls (age, 5–13 years). TCA cycle analysis showed that basal plasma citrate and α-ketoglutarate levels were significantly higher in the affected than the control group ($p < 0.01$). Conversely, basal plasma fumarate and malate levels were significantly lower than those for the control ($p < 0.001$). The plasma level of 3-OH-butyrate derived from fatty acid β-oxidation was significantly higher in the affected group ($p < 0.01$). Ten patients underwent sodium pyruvate therapy. However, this therapy did not correct or attenuate such deviations in both cycles. Sodium pyruvate therapy significantly increased fasting insulin secretion ($p < 0.01$); the fasting sugar level remained unchanged. Our results suggest that citrin-deficient children show considerable deviations of TCA cycle metabolite profiles that are resistant to sodium pyruvate treatment. Thus, long-standing and considerable TCA cycle dysfunction might be a pivotal metabolic background of CTLN-2 development.

1. Introduction

Adult-onset type II citrullinemia (CTLN2), a fatal metabolic disease, presents with frequent bouts of hyperammonemia, liver steatosis, mental derangement, sudden episodes of unconsciousness, and, ultimately, death within a few years of onset [1,2]. Mutations in the *SLC25A13* gene, which is located on chromosome 7q21.3 and encodes the

Abbreviations: AGC2, aspartate–glutamate carrier-2; CTLN-2, adult-onset type II citrullinemia; FFA, free fatty acid; MA shuttle, malate–aspartate shuttle; MC shuttle, malate–citrate shuttle; TCA, tricarboxylic acid.

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calcium-binding mitochondrial protein citrin, are responsible for CTLN-2 [3–5]. Citrin, a liver-type aspartate–glutamate carrier, plays an important role in the malate–aspartate (MA) NADH shuttle and urea synthesis [6,7]. Impairment of citrin function leads to increased NADH/NAD^+ ratios in the cytosol and failure of aspartate supply from the mitochondria to the cytoplasm for argininosuccinate synthesis, leading to hypercitrullinemia and hyperammonemia. An increasing amount of information on other metabolic abnormalities has been accumulating, but sufficient information is not available yet.

In early life, citrin deficiency presents with diverse clinical manifestations, namely, neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), which manifests as considerable liver dysfunction along with cholestasis, citrullinemia, mild hyperammonemia, galactosemia, and hypoglycemia [6,8–10]. The clinical presentations of NICCD resolve from 6 months to 1 year of life, probably due to metabolic adaptation. However, among patients who manifest NICCD, only one-fifth or less develop CTLN2 in later life. Serious and long-standing energy deficit of the liver and brain, together with unfavorable lipid metabolism may engender CTLN-2.

In the current study, to establish a suitable treatment for children exhibiting metabolic adaptation to citrin deficiency, we examined metabolites involved in the tricarboxylic acid (TCA) cycle and fatty acid β-oxidation, which generate NADH or FADH in terms of energy, in blood samples. Further, metabolic effects of pyruvate that is transformed into lactate by lactate dehydrogenase, converting NADH to NAD^+, or into oxaloacetate and citrate as members of TCA cycle, were examined. It was expected that the decrease of cytoplasmic NADH enhances carbohydrate utility.

We found considerable and persistent deviations in the TCA cycle and fatty acid β-oxidation that are resistant to sodium pyruvate therapy in citrin-deficient children exhibiting metabolic adaptation.

2. Subjects and methods

2.1. Subjects, sample collection, and sodium pyruvate treatment

For 2009 to 2014, 22 children with citrin deficiency (12 boys and 10 girls) were enrolled; their ages ranged from 3 years 2 months to 13 years 3 months. Of these children, 15 were found to have metabolic abnormalities (hypergalactosemia, n = 9; hyperphenylalaninemia, n = 4; hypermethioninemia, n = 2) on performing neonatal mass screening at around the age of 5 days. Thereafter, they developed considerable liver dysfunction along with cholestasis, causing hyperbilirubinemia, hypoproteinemia, and prolonged coagulation. Precise metabolic examination revealed that they had markedly elevated plasma citrulline levels accompanied by increased plasma arginine, threonine, tyrosine, and phenylalanine levels. The remaining seven patients developed hyperbilirubinemia and admitted to eligible hospitals at the age of 1–6 months. Metabolic examination revealed prominent citrullinemia accompanied by increased plasma arginine, threonine, tyrosine, and phenylalanine levels.

The children were confirmed to have citrin deficiency at ages ranging from 3 weeks to 4 years 1 month by gene analyses for *SLC25A13*. The genotypes were determined to be as follows: [I] 851del4; [II] IVS11 + 1G > A; [III] 1638ins23; [IV] S225X; [V] IVS13 + 1G > A; [VI] 1800ins1; [VII] R605X; [VIII] E601X; [IX] E601K; [X] IVS6 + 5G > A; [XI] R184X; and [XIV] IVS6 + 1G > C: genotypes, number: I/I, five; I/II, five; II/II, four; II/V, four; I/VI, two; and II/VIII, two [3–5]. The results of liver function tests normalized at ages ranging from 7 to 18 months. The blood levels of aspartate transaminase, alanine aminotransferase, gamma-glutamyl transpeptidase, total bile acids, and total bilirubin were normal at the time of the study. We also obtained age-matched control data for non-obese healthy 37 children consisting 16 boys and 21 girls aged 5–13 years for comparison with the basal data of the 22 citrin-deficient children at the time of inclusion in the study.

Ten of the 22 affected children underwent sodium pyruvate therapy; the daily dosage of 300 mg/kg/day was orally administered over three doses. A few children complained of nausea soon after initiation of therapy; the remaining children did not develop adverse effects during therapy. We monitored the parameters at 2–4 weeks before initiation and 3–6 months after initiation of therapy.

Blood samples were collected before lunch (i.e., at 10:30–11:30 am) under 4–5 h fasting. The methods and purpose of the study were explained to the parents, and their informed consent was obtained prior to enrollment. The project was approved by the institutional medical ethics committee.

2.2. Lipid and carbohydrate analyses

Total cholesterol (TC) and triglyceride (TG) levels were determined enzymatically. The serum level of free fatty acids (FFAs) was measured by enzymatic methods using the NEFA-SS kit EIKEN (Eiken Chemicals Co. Ltd., Tokyo, Japan). High-density lipoprotein cholesterol (HDL-C) and LDL-C were measured by homogeneous assays (Choletest HDL and Choletest LDL, respectively, Sekisui Medical, Tokyo).

The plasma glucose level was determined enzymatically. The whole blood hemoglobin A_{1c} (HbA_{1c}) level was determined with high-performance liquid chromatography (HPLC; HLC-723 G8, Tosoh Co. Ltd., Tokyo). The serum insulin level was determined with an enzyme immunoassay performed using a commercial kit (TOSOH-II, Tosoh Co. Ltd.).

2.3. TCA cycle metabolite, carnitine, and 3-OH-butyrate analyses

The serum concentrations of metabolites involved in the TCA cycle were measured with HPLC–mass spectrometry (MS)/MS. Fifty microliters of serum was added to a microcentrifuge tube (1.5 ml, Eppendorf, Hamburg, Germany), and 934 pmol (100 ng) of [$^{13}C_3$] malonate in 200 μl of acetonitrile was added as an internal standard. The sample tube was vortexed for 1 min and centrifuged at 2000 g for 1 min. The solution of the internal standard in acetonitrile caused deproteinization of the sample; the liquid phase was collected and evaporated to dryness at 55 °C under a nitrogen stream. The residue was redissolved in 30 μl of water and centrifuged again at 2000 g for 1 min. The supernatant was collected and an aliquot (3 μl) was injected into an HPLC–electrospray ionization (ESI)–MS/MS system. The system consisted of a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan). Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) at 40 °C. The mobile phase was composed of methanol-water (1:19, v/v) containing 0.1% formic acid and was used at a flow rate of 300 μl/min. The MS/MS conditions were as follows: spray voltage, 2500 V; vaporizer temperature, 450 °C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 15 arbitrary units; ion transfer capillary temperature, 220 °C; collision gas (argon) pressure, 1.0 mTorr; ion polarity, negative; and selected reaction monitoring (SRM) and collision energy, m/z 106 → m/z 61 (13 V) for [$^{13}C_3$]malonate, m/z 115 → m/z 71 (13 V) for fumarate, m/z 117 → m/z 73 (17 V) for succinate, m/z 133 → m/z 115 (15 V) for malate, m/z 145 → m/z 101 (12 V) for α-ketoglutarate, m/z 191 → m/z 87 (18 V) for citrate, and m/z 191 → m/z 155 (15 V) for isocitrate.

Serum carnitine and 3-OH-butyrate concentrations were also determined by HPLC–ESI–MS/MS. Five microliters of serum was added to a microcentrifuge tube, and 769 pmol (100 ng) of sodium DL-[$^{13}C_4$]3-OH-butyrate, 147 pmol (25 ng) of DL-[$^2H_9$] carnitine and 51 pmol (12.5 ng) of acetyl-L-[$^2H_3$]carnitine HCl in 100 μl of acetonitrile-water (19:1, v/v) was added as an internal standard. The sample tube was vortexed for 1 min and centrifuged at 2000 g for 1 min. The liquid phase was collected and evaporated to dryness at 55 °C under a nitrogen stream. The residue was redissolved in 70 μl of water containing 0.1%