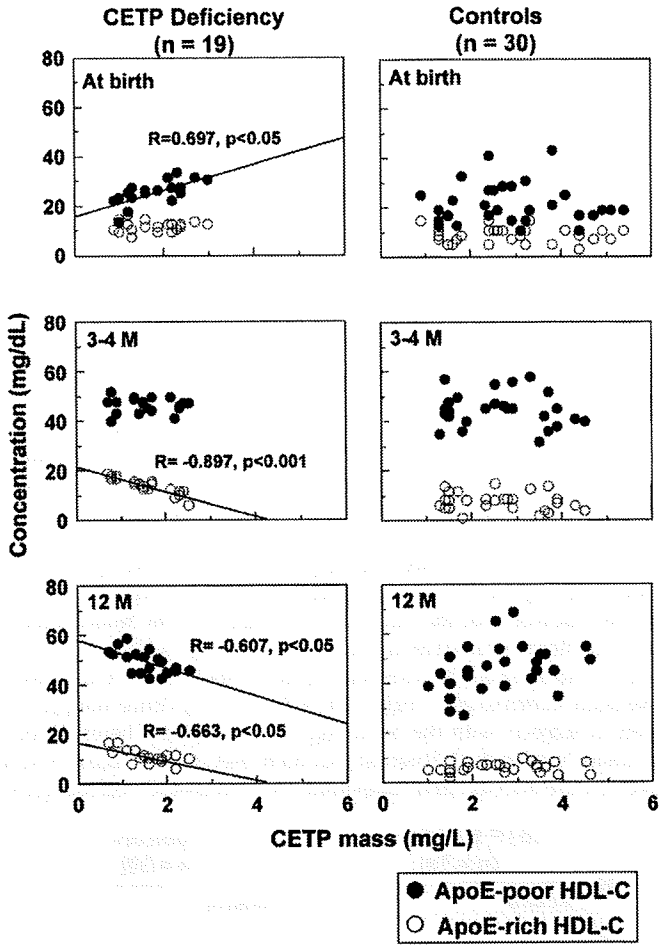

Fig. 2. Scatterplots of apoE-rich HDL-C and apoE-poor HDL-C against CETP mass levels in 19 heterozygous CETP-deficient subjects (left panels) and 30 controls (right panels). Blood samples were obtained at birth (top panels), 3–4 months (middle panels), and 12 months (bottom panels).

(which have very low CETP activity) have normal LDL-C levels, whereas Akita et al. reported that these subjects have lower LDL-C levels [8,9].

After birth, LDL-C increases rapidly and intensively with growth and development, but the level at 12 months is still considerably low compared with the adult level [1–5]. It is likely that the LDL receptor is less saturated in young children than in adults. The present study provided evidence that LDL-C concentrations in neonates and infants, in whom LDL production is limited, are dependent on the cholesterol transferred from HDL-C by CETP. Taken together, these findings suggest that LDL metabolism in neonates and infants is greatly different from that in adults. The differences in LDL metabolism between young children and adults may underpin the effects of CETP mutations on the lipoprotein profile.

The LDL particles from CETP-deficient subjects are small and very heterogeneous, and therefore exhibit reduced affinity for LDL receptors [14–16]. Higher LDL-C levels in CETP-deficient adults may be explained by decreased LDL receptor binding, leading to the subsistence of LDL in plasma. However, this may not be sufficient to raise LDL-C levels in neonates and infants, because their LDL-C receptors are less saturated. Moreover, recent studies have shown that the suppression of CETP activity results in decreased LDL and apoB production [17–19]. If this holds true for neonates and infants, their lower LDL and apoB levels could be explained in part by suppressed CETP activity.

There are also limited data concerning the relationship between CETP mutations and HDL-C in children [12,13]. In Japanese children 10 years of age, the D442G mutation had no effect on HDL-C, apoA-I, or apoA-II levels [12]. In contrast, the D442G mutation was associated with high HDL-C levels in Vietnamese girls aged 7–9 y [13]. In the present study, the HDL-C level at birth was similar between heterozygous CETP-deficient and control children. Even in the subject with compound heterozygous CETP deficiency, the HDL-C level at birth was similar to that in the control group (Table 1). At 3–4 and 12 months, however, HDL-C was significantly higher in heterozygous CETP-deficient subjects compared with controls (Table 1). A typical CETP-deficient adult characterized by the I14A or D442G mutation exhibits high HDL-C [6–9].

The effects of CETP mutations on LDL-C and HDL-C levels should be based on the balance between CETP mass and acceptor lipoproteins (VLDL and LDL) of cholesteryl esters. CETP mass is likely sufficient for acceptor lipoproteins in control children but insufficient in CETP-deficient children. With the exception of the compound heterozygote, apoE-poor HDL-C levels were similar between heterozygous CETP-deficient subjects and healthy controls during the first year of life (Table 1). Since HDL-C levels were higher in CETP-deficient children compared with controls after 3–4 months, differences in the level of HDL-C are attributable to the high apoE-rich HDL levels that occur in CETP-deficient subjects. CETP mass exhibited a strong inverse correlation with apoE-rich HDL-C at 3–4 months and with apoE-rich and apoE-poor HDL-C at 12 months. These results strongly suggest that cholesteryl esters accumulate in HDL because of impaired cholesterol ester transfer.

Unexpectedly, CETP mass correlated positively with apoE-poor HDL-C at birth (Fig. 2). The liver is one of the main organs synthesizing CETP and apo A-I. Therefore, at birth, these levels may reflect the amount of lipoprotein synthesis in CETP-deficient subjects. Consistent

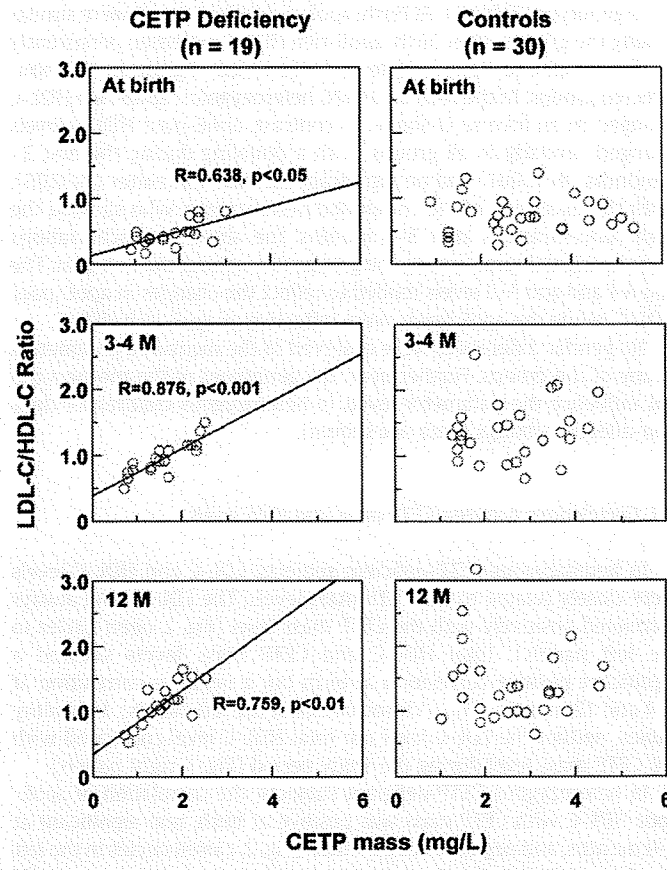

Fig. 3. Scatterplots of the LDL-C/HDL-C ratio against CETP mass levels in 19 heterozygous CETP-deficient subjects (left panels) and 30 controls (right panels). Blood samples were obtained at birth (top panels), 3–4 months (middle panels), and 12 months (bottom panels).

with our hypothesis, CETP mass at birth correlated positively with apoE-poor HDL but not with apoE-rich HDL, which is considered a better substrate for CETP [5,6].

The absence of an increase in apoE-rich HDL-C in CETP-deficient subjects at birth may be explained by the abundance of apoE receptors that bind HDL. Given the low LDL concentration in fetuses, impaired cholesterol delivery by LDL to systemic tissues must be compensated for by other carrier systems. The ubiquitous distribution of apoE and apoE receptors strongly suggests that apoE plays a crucial role in the growth and development of various organs, including the central nervous system, in fetuses [20–23].

The clinical implications or adverse effects of CETP deficiency in early life remain unknown. However, some of the CETP-deficient subjects enrolled in this study, particularly those with considerably low TC and LDL-C levels, showed lower plasma vitamin E levels a few months after birth. Recently, vitamin E deficiency associated with abeta- or hypobetalipoproteinemia has been reported [24,25]. It is, therefore, likely that CETP deficiency influences vitamin E metabolism in early life.

We conclude that CETP is a determinant for LDL-C and HDL-C in CETP-deficient children in their first year of life. We speculate that the lack of these associations in normal children may involve the altered production and/or clearance of LDL and HDL particles. More detailed kinetic studies are required to elucidate the underlying mechanisms.

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Sustaining hypercitrullinemia, hypercholesterolemia and augmented oxidative stress in Japanese children with aspartate/glutamate carrier isoform 2-citrin-deficiency even during the silent period

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ABSTRACT

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) shows diverse metabolic abnormalities such as urea cycle dysfunction together with citrullinemia, galactosemia, and suppressed gluconeogenesis. Such abnormalities apparently resolve during the first year of life. However, metabolic profiles of the silent period remain unknown. We analyzed oxidative stress markers and profiles of amino acids, carbohydrates, and lipids in 20 asymptomatic children with aspartate/glutamate carrier isoform 2-citrin-deficiency aged 1–10 years, for whom tests showed normal liver function. Despite normal plasma ammonia levels, the affected children showed higher blood levels of ornithine ($p < 0.001$) and citrulline ($p < 0.01$)—amino acids involved in the urea cycle—than healthy children. Blood levels of nitrite/nitrate, metabolites of nitric oxide (NO), and asymmetric dimethylarginine inhibiting NO production from arginine were not different between these two groups. Blood glucose, galactose, pyruvate, and lactate levels after 4–5 h fasting were not different between these groups, but the affected group showed a significantly higher lactate to pyruvate ratio. Low-density and high-density lipoprotein cholesterol levels in the affected group were 1.5 times higher than those in the controls. Plasma oxidized low-density lipoprotein apparently increased in the affected children; their levels of urinary oxidative stress markers such as 8-hydroxy-2′-deoxyguanosine and acrolein-lysine were significantly higher than those in the controls. Results of this study showed, even during the silent period, sustained hypercitrullinemia, hypercholesterolemia, and augmented oxidative stress in children with citrin deficiency.

Introduction

Adult-onset type II citrullinemia (CTLN2, OMIM 603471), a disease caused by a mitochondrial transporter, is characterized by frequent bouts of hyperammonemia, liver steatosis, mental derangement, sudden unconsciousness, and ultimately death within a few years of onset [1–3]. In fact, CTLN2 results from mutations of the SLC25A13 gene on chromosome 7q21.3 encoding a calcium-binding mitochondrial protein: a liver-type aspartate/glutamate carrier isoform 2 (AGC2), so-called citrin [3–6]. In the malate-aspartate NADH shuttle and urea synthesis, AGC2 plays an important role [3,7,8]. Impairment of AGC2 function can engender an increased NADH/NAD^+ ratio in cytosol. Failure of the aspartate supply from the mitochondria to the cytoplasm for synthesis of argininosuccinate engenders hypercitrullinemia and hyperammonemia.

Clinical characteristics of citrin deficiency vary dramatically by age [1–6,8–12]. About half of the Japanese children diagnosed with citrin deficiency were found to have metabolic abnormalities such as hypergalactosemia, hyperphenylalaninemia, and hypermethioninemia

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by newborn mass screening (NMS) at the age of 5 days. The remaining children visited eligible hospitals to receive precise examinations for prolonged jaundice, acholic stool, and/or failure to thrive during early infancy [9–12]. These children present diverse clinical manifestations, namely neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) such as considerable liver dysfunction, along with cholestasis, citrullinemia, mild hyperammonemia, galactosemia, and hypoglycemia.

The clinical presentations of NICCD resolve from 6 months to one year of life. However, among patients who have manifested NICCD, only one-fifth of patients develop CTLN2 [8–12].

Prompted by the fact that onset of CTLN2 is fatal, we sought the metabolic profiles of affected children from the silent stage: before the onset of CTLN2. Correction of metabolic anomalies at this stage would be expected to prevent the onset of CTLN2.

For this study, we examined the profiles of amino acids, carbohydrates, and lipids. We also examined NO synthesis, which shares processes with the urea cycle [13,14]. Furthermore, the status of oxidative stress, which is related closely to the development of liver steatosis, was evaluated using biomarkers.

In this report, we describe hypercitrullinemia, dyslipidemia and augmented oxidative stress in the affected children. The metabolic abnormalities underlying the development of CTLN2 will be discussed.

Subjects and methods

Subjects and sample collection

This study enrolled 20 children with citrin deficiency (10 males and 10 females, 1 year 10 months – 10 years 5 months) and 32 age-matched healthy children (16 female and 16 males, 2 year 2 months – 9 years 5 months) as controls.

The affected children's blood levels of transaminase, gamma-glutamyl transpeptide, total bile acids, and total bilirubin at that time were entirely normal. Of the 20 affected children, 12 were found to have metabolic abnormalities (hypergalactosemia, $n = 7$; hyperphenylalaninemia, $n = 4$; hypermethioninemia, $n = 2$) by NMS performed at the age of 5 days (Table 1). Thereafter, they developed considerable liver dysfunction along with cholestasis manifesting hyperbilirubinemia, hypoproteinemia, and prolonged coagulation. Precise examination revealed that they had markedly elevated plasma citrulline levels accompanying higher plasma levels of arginine, threonine, tyrosine, and phenylalanine. The remaining eight patients developed hyperbilirubinemia and visited their respective hospitals at the ages of 1–4.5 months. Precise examination detected prominent citrullinemia accompanying higher plasma arginine, threonine, tyrosine, and phenylalanine levels.

They were diagnosed as having citrin deficiency at ages of 3 weeks – 2 years 2 months based on gene analyses for the SLC25A13 determining the genotypes as follows: [I] 851del4, [II] IVS11 + 1G > A, [III] 1638ins23, [IV] S225X, [V] IVS13 + 1G > A, [VI] 1800ins1, [VII] R605X, [VIII] E601X, [IX] E601 K, [X] IVS6 + 5-G > A, [XI] R184X and [XIV] IVS6 + 1G > C (Table 1) [4–6].

The liver function tests at the ages of 4–12 months yielded normal results. Their blood levels of transaminase, gamma-glutamyl transpeptidase, total bile acids, and total bilirubin at presentation were entirely normal.

Blood and urine samples were collected at 10:30–11:30 before lunch after 4–5 h fasting. The methods and purpose of the study were explained to each child's parents. Their informed consent was obtained before enrollment of the child. Approval of the project was obtained from the institutional medical ethics committee.

Methods

Estimation of amino acids metabolism in terms of urea cycle

The urea cycle is initiated by carbamoylphosphate synthesis from ammonia via carbamoylphosphate synthetase, a limiting enzyme in the urea cycle [13]. Carbamoylphosphate is subsequently transformed into citrulline, which is ultimately transformed into arginine via argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) under a supply of aspartate by citrin [7,8,13]. To estimate the urea cycle function, plasma levels of ammonia and amino acids––including citrulline, arginine and ornithine––were examined. Plasma amino acids were determined using routine ion-exchange chromatography with an auto-analyzer (L822; Hitachi High-Technologies Corp., Tokyo, Japan).

Estimation of NO pathway

Arginine and citrulline are also involved in the nitric oxide (NO) pathway [13,14]. In fact, NO is synthesized from arginine by NO

Table 1
Background and present liver functions in patients.

Pt	Age at present	M/F	Gene mutations	NMS	Age at diagnosis	Liver dysfunction	AST (IU/L)	ALT (IU/L)	Γ-GTP (IU/L)	TBA (μmol/L)
1	2y7m	F	II/II	Met	3w	3w–6m	31	13	12	3.3
2	5y3m	M	II/V	Gal	5m	1m–5m	42	23	14	4
3	5y4m	M	II/V	(–)	4.5m	4.5m–7.5m	35	22	14	7.6
4	6y7m	M	I/II	Phe	1m	1m–5.5m	34	30	16	3.4
5	4y10m	F	I/II	Phe	1m	1m–4.5m	36	15	11	4.9
6	1y8m	M	I/V	Gal	22d	3w–7m	48	20	21	3.2
7	10y5m	M	II/VIII	(–)	1m	1m–4m	26	16	10	4.9
8	5y9m	M	II/II	(–)	1y2m	1m–12m	29	16	13	16.2
9	4y1m	F	II/V	Gal	3m	1m–8m	33	23	15	3.1
10	3y3m	M	II/II	Gal	4m	3w–11m	19	27	9	6.5
11	5y6m	M	II/V	(–)	2m	2m–9m	40	35	11	7.1
12	4y9m	F	III/V	Phe	2m	1m–7m	29	29	18	5.2
13	3y7m	F	II/VI	(–)	3m	3m–11m	37	34	15	5.5
14	6y2m	M	I/I	Gal	3m	1m–6m	31	16	11	4.1
15	4y0m	M	I/VI	Met	4m	3w–10m	39	19	15	8.2
16	2y11m	F	I/II	(–)	5m	2m–7m	23	20	10	3.2
17	3y11m	F	II/II	Phe	1m	1m–4m	37	27	16	6.5
18	7y1m	M	V/XIX	(–)	2y2m	2m–11m	28	14	14	5.9
19	5y5m	F	I/II	Gal	5m	1m–7m	22	11	11	2.9
20	5y6m	M	I/I	Gal	4m	3w–8m	39	26	14	4.9

NMS, newborn mass screening at 5 days of age; AST, aspartate aminotransferase (normal range: 5–40 IU/L); ALT, alanine aminotransferase (5–40 IU/L); Γ-GTP, gamma-glutamyl-transpeptidase (5–60 IU/L); TBA, total bile acids (2–15 μmol/L); Gal, hypergalactosemia; Phe, hyperphenylalaninemia; Met, hypermethionemia.

synthase (NOS). The availability of arginine is a rate-limiting factor in cellular NO production. Citrulline, a by-product of the NOS reaction, is recycled to arginine through successive actions of ASS and ASL, forming the citrulline-NO cycle. Therefore, in this study, blood levels of nitrite/nitrate (NO_x^-) as stable metabolites of NO and asymmetric dimethylarginine (ADMA) as a putative inhibitor of NOS were determined to estimate the NO pathway activity [15].

Serum levels of (NO_x^-) and ADMA were measured using the Griess method (nitrate/nitrite colorimetric assay; Cayman Chemical, Ann Arbor, ML, USA) and a recently developed enzyme-linked-immunosorbent assay method (ADMA–ELISA; DLD Diagnostika GmbH, Hamburg, Germany) [16]. Competitive ADMA–ELISA uses the microtiter plate format. Briefly, serum samples, as well as standards and kit controls, are acetylated in 96-well plates. The acetylated samples, standards and kit controls are pipetted into the respective wells of the ADMA-coated microtiter strips and incubated with a polyclonal antibody (rabbit-anti-*N*-acetyl-ADMA). After incubation, the antiserum solution is discharged and the wells are washed with washing buffer. A peroxidase-conjugated secondary antibody is added; then all wells are washed and incubated with tetramethylbenzidine solution as the substrate for peroxidase. The enzymatic reaction is stopped using an acidic stop solution; the absorbance is then measured using a microplate reader at 450 nm. The amount of antibody bound to the solid-phase ADMA is inversely proportional to the ADMA of the sample concentration.

Estimation of carbohydrate metabolism

Carbohydrate metabolism was estimated indirectly using blood glucose, galactose, lactate, and pyruvate levels. Blood levels of glucose, lactate, and pyruvate were determined using their respective enzymatic methods. Blood galactose concentrations in dried blood spots were determined with microassay using a fluorometric microplate reader, as described by Yamaguchi and colleagues [17].

Estimation of lipid metabolism

Lipid metabolism was estimated according to the blood levels of free fatty acids (FFA), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), phospholipids (PL), triglycerides (TG), and apolipoprotein (apo) levels. Malondialdehyde-modified LDL (MDA-LDL) as oxidized LDL (Ox LDL) was also determined.

Serum levels of TC, PL, and TG were determined with commercial kits (Kyowa Medex Co. Ltd., Tokyo, Japan) using enzymatic methods. Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd., Osaka, Japan.) [18]. The serum FFA level was measured using enzymatic methods (NEFA-SS kit Eiken; Eiken Chemical Co. Ltd., Tokyo, Japan). Furthermore, LDL-C was measured using an enzyme immunoassay with a commercial kit (LDL-C Daiichi; Daiichi Pure Chemicals Co. Ltd.). Serum levels of apoA-I, apo-B, apo-CII, and apo-E were determined using turbidimetric immunoassay (Apo-AI, apo-B, apo-CII, and Apo-E Auto·N 'Daiichi'; Daiichi Pure Chemicals Co. Ltd.). A sensitive enzyme-linked immunosorbent assay for detection of MDA-LDL in serum was used for determination of oxidized LDL [19]. In this assay, a monoclonal antibody interacts with MDA-apo-B.

Western blot analyses of biopsy specimens

Liver samples were obtained from two affected children (patients 19 and 20) by percutaneous liver biopsy (Table 1). Liver expressions of three important lipoprotein regulators were examined: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase as a key enzyme of cholesterol synthesis, LDL-receptor and scavenger receptor B-I (SR-BI) as a major HDL receptor. As control livers, liver fragments obtained from two non-related donors for liver transplantation (1 male and 1 female) with ages of 28 and 33 years were used; they were entirely healthy. The donors had no history of smoking.

The frozen samples (50–100 mg) were divided into cytoplasmic and nuclear fractions using nuclear and cytoplasmic extraction reagent kits (NE-PER™; Pierce Biotechnology Inc., Rockford, IL). The former was used for analyses of SR-BI, LDL-receptor, and HMG-CoA reductase.

These samples were separated using 10% SDS–polyacrylamide gel electrophoresis. Then they were transferred to nitrocellulose membranes using a semi-dry transfer unit.

After blocking with Tris-buffered saline containing 10% non-fat dried milk, the membranes were reacted with primary antibodies and then with peroxidase-conjugated secondary antibodies. After vigorous washing, the membranes were incubated with an enhanced chemiluminescence reagent (ECL; GE Healthcare Life Sciences, Tokyo, Japan), and exposed to X-ray film. The following primary antibodies were purchased from two companies: HMG-CoA reductase (mouse, polyclonal; Abcam plc., Cambridge, UK), SR-BI (goat, polyclonal; Lifespan Biosciences Inc., Seattle, WA), and LDL-receptor (chicken, polyclonal; Abcam plc.).

Estimation of oxidative stress

As the marker for oxidative stress, urinary acrolein-lysine reflecting the amounts of lipid peroxidation products in plasma and urine and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) reflecting oxidative DNA damage were examined. Furthermore, vitamin E, functioning as an antioxidant in plasma and activities of anti-oxidative enzymes such as catalase and superoxide-dismutase (SOD) in erythrocytes, was examined.

Concentrations of urinary acrolein-lysine and 8-OHdG were determined, respectively, using competitive ELISA kits: ACR-Lysine Adduct ELISA (NOF Corp., Tokyo, Japan) and 8-OHdG Check (the Institute for the Control of Aging, Shizuoka, Japan) [20]. Plasma vitamin E levels were measured using high-performance liquid chromatography (HPLC), as described in a previous report [21].

The SOD activity was determined using spectrophotometry at 505 nm (RANSOD kit; Randox Laboratories Ltd.; Antrim, United Kingdom), as described in a previous report [22]. Catalase activity was determined using the method described by Aebi [23]. In brief, we monitored the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 M potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml.

Results

Effects on amino acids involved in the urea cycle and NO pathway

Among amino acids, despite the normal plasma ammonia level, ornithine and citrulline levels of the affected children were, respectively, 1.7 times ($p < 0.001$) and 1.4 times ($p < 0.01$) higher than those of the controls. Although their arginine level was 0.87 times as high as the controls' level, no significant difference was found between these two groups (Table 2).

The other amino acid levels of the affected patients were comparable to those of the control levels. Blood NO_x^- and ADMA levels were not different between the two groups, suggesting that the NO pathway in the affected children remained normal (Table 2).

Effects on carbohydrate metabolism

No significant difference was found between these two groups' blood glucose, galactose, lactate, or pyruvate levels at fasting

Table 2
Blood levels of amino acids involved in the urea cycle, NO_x^- and ADMA.

	Arginine (μmol/L)	Ornithine*** (μmol/L)	Citrulline** (μmol/L)	NO_x^- (μmol/L)	ADMA (μmol/L)	Ammonia (μg/dl)
20 patients	74.2(14.4)	105.1(24.2)	40.8(6.3)	31(5)	0.78(0.11)	35(14)
Ranges	45.4–137.8	65.0–193.4	25.3–56.4	22–49	0.60–1.12	20–91
32 controls	85.0(13.2)	61.3(13.6)	28.2(6.3)	30(9)	0.63(0.17)	31(9)
Ranges	52.8–106.8	40.1–90.0	14.4–41.4	22–49	0.42–0.97	18–49

NO_x^-, nitrite/nitrate; ADMA, asymmetric dimethylarginine.
Presented data are mean (SD) values and ranges.
** $p < 0.01$ versus controls.
*** $p < 0.001$ versus controls.

Table 3
Blood levels of carbohydrate at 4–5 h fasting.

	Glucose (mg/dl)	Galactose (mg/dl)	Pyruvate (mg/dl)	Lactate (mg/dl)	*L/P**
Patients ($n = 20$)	84(5)	0.3(0.1)	0.8(0.3)	12(3)	15(2)
Ranges	72–95	0.1–0.6	0.2–1.7	6–26	9–18
Controls ($n = 32$)	85(5)	0.3(0.1)	0.8(0.2)	10(4)	11(1)
Ranges	76–99	0.1–0.5	0.3–1.1	7–19	7–13

L/P: ratio of lactate to pyruvate.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.

(Table 3). The *L/P* ratio in the affected children was significantly higher than that in the controls ($p < 0.05$), suggesting a high ratio of NADH to NAD^+ and/or suppressed mitochondrial functions in the affected children [24].

Effects on lipid metabolism

Serum LDL-C and HDL-C levels in the affected children were 1.5 times higher than those in the age-matched controls, resulting in high total cholesterol levels (Table 4). Triglycerides and FFA levels were not different between these two groups. The apo-AI and apo-B levels in the affected children were apparently higher than those in the controls, respectively, reflecting the higher LDL-C and HDL-C levels (Table 4). Surprisingly, oxidized LDL levels were much higher in the affected patients.

Western blot analyses showed that liver HMG-CoA reductase expression was elevated in the two affected children, although their liver LDL-receptor and SR-BI expressions were similar to those in the control subjects (Fig. 1).

Effects of oxidative stress

Urinary acrolein-lysine and urinary 8-OHdG in the affected children were significantly higher than those in the age-matched con-

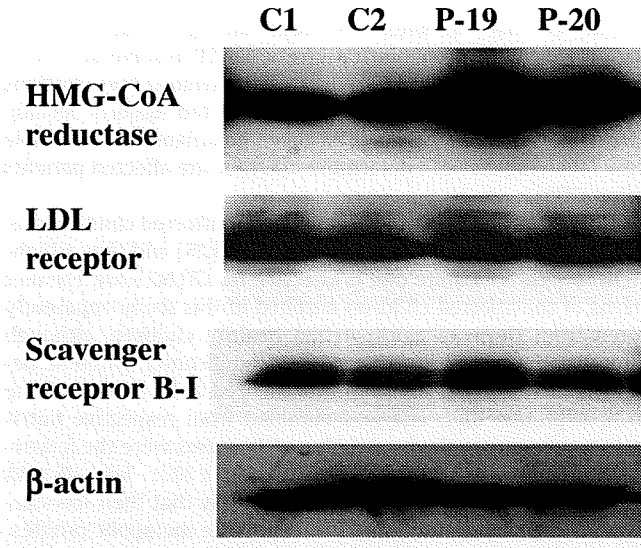

Fig. 1. Western blots of liver biopsy specimens against 3-hydroxy-3-methylglutaryl-coenzyme A reductase, low-density lipoprotein receptor and scavenger receptor B-I. HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL-receptor, low-density lipoprotein receptor. Liver biopsy specimens were obtained from healthy controls (C1 and C2) and two affected children (P19 and P20).

Table 4
Blood lipid and apolipoprotein levels at a fasting state.

Lipids	TC*** (mg/dl)	LDL-C*** (mg/dl)	HDL-C*** (mg/dl)	PL*** (mg/dl)	TG (mg/dl)	FFA (mmol/L)
Patients ($n = 20$)	213(32)	116(23)	79(7)	237(40)	77(19)	0.8(0.2)
Ranges	153–319	76–196	54–108	189–333	38–124	0.4–1.2
Controls ($n = 32$)	169(22)	85(13)	54(11)	193(25)	80(25)	0.9(0.2)
Ranges	111–207	42–106	39–77	123–239	35–139	0.4–1.5
Apoproteins & Ox LDL	Apo-AI*** (mg/dl)	Apo-AII *** (mg/dl)	Apo-B*** (mg/dl)	Apo-CII* (mg/dl)	Apo-E* (mg/dl)	Ox LDL*** (U/L)
Patients (n=20)	169(20)	36(5)	111(22)	3.5(1.1)	5.6(1.5)	82(24)
Ranges	133–213	30–50	79–192	1.5–7.5	3.1–9.4	39–156
Controls (n=32)	127(16)	30(4)	78(13)	3.1(1.1)	4.6(1.1)	25(7)
Ranges	88–165	20–42	48–109	0.8–5.5	1.9–7.5	5–50

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PL, phospholipids; TG, triglycerides; FFA, free fatty acids; Ox LDL, oxidized LDL.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.
*** $p < 0.001$ versus controls.

Table 5
Levels of urinary biomarkers for oxidative stress, anti-oxidant enzyme activities in erythrocytes and blood vitamin E level.

	3-OHdG*** (ng/mg Cr)	Acrolein-lysine** (nmol/mg Cr)	SOD*	Cat*	Vitamin E** (mg/dl)
Patients ($n = 13$)	67(21)	481(125)	1.49(0.34)	3.60(0.52)	0.60(0.21)
Ranges	32–100	220–686	0.92–1.92	2.77–4.44	0.32–1.29
Controls ($n = 32$)	19(5)	272(90)	1.06(0.18)	2.96(0.21)	0.98(0.14)
Ranges	11–29	70–424	0.80–1.50	2.55–3.56	0.67–1.45

3-OHdG; urinary 8-hydroxy-2′-deoxyguanosine; SOD: superoxide-dismutase (U/mg protein); Cat, catalase (pmol/mg protein); Cr, creatinine.
Presented data are mean (SD) values and ranges.
* $p < 0.05$ versus controls.
** $p < 0.01$ versus controls.
*** $p < 0.001$ versus controls.

trols (acrolein-lysine, $p < 0.01$; 8-OHdG, $p < 0.001$). In contrast, blood vitamin E levels in the affected patients were significantly lower than those in the controls (Table 5). Erythrocyte SOD and catalase activities in the affected patients were significantly higher than those in the age-matched controls ($p < 0.05$) (Table 5).

These findings suggest that the affected children were substantially influenced by oxidative stress.

Discussion

Citrin deficiency manifests as NICCD during neonatal and infancy periods. Clinical manifestations of NICCD resolve at around 12 months of age, with no subsequent overt clinical presentations [1,3,6,8–12]. However, about one-fifth of affected subjects develop CTLN2 at ages of 11–79 years. It has been postulated that multiple factors involving diet can determine whether the affected patients develop CTLN2.

Results of recent studies indicate that the affected children prefer a low-carbohydrate high-fat/protein diet [25] and that a high-protein low-carbohydrate diet is effective for CTLN2 [26]. The diet patterns of the affected children enrolled in this study apparently differed from those of age-matched healthy children, although the total daily energy intake was not significantly different between these two groups. The former favored a low-carbohydrate high-lipid/protein diet: energies obtained from respective nutritional components to the total daily energy ratios were the following: carbohydrates, 35 ± 5% (control, 55 ± 3%); fat, 45 ± 4% (29 ± 2%); protein, 20 ± 3% (14 ± 2%). It is likely that their low-carbohydrate high-lipid/protein diets affected the metabolic profiles.

Results of this study suggest that the affected children at the silent stage differed from age-matched healthy children in many metabolic aspects.

Despite the high-protein intake, plasma ammonia levels in the affected children were comparable to those in healthy age-matched controls. However, plasma citrulline and ornithine levels were substantially higher in the affected children than in the healthy children. Citrulline is synthesized from ornithine and carbamoylphosphate by ornithine transcarbamylase. It is subsequently transformed by ASS into argininosuccinate under the supply of aspartate from the mitochondria by AGC2-citrin [3,7,8]. From this context, amino-profiles of affected children enabled us to assume that the supply of aspartate to cytosol from mitochondrial fraction remains at lower levels in them. Strikingly high plasma citrulline levels in NICCD and CTLN2 engender high synthesis of arginine by ASS and ASL in kidney or intestine, resulting in a high plasma arginine level [1,2,27]. As compared to NICCD and CTLN2, the increase in plasma citrulline level at the silent stage was too minute to increase plasma arginine levels.

The NO synthesis in the affected patients remained normal. Their NO_x^- and ADMA levels resembled those in the age-matched healthy controls. We recently reported that urea cycle defects exhibiting markedly abnormal arginine and citrulline levels show abnormal NO synthesis [28]. The citrulline level abnormalities in the affected children during the silent period might be too slight to affect NO synthesis.

The *L/P* ratio in the affected children was significantly higher than that in the controls, suggesting that their NADH to NAD^+ ratio remains high even during the silent period. The *L/P* ratio is determined by the NADH to NAD^+ ratio in cytosol [24]. It has been postulated that citrin plays a crucial role in the regulation of the NADH to NAD^+ ratio in the cytosol and that citrin deficiency presents a high NADH to NAD^+ ratio [3,7,8].

The affected children showed high levels of serum total cholesterol, LDL-C, and HDL-C. The mechanistic explanation for the hypercholesterolemia remains unclear. Affected patients favor a considerably high-lipid diet [25]. Accordingly, their hypercholesterolemia is expected to be at least partly attributable to such a dietary habit. Using Western blot analysis, we examined HMG-CoA reductase expressions in liver samples that had been obtained by percutaneous liver biopsies from a few affected patients. The results suggest that liver HMG-CoA reductase expression was increased in such patients, although expressions of LDL-receptor and HDL receptor such as SR-BI in their liver tissues were comparable to those in the controls (Fig. 1). For that reason, we now assume that hypercholesterolemia in the affected children was at least partly attributable to increased cholesterol synthesis.

On the other hand, their triglyceride levels remained at values comparable to those of the age-matched healthy children. As described above, our patients consumed a considerably low-carbohydrate diet during this study. Probably, such a diet prevented hypertriglyceridemia. A high-carbohydrate diet has been proven to promote production of NADH and thereby stimulate productions of triglycerides [1,8,29,30]. In particular, citrin-deficient individuals are apparently directed easily to hypertriglyceridemia [1,8,26]. We found in many cases that CTLN2 patients developed hypertriglyceridemia when consuming a high-carbohydrate diet. Furthermore, we are now following two citrin-deficit adults with postprandial hypertriglycemia but without overt liver dysfunction.

We inferred that the affected children were subjected persistently to considerable oxidative stress, although is difficult to judge the magnitude of the oxidative stress merely using oxidative stress biomarkers. Nevertheless, decreased blood vitamin E levels and increased erythrocyte anti-oxidant enzyme activities implied augmented oxidative stress in the affected patients. The increased SOD and catalase activities in erythrocytes can be interpreted as responses to increased plasma oxidants. The considerable increase in oxidized LDL supports this notion.

We speculate that the augmented oxidative stress might be partly attributable to the increased cytosolic NADH. Accumulation of cytosolic NADH has been shown to have some probability of causing oxidative stress [7,31,32].

Evidence that dyslipidemia and oxidative stress are closely related to development of liver steatosis or steatohepatitis has been accumulating [33,34]. Considering that citrin deficiency often

develops liver steatosis as a clinical presentation of CTLN2 in later life, it might be important to improve dyslipidemia and to reduce oxidative stress for management of citrin deficiency.

Results of this study show that metabolic abnormalities such as hypercitrullinemia and hypercholesterolemia were sustained in children with citrin deficiency, even during the silent stage. Results provide evidence that the affected children were subjected persistently to oxidative stress.

Further study is necessary to determine whether such sustained metabolic abnormalities might induce development of CTLN2.

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