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RESEARCH ARTICLE

A Missense Mutation in the OCTN2 Gene Associated With Residual Carnitine Transport Activity

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Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation caused by defective carnitine transport. This disease can present early in life with hypoketotic hypoglycemia and acute metabolic decompensation, or later in life with skeletal or cardiac myopathy. Mutations abolishing the function of OCTN2, an organic cation/carnitine transporter with twelve putative transmembrane spanning domains, were recently demonstrated in patients with early- and late-onset (up to seven years of age) presentation of this syndrome. Most of the reported mutations are null alleles. Here we evaluate the OCTN2 gene in a male patient who presented at seven years of age with severe dilated cardiomyopathy. Plasma carnitine levels were undetectable and carnitine transport by his fibroblasts was reduced to about 3% of normal controls. This patient was homozygous for a single base pair change in exon 8 of the OCTN2 gene (1354G>A) converting the codon for Glu 452 to Lys (E452K) in the predicted intracellular loop between transmembrane domains 10 and 11. Stable expression of the mutant E452K-OCTN2 cDNA in Chinese hamster ovary (CHO) cells caused a partial increase in carnitine transport to 2–4% of the levels measured in the wild type transporter. This reduced transport activity was associated with normal Km toward carnitine (3.1 ± 1.1 μM), but markedly reduced Vmax. These results indicate that primary carnitine deficiency can be caused by mutations encoding for carnitine transporters with residual activity, and that the E452K affects a domain not involved in carnitine recognition. Hum Mutat 15:238–245, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: **carnitine transport; carnitine deficiency; fatty acid oxidation; cardiomyopathy; SLC22A5; OCTN2**

INTRODUCTION

Primary carnitine deficiency (MIM# 212140) is a recessively inherited disorder of fatty acid oxidation due to defective carnitine transport [Roe and Coates, 1995; Scaglia and Longo, 1999]. Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent beta oxidation, and the lack of carnitine impairs the ability to use fat as fuel during periods of fasting or stress. This can result in hypoketotic hypoglycemia, Reye syndrome, and sudden infant death in younger children or in skeletal or heart myopathy with insidious onset later in life.

The gene for primary carnitine deficiency encodes a functional carnitine transporter named OCTN2 (SLC22A5; MIM# 603377) [Wu et al., 1998; Tamai et al., 1998] which maps to chromosome 5q31.1-32 [Shoji et al., 1998; Wu et al., 1998]. Mutations in this gene have been reported in a few patients with primary carnitine deficiency [Nezu et al., 1999; Wang et al., 1999; Tang et al., 1999; Burwinkel et al., 1999], most of whom presented early in life with a severe metabolic decompensation. Most mutations reported to date cause the premature insertion of STOP codons due

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to single bp changes, insertions, or deletions [Nezu et al., 1999; Wang et al., 1999, Tang et al., 1999]. The only missense mutation identified and transiently expressed in heterologous cells completely abolishes carnitine transport, confirming its causative role in this condition [Tang et al., 1999].

Myopathic involvement is the most frequent presentation of primary carnitine deficiency [Stanley et al., 1991]. In at least two families, both the hypoglycemic and cardiomyopathic presentations have been reported in siblings [Stanley et al., 1991], indicating that environmental factors such as supply of carnitine in the diet or intercurrent infections which increase energy demand play an important role in determining time and type of presentation. This is further underscored by the recent report of fulminant death in the child of a vegetarian mother with limited assumption of carnitine during pregnancy [Rinaldo et al., 1997]. However, it is unclear whether there are mutations retaining residual carnitine transport activity, and whether these could contribute to a milder phenotype with late-onset presentation.

We report a new patient with primary carnitine deficiency who presented at seven years of age with cardiomyopathy. This patient, whose parents were consanguineous, was homozygous for a single missense mutation converting the codon for Glu 452 to Lys (E452K). Expression of the mutant transporter in CHO cells confirmed the causative role of this mutation in reducing carnitine transport, but also indicated that the mutant transporter retained minimal but significant residual carnitine transport activity.

MATERIALS AND METHODS

Subjects

The proband, a Pakistani male, is the first and only child of a healthy 38-year-old mother. Birth weight was 2.9 Kg after 38.5 wk of uncomplicated pregnancy. The child had mild jaundice which resolved with phototherapy, and he was discharged home in 48 hr. He was noted to have nystagmus and was diagnosed with oculocutaneous albinism, a condition also affecting his father. He was otherwise healthy until six years of age, when he presented with weakness and decreased exercise tolerance. This condition was initially attributed to a viral illness, but persisted after the infection resolved. A chest X-ray demonstrated enlarged heart, for which a cardiology consultation was obtained. This revealed a heart murmur, in addition to fair skin with a number of moles, fair hairs, horizontal nystagmus, and an enlarged liver which was about 3 cm below the right costal margin. Growth parameters and development were age-appropriate. Echocardiography indicated a dilated cardiomyopathy, with a markedly reduced shortening fraction of 9% (normal 28–38%) and mild valvular regurgitation. An endomyocardial biopsy of the right ventricle was consistent with metabolic cardiomyopathy, with cytoplasmic lipids and phospholipid lamellar bodies by electron microscopy. The child was started on diuretics and angiotensin-converting enzyme inhibitors in addition to cardiac glycosides, with minimal improvement. Given the poor response, the child was evaluated for cardiac transplantation. Plasma carnitine obtained at that time was undetectable (normal 30–50 μM) with normal urine organic acids and plasma amino acids. A diagnosis of primary carnitine deficiency was considered and fibroblasts were sent to confirm the diagnosis. The child was given carnitine supplements which slowly restored cardiac function.

Family history was significant for consanguinity, since the parents are first cousins (Fig. 1). The father has two healthy boys from a previous marriage with a second cousin once removed. The father has oculocutaneous albinism and underwent cardiac bypass for atherosclerotic cardiovascular disease. Total plasma carnitine levels were 37.4 (24 free) and 47.1 (29.4 free) μM in the mother and father, respectively.

Cell Strains and Carnitine Transport

Fibroblasts from patient Balt-1 with primary carnitine deficiency were obtained by skin biopsy for diagnostic purposes. Fibroblasts from patient 10665 were obtained from the NIGMS Human Genetic Mutant Cell Repository, Coriell Cell Repositories (Camden, NJ). Fibroblasts from patient 2996 were obtained as previously described [Scaglia et al., 1998]. Fibroblasts were grown in Dulbecco-modified minimal essential medium (MEM) supplemented with 15% fetal bovine serum. CHO cells were grown in Ham F12 medium supplemented with 6% fetal bovine serum.

Carnitine transport was measured at 37°C with the cluster-tray method, as described previously [Scaglia et al., 1999]. Cells were grown to confluence in 24-well plates (Costar, Cambridge, MA) and depleted of intracellular amino acids by incubation for 90 min in Earle's balanced salt solution containing 5.5 mM D-glucose and supplemented with 0.5% bovine serum albumin. Carnitine (0.5 μM, 0.5 μCi/ml) was then added to the cells for the time indicated. Nonsaturable carnitine transport was measured in the presence of 2 mM cold carnitine.

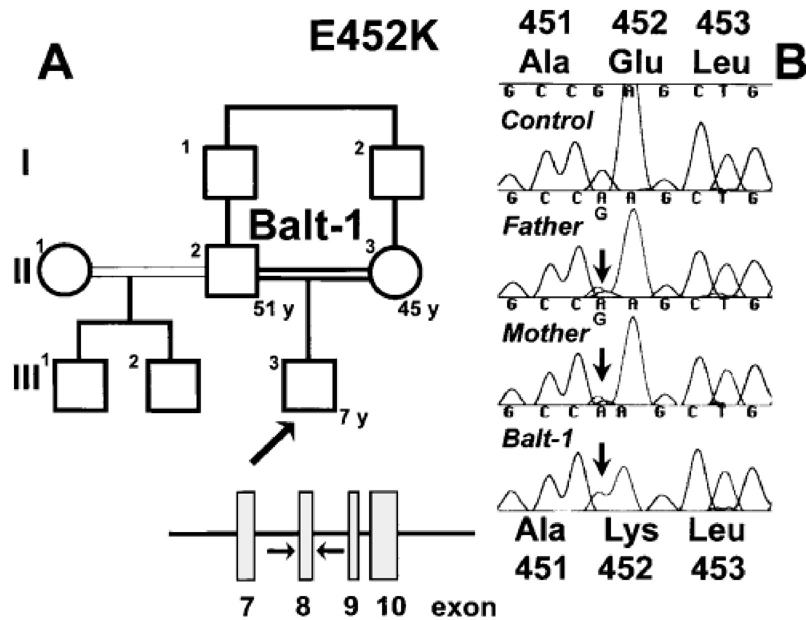

FIGURE 1. **Pedigree (A) and mutation in exon 8 of the OCTN2 gene (B) of patient Balt-1 (and his parents) with cardiomyopathy and primary carnitine deficiency. Exon 8 was amplified by PCR and sequenced directly without cloning. The mutation was confirmed by sequencing in both directions and in independent PCR products.**

Kinetic experiments were performed in the presence of increasing concentrations of cold carnitine. The transport reaction was stopped by rapidly washing the cells four times with ice-cold 0.1 M $MgCl_2$. Intracellular carnitine was then corrected for intracellular water content and expressed as nmol/ml cell water [Scaglia et al., 1998]. Saturable carnitine transport was calculated by subtracting nonsaturable carnitine transport from total transport, and values are reported as means ± SE of 3–6 independent determinations.

Kinetic experiments were analyzed by nonlinear regression analysis according to a Michaelis-Menten equation [Scaglia et al., 1999]. Parameters are expressed as means ± 95% confidence intervals, unless otherwise indicated.

Site-Directed Mutagenesis

The OCTN2 expression vector was generated by inserting the OCTN2 cDNA [Wu et al., 1998] in pcDNA3 as previously described [Wang et al., 1999]. The E452K mutation was introduced by site-directed mutagenesis using the Quick Change system (Stratagene, La Jolla, CA), following the manufacturer's instructions. The final clone was sequenced to confirm the presence of the mutation and the absence of PCR artifacts. The clone obtained was transfected into CHO cells using lipofectamine [Wang et al., 1999]. Cells were selected for 2 wk in 0.8 mg/ml of G418 and then used for the transport assay.

DNA Analysis

Genomic DNA was extracted from fibroblasts by standard methods and amplified using PCR and primers flanking each of the ten exons (Table I). PCR amplification of genomic DNA was performed according to standard conditions, except that 10% DMSO was included in the reaction when exon 1 was amplified. PCR products were

TABLE 1. Primers for Amplification and Sequencing of the Ten Exons of the OCTN2 Gene in Patients With Primary Carnitine Deficiency

5′ primer	Exon	bp	3′ primer
GGAACGTTCTAACATCCTTGG	1	551	TGACGCAGAGGGAGGGTCAG
TGGCAGGATGTTCTGACTTC	2	282	ACTACCAACTGAAATCAAGGG
ACTTGGTGGAGCCCATTCC	3	309	GACATCACAGCTGTCTCCAG
ACTGCTAACTCGACCTCCC	4	248	AATCATCCTGCCAGTGGGCA
CTATGGCTGTGCTCTACCTG	5	256	CTCAAATCACGGTCAGTCTG
CTGAGTCTCTGACCACCTC	6	191	TTGTCTGGAAGCCTCAGGCA
TGGGAAAGATGTGGATACTGC	7	334	GAGACAGCCTGGTAGACAG
TCAATAGCTGCATGCCATGG	8	311	GCTCACATTCAAGCCAGTTAG
GCATAAAGGGGTAGATGAGAG	9	282	TATTGTGAGGGGCTCCTGAG
AGGATTCTCTTCCCAGGGAAG	10	238	GCAAGACAGTCTTTCCTCTTCAG

The size of the amplified DNA fragment is indicated.

visualized by agarose gel electrophoresis, purified by Qiagen column, and sequenced directly without subcloning [Longo et al., 1992, 1993; Wang et al., 1999] using an ABI automated DNA sequencer (Perkin Elmer, Wellesley, MA). The mutation was confirmed by sequencing in both directions in two independent PCR products. The E452K mutation did not affect known restriction sites.

RESULTS

Carnitine Transport in Fibroblasts From Patients With Primary Carnitine Deficiency

Figure 2 shows carnitine transport by fibroblasts of patients with primary carnitine deficiency. Saturable carnitine transport was indistinguishable from zero in patients with primary carnitine deficiency, number 2996 and 10665 (who carry null OCTN2 alleles, [Wang et al., 1999]), and was reduced to about 3% of controls in fibroblasts from patient Balt-1 (Fig. 2A). This confirmed the diagnosis of primary carnitine deficiency.

The small but significant residual levels of carnitine transport allowed kinetic analysis. Normal fibroblasts had a Vmax of 45.4 ± 1.5 nmol/ml cell water/h and a Km toward carnitine of 6.2 ± 0.8 μM (Fig. 2B). Cells from patient Balt-1 had marked reduction in the Vmax value (1.9 ± 0.1 nmol/ml cell water/h, $P < 0.05$ versus control) and a mild increase in the Km value (9.4 ± 1.5 μM) which overlapped with the range of normal controls (6–9 μM, [Scaglia et al., 1998, 1999]). Fibroblasts from patient 2996, carrying two nonsense alleles [Wang et al., 1999], lacked saturable carnitine transport and had a linear increase in carnitine transport with carnitine concentration.

Identification of Mutations in the OCTN2 Gene

Analysis of genomic DNA by PCR and primers flanking the ten exons of the OCTN2 gene indicated that patient Balt-1 was homozygous and both parents heterozygous for a G to A transition in exon 8 converting the codon for Glu 452 to a Lys (E452K) (Fig. 1B). This non-conservative substitution is located in the intracellular loop between the predicted transmembrane domains 10 and 11. This residue is conserved in the human, rat, and mouse OCTN2 cDNA.

Expression of the E452K Mutant OCTN2 cDNA

To confirm its causative role, the E452K mutation was stably expressed in CHO cells and evaluated for its effects on carnitine transport (Fig. 3A). CHO cells had low levels of endog-

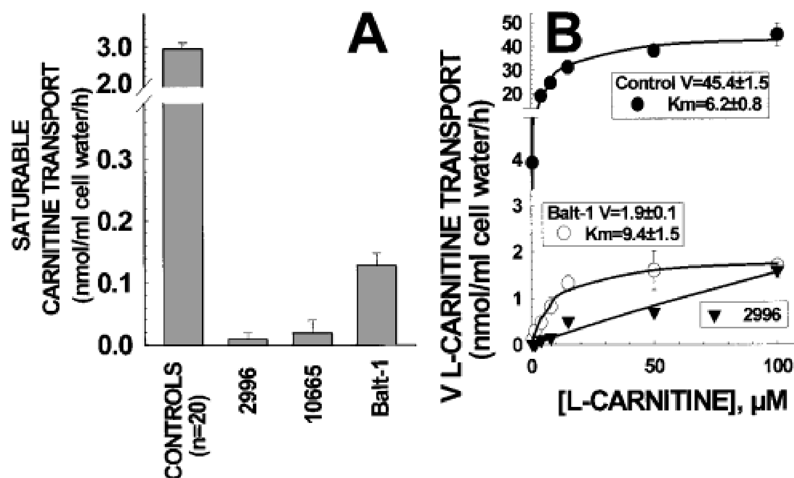

FIGURE 2. **Carnitine transport by fibroblasts obtained from patient Balt-1 with primary carnitine deficiency. A: Carnitine (0.5 μM) transport was measured for 4 hr at 37°C. Nonsaturable transport, measured in the presence of 2 mM cold carnitine, was subtracted from total transport to obtain saturable carnitine transport. The nonsaturable component was similar in normal and mutant cells. It corresponded to about 10% of total carnitine transport in control cells, while it accounted for 90% or more of total transport in patients' cells. Data are means ± SE of six observations. Carnitine transport by the patients' cells was significantly ($P < 0.01$) different from that of controls (note the broken scale on the Y axis). B: Kinetic analysis of carnitine transport in fibroblasts of patients Balt-1 and 2996. Carnitine transport was measured for 4 hr in the presence of increasing concentrations of carnitine (0.5–100 μM). Non saturable carnitine transport (measured in the presence of 2 mM carnitine) was subtracted from all points prior to plotting. Data are means of triplicates ± SE which is not shown when within the symbol. Lines represent the best fit to a Michaelis-Menten equation. In patient 2996, the low levels of carnitine transport prevented the estimation of kinetic parameters.**

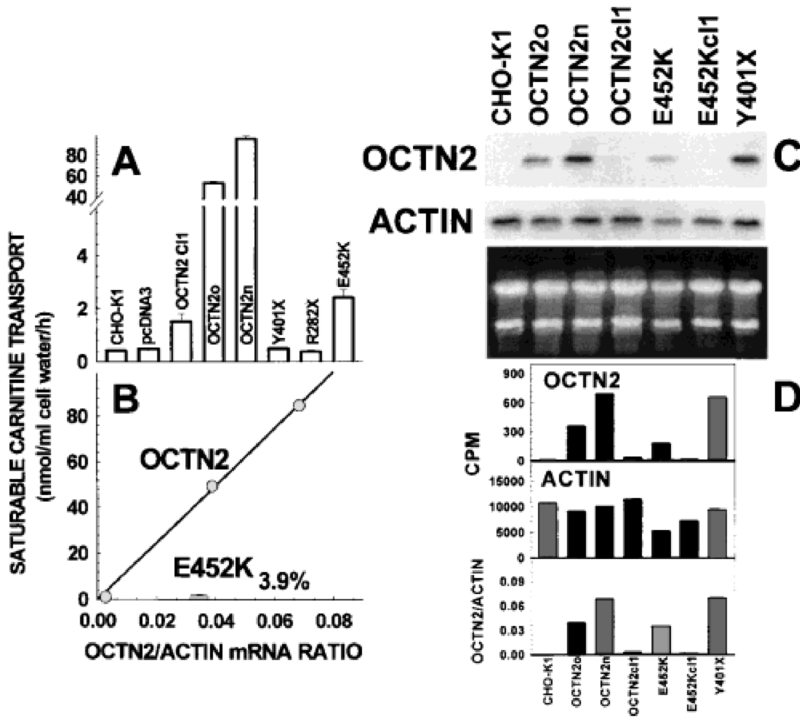

FIGURE 3. **Carnitine transport and OCTN2 mRNA levels in CHO transfected with normal and mutant OCTN2 cDNA. CHO were transfected with the OCTN2 cDNA in pcDNA3 and selected for resistance to 0.8 mg/ml of G418 for 2 wk. A: Carnitine (0.5 μM) transport was measured for 1 hr. Data are means ± SE of triplicates. OCTN2Cl1, OCTN2o, and OCTN2n are different clones of CHO cells transfected with the normal OCTN2 cDNA. B: Carnitine transport was corrected for endogenous carnitine transport by CHO cells and plotted against the OCTN2/actin mRNA ratio (panel D) measured in the same cells. Points are means ± SE of triplicates (SE was within the symbol for OCTN2). C: RNA was extracted from CHO cells and analyzed by Northern blot analysis with hybridization to the full-length OCTN2 cDNA and to a human actin cDNA fragment. The actual agarose gel is shown below the autoradiograms. D: Quantification of the Northern blot by counting. CPM in each band were counted on an Instant Imager (Packard Instruments, Downers Grove, IL) and normalized to actin.**

enous carnitine transport which, at 0.5 μM, corresponded to about one-sixth of that measured in human fibroblasts (0.43 ± 0.05 versus 3.0 ± 0.2 nmol/ml cell water/h in human fibroblasts). Stable transfection of OCTN2 increased carnitine transport up to 220 times (Fig. 3A). By contrast, transfection with the vector alone (pcDNA3) or with OCTN2 mutagenized to contain STOP codons identified in patients 2996 and 10665 (Y401X and R282X [Wang et al., 1999]) failed to increase carnitine transport. Transfection with the E452K-mutant OCTN2 significantly increased carnitine transport. This increase ranged between three and six times above the levels measured in untransfected CHO cells in four different experiments.

To define the amount of residual carnitine transport left by the E452K mutation, carnitine transport was corrected for the endogenous levels of carnitine transport in untransfected CHO cells and normalized to OCTN2 mRNA levels. OCTN2 mRNA levels were measured by Northern blot analysis and normalized to the actin mRNA (Fig. 3C). CPM in each band were quantified by a micro array detector (Fig. 3D). Different clones of cells (OCTN2Cl1, OCTN2o, and OCTN2n) transfected with the normal OCTN2 cDNA had a linear increase of carnitine transport with OCTN2 mRNA levels (Fig. 3B). The increase in saturable carnitine transport by cells expressing the mutant E452K OCTN2 cDNA corresponded to about 3.9% of the increase measured with the normal OCTN2 cDNA when compared to cells expressing similar amounts of normal OCTN2 mRNA.

Kinetic analysis indicated that parental CHO cells (CHO-K1) transported carnitine with a Km of 74 ± 11 μM (range 74–115 μM in five different experiments) and a Vmax of 27.8 ± 1.8 nmol/ml cell water/h (Fig. 4A). The value of Km is significantly above that of human fibroblasts (Fig. 2) and that of the human OCTN2 cDNA expressed in kidney 293 cells (4–9 μM, [Tamai et al., 1998]). Different clones of CHO cells (OCTN2, OCTN2o, OCTN2n, OCTN2cl3) transfected with the normal OCTN2 cDNA had a Vmax ranging from 180–1,043 nmol/ml cell water/h and a Km of 3.3–5.1 μM (Fig. 4A, B).

CHO cells transfected with the Y401X mutation had no significant changes in Vmax or Km from untransfected CHO cells (not shown). Initial experiments with the E452K-CHO cells studied in Figure 3 suggested that this mutation did not affect the Km for carnitine recognition, but markedly decreased the Vmax. However, the relatively small increase in carnitine transport in these cells as compared to parental CHO cells rendered interpretation of these data problematic. Therefore, we selected a stable transformant (E452K2) with higher levels of carnitine transport whose ki-

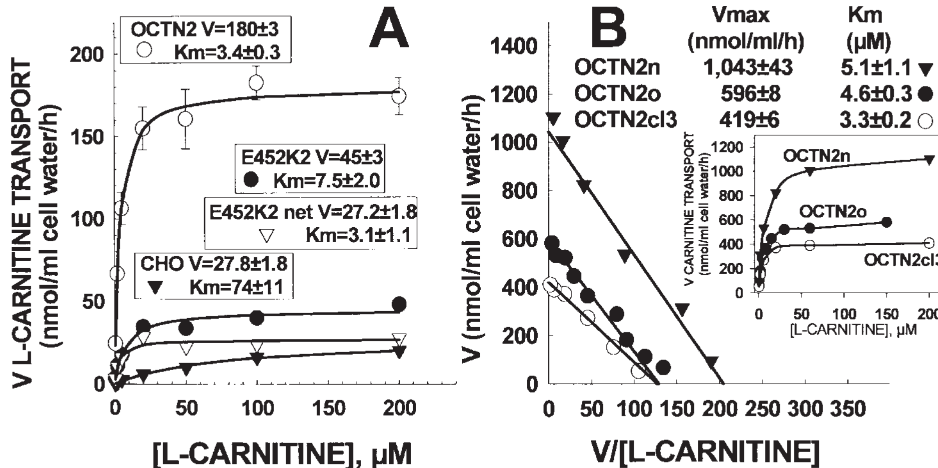

FIGURE 4. **Kinetic analysis of carnitine transport by CHO cells transfected with normal (B) and mutant (A) OCTN2 cDNA. Carnitine (0.5–200 μM) transport was measured for 1 hr (10 min for OCTN2). Points are average of triplicates. Data were corrected for nonsaturable uptake (measured in the presence of 2 mM cold carnitine) prior to nonlinear regression analysis according to a Michaelis Menten equation. Nonsaturable carnitine (0.5 μM) transport was negligible in cells transfected with the normal OCTN2 cDNA and was less than 10% of total in E452K2 cells. A: Data are reported in a V versus [S] plot. E452K2 cells are a second clone of CHO cells transfected with the E452K mutant cDNA, with higher carnitine transport activity than that of the cells presented in Figure 3. B: Data are reported according to an Eadie-Hofstee graphical representation, with the inset showing the V versus [S] plot. Km values are in μM, Vmax in nmol/ml cell water/hr.**

netic constant is shown in Figure 4A. These cells had a significantly ($P < 0.05$) decreased Km toward carnitine (7.5 ± 2 μM) as compared to untransfected CHO cells. When endogenous carnitine transport by CHO cells was subtracted from total uptake, the residual transport by E452K2 cells (net uptake) had a Km of 3.1 ± 1.1 μM, indistinguishable from that of cells expressing the normal OCTN2 (Fig. 4B), but the Vmax (27.2 ± 1.8 nmol/ml cell water/h) corresponded to only 2.7% that of CHO cells expressing similar amounts of normal OCTN2 mRNA (OCTN2n). Four independent clones of E452K transfectants had variably reduced Vmax, but the Km ranged between 3.1 and 9.7 μM. This result indicates that the increase in carnitine transport caused by the E452K mutation is due to transporters retaining normal affinity toward carnitine.

DISCUSSION

Primary carnitine deficiency is an autosomal recessive disorder caused by impaired carnitine transport. Mutations in the organic cation/carnitine transporter OCTN2 have been demonstrated in a few families, but so far none has been shown to retain significant residual transport activity. Here we report the first patient homozygous for a missense mutation retaining 2–4% of the normal OCTN2 transport activity. This mutation converted the codon for Glu452 to Lys (E452K) in the portion of transporter encoded by exon 8 of the gene (Fig. 1). Residual carnitine transport was demonstrated both in fibroblasts homozygous for this mutation (Fig. 2) and in CHO cells transfected with the E452K-mutant cDNA (Figs. 3, 4). It is unclear at present whether the residual carnitine transport may account for the relatively milder cardiomyopathic presentation during school-age years compared to the acute metabolic decompensation observed in other children with the same condition earlier in life. In a few families, with presumably the same identical mutation in related individuals, both types of presentations have been described [Stanley et al., 1991], indicating that environmental factors, such as the supply of carnitine with the diet or infections and fasting which increase the requirements for fatty acid oxidation, play a major role in determining the timing of phenotypic expression. In the families in which a mutation has been identified, the majority presented with Reye-like episodes [Tang et al., 1999; Wang et al., 1999; Nezu et al., 1999; Burwinkel et al., 1999], and only a few presented with cardiomyopathy. Homozygosity for the same null allele (R282X) was identified in two unrelated patients, one presenting with acute metabolic decompensation and one with cardiomyopathy [Wang et al., 1999; Burwinkel et al., 1999], suggesting that the same primary genetic defects may cause different phenotypic expression.

Nevertheless, it is possible that "leaky" mutations, such as E452K, may result only in late-onset cardiomyopathy, with the presentation of our patient representing the severe end of the spectrum possibly due to insufficient carnitine in the diet. The identification of similar mutations, leaving residual carnitine transport activity in a larger cohort of patients may further clarify this issue.

Expression of the E452K mutant carnitine transporter in CHO cells (Figs. 3, 4) confirmed the causative role of this mutation in reducing carnitine transport in this patient's fibroblasts (Fig. 2). CHO cells transfected with the normal OCTN2 cDNA had a linear increase in the Vmax for carnitine transport correlating with OCTN2 mRNA levels (Fig. 3), suggesting (but not proving) that OCTN2 encodes for all the components of the carnitine transporter. This differs from the cationic amino acid transporter responsible for lysinuric protein intolerance (y^+LAT-1), which must associate with the cell-surface antigen 4F2 to encode a fully functional transporter [Borsani et al., 1999; Torrents et al., 1999]. Alternatively, if a second gene is involved in carnitine transport, this must be constitutively expressed by CHO cells to explain our data.

An unexpected finding of the present study was that the E452K mutant transporter had normal recognition properties toward carnitine. The Km of the mutant transporter toward carnitine was in the micromolar range, identical to that of the normal OCTN2 transporter. This was demonstrated both in fibroblasts and in transfected CHO cells (Figs. 2–4). The most likely explanation of this behavior is that the mutation affects insertion of transporters into the plasma membrane, as suggested for human mutations in the Na^+-glucose cotransporter SGLT1 [Martin et al., 1996; Wright, 1998], with, in the case of OCTN2, the transporters which escape intracellular sequestration having normal affinity toward the substrate. The other possibility is that the mutation, while not affecting carnitine recognition, affects the rate at which carnitine moves across the membrane. This could be caused by a decreased rate of turnover of the transporter or by abnormal interaction of the E452K mutant transporter with sodium, whose electrochemical potential energizes carnitine transport [Scaglia et al., 1999]. Further experiments are required to define the precise mechanism for this kinetic anomaly.

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