Molecular and biochemical aspects of carnitine biosynthesis
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Identification of two novel mutations in OCTN2 of three patients with systemic carnitine deficiency

Identification of two novel mutations in OCTN2 of three patients with systemic carnitine deficiency

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SUMMARY
Systemic carnitine deficiency is a potentially lethal, autosomal recessive disorder characterised by cardiomyopathy, myopathy, recurrent episodes of hypoketotic hypoglycaemia, hyperammonemia and failure to thrive. This form of carnitine deficiency is caused by a defect in the active cellular uptake of carnitine, and it has recently been shown that the gene encoding the high affinity carnitine transporter OCTN2 is mutated in patients suffering from this disorder. Here we report the underlying molecular defect in three unrelated patients. Two patients were homozygous for the same missense mutation 632A>G, which changes the tyrosine at amino acid position 211 into a cysteine (Y211C). The third patient was homozygous for a nonsense mutation, 844C>T, which converts the arginine at amino acid position 282 into a stop codon (R282X). Re-introduction of wild type OCTN2 cDNA in fibroblasts of the three patients by transient transfection restored the cellular carnitine uptake, confirming that mutations in OCTN2 are the cause of systemic carnitine deficiency.

INTRODUCTION
Carnitine (3-hydroxy-4-trimethylaminobutyric acid) plays an indispensable role in the transport of activated long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix where β-oxidation takes place (1). Tissues including heart, muscle, liver and kidney are highly dependent on the energy generated by β-oxidation, and it is therefore essential that these tissues have sufficient amounts of carnitine. Because the carnitine concentration in tissues is generally 20- to 50-fold higher than in plasma (2,3) whereas only kidney, liver and brain have the complete set of enzymes to synthesise carnitine (2,4), most tissues depend on carnitine uptake from the blood via active transport. Active transport is also involved in the renal tubular reabsorption (5) and intestinal absorption of carnitine (6). The dependence on carnitine uptake is evident from patients suffering from systemic carnitine deficiency (SCD: MIM: 212140) who show excessive renal and intestinal wastage of carnitine, which results in very low plasma and tissue carnitine concentrations. Studies in cells of SCD patients indicated that this disorder is caused by a defect in the active cellular uptake of carnitine into the cell (7-11). Recently, a human cDNA
encoding a sodium-dependent, high affinity carnitine transporter \textit{OCTN2} was cloned from human kidney (12) and mapped to chromosome 5q31 (13). Analysis of the \textit{OCTN2} gene (\textit{SLC22A5}) in three SCD pedigrees identified four mutations, which cause the disorder (14). In addition, the murine orthologue of \textit{OCTN2} was shown to be mutated in the juvenile steatosis mouse, which presents with similar symptoms as SCD patients and is considered as the murine equivalent of human SCD (15-19,14). Here, we report the identification of two novel mutations in \textit{OCTN2} in three unrelated SCD patients. Furthermore, we show that the deficient cellular carnitine uptake in fibroblasts of the patients can be restored by reintroduction of wild type \textit{OCTN2} cDNA.

**EXPERIMENTAL PROCEDURES**

**Patients**

All three patients presented with classical SCD symptoms which are summarised in TABLE I.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Origin of family</th>
<th>Consanguinity</th>
<th>Cardiomyopathy</th>
<th>Myopathy</th>
<th>Death of patient</th>
<th>Free plasma carnitine ((\mu M)) (Normal range 19-59 (\mu M))</th>
<th>Total plasma carnitine ((\mu M)) (Normal range 26-70 (\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>Morocco</td>
<td>No(\ddagger)</td>
<td>18 m</td>
<td>No</td>
<td>No(\dagger)</td>
<td>1.4 (Normal range 19-59 (\mu M))</td>
<td>1.8 (Normal range 26-70 (\mu M))</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>Cape Verde</td>
<td>No(\ddagger)</td>
<td>8 m</td>
<td>No</td>
<td>No(\dagger)</td>
<td>4.4 (Normal range 19-59 (\mu M))</td>
<td>7.7 (Normal range 26-70 (\mu M))</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>Germany</td>
<td>Yes</td>
<td>24 m</td>
<td>Yes</td>
<td>No(\dagger)</td>
<td>0.5 (Normal range 19-59 (\mu M))</td>
<td>1.4 (Normal range 26-70 (\mu M))</td>
</tr>
</tbody>
</table>

\(\ddagger\)Parents are from a secluded area, consanguinity is likely

\(\dagger\)Five children died from a previous marriage of the father with a women from the same village

A full clinical description of patient 1 has been reported previously (6,20). Patient 2 was admitted to the hospital at 8 months of age, because of failure to thrive. Physical examination revealed a dilated cardiomyopathy. Treatment with digoxine and diuretics was started. At the age of 20 months she presented with lowered consciousness, respiratory insufficiency, hypoglycaemia (2.5 mM), hyperammonemia (1000 \(\mu M\)), elevated transaminases and low plasma carnitine concentrations (see TABLE I). Following a cardiac arrest, a full cardiac resuscitation was necessary after which the levels of ammonia and transaminases normalised rapidly. She was started on carnitine (500 mg 3 times a day) which led to the increase of her plasma free carnitine to 16 \(\mu M\), and the total carnitine concentration to 25 \(\mu M\). A long-chain triglyceride (1.5 g/kg bodyweight) loading test showed a normal production of ketone bodies. At the age of 2 years, improvement of the echo-cardiographic abnormalities was noted. All medication was gradually stopped except for the carnitine supplementation. At the age of 5½ years the echo-cardiograph was almost normal, with minimal tricuspid and mitralic insufficiency. ECG and contractility (shortening fraction of 0.38) were normal.

From the age of 2 years, Patient 3 suffered exercise intolerance and muscle weakness. Physical examination showed a dilated cardiomyopathy and low plasma carnitine levels. He
was started 50 and later 180 mg carnitine per kg bodyweight per day which led to a rapid normalisation of muscle functioning and the complete disappearance of the cardiac abnormalities in 6 years. A recent echo-cardiography revealed normal ventricular myocardial thickness and a good ventricular function. At present, the patient receives 65 mg carnitine per kg bodyweight per day. His plasma free carnitine level is now 16 μM.

**Carnitine uptake assay**

Carnitine uptake was measured essentially as described previously (9,21) with slight modifications as described below. Fibroblasts of patients and control subjects were cultured for 16 h on F-10 HAM medium containing 10% foetal calf serum (Life Technologies, Rockville, MA) in 6-well tissue culture dishes in a humidified incubator at 37°C under 5% CO₂. Subsequently, fibroblasts of each well were washed three times with phosphate-buffered saline (PBS) and incubated for 4 hours at 37°C under 5% CO₂ in 1 ml serum-free RPMI medium (Life Technologies, Rockville, MA) containing 5 μM [CH₃⁻¹⁴C]-carnitine (1.85 kBq/μmol; Amersham Pharmacia Biotech, Uppsala, Sweden). The validity of these assay conditions was checked by showing linearity of carnitine uptake for up to 5 hours (not shown). To correct for aspecific binding of carnitine to the cells, parallel experiments were performed at 4°C. After washing three times with cold PBS, cells were lysed in 1 ml PBS containing 0.1% triton-X-100 and the radioactivity in an 800 μl aliquot was determined in a liquid scintillation counter. The remaining 200 μl was used to determine the protein concentration using the bicinchoninic acid assay (Sigma, St. Louis, USA) (22) with bovine serum albumin as standard.

**Reverse transcription-PCR amplification and analysis of OCTN2 cDNA and genomic DNA**

First strand cDNA was synthesised from total RNA isolated from cultured primary skin fibroblasts as described previously (23). Using first strand cDNA as template, the OCTN2 cDNA was amplified by PCR using three overlapping primer sets: OCT₁₁₉₉f (5'[-21M13]-GCTCTGTGGCCTCTGAGG-3') and OCT₆₃₂r (5'[-M13rev]-ATGGTCACTGAAACACATTCC-3'), OCT₅₈₁f (5'[-21M13]-CTCTCTGTCTCTGGTGTTG-3') and OCT₁₂₃₂r (5'[-M13rev]-GAAGCAGGACATATCAGTG-3'), OCT₁₁₉₉f (5'[-21M13]-CTGGTGATTGACCATATCAGTG-3') and OCT₁₈₀₀₉r (5'[-M13rev]-CTTTCTCATCTTCTAGGACCGG-3'). DNA was isolated from cultured primary skin fibroblast as described elsewhere (24). Using the DNA as template, exon 3 and 5 of OCTN2 were amplified in a PCR reaction by means of primer sets OCTEX₃f (5'[-21M13]-CAGTATTCTGGCAACACTGTTC-3') and OCTEX₃r (5'[-M13rev]-TTAGAAGGCTGTGCTTTAAGG-3') and OCTEX₅f (5'[-21M13]-CTGACCTGCACTGTGACCTTGCACTG-3') and OCTEX₅r (5'[-M13rev]-TAAATACGGTCAGTCTGTC-3'), respectively. All primers were tagged with either a -21M13 (5'-TGTAACGACCGGCGGACGT-3') or an M13rev (5'-CAGGAAACAGCTATAGGACC-3') extension to enable sequencing of the amplified fragments by means of the Big Dye Primer kit (PE Applied Biosystems; Foster City, CA). Sequence reactions were analysed on an ABI377 automated sequencer (PE Applied Biosystems; Foster City, CA).

**Cloning of OCTN2 and transient transfection of primary skin fibroblasts**

The complete open reading frame of OCTN2 was amplified by PCR from fibroblast cDNA of control subjects using a BamHI-tagged forward primer 5'tataggatatcctggttggactacgacgagg-3' and a NotI-tagged reverse primer 5'tatataggagtactggcctgtagaaggctgtgcttttaagg-3'. The PCR product was cloned downstream of the CMV promoter into the BamHI and NotI sites of the mammalian expression vector pcDNA3.
(Invitrogen; San Diego, CA). To assess the integrity of the PCR process the entire open reading frame was sequenced. The pcDNA+OCTN2 construct was used to transfect primary human fibroblasts from SCD patients and control subjects using the LipofectaminePLUS reagent (Life Technologies; Rockville, MA) according to the manufacturer's recommendations. Cells were cultured on F-10 HAM medium containing 10% foetal calf serum in 6-well tissue culture dishes for 16 h in a humidified incubator at 37°C under 5% CO₂ and then transfected with pcDNA3 plasmid containing full-length OCTN2 cDNA or with the pcDNA vector without insert. At 48 h after transfection, carnitine uptake was measured as described above.

**RESULTS**

All three patients studied in this paper presented with typical SCD symptoms (TABLE I). The diagnosis of SCD was confirmed by measuring the carnitine uptake in cultured skin fibroblasts which was <10% of control values for all three patients. Sequencing of the entire coding region of OCTN2 cDNA from patient 1 showed that this patient was homozygous for a single missense mutation, 632A>G which changes the tyrosine at amino acid position 211 into a cysteine (Y211C). The homozygosity of the 632A>G mutation was confirmed by the finding that both parents of the patient were heterozygous for this mutation. The heterozygosity of the parents was biochemically reflected by a significantly lower carnitine uptake in their skin fibroblasts (30-60% of control values). Finally, the homozygosity of the 632A>G mutation was demonstrated at the genomic level by sequence analysis of exon 3 of the OCTN2 gene amplified by PCR from genomic DNA of patient 1 and his parents (FIG. 1).

Sequence analysis of both the coding region of OCTN2 cDNA and, subsequently, exon 3 of the OCTN2 gene from patient 2 showed that this patient was also homozygous for the same 632A>G mutation as found in patient 1. No material of the parents of patient 2 was available for investigation.

Sequence analysis of OCTN2 cDNA from patient 3 showed that this patient was homozygous for a nonsense mutation, 844C>T, which changes the codon for the arginine at amino acid position 282 into a stop codon (R282X). Upon translation, this mutation will lead
to the production of a truncated protein shortened by 275 amino acids. Sequence analysis of exon 5 of the \textit{OCTN2} gene amplified from genomic DNA of this patient confirmed the homozygosity of this mutation. The mother of patient 3 was heterozygous for the 844C>T mutation at the genomic level which was biochemically reflected by a significant reduction of the carnitine uptake in her skin fibroblasts (45\% of control values). No material of the patient's father was available for investigation.

To confirm that the carnitine uptake defect was caused by mutations in \textit{OCTN2}, we examined whether wild type \textit{OCTN2} could correct this defect. To this end, fibroblasts of the patients were transiently transfected with either pcDNA3+OCTN2 or with pcDNA3 without insert.

In fibroblasts of a control subject transfected with pcDNA3+OCTN2, carnitine uptake was increased 12-fold, whereas after transfection with pcDNA3 without insert no increase in carnitine uptake was observed (results not shown). As with control fibroblasts, the transfection of fibroblasts of the patients with pcDNA3 did not increase the carnitine uptake compared to untransfected cells. However, transfection with pcDNA3+OCTN2 resulted in complete restoration of carnitine uptake in fibroblasts from all three patients (FIG. 2).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Transient transfection of wild type \textit{OCTN2} cDNA in patients' fibroblasts.}
\end{figure}

\section*{DISCUSSION}

Following the recent identification of \textit{OCTN2} as the high affinity human carnitine transporter (12,13), we initiated studies to determine whether mutations in the \textit{OCTN2} gene could be responsible for SCD. After analysing three unrelated SCD patients, we discovered two mutations in \textit{OCTN2}: an A>G transition at nucleotide position 632 and a C>T transition at position 844 leading to amino acid changes Y211C and R282X, respectively. Furthermore, as shown in Fig. 2, the carnitine uptake defect in cells from these patients could be corrected by wild type \textit{OCTN2} cDNA. These results provide unequivocal evidence for \textit{OCTN2} as the defective gene in SCD.

While this work was in progress, a paper appeared that reported the first mutations in \textit{OCTN2} of SCD patients (14). After analysis of three pedigrees, Nezu \textit{et al.} identified four different mutations, including a 113 basepair deletion in exon 1, a G>A nonsense mutation at nucleotide position 132, a cytosine insertion after the fourth nucleotide that causes a frameshift, and a mutation in the splice acceptor site at the 3' end of intron 8 (14). However, the authors did not demonstrate that wild type \textit{OCTN2} could restore the carnitine uptake in their patients' cells.

A second recently published paper also claims to show that mutations in \textit{OCTN2} are responsible for SCD (25). However, this paper only reported two aberrantly spliced mRNA
species found in two SCD patients without demonstrating the actual mutations or excluding that these splice variants also occur in control cells.

While this manuscript was under review, two articles were published in which four mutations in OCTN2 are reported in three SCD patients (26,27). One patient has the same R282X mutation which has also been found in this study (27). The two mutations we identified are both predicted to have a deleterious effect on the function of the protein. The 632A>G mutation found in patients 1 and 2, which results in a tyrosine to cysteine substitution at position 211 of the OCTN2 protein, is located in the middle of the fourth of a total of twelve putative transmembrane regions. Sequence alignment of various organic cation transporters from different organisms showed in all cases an aromatic amino acid on this position, suggesting that the conversion to a hydrophilic cysteine may affect the topology and/or protein function.

Although patient 1 and 2 have the same mutation, their families are unrelated and have different geographical backgrounds. Recently, two silent polymorphisms have been reported in the OCTN2 cDNA (26). Both polymorphisms are different for patient 1 and 2 and are present in a homozygous form. This strongly suggests that these patients do not have a common ancestor and that the mutation has occurred independently.

The homozygous 844C>T mutation in patient 3 converts the arginine at position 282 into a stop codon, which results in a truncated protein shortened by 275 amino acids. Compared to the other two patients, patient 3 had the lowest plasma carnitine concentrations, showed the lowest carnitine uptake and, apart from the cardiomyopathy also observed in all the other patients, also presented with myopathy. This more severe phenotype may well be explained by the presence of the nonsense mutation which most likely leads to a non-functional protein.

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REFERENCES


