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OCTN2-Mediated Carnitine Uptake in a Newly Discovered Human Proximal Tubule Cell Line (Caki-1)

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Abstract: The proximal tubular reabsorption of carnitine in the human kidney is significant because more than 95% of the carnitine filtered in the kidney is reabsorbed by the proximal tubules therefore maintaining the homeostatic balance of carnitine in the body. Objectives of this study include the characterization of OCTN2 function in the Caki-1 cell line and the potential interactions of carnitine uptake with renally secreted drugs, including drugs of quaternary ammonium structure. Caki-1 cells were additionally characterized to be of proximal tubule nature, and an apical membrane expression pattern of OCTN2 in Caki-1 cells was discovered. Uptake studies with radiolabeled L-carnitine in Caki-1 cells revealed a Na^+- and temperature-dependent carrier-mediated process ($K_m = 15.90\ \mu M$) which was unaffected by pH in a range from 6.5 to 8.5. All drugs tested were able to inhibit the carnitine uptake process to different degrees. The quaternary ammonium compounds ciclotropium bromide and ipratropium bromide were strong inhibitors with IC_{50} values of 30 μM and 95 μM, respectively. The observed kinetics, immunohistolocalization, and inhibition studies indicate that the high-affinity uptake of carnitine in the Caki-1 cell line is most likely mediated by OCTN2. The interaction of drugs at the renal level with OCTN2 indicates a possible pathway for the final step of cationic secretion into the urine.

Keywords: OCTN2; L-carnitine; proximal tubule; Caki-1; renal secretion

Introduction

L-Carnitine (β-hydroxy-γ-trimethylaminobutyrate), a small water soluble zwitterionic molecule, plays an essential physiological role in lipid metabolism via the translocation of long-chain fatty acids across the mitochondrial inner membrane, where they are subjected to fatty acid β-oxidation for the production of cellular energy.[1,2] Carnitine is endogenously produced in very few tissues (brain, kidney, and liver); therefore it is primarily supplied from the diet (meat and dairy products) and is absorbed in the intestine, where it is further transported to several other tissues in the body via putative transporters.[3,4] The OCTN transporter family has been characterized to be organic cation/carnitine transporters, and members of this subfamily include OCTN1, OCTN2, and OCTN3, each with unique transport characteristics for carnitine and organic cations.[5,6] OCTNs form part of an

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organic ion transporter superfamily consisting of OCTs (SLC22A1−3) and OATs (SLC22A6−8 and 11).[7] All of these transporters play significant roles in drug disposition. OCTN2 has been characterized to be a high-affinity carnitine transporter.[8,9] The impaired function of this transport protein leads to the autosomal recessive disease known as systemic carnitine deficiency (SCD), a syndrome characterized by an increased renal excretion of carnitine and resulting low carnitine plasma and tissue concentrations.[10,11] This illustrates the importance of OCTN2 in the proximal tubular reabsorption of L-carnitine. Structurally, OCTN2 has 12 putative transmembrane domains and is expressed in several tissues at the apical membrane of epithelial cells, including the kidney, skeletal muscle, heart, and placenta.[12] The kidney plays an important role in the homeostatic balance of carnitine in body fluids. Greater than 95% of carnitine is filtered and reabsorbed in the kidney, and a small amount is eliminated as free carnitine or acylcarnitine in the urine.[6,13] Functionally, OCTN2 mediates the uptake of L-carnitine in a Na^+-coupled fashion and several organic cations in a Na^+-independent fashion.[9,12,14−16] Pharmacologically, OCTN2 plays a significant role because of its interaction with several commonly prescribed drugs.[1,17]

In the kidney, L-carnitine transport has been well characterized and the reabsorption of L-carnitine takes place primarily via OCTN2,[4,18] and interactions with renally secreted drugs could lead to severe complications. Human derived proximal tubule cell lines endogenously expressing transport proteins are a rare commodity. To date, a few publications have described human proximal tubule carnitine transport, however, only with cell systems artificially expressing relevant transport proteins or in cell systems only suspected to contain the specific transporter of interest.[19] Caki-1 cells, a renal clear cell carcinoma cell line, represent the first human proximal tubule cell line characterized to endogenously express the OCTN2 transporter among other important transport proteins.

With the use of these cells, in the present study, we have characterized a Na^+-dependent transporter of carnitine via functional and molecular techniques. The L-carnitine uptake in the human proximal tubule (Caki-1 cells) involves a carrier-mediated system ($K_m = 15.90\ \mu M$) that is Na^+ and temperature dependent, pH independent, and inhibited by several clinically administered drugs which are partially renally excreted unmodified. Previously tested drugs, including cephaloridine, cimetidine, valproic acid, tetraethylammonium bromide, famotidine, and 1-methyl-4-phenylpyridinium, showed inhibitory effects, and, in addition, ciclotropium bromide and ipratropium bromide, drugs with quaternary ammonium structures, which are as well renally excreted, were tested as potential inhibitors. Quaternary ammonium derivatives were tested, since they are known to interact with other members of the OCT family and because of the fact L-carnitine possesses a quaternary ammonio group in its structure. This cell line represents a plausible in vitro screening technique for

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currently administered drugs, as well as new active ingredients, still in their developmental stages, for their possible interference with the reabsoprtion of L-carnitine in the human kidney.

Materials and Methods

Cell Culture. Caki-1 cells were obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (Braunschweig, Germany). Cells between passages 18 and 26 were used for these studies. Cells were routinely seeded out at a density of 80 000 cells into 75 cm^2 plastic flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and cultivated in McCoy's 5A (Biochrom AG, Berlin, Germany), supplemented with FBS 10% (Biochrom AG), penicillin/streptomycin 1.14% (Biochrom AG), nonessential amino acid solution 1.14% (Biochrom AG), and stabile L-glutamine 0.7% (Biochrom AG). The cells were maintained at 37 °C, 5% CO_2, and 95% relative humidity. After confluence (95−100%), cells were harvested using 0.25% trypsin−EDTA solution (Biochrom AG).

γ-Glutamyl-transferase Proximal Tubule Staining. Caki-1 cells were grown in 24 multiwell plates with McCoy's 5A complete medium at a seeding density of 12 000 cells/well until 70−90% confluence (approximately 5 days in culture). The medium was then aspirated off, and the cells were washed 3× with 1 mL of 0.85% saline solution. To each well was added 0.5 mL of completed reaction mixture (14 mL of 0.85% saline, 5 mL of 0.1 M Tris buffer pH 7.5, 10 mg of Gly-Gly, 10 mg of Fast Blue, and 1 mL of L-glutamic acid γ-(4-methoxy-β-naphthylamide) (GMNA) stock solution (15.2 mL of distilled water, 0.4 mL of DMSO, 0.1 mL of NaOH, and 10 mg of GMNA), and the mixture was incubated at room temperature for 3 h in the dark. The cells were then carefully washed with 2 mL of 0.85% saline solution. Subsequently, each well was incubated with 1 mL of a 2.5% $CuSO_4$ solution for 2 min. The cells were washed with 2 mL of 0.85% saline solution, and 0.5 mL of a 50:50 glycerol:water solution was added to each well (fixation). Controls were carried out with a reaction mixture containing serine borate. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

Carnitine Uptake. L-Carnitine uptake studies were carried out as described previously with minor modifications.[20] Caki-1 cells (between passages 18 and 26) were plated (0.012 × 10^6/well) onto 24 multiwell plates (Nunc, Wiesbaden, Germany) in McCoy's 5A complete medium, and the medium was changed every third day. The cells were used for uptake studies at confluence (6−8 days), and all experiments were conducted at 37 °C with the exception of temperature dependence studies, which were carried out at 4 °C. The cells were washed twice with 1.5 mL of buffered Hanks balanced salt solution (HBSS) (5 mM HEPES, pH 7.4) before starting the experiment. The confluent cells were incubated at 37 °C with a final volume of 0.5 mL of buffered HBSS containing methyl-L-[^{3}H] carnitine (specific radioactivity 50−60 mCi/mmol, Hartmann Analytic GmbH, Braunschweig, Germany) at a concentration of 10 nM with (or without) inhibitors. At the end of the incubation period (10 min), the solutions were aspirated off and the monolayers were immediately washed five times with 1 mL of ice-cold buffered HBSS and thereafter dissolved at room temperature with 0.5 mL of 1 N NaOH. The cells were incubated for approximately 3 h with shaking (75 rpm) at room temperature. The complete volume of dissolved cells was collected, 4 mL of Rotiszint Ecoplus scintillation fluid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added, and radioactivity was quantified with a liquid scintillation counter. In another series of experiments the Na^+, pH, and temperature dependence on carnitine uptake was tested. For Na^+ dependence the experiments were conducted in a buffer composed of either 25 or 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5 mM HEPES. For pH dependence studies buffered HBSS in a pH range from 6.5 to 8.5 was employed (HBSS/10 mM MES (pH 6.5) and HBSS/5 mM HEPES (pH 7.5−8.5); pH values were adjusted with 1 M HCl and 1 M NaOH). Temperature dependence studies were carried out as described above at either 4 or 37 °C. All experiments were carried out in triplicate.

Assays with Xenobiotic Inhibitors. For the inhibitor experiments, confluent Caki-1 cells were incubated with 0.5 mL of buffered HBSS (pH 7.4) containing methyl-L-[^{3}H] carnitine at a concentration of 10 nM plus varying concentrations (50, 100, 500, 1000, and 5000 μM) of valproic acid, famotidine, cimetidine, 1-methyl-4-phenylpyridinium (MPP^+) iodide, tetraethylammonium (TEA), cephaloridine, cetirizin, metformin, ipratropium bromide, ciclotropium bromide, or (−)-*N*-butylscopolamine bromide. All substances were purchased from Sigma Aldrich with the exception of ciclotropium bromide, which was kindly provided by Prof. Hildegard Spahn-Langguth.

Protein Determination. The cellular protein content per well (24 multiwell plate) was determined according to the bicinchoninic acid (BCA) protein assay.

Preparation of cDNA and Real Time RT-PCR. RNA was isolated from confluent cultures after 6−8 days in culture, using RNA STAT-60 (Tel-Test Inc., Friendswood, TX) and performed according to the product manual. RNA preparations were treated with DNA-free (Ambion Ltd., Huntingdon, Cambridgeshire, U.K.), and the quantity and purity of the RNA samples was determined using a GenQuant pro RNA/DNA calculator. The RNA integrity was assessed by visualizing the sharpness of ribosomal RNA bands on a 1% agarose gel. cDNA was prepared from total RNA using the Superscript First-Strand Synthesis System for real-time PCR (Invitrogen Ltd., Paisly, U.K.) according to the product manual. The two step reaction mixture contained 2 μg of RNA, 100 ng of random hexamers, 0.5 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCL (pH 8.4), 25 mM KCL, 5 mM $MgCl_2$ 10 mM DTT, and 40

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units of RNAseOUT recombinant ribonuclease inhibitor. Incubation was performed at 65 °C for 5 min and 25 °C for 2 min, with subsequent incubation after the addition of Superscript II, for 10 min at 25 °C, followed by 42 °C for 50 min. The reaction was terminated by heating at 70 °C for 15 min.

TaqMan real-time RT-PCR was carried out in 384 well reaction cards and an ABI PRISM 7900ht sequence detection system. Assay-on-Demand gene expression products were used for all genes tested. All TaqMan equipment was obtained from Applied Biosystems (Foster City, CA). TaqMan analysis was performed in a 1 μL reaction mixture containing 2 ng of RNA converted to cDNA, 1x TaqMan Universal PCR Master Mix (containing AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference, and optimized buffer), 900 nM each of custom designed forward and reverse primer, and 250 nM of custom-designed probe. Cycling conditions were as follows: 2 min at 50 °C, 10 min polymerase activation, at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

For normalization of gene levels, Cyclophillin-A (cytoplasmic protein) was used to correct for minor variations in the input RNA or inefficiencies in reverse transcription. The cycle number and the threshold cycle (C_T) were used to quantify the PCR product. The relative expression of the target gene normalized to the endogenous control was calculated as follows:

$$\Delta C_T = C_T \text{ target gene} - C_T \text{ control gene}$$

and they were converted according to Applied Biosystems (1997) to

$$2^{-\Delta C_T}$$

Immunofluorescence/Membrane Localization of h-OCTN2. Caki-1 cells were grown on glass coverslips and were maintained in a 95% air−5% CO_2 atmosphere at 37 °C in complete McCoy's 5A medium. The cells were used between 70% and 100% confluence (no differences in expression patterns were observed within this range). They were fixed in −20 °C methanol for 10 min and subsequently dried at room temperature for 30 min. The cells were washed 2×/10 min with PBS and incubated with Tween 20 (0.04% in PBS) for 20 min. After washing with PBS and blocking with blocking solution (0.5% cold-water fish gelatine (Sigma) plus 0.1% ovalbumin (Sigma) in PBS) for 30 min, the cells were incubated with primary antibody, anti-OCTN2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or E-cadherin (BD Biosciences, Heidelberg, Germany) diluted 1:25 and 1:200, respectively, in blocking solution, at 4 °C overnight. The cells were washed with PBS and subsequently incubated with the secondary antibody conjugated to Alexa 488 (Molecular Probes) (1:400) and DAPI (1:8000) in blocking solution for 2 h in the dark. Washed cells were mounted in Mowiol 4.88 (Hoechst, Frankfurt, Germany). Mounted sections were examined by fluorescence microscopy with a Leica DMRP fluorescence microscope. Images were obtained with a Hamamatsu Orca ER CCD camera and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). Double stainings were performed with cells grown on 8-well culture slides (BD Biosciences, Heidelberg, Germany). Primary antibodies, hOCTN2 and Na^+/K^+-ATPase, diluted in PBS 1:25 and 1:300 respectively, were applied overnight. Secondary antibodies were applied for 1 h at room temperature (Texas Red donkey anti-goat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse (Dianova, Hamburg, Germany). Cells were PBS-rinsed, fixed (4% PFA), and transferred to cover slips with carbonated glycerol (pH 8.6). Samples were evaluated with a confocal laser scanning microscope (TCSSP2 AOBS, Leica, Mannheim, Germany).

Statistical Analysis. Initial uptake rates of methyl-L-[^{3}H] carnitine were obtained by measuring the uptake at 10 min (linear range). To estimate kinetic parameters K_m and IC_{50} values, the uptake rate was fitted by means of nonlinear regression analysis using GraphPad Prism software. Results are reported as means ± SEM of at least triplicate determinations. All statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Determination of the Proximal Tubule Nature of Human Renal Caki-1 Cells. Caki-1 cells were stained to determine the expression of γ-glutamyl transferase, a characteristic marker of proximal tubule cells, used to distinguish this cell type from another major epithelial cell type, distal tubule cells, which only weakly express this enzyme. In this assay, γ-glutamyl transferase converts GMNA, to a product which gives results of a reddish brown stain for positive cells, and cells which are negative for γ-glutamyl transferase take on an unstained or yellow appearance. The positive reddish brown staining of Caki-1 cells can be seen in Figure 1.

Methyl-L-[^{3}H] Carnitine Uptake. The uptake of methyl-L-[^{3}H] carnitine (10 nM) into confluent Caki-1 cell monolayers grown on 24 multiwell plates was studied. Confluency was observed after 6−8 days in culture, via microscopy and E-cadherin immunostainings. Protein concentration after 7 days was determined to be 1.25 mg/well. L-Carnitine uptake was in the linear phase up to 30 min (data not shown), and in order to represent the linear uptake phase, an incubation period of 10 min was chosen, which as well resulted in an adequate level of DPM counts.

L-Carnitine uptake was significantly affected by a reduction in the NaCl concentration in the uptake buffer from 125 mM to 25 mM (Figure 2). These results strongly suggest that L-[^{3}H] carnitine uptake is Na^+ dependent. In addition, the influence of temperature on L-carnitine upake was tested, where a significant decrease in the uptake was observed at 4 °C (Figure 3), a factor indicating carrier-mediated transport. Varying the pH between 6.5 and 8.5 had no significant effects on the uptake of L-carnitine into Caki-1 cells; uptake remained independent with changes in pH, which is in accordance with previous studies[1,20](Figure 4).

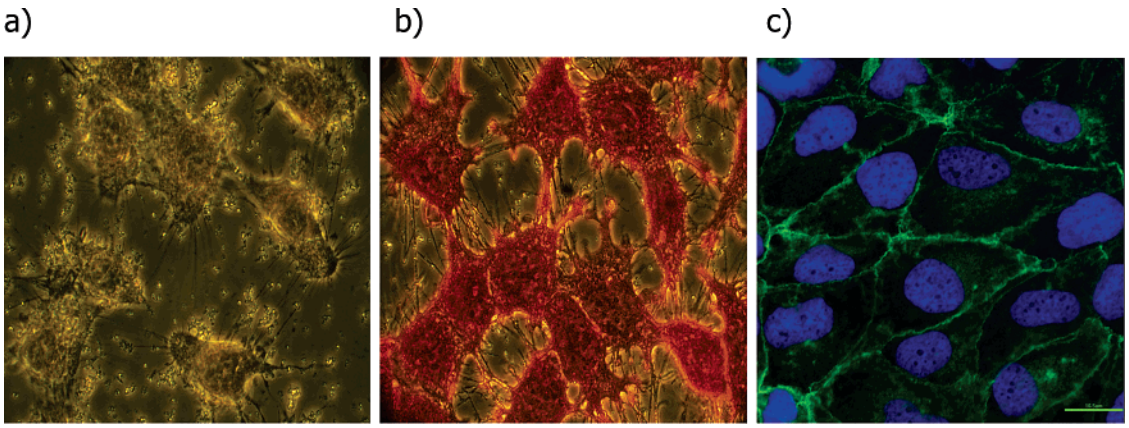

Figure 1. Proximal tubule staining and expression of E-cadherin in Caki-1 cells. For proximal tubule staining, Caki-1 cells were grown in 24 multiwell plates and maintained at 37 °C, 5% CO_2, and 95% humidified atmosphere. Cells were stained for the presence of γ-glutamyl transferase, a characteristic marker of human proximal tubule cells. Figure 1a is a negative control of Caki-1 cells (solution containing serine borate) (yellowish brown) and Figure 1b a positive staining of the Caki-1 cells (red). Figure 1c illustrates the organized expression of E-cadherin (green) and the cell nuclei (blue).

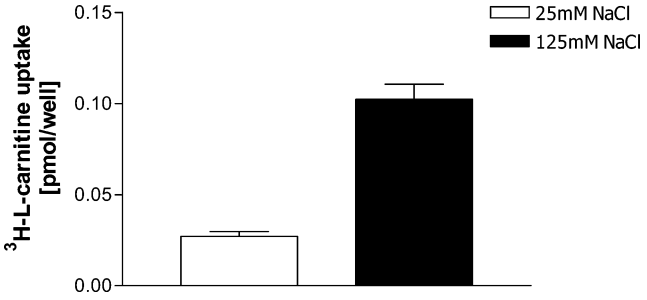

Figure 2. Na^+ dependence of Caki-1 cell L-carnitine uptake. Caki-1 cells were grown in 24 multiwell plates and maintained at 37 °C, 5% CO_2, and 95% humidified atmosphere. Methyl-L-[^{3}H] carnitine uptake was measured in buffer containing either 25 or 125 mM NaCl. The data represents the mean ± SEM of three separate determinations.

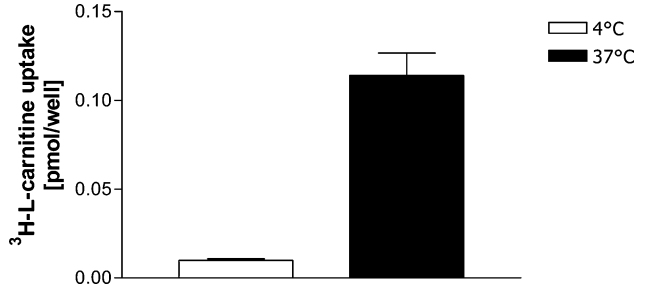

Figure 3. Temperature dependence of L-carnitine uptake into Caki-1 cells. Cells were grown in 24 multiwell plates as previously descibed. Methyl-L-[^{3}H] carnitine uptake was measured in buffered HBSS at pH 7.4 at 4 and 37 °C for 10 min. The data represents the mean ± SEM of three separate determinations.

The uptake of methyl-L-[^{3}H] carnitine was measured at concentrations between 0.01 and 250 μM to provide evidence for the saturability of the uptake process and to determine kinetic parameters. Figure 5 shows the saturable uptake process of methyl-L-[^{3}H] carnitine with a K_m of 15.90 μM (95% confidence interval of 8.502 to 23.31).

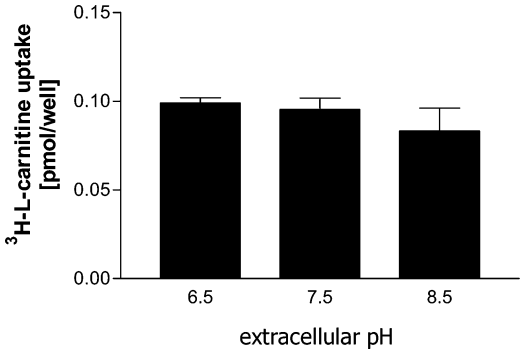

Figure 4. pH independence of methyl-L-[^{3}H] carnitine uptake by Caki-1 cell monolayers. Uptake was measured at 37 °C for 10 min in buffered HBSS; each data point and error bar represents the mean ± SEM for three individual determinations.

Effect of Renally Secreted Drugs on L-Carnitine Uptake. All subsequent uptake experiments were performed using Caki-1 cells, in the presence of Na^+ (125 mM NaCl). The uptake of methyl-L-[^{3}H] carnitine by Caki-1 cells was characterized in the presence of various renally secreted drugs known to inhibit carnitine uptake in other systems. Caki-1 cells were incubated with 10 nM methyl-L-[^{3}H] carnitine together with varying concentrations (50, 100, 500, 1000 and 5000 μM) of valproic acid, famotidine, cimetidine, 1-methyl-4-phenylpyridinium (MPP^+) iodide, tetraethylammonium (TEA), cephaloridine, cetirizin, and metformin. Caki-1 cells were additionally incubated together with drugs of quaternary ammonium structure: ipratropium bromide, ciclotropium bromide, or (−)-*N*-butylscopolamine bromide to determine the influence these substances have on the uptake of methyl-L-[^{3}H] carnitine into Caki-1 cells. In all cases, the methyl-L-[^{3}H] carnitine uptake was inhibited in a concentration-dependent manner, however with varying intensities as summarized in Table 1 and Figure 6. Cationic compounds such as TEA showed moderate effects as well as anionic substances such as valproic acid. Ipratropium bromide and

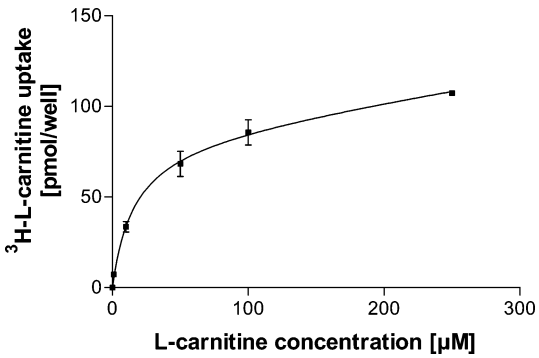

Figure 5. Concentration-dependent uptake of L-carnitine by Caki-1 cells. Cells were grown in 24 multiwell plates and maintained at 37 °C, 5% CO_2, and 95% humidified atmosphere. Uptake of L-carnitine was measured at 37 °C for 10 min in buffered HBSS, in the presence of different concentrations of L-carnitine. Each point represents the mean ± SEM of three separate determinations.

Table 1. Inhibitory Effects of Various Compounds on L-[^{3}H] Carnitine Uptake by Caki-1 Cells

inhibitor	IC_{50} [μM]	95% confidence interval
valproic acid	139	88.07−221.2
famotidine	1920	890.8−4138
cimetidine	336	243.1−466.3
MPP^+	1713	1231−2384
TEA	551	395.8−767.3
cephaloridine	248	187.4−328.1
cetirizin	27	16.04−45.70
metformin	4963	3541−6956
ipratropium bromide	95	67.43−136.2
ciclotropium bromide	30	22.37−42.42
(−)-*N*-butylscopolamine bromide	1007	615.7−1648

ciclotropium bromide, quaternary compounds, strongly inhibited the L-carnitine uptake into Caki-1 cells.

OCTN2 Identification and Localization. OCTN2 mRNA expression, as well as OCT1, OCT3, and OCTN1, was investigated in Caki-1 cells by real-time RT-PCR for younger (P5) and older (P49) passages (Figure 7). Various potential endogenous controls were tested; cyclophillin A was chosen as the endogenous control since it showed C_T values closest in range to those of the Caki-1 samples (data not shown). A definite expression of OCT3 and OCTN2 was demonstrated, with relatively constant expression over an extended passaging period (passages 5−49). Immunostaining with an anti-hOCTN2 polyclonal antibody revealed a strong positive fluorescence (Figure 8). The distribution of the fluorescence was localized to one side of the cell membrane, which is in accordance with the restricted apical nature of OCTN2 in human epithelial cells (Figure 8). Further double stainings with the basolaterally located Na^+/K^+-ATPase revealed the apical location of OCTN2 in Caki-1 cells (Figure 8b).

Discussion

L-Carnitine has long been known for its role in fatty acid oxidation, and more recently it has been shown that the highly specific transporter OCTN2 is responsible for the transport of carnitine in many organs including the brain, placenta, intestine, and kidney.[21] Several cell culture models exist for studying the transport processes in the placenta, intestine, and brain, providing rapid characterization of L-carnitine transport processes.[1,17,20] However, a human proximal tubule cell line that endogenously expresses *several* relevant drug transporters had not yet been discovered. Caki-1 cells express OCTN2 and additionally OCT3, a very relevant organic cation transporter in the kidney. It represents the first human proximal tubule cell line confirmed to contain not only the gene but as well the apically localized OCTN2 protein in potentially active form.

The current study demonstrates the presence of a specific transporter for the uptake of L-carnitine into Caki-1 cells. We have characterized carnitine transport in Caki-1 cells with and without the presence of known inhibitors of OCTN2-mediated transport. No inhibition was observed with the OCT3 specific inhibitor decynium22 (data not shown here), indicating no involvement of OCT3. Via real-time RT-PCR and immunofluorescence analysis we have determined the presence of the mRNA and protein of OCTN2 within Caki-1 cells. The Caki-1 cells were as well characterized to be of epithelial proximal tubule nature due to the presence of γ-glutamyl transferase and E-cadherin, specific proximal tubule and epithelial markers respectively. It has been often reported that L-carnitine reabsorption in the kidney is mainly carried out via OCTN2, and our results are consistent with these reports.[12,22]

L-Carnitine uptake in confluent Caki-1 cells was Na^+ dependent (Figure 2). At a lower concentration of NaCl in the uptake buffer (25 mM) the uptake was reduced to approximately 26% as compared to standard uptake buffer containing 125 mM NaCl. These results indicate the presence of a Na^+ dependent transport pathway for L-carnitine in Caki-1 cells. In addition, the effect of variation in the extracellular pH on the process of L-carnitine uptake in Caki-1 cells was studied and revealed that, in a pH range of 6.5−8.5, L-carnitine uptake is independent of pH, as has been previously reported for OCTN2.[1,20]

L-Carnitine uptake into Caki-1 cells is a saturable process (Figure 5) with a high affinity (K_m = 15.90 μM), similar to the known kinetics of hOCTN2.[8,14] hOCTN2 has been previously shown to transport L-carnitine in the human kidney with a high affinity (K_m = 4.3 μM) and Na^+ dependency.[8]

In this study clinically relevant cationic and anionic drugs were tested for their ability to inhibit the saturable L-carnitine

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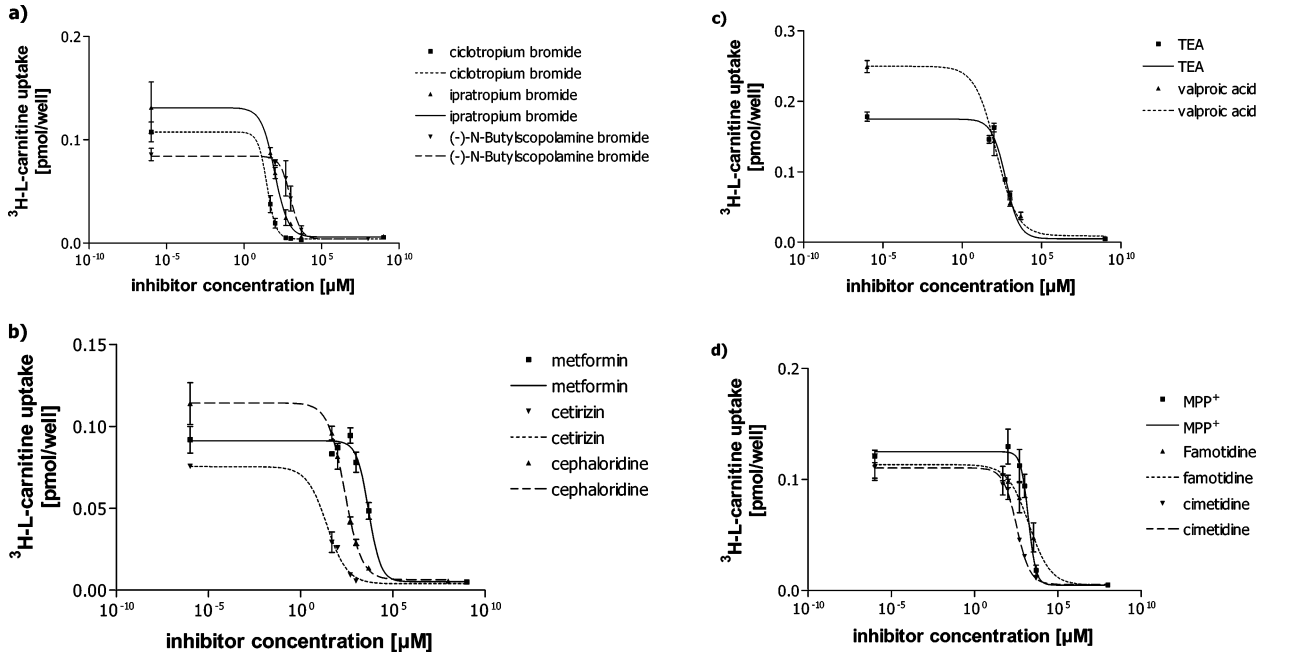

Figure 6. Inhibition of methyl-L-[^{3}H] carnitine uptake in Caki-1 cells by various concentrations of inhibitors: (a) ciclotropium bromide, ipratropium bromide, and (−)-*N*-butylscopolamine (quaternary compounds), (b) metformin, cetirizin, and cephaloridine, (c) tetraethylammonium (TEA) bromide and valproic acid, and (d) MPP^+, famotidine, and cimetidine. Each point and error bar represents the average ± SEM for three separate determinations of uptake. Fitted lines reflect the IC_{50} values displayed in Table 1.

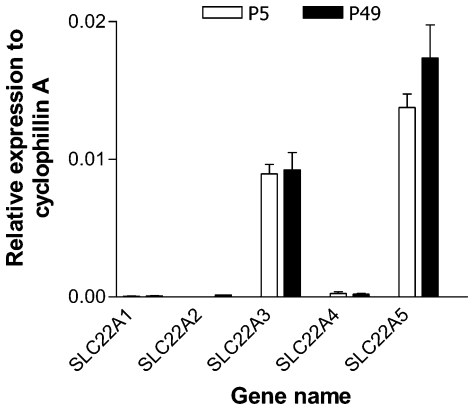

Figure 7. Relative gene expression levels ($2^{-\Delta C_T}$) of OCT1, OCT2, OCT3, OCTN1, and OCTN2 in two passages (P5 and 49) of Caki-1 cells. Each value represents the mean ± SEM for three separate determinations.

uptake process in Caki-1 cells. All of the drugs tested are renally excreted to different percentages, and can therefore possibly lead to problems in the OCTN2-mediated reabsorption of L-carnitine in the kidney. It has been previously proven that administration of valproic acid, an antiepileptic drug, induced a reduction in serum and tissue levels of L-carnitine,[22−24] and in the current studies valproic acid was able to inhibit L-carnitine uptake with an IC_{50} value of approximately 139 μM in Caki-1 cells, which is in accordance with previous results from cell culture systems.[22] Furthermore, in Caki-1 cells all cationic drugs were able to inhibit the L-carnitine uptake process to different degrees (Table 1), indicating that there is a potential for these drugs to interfere with the in vivo process of L-carnitine reabsoprtion in the human kidney. Additionally, a new group of cationic drugs, possessing quaternary ammonium structures, were tested for a potential interaction with L-carnitine uptake in Caki-1 cells. Ipratropium bromide, ciclotropium bromide, and (−)-*N*-butylscopolamine are drugs commonly or previously prescribed for chronic bronchitis, muscle relaxation, and irritable bowel syndrome, respectively. All three quaternary compounds were able to interfere with L-carnitine uptake. Ciclotropium bromide and ipratropium bromide represented strong inhibition profiles with IC_{50} values of 30 μM and 95 μM, respectively, and (−)-*N*-butylscopolamine was a weaker inhibitor with an IC_{50} value of 1007 μM.

To confirm that the saturable uptake process of L-carnitine in Caki-1 cells is likely mediated by OCTN2, the gene and protein expression of OCTN2 was determined in Caki-1 cells. In order to eliminate the possibility of an influence of OCTN1 (a known carnitine transporter in the same family as OCTN2) on the L-carnitine uptake in Caki-1 cells, the gene expression was also determined for this transporter.[6] The lack of expression of OCTN1 in Caki-1 cells eliminated the possibility of OCTN1 being an active transporter of

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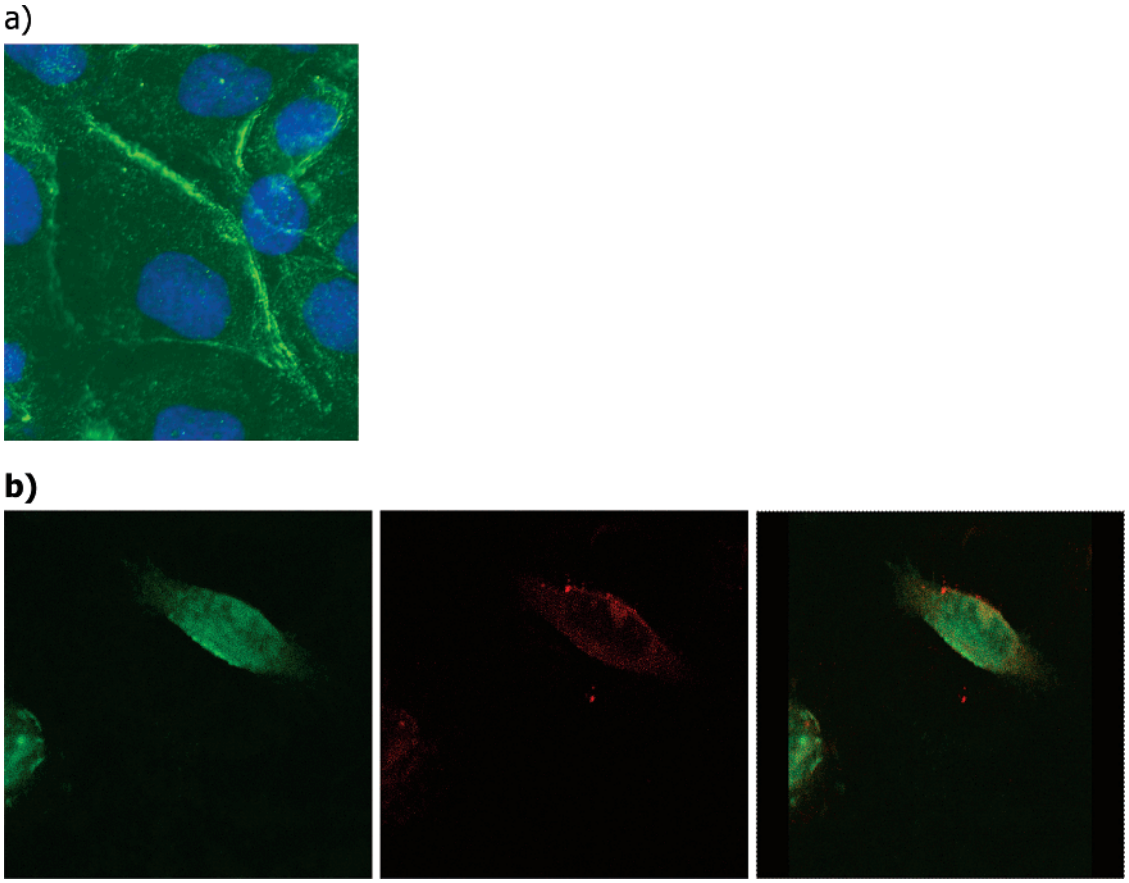

Figure 8. Detection of OCTN2 by immunofluoresence in Caki-1 cells: (a) Caki-1 cells were grown on glass coverslips and maintained in a 5% CO_2 and 95% humidified atmosphere at 37 °C. Cells were used between 5 and 7 days (70−90% confluence). They were fixed in −20 °C methanol, immunostained for hOCTN2, and viewed by fluorescence microscopy. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (blue) and OCTN2 with the anti-hOCTN2 polyclonal antibody (green); (b) localization of OCTN2 (red) to the apical membrane and Na^+/K^+-ATPase (green) to the basolateral membrane.

interest here. Another known transporter of carnitine, $B^{0,+}$, was confirmed not to be expressed in Caki-1 cells[25] (data not shown). Real-time PCR revealed a definite expression of OCTN2, and in addition, the expression of the hOCT3 transporter gene was observed. No expression of OCT2, the supposed main organic cation transporter in the human kidney, was observed. To confirm that the OCTN2 transporter protein is as well actively expressed in Caki-1 cells, a double immunohistochemical staining was undertaken. Staining with a polyclonal anti-hOCTN2 antibody revealed the presence of positive fluorescing on a single side of the membrane. Costaining with Na^+/K^+-ATPase, a known basolateral membrane marker, revealed a clear separation in the staining pattern, concluding that OCTN2 is located at the apical membrane in Caki-1 cells. This is in accordance with many previous publications locating OCTN2 in various species to the apical membrane[1,14]

In conclusion, these results are consistent with the prior hypothesis that OCTN2 plays a major role in carnitine transport in the kidney.[6,21] Based on the transport characteristics determined by the current and previous studies of hOCTN2, we conclude that L-carnitine uptake in Caki-1 cells and the human kidney is probably primarily mediated by OCTN2. It has been demonstrated that L-carnitine is taken up into Caki-1 cells with a high affinity, in a Na^+- and temperature-dependent fashion. The results of these studies have clinical implications in the fact that many commonly prescribed drugs of cationic and anionic nature have the potential to significantly reduce the reabsorption of L-carnitine via inhibition of OCTN2 in the kidney, therefore, reducing the serum and tissue levels of L-carnitine. Additionally, it has been previously proposed that OCTN2 may be responsible for the reabsorption of cations in the kidney, and these interaction studies point in this direction. However, the exact functional activity of OCTN2 in the kidney is still unknown, and therefore, a secretory function should not be excluded. This would lead to speculation that OCTN2 plays the last step in renal secretion of cations across the apical membrane into the urine. An overall reduction in L-carnitine serum levels can lead to systemic carnitine deficiency (SCD) if not treated with high doses of carnitine.[26] Due to the fact of the proximal tubular nature and endogenous expression

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of OCTN2 in Caki-1 cells, it is to date the easiest, most natural and perhaps most accurate of all in vitro cell culture models existing for studying L-carnitine−drug interactions. This system has the potential to be a renal equivalent to the intestinal Caco-2 cell model, the most commonly used in vitro screening method for currently administered and newly developing drugs. Further studies need to be carried out to determine the potential these interactions may have in vivo. However, it is important to note that information discovered here should not be limited to the kidney but rather expanded to other organs where OCTN2 is known to be expressed, since it has been proven that within a species inhibition and kinetic profiles are often similar between organs.

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