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Species specificity profiling of rat and human organic cation/carnitine transporter Slc22a5/SLC22A5 (Octn2/OCTN2)

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ABSTRACT

The purpose of this study was to characterize the uptake of carnitine, the physiological substrate, and the uptake of 3-(2,2,2-trimethylhydrazinium)propionate, a consensus substrate by rat Octn2 and human OCTN2 transporters as well as to characterize drug-mediated inhibition of L-carnitine uptake by the rat and human orthologs overexpressed in CHO-K1 cells. L-carnitine and 3-(2,2,2-trimethylhydrazinium)propionate were found to be a lower affinity substrate for rat Octn2 ($K_M = 32.66 \pm 5.11$ μM and 23.62 ± 4.99 μM respectively) than for human OCTN2 ($K_M = 3.08 \pm 0.74$ μM and 7.98 ± 0.63 μM). The intrinsic clearance (CL_{int}) value for carnitine was higher for the human than for the rat transporter (22.82 ± 5.57 ml/min*mg vs 4.008 ± 0.675 ml/min*mg). For 3-(2,2,2-trimethylhydrazinium)propionate, in contrast, the CL_{int} value for rat Octn2 was higher than for human OCTN2 (323.9 ± 72.8 ml/min*mg vs 65.11 ± 5.33 ml/min*mg).

Furthermore, many pharmacologically important drugs were shown to affect L-carnitine transport by Octn2/OCTN2. The correlation between the IC_{50} datasets for the rat and human transporter resulted in an r value of 0.47 ($p > 0.05$). However, the greatest difference was less than seven-fold and 13 of 15 compounds yielded a difference less than 3-fold.

Thus, the transporters from these two species showed an overlapping but somewhat different substrate and inhibitor specificity.

1. Introduction

OCTN2/Octn2 ("organic cation/carnitine transporter novel type 2") is a specific Na^+/L-carnitine co-transporter [1–3]. Carnitine plays a crucial role in mitochondrial transport of fatty acids and, thus, is essential in many tissues [4]. Carnitine is required in high quantities and 10–100-fold accumulations in different organs have been reported [4]. As expected, OCTN2/Octn2 is expressed in many tissues such as intestine [5–7], pancreas [8], liver [5], muscle [9], heart [5], kidney [5, 7] and brain [5, 10, 11]. Furthermore, OCTN2 is a homeostasis regulator of the heart and the brain [5]. OCTN2/Octn2 was one of the first uptake transporters cloned from rat [1, 12, 13], human [2,14] and mouse [13,15] tissues underlining the importance of the protein.

A role in the pH-dependent transport of organic cations such as drugs and physiological substrates has been suggested [16–18]. However, *in vitro* assays using active pharmaceutical ingredients (API) or drugs other than 3-(2,2,2-trimethylhydrazinium)propionate [19] and, perhaps, oxaliplatine [20] as substrate is a challenge [19] as drug transport data from different groups are controversial.

Missense mutations of OCTN2 with impaired transport function cause recessive primary systemic carnitine deficiency (SCD) [7]. SCD patients display impaired L-carnitine uptake across the plasma membrane [21,22]. The malfunction of L-carnitine uptake blocks the

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fatty acid b-oxidation and causes hypoketotic hypoglycemia, cardiomyopathy and progressive atrophy and malfunctions in organic cation metabolism. It is manageable by L-carnitine administration [5]. The secondary carnitine-deficiency might have genetic, therapeutic (dialysis, medical treatment) and nutritional reasons. It may cause cardiomyopathy, skeletal myopathy, hypoketotic coma, hypoglycemia and hyperammonemia [23].

Knocking out the gene of OCTN2/Octn2 is not lethal. Probably other transporters – like OCTN1/Octn1 – can transport L-Carnitine with lower affinity. Mouse Octn3 can also transport carnitine in the testis, although the main function of this protein is in the peroxisomal fatty-acid transport [24]. In humans the testis specific CT2 (SLC22A16) that is not classified as an OCTN family member is also a sodium independent high affinity carnitine transporter [25].

Several focused studies implicated drug [26–28] or toxicant-mediated [29] inhibition of OCTN2 in toxicity or adverse events. Only three broader scale studies monitored drug-mediated inhibition of the transporter [18, 21, 30]. All studies were focused on the human transporter but only one of them provided values (IC_{50}/K_I) important for drug discovery and development [30].

Our aim was to correlate transport properties of rat Octn2 and human OCTN2 expressed on the same genetic background using the carnitine, a physiological substrate and 3-(2,2,2-trimethylhydrazinium)propionate, an API and an efficiently transported substrate [19]. Our *in vitro* data show similar K_M but different CL_{int} (intrinsic clearance) values for the rat and human transporter for substrates and an overlapping inhibitor specificity. These data suggest that *in vivo* rat models may be applicable to predict human OCTN2 mediated toxicity and adverse events in the clinic but *in vitro* differences between Octn2/OCTN2 transporter profile should be taken into account.

2. Materials and methods

Cell lines: The CHO-K1 cells were provided by Prof. Bruno Stieger (University of Zurich, Zurich, Switzerland).

Cell culturing: Cells were stored at 37 °C with 5% carbon-dioxide. Cells were cultured on 75 cm^2 flasks in 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 (DMEM F-12), from Lonza (Basel, Switzerland) containing GIBCO™ fetal bovine serum (10%), Thermo Fisher Scientific, (Waltham, MA, USA), L-Proline (34 μg/ml), L-Glutamine (2 mM, from Sigma–Aldrich (St. Louis, MO, USA)) and Penicillin-Streptomycin (100 unit/ml Penicillin, 100 μg/ml Streptomycin, from Lonza, (Basel, Switzerland)).

Generation of CHO-OCTN2 and CHO-rOctn2 cell lines: The CHO-rOctn2 and the CHO-OCTN2 stably transfected cells lines were prepared at SOLVO Biotechnology (Szeged, Hungary) using the CHO-K1 cell line.

Sequence verified complementary DNA (cDNA) encoding human OCTN2 (SLC22A5; GenBank accession no. NM_001308122) was ordered from Open Biosystems (Huntsville, AL, USA) distributing public cDNA collection of the I.M.A.G.E. Consortium. OCTN2 cDNA was originally cloned into pOTB7 vector. cDNA of OCTN2 was subcloned into a pcDNA3.1(+)Neo eukaryotic expression vector under the control of a CMV (cytomegalovirus) promoter at BamHI and XhoI sites. The resulting construct was fully sequence verified. CHO-K1 cells (200 000 per well) were transfected with 0.1 μg of pcDNA3.1 construct with the Lipofection method using Lipofectamine LTX from Thermo Fisher Scientific (Waltham, MA, USA), and stable transfected clones were isolated in the presence of 800 μg/μl Geneticin from Sigma–Aldrich (St. Louis, MO, USA) in the media. The activities of the isolated clones were quantitated by functional uptake measurements of radiolabeled ^{14}C-L-Carnitine from ARC American Radiolabeled Chemicals (Saint Louis, MO, USA) purchased from IZINTA (Budapest, Hungary).

Rat Octn2 (SLC22A5) was cloned from rat (Rattus norvegicus; 250–300 g male Wistar, from Toxi-Coop Ltd. (Dunakeszi, Hungary)) liver tissue. mRNAs were extracted from fresh tissue, cDNA library was generated by retrotranscription of mRNAs. cDNA library was then used to target the SLC22A5 gene using primers targeting both ends of the gene (rSlc22A5-fw-1: 5′- GCCAAGCTTGCCACCATGCGGGACTACGACGAGGT -3′, rSlc22A5-rev-1: 5′- GCCGGATCCTTAGAAGGCTGTGCTCTTTAGG -3′; from Avidin Ltd. (Szeged, Hungary)). cDNA of rat Octn2 was cloned into a pcDNA3.1(+)Neo eukaryotic expression vector under the control of a CMV promoter using HindIII and BamHI sites. The resulting construct was fully sequence verified (GenBank accession no. NM_019269). CHO-K1 cells (200 000 cell/well) were transfected with 0.1 μg of pcDNA3.1 construct containing rat Octn2 with the Lipofection method using Lipofectamine LTX from Thermo Fisher Scientific (Waltham, MA, USA), and stably transfected clones were selected in the presence of geneticin (800 μg/μl).

The activities of the isolated clones were quantitated by functional uptake measurements of radiolabeled ^{14}C-L-Carnitine.

qRT-PCR: RNA was isolated from CHO-rOctn2, CHO-OCTN2 and CHO-K1 cell lines with Invitrogen PureLink RNA MiniKit from Thermo Fisher Scientific (Waltham, MA, USA), cDNA was synthetized from RNA using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA). PCR (polymerase chain reaction) was prepared with IQ SYBR Green Supermix from Bio-Rad (Hercules, CA, USA). rOctn2 primers (rSlc22a5 fw: 5′-GCTCAGGGTCAAAGGAATAAAACA-3′; rSlc22a5 rev: 5′-GGGTGTTAGAAGGCTGTGCT-3′; from Thermo Fisher Scientific (Waltham, MA, USA)), and OCTN2 primers (hSLC22A5 fw: 5′-CCCTACTTCGTTTACCTTGGTG-3′; hSLC22A5 rev: 5′-GCTGTCAGGATGGTCAGACTT-3′; from Avidin Ltd. (Szeged, Hungary)) were used at 10 μM final concentration in the reaction mix and 1 μg cDNA was used in each reaction. PCR reactions were run with iQ5 Real-Time PCR System from Bio-Rad (Hercules, CA, USA). Each sample was run in triplicate.

2.1. Uptake assays

L-Carnitine uptake substrate time dependence assays: 50 000 cells per well were plated into 96-well plates and were incubated for 48 h at 37 °C with 5% carbon-dioxide before measurement. Untransfected CHO-K1 cells were used as controls. Medium was removed and the cells were washed twice with 200 μl transport buffer (Henseleit-Krebs buffer, pH 7.4) per well. Cells were incubated with 50 μl reaction mixture containing Henseleit-Krebs buffer, 1.8 μM L-carnitine for 0–30 min at 37 °C. Measurements were performed with radiolabelled L-carnitine hydrochloride L-[N-methyl- ^{14}C] from ARC American Radiolabeled Chemicals (Saint Louis, MO, USA) purchased from IZINTA (Budapest, Hungary; 2.035 GBq/mmol; 3.7 MBq/ml) and unlabeled L-carnitine hydrochloride from Sigma–Aldrich (St. Louis, MO, USA). Reaction mixture was removed and the cells were washed twice with 200 μl transport buffer per well. Cells were incubated with 50 μl 0.1 M sodium-hydroxide per well for 10 min at 37 °C for lysis. The quantity of cell-associated L-carnitine was determined with a Microbeta^2 liquid scintillation counter from Perkin Elmer (Norwalk, CT, USA) purchased from Perform Hungary Ltd. (Budapest, Hungary).

L-Carnitine uptake substrate concentration dependence assays: 50 000 cells per well were plated into 96-well plates and were incubated for 48 h at 37 °C with 5% carbon-dioxide before measurement. Sodium-free Henseleit-Krebs buffer was used with the transfected cells as controls. Medium was removed and the cells were washed twice with 200 μl transport buffer (Henseleit-Krebs buffer, pH 7.4) per well. Cells were incubated with 50 μl reaction mixture containing Henseleit-Krebs buffer, 0–83 μM (for rOctn2)