molecular pharmaceutics

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Kidney Transplantation Down-Regulates Expression of Organic Cation Transporters, Which Translocate β-Blockers and Fluoroquinolones

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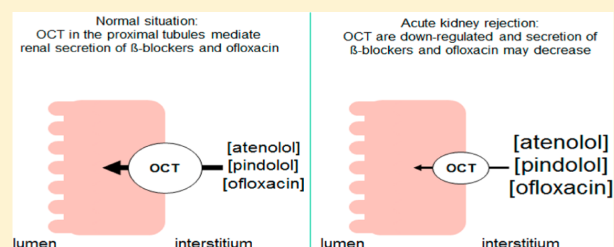
Supporting Information



ABSTRACT: Kidney transplanted patients are often treated with immunosuppressive, antihypertensive, and antibiotic drugs such as cyclosporine A (CsA), β-blockers, and fluoroquinolones, respectively. Organic cation transporters (OCT) expressed in the basolateral membrane of proximal tubules represent an important drug excretion route. In this work, the renal expression of OCT after syngeneic and allogeneic kidney transplantation in rats with or without CsA immunosuppression was studied. Moreover, the interactions of CsA, β-blockers (pindolol/atenolol), and fluoroquinolones (ofloxacin/norfloxacin) with rOCT1, rOCT2, hOCT1, and hOCT2 in stably transfected HEK293-cells were studied. Kidney transplantation was associated with reduced expression of rOCT1, while rOCT2 showed only reduced expression after allogeneic transplantation. All drugs interacted subtype- and species-dependently with OCT. However, only atenolol, pindolol, and ofloxacin were transported by hOCT2, the main OCT in human kidneys. While CsA is not an OCT substrate, it exerts a short-term effect on OCT activity, changing their affinity for some substrates. In conclusion, appropriate drug dosing in transplanted patients is difficult partly because OCT are down-regulated and because concomitant CsA treatment may influence the affinity of the transporters. Moreover, drug−drug competition at the transporter can also alter drug excretion rate.

KEYWORDS: *organic cation transporters, acute rejection, kidney transplantation, drugs*

■ INTRODUCTION

Kidney transplantation is the treatment of choice for patients suffering from end-stage renal disease. However, despite improvements in prophylaxis and treatment, graft rejection, hypertension, and infectious complications continue to contribute to substantial morbidity and mortality after kidney transplantation. For this reason, drug therapy plays a significant role in the management of many transplant recipients. Patients suffering from hypertension after kidney transplantation are usually treated with multiple drugs such as calcium channel-, renin−angiotensin−aldosterone system-, and β-blockers,[1] and those suffering from infectious complications are treated with oral antibiotics with excellent absorption, for instance trimethoprim/sulfamethoxazole or fluoroquinolone.[2] To reduce the incidence of organ rejection, immunosuppressive agents such as cyclosporine A (CsA), tacrolimus, or mycophenolate mofetil are used. However, because renal dysfunction alters drug pharmacokinetics,[3] appropriate drug dosing in renal transplant recipients is difficult.

A variety of commonly used drugs are actively secreted by specific transporters in the kidney, yet the functional expression and activity of these proteins after kidney transplantation have not been investigated. Organic cation transporters (OCT) have a particular importance for renal drug excretion because up to 40% of the prescribed drugs are organic cations[4] and because OCT are highly expressed in the kidney.[5] OCT are polyspecific transport proteins, which are mainly localized in the basolateral membrane of renal proximal tubule cells and therefore mediate the first step in renal excretion of organic cations. Of translational importance is the observation that kidneys from rodents express the OCT subtypes 1 and 2 in the proximal tubules,[6−9] while human kidneys express mainly the subtype 2 (hOCT2).[10,11] The subtype 1 (OCT1) is typically expressed in the liver.[6,10]

Received: January 15, 2013
Revised: April 19, 2013
Accepted: April 22, 2013
Published: April 22, 2013

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In this work, the renal expression of OCT1 and OCT2 in a rat model of allogeneic kidney transplantation undergoing acute rejection with or without immunosuppressive therapy with CsA has been compared with that in control animals and after syngeneic kidney transplantation. Moreover, the transport of β-blockers such as atenolol and pindolol, of fluoroquinolones such as ofloxacin and norfloxacin, and of CsA as examples of drugs commonly used in renal transplant recipients has been measured in cells stably transfected with rat (rOCT1 and rOCT2) or human (hOCT1 and hOCT2) OCT subtypes.

■ EXPERIMENTAL SECTION

Kidney Transplantation. Male Lewis−Brown−Norway (LBN) and Lewis (LEW) rats (250−300 g, Charles River, Sulzfeld, Germany) with free access to standard rat chow (Ssniff, Soest, Germany) and tap water were used. Experiments were approved by a governmental committee on animal welfare and were performed in accordance with national animal protection guidelines. Transplantation was performed as published before.[12,13] In short, the left kidney including ureter, renal artery, a piece of aorta, and renal vein was transferred into the recipient. For acute rejection, kidneys of LBN rats were transplanted into LEW rats, which were bilaterally nephrectomized immediately before transplantation (allogeneic transplantation model). This transplantation model leads to histological changes typical for acute transplant rejection within 2−4 days.[12] A second group of transplanted animals received CsA 5 mg/kg/day (ip) as immunosuppressive therapy. Kidneys of LBN rats with and without CsA therapy and syngeneically transplanted LBN kidneys served as controls. When possible, at least four kidney samples were used for each analysis. Transplanted kidneys were harvested on day 4 after transplantation.

Real-Time PCR Analysis. Gene expression profiles were analyzed by real-time PCR. After graft removal, the total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). For cDNA synthesis, 2 μg of total RNA was used with the SuperScript-III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master Mix on an ABI PRISM 7700 sequence detection system. Specific primer pairs as listed in Table 1 were used. All instruments and reagents were purchased from Applied Biosystems (Darmstadt, Germany). Relative gene expression values were evaluated with the $2^{-\Delta\Delta Ct}$ method using Gapdh as a housekeeping gene.[14]

Table 1. Primers Used for Real-Time PCR

Gapdh	sense primer	CAT CAA CGA CCC CTT CAT TGA C
	antisense primer	ACT CCA CGA CAT ACT CAG CAC C
rOCT1	sense primer	CAA ACA GGC AAG TCC TCA AGT AC
	antisense primer	GGT TGG TAG TTC ATT TGG AAC C
rOCT2	sense primer	GCA AGC AGA CCG TCC GCT AAG
	antisense primer	CAG ACC GTG CAA GCT ACA GCA C

Immunocytochemistry. The rabbit-raised, affinity-purified polyclonal antibodies against the C-terminal domain of the rat OCT1 (rOCT1-Ab) and OCT2 (rOCT2-Ab) were kindly provided by Prof. H. Koepsell (Institut für Anatomie and Zellbiologie, Universität Würzburg). The use of these antibodies in immunochemical studies has been described before.[9] The working dilutions of the antibodies (in PBS) were 1:300 for rOCT1-Ab and 1:50 for rOCT2-Ab. Secondary antibody was the CY3-labeled goat antirabbit IgG (Jackson Immunoresearch, West Grove, PA USA) at concentration of 1.6 μg/mL.

Rats were anesthetized by injecting ip the mixture of Narketan (80 mg kg^{-1} bm) and Xylapan (12 mg kg^{-1} bm), and their circulatory system was perfused manually (using 250 mL syringes) via left ventricle, first with temperature equilibrated (37 °C) PBS to remove blood, followed by 150 mL of fixative (4% paraformaldehyde; PFA). Kidneys were removed, decapsulated, cut in ∼2 mm-thick sagittal slices, postfixed in PFA for 24 h at 4 °C, and then extensively washed with PBS to remove PFA and stored in PBS containing 0,02% $NaNO_3$ until use. Before cryosectioning, tissue samples were left in 30% sucrose overnight at 4 °C, then embedded in Tissue-Tek OCT compound (Tissue-Tek, Sakura, Japan) and frozen at −25 °C. Leica CM 1850 cryostat (Leica instruments GmbH, Nussloch, Germany) was used to cut 4 μm thick cryosections, which were collected on SuperFrost Plus microscope slides (Thermo Scientific, Gerhard Menzel GmbH, Braunschweig, Germany), dried at room temperature for 2 h, and stored refrigerated until use. In preliminary studies, cryosections were tested for optimal antigen retrieval protocol;[15] microwave heating in citrate buffer pH 6 yielded the best staining intensity and was used for presenting both OCTs. In short, cryosections underwent the following steps: rehydration in PBS for 15 min, heating in citrate buffer pH 6 in a microwave oven at 800 W for 20 min, washing in PBS (3 × 5 min), incubation in 0.5% Triton-X-100 (in PBS) for 15 min and 2% Triton-X-100 (in PBS) for 30 min, washing in PBS (3 × 5 min), incubation in bovine serum albumin (1% solution in PBS) for 30 min, incubation in primary antibody at 4 °C overnight, incubation in secondary antibody at room temperature for 60 min, and washing in 0.1% Triton-X-100 (10 min) and PBS (3 × 5 min). The cryosections were finally covered with the fluorescence fading retardant Vectashield (Vector Laboratories Inc., CA, USA), coverslipped, and sealed with nail polish. The fluorescence was examined with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) and images captured using the Spot RT slider camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). Each set of images, related to the specific antibody, was captured under the same parameters. The original images were imported into Adobe Photoshop 6.0, assembled in one panel, the CY3-related red fluorescence was converted into black and white mode, and the whole panel was further processed and labeled as a single picture using the same software.

Western Blot Analysis. Proteins were separated by SDS-polyacrylamide gel (4−20%) electrophoresis and transferred to a PVDF membrane incubated with 3% gelatin (Sigma, Steinheim, Germany) in Tris-buffered saline with 0.1% Tween. After incubation with the primary antibody (anti-rOCT1 or anti-rOCT2, both from Alpha Diagnostic Int., San. Antonio, TX, USA, or anti Gapdh from New England Biolabs, Frankfurt, Germany), membranes were covered with Super-Signal (Pierce, Bonn, Germany) before exposure (Roche Diagnostics, Mannheim, Germany). Semi quantification of specific signals with the internal standard Gapdh blotted in parallel was performed using BioDoc analysis software (Biometra).

HEK293 Cell Culture. Some experiments were performed with human embryonic kidney (HEK) 293 cells (CRL-1573; American Type Culture Collection, Rochville, MD), which

stably express rOCT1, rOCT2, hOCT1, or hOCT2 (kind gift of Prof. H. Koepsell, University Würzburg). Stable transfection was performed as described earlier.[16−20] Cells were grown at 37 °C in 50 mL cell culture flasks (Greiner, Frickenhausen, Germany) in DMEM (Biochrom, Berlin, Germany) containing 3.7 g/L $NaHCO_3$, 1.0 g/L D-glucose, and 2.0 mM L-glutamine (Biochrom) and gassed with 8% CO_2. Penicillin (100 U/mL), 100 mg/L streptomycin (Biochrom), 10% fetal calf serum, and, only for OCT-transfected cells, 0.8 mg/mL geneticin (PAA Laboratories, Coelbe, Germany) were added to the medium. Experiments were performed with cells grown to confluence on glass coverslips for 5−8 days from passages 12−60. Culture and functional analyses of these cells were approved by the state government Landesumweltamt Nordrhein-Westfalen, Essen, Germany (no. 521-M-1.14/00).

Microdissection of Proximal Tubules. Human proximal tubules were isolated for functional analyses using the procedure customary in our laboratory.[21] Human kidneys were obtained from patients undergoing tumor nephrectomy. Written informed consent of the patients was obtained. This procedure was specifically approved by the Ethics Committee of the University of Münster. Pieces of normal kidney tissue distant from the tumor were used. Thin corticomedullary slices were cut from the kidney pieces using a scalpel and immediately transferred into a dissection dish containing minimum Eagle's culture medium and 5 mM glycine at 4 °C. Single proximal tubules (S2/S3 segments) were mechanically isolated using fine forceps under stereomicroscopic observation and then transferred into a perfusion chamber with a glass bottom mounted in the focus of the microscope and finally fixed with two holding pipettes.

Fluorescence Measurements. As substrate for OCT, the fluorescent organic cation 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP^+) at a concentration of 1 μM was used. The ASP^+ uptake by OCT expressing cells and freshly isolated human proximal tubules was measured as customary in our laboratory.[21−23] Measurements were performed in the dark with an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany) equipped with a 100× oil immersion objective. Excitation light (450−490 nm) was reflected by a dichroic mirror (560 nm) to a perfusion chamber. Empty coverslips (in experiments with isolated tubules) or those with cell monolayers formed the bottom of the chamber. The preparations were superfused at a rate of 10 mL/min with a HCO_3^- free Ringer-like solution containing (in mM): NaCl 145, K_2HPO_4 1.6, KH_2PO_4 0.4, D-glucose 5, $MgCl_2$ 1, calcium gluconate 1.3, and pH adjusted to 7.4 at 37 °C. Fluorescence emission (575−640 nm) was measured after specific excitation (450−490 nm) by a photon counting tube (Hamamatsu H 3460−04; Herrsching, Germany). To study interaction of β-blockers and fluoroquinolones with OCT, ASP^+ uptake was evaluated in the continued presence of these substances and compared to what was observed in control experiments performed under the same conditions without these drugs. For proximal tubules, the cellular fluorescence increase upon addition of ASP^+ to the perfusion chamber reflected organic cation transport across basolateral membrane of the tubule only because the ends of the tubules were kept inside the holding pipettes and tubules were collapsed.[21] Results are expressed as changes of ASP^+ uptake in percentage of control experiments.

For the study of the regulation of OCT by CsA, cells or freshly isolated human proximal tubules were incubated with 10 μM CsA for 10 min at 37 °C before ASP^+ was added in the continued presence of CsA. We evaluated the initial slope of the first 10−60 s as transport parameter because it directly represents uptake of organic cations across the plasma membrane.[24]

Uptake of β-Blockers and Quinolones by hOCT1-, hOCT2-HEK293, and HEK293 Cells. To determine whether the presence of hOCT1 or hOCT2 is important for the uptake of these substances, hOCT1, hOCT2, and HEK293 WT cells were incubated for 10 min at 37 or 4 °C with 100 μM β-blockers or 500 μM quinolones, respectively. After incubation, cells were washed three times with ice-cold Ringer-like solution and hypoosmotic lysis was induced by addition of water. Lysates were sonicated for 10 min and centrifuged 10 min at 12100*g*. For the precipitation of proteins, two volumes of acetonitrile were added to one volume of cell lysate, mixed, and centrifuged. Then 10 μL of the supernatant was injected into a HPLC system (HP 1100 series liquid chromatograph connected to a HP 1100 MSD model G1946A mass spectrometer, Hewlett-Packard, Camas, WA, USA) with a reversed phase column (PUROSPHER RP 18ec, 55 × 2, 3 μ, Merck, Darmstadt, Germany). Pindolol and ofloxacin were eluted isocratically over a time period of 4 min with a mobile phase consisting of 80% eluent A (water with 0.1% formic acid) and 20% eluent B (methanol), a flow rate of 0.4 mL/min, and a column temperature of 35 °C. Norfloxacin was eluted within 5 min with 87% eluent A and 13% eluent B. For the determination of atenolol, gradient conditions were used where within the first 2 min eluent B was increased from 0% to 30%, kept at that concentration for 1 min, increased to 90% eluent B within half a minute to wash the column, again kept at that concentration for 1 min, and then decreased within half a minute to the initial conditions to prepare the column for the next measurement (total run time 10 min).

For detection, the mass spectrometer with electrospray interface was used in the positive ion mode. As drying gas ultrapure nitrogen with a flow of 10 L/min was used at a temperature of 350 °C. The nebulizer pressure was set at 20 psi, and the electrospray needle was maintained at −4000 V. Atenolol was measured at m/z 267.1, pindolol at m/z 249.4, ofloxacin at m/z 362.0, and norfloxacin at m/z 320.3. The measurements were linear at least over a concentration range of 0−10 μM.

Solution and Chemicals. ASP^+ was obtained from Molecular Probes (Leiden, The Netherlands). All standard substances were obtained from Sigma (Munich, Germany) or Merck (Darmstadt, Germany) at highest purity available. CsA, atenolol, pindolol, ofloxacin, and norfloxacin were obtained from Sigma.

Statistical Analysis. Data are presented as means ± SEM. IC_{50} values were calculated and compared from curve fittings with GraphPad Prism (San Diego, USA). Unpaired Student's *t* test or ANOVA analysis with Tukey posthoc test was used to test for statistical significance of the effect. A P value <0.05 was set as the significance level.

■ RESULTS

As published before,[12,25,26] in the allogeneic transplantation model signs of severe acute rejection were present after transplantation when compared with control conditions. On day 4, marked glomerulitis and cellular interstitial infiltrations were evident. In addition, perivascular edema and endothelialitis were found. These changes were significantly reduced in kidneys from allogeneically transplanted rats under CsA

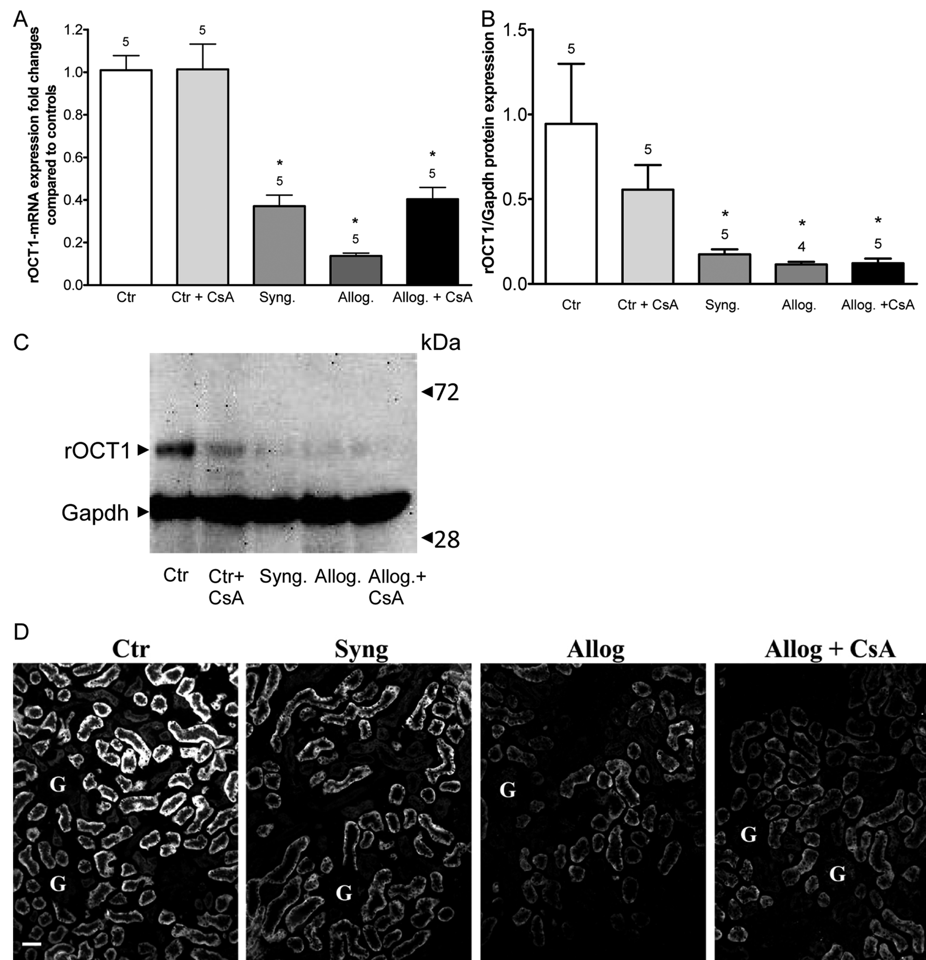

Figure 1. Effects of syngeneic (Syng) and allogeneic (Allog) kidney transplantation on the mRNA (A) and protein (B−D) expression of rOCT1 compared to untreated (Ctr) and CsA-treated (Ctr + CsA) control kidneys. The effect of immunosuppression with CsA in the allogeneic model (Allog + CsA) on rOCT1 expression is also shown. Results are expressed as mean ± SEM; the number of experiments is indicated on the top of each column. * Statistically significant difference from Ctr and Ctr + CsA ($P < 0.05$, ANOVA with Tukey post-test). (B) Results of Western blot analysis and (C) a typical Western blot for kidney expression of rOCT1 and Gapdh proteins in the animal groups under study. rOCT1 protein band appears at an apparent molecular weight of around 55 kDa and Gapdh at around 36 kDa. The immunocytochemical data in (D) show the OCT1-related staining in kidney cortex. In control kidneys (Ctr), the rOCT1-Ab strongly stained the basolateral membrane in proximal convoluted tubules (S1/S2 segments). Following syngeneic (Syng) and allogenic (Allog) transplantation, the expression of OCT1 in the transplanted kidneys was strongly downregulated (Syng < Allog). A weakly stained OCT1 in the tubules of allogeneically transplanted kidneys was not improved by treating rats with CsA (Allog + CsA). G, glomeruli. Bar, 40 μm.

treatment.[12,25,26] In syngeneically transplanted animals, no such findings were visible.[12,25,26]

Real-time PCR analysis showed a strong reduction of rOCT1 mRNA expression in kidneys of syngeneically and allogeneically transplanted rats (Figure 1A). Treatment with CsA was not able to prevent this effect. CsA had no effect on rOCT1 expression in control kidneys. The down-regulation of rOCT1 mRNA expression was apparently not caused by the rejection reaction because it was present in both groups of transplanted rats. Western blot analysis of whole kidney lysates (Figure 1B) showed that the renal protein expression of rOCT1 was decreased to the same levels in allogeneically and syngeneically transplanted rats and that treatment with CsA did again not rescue this effect. Treatment with CsA of control animals was also without any significant effect. Figure 1C shows a representative Western blot analysis. The Western blotting data are supported by the data in immunocytochemical studies; as shown in Figure 1D, in accordance with the previous data,[9] the rOCT1-Ab strongly stained the basolateral membrane in proximal convoluted tubules (S1/S2 segments) of the control kidney cortex. In the tubules of syngeneically and allogenically transplanted kidneys, the staining intensity was strongly downregulated and remained unchanged in allogeneically transplanted kidneys after treating animals with CsA.

The mRNA expression of rOCT2 (Figure 2A) was down-regulated by allogeneic but not by syngeneic transplantation. This effect was also present in allogeneically transplanted rats treated with CsA. The results of Western blot analysis (Figure 2B) confirm what was observed at the mRNA level: the renal protein expression of rOCT2 was significantly decreased in allogeneically transplanted rats with or without CsA treatment. CsA alone had no influence on the protein expression of rOCT2. Figure 2C shows a representative Western blot analysis. Figure 2D shows the rOCT2-Ab-related immunostaining in the basolateral membrane of proximal tubule straight segment (S3) in the renal outer stripe. In control kidneys, the basolateral membrane of S3 segments was strongly stained, which agrees with the original findings by Karbach et al.[9] The

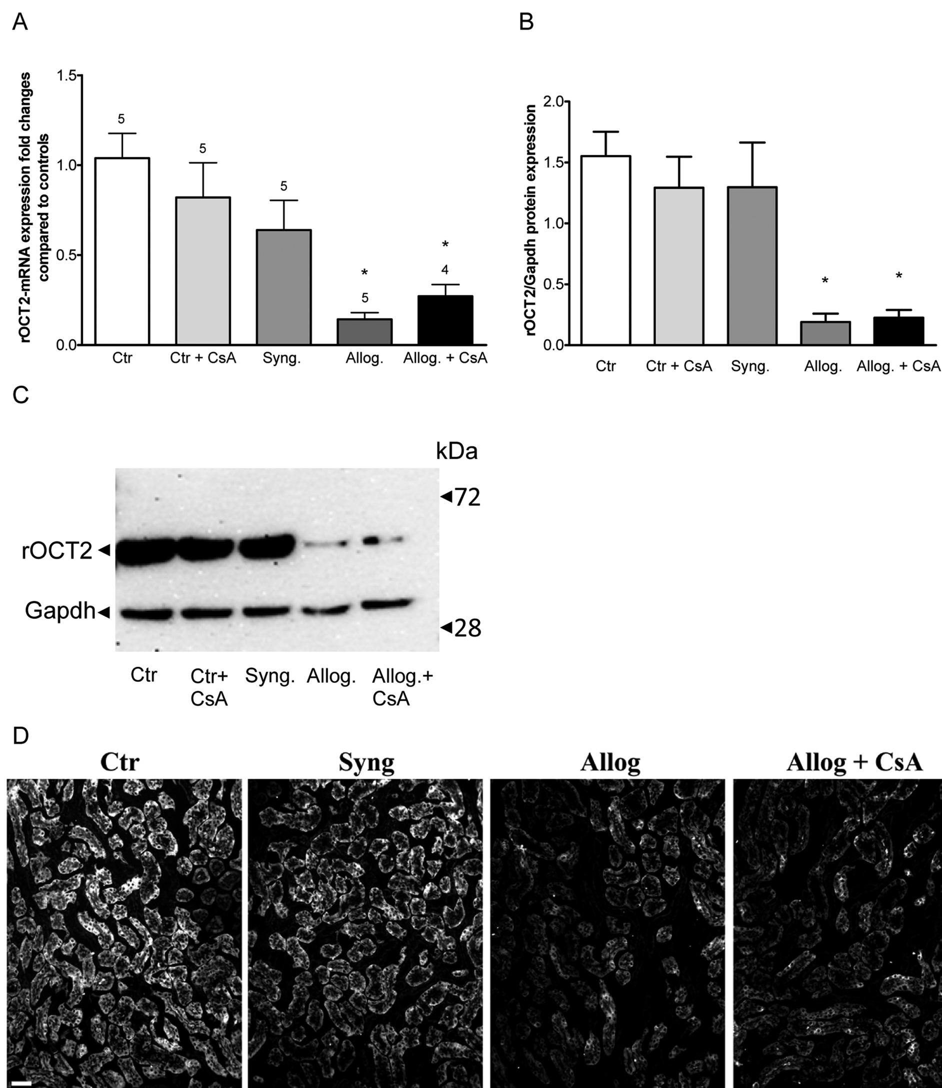

Figure 2. Effects of syngeneic (Syng) and allogeneic (Allog) kidney transplantation on the mRNA (A) and protein (B−D) expression of rOCT2 compared to untreated (Ctr) and CsA-treated (Ctr + CsA) control kidneys. The effect of immunosuppression with CsA in the allogeneic model (Allog + CsA) on rOCT2 expression is also shown. Results are expressed as mean ± SEM, the number of experiments is indicated on the top of each column. *Indicates in (A) and (B) a statistically significant difference from Ctr, Ctr + CsA, and Syng ($P < 0.05$, ANOVA with Tukey post-test). (B) Results of Western blot analysis and (C) a typical Western blot for kidney expression of rOCT2 and Gapdh proteins in the animal groups under study. rOCT2 protein band appears at an apparent molecular weight of around 55 kDa and Gapdh at around 36 kDa. In (D) the OCT2-related immunostaining in outer stripe is shown. In control kidneys (Ctr), rOCT2-Ab strongly stained the basolateral membrane of proximal tubule S3 segments. The tubules in syngeneically transplanted kidneys show slightly lower staining intensity (Syng), whereas the tubules in allogeneically transplanted and allogeneically transplanted + CsA-treated kidneys exhibited considerable and similar downregulation in staining intensity. Bar = 40 μm.

staining was similar-to-slightly weaker in the tubules of syngeneically transplanted kidneys and strongly diminished in the tubules of allogeneically transplanted kidneys. In the latter, the CsA treatment in animals did not affect the intensity of OCT2 staining in S3 segments.

Because transplantation and acute rejection massively downregulated the expression of rOCT1 and rOCT2, respectively, we investigated whether these transporters interact with the β-blockers atenolol and pindolol and the fluoroquinolones ofloxacin and norfloxacin, as examples for drugs that are often prescribed after transplantation in humans. These experiments have been performed in HEK293 cells stably transfected with rOCT1 or rOCT2. Moreover, in order to extend this study to a human model, the same experiments were performed in cells stably transfected with hOCT1 or hOCT2. Atenolol (Figure 3A) showed low apparent affinities for rOCT1 and hOCT1 (IC_{50} values: 1400, and 3200 μM, respectively). Conversely, its apparent affinities for rOCT2 and hOCT2 were higher, as shown by the lower IC_{50} values of 373 and 93 μM, respectively. Because the inhibition of ASP^+ uptake does not necessarily mean that atenolol is also transported into the cells by OCT, the accumulation of atenolol at 37 and 4 °C in cells transfected with hOCT was compared with that observed in WT cells (Figure 3B). Atenolol uptake significantly depended on temperature, being at 4 °C significantly lower than at 37 °C, indicating the probable involvement of a transport system in this process. Cells overexpressing hOCT2 had a significantly higher atenolol uptake than WT cells and cells expressing hOCT1 (Figure 3B). Atenolol accumulation

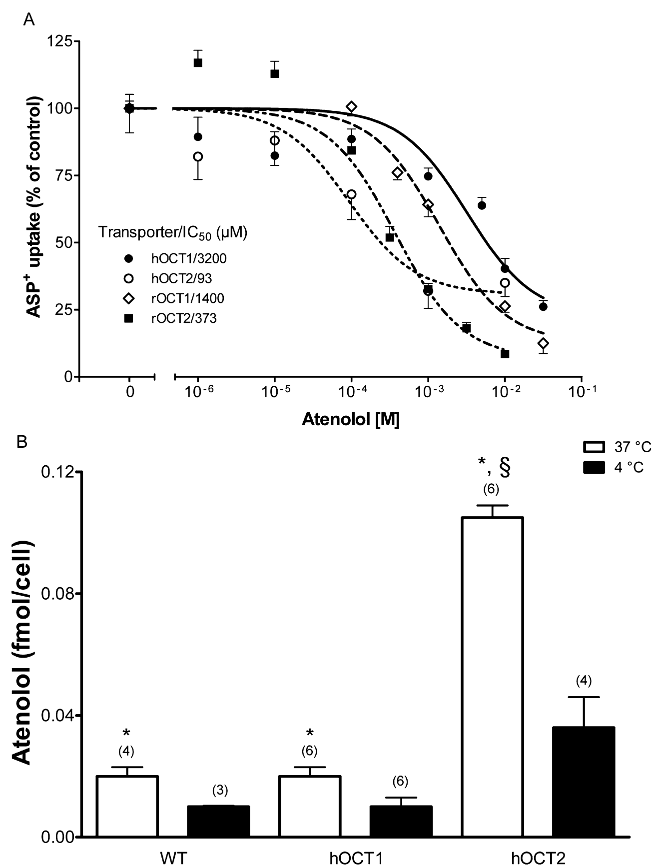

Figure 3. (A) Concentration-dependent inhibition of ASP^+ uptake by atenolol in HEK293 cells stably transfected with rOCT1 (◇, n = 4–21), rOCT2 (■, n = 4–12), hOCT1 (●, n = 3–16), or hOCT2 (○, n = 8–21). In the inset, the IC_{50} values calculated from the inhibition curves are shown. (B) Atenolol accumulation in wild-type (WT), hOCT1, and hOCT2 expressing HEK293 cells after 10 min incubation at 37 °C (white columns) with 100 μM atenolol. Experiments performed at 4 °C are also shown (black columns). Values are mean ± SEM expressed as fmol atenolol per cell. *Indicates a statistically significant higher atenolol accumulation at 37 °C compared with experiments performed at 4 °C (P < 0.05, unpaired t test). § indicates a statistically significant higher atenolol accumulation in hOCT2- compared with WT- and hOCT1-expressing cells (P < 0.05, ANOVA with Tukey post-test). Above the columns are the number of experiments.

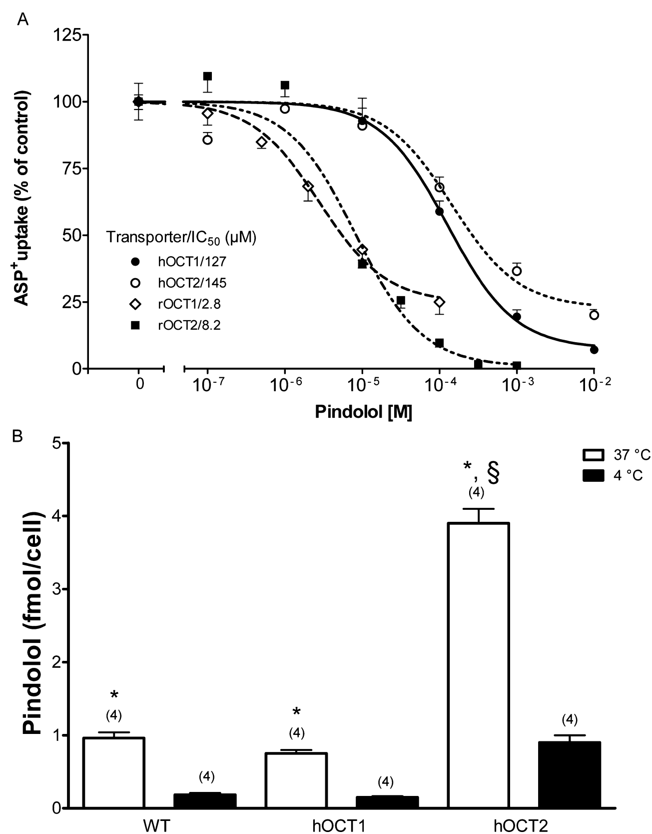

Figure 4. (A) Concentration-dependent inhibition of ASP^+ uptake by pindolol in HEK293 cells stably transfected with rOCT1 (◇, n = 7–30), rOCT2 (■, n = 8–24), hOCT1 (●, n = 6–11), or hOCT2 (○, n = 3–30). In the inset, the IC_{50} values calculated from the inhibition curves are shown. (B) Pindolol accumulation in wild-type (WT), hOCT1, and hOCT2 expressing HEK293 cells after 10 min incubation at 37 °C (white columns) with 100 μM pindolol. Experiments performed at 4 °C are also shown (black columns). Values are mean ± SEM expressed as fmol pindolol per cell. *Indicates a statistically significant higher pindolol accumulation at 37 °C compared with experiments performed at 4 °C (P < 0.05, unpaired t test). § indicates a statistically significant higher pindolol accumulation in hOCT2- compared with WT- and hOCT1-expressing cells (P < 0.05, ANOVA with Tukey post-test). Above the columns are the number of experiments.

was not significantly increased in hOCT1 transfected cells compared to WT cells.

Pindolol (Figure 4A) showed a higher apparent affinity for rat than for human OCT (IC_{50} values: 2.8, 8.2, 127, and 145 μM for rOCT1, rOCT2, hOCT1 and hOCT2, respectively). Even though the IC_{50} values for inhibition of ASP^+ uptake for hOCT1 and hOCT2 were similar, pindolol was much more efficiently transported into the cell by hOCT2 than hOCT1 (Figure 4B).

The rat OCT showed a higher affinity for ofloxacin (Figure 5A) than the human OCT (IC_{50} values: 235, 365, 3740, and 686 μM for rOCT1, rOCT2, hOCT1, and hOCT2, respectively). The accumulation of ofloxacin in HEK293 cells was a temperature-dependent process, which was significantly stimulated only by stable expression of hOCT2 (Figure 5B). On the contrary, human OCT exerted a slightly higher affinity for norfloxacin than rat OCT (Figure 6A, IC_{50} values: 525, 639, 263, and 132 μM, for rOCT1, rOCT2, hOCT1, and hOCT2, respectively). While the accumulation of norfloxacin in HEK293 cells was temperature-dependent, it was not further stimulated by stable transfection with hOCT1 or hOCT2 (Figure 6B).

Next, we investigated whether CsA has an acute effect on the drug transport mediated by rOCT1 and hOCT2 as representatives of this transporter family. After 10 min incubation with 10 μM CsA, the ASP^+ uptake by rOCT1, rOCT2, and hOCT2 was inhibited by 29 ± 5% (n = 6), 35 ± 3% (n = 8), and 28 ± 7% (n = 10), respectively (Figure 7A). The same CsA concentration (10 μM) had no acute effect on the ASP^+ uptake by rOCT1, rOCT2, hOCT2, or freshly isolated human proximal tubules (Supporting Informations, Figure 1), suggesting that CsA is not a substrate for these transporters. The effect of 10 min incubation with 10 μM CsA was also tested in freshly isolated human proximal tubules, which express mainly hOCT2 on the basolateral membrane.[11] Also, in this ex vivo preparation, a similar inhibition of ASP^+ uptake was observed (Figure 7A, −25 ± 7%, n = 3). Because

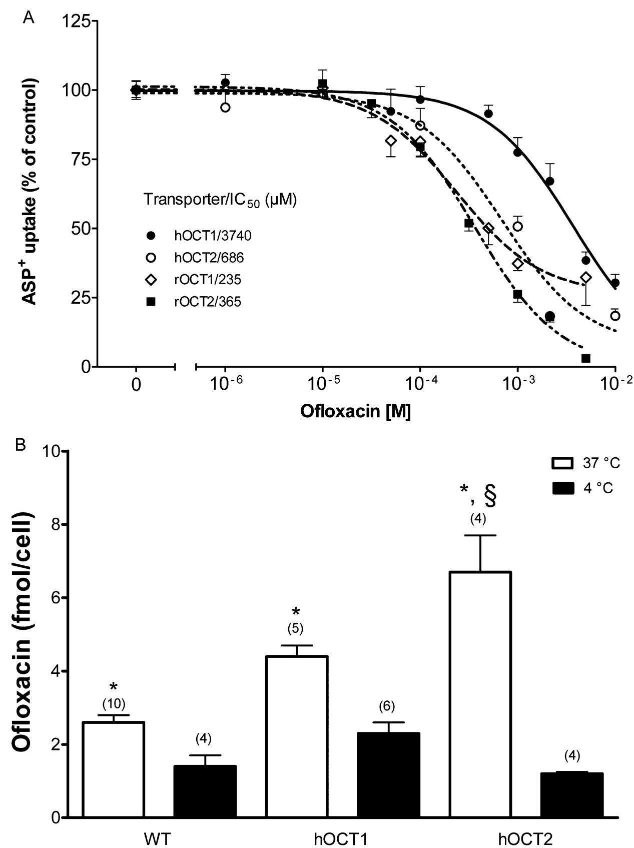

Figure 5. (A) Concentration-dependent inhibition of ASP^+ uptake by ofloxacin in HEK293 cells stably transfected with rOCT1 (◇, n = 3–29), rOCT2 (■, n = 7–24), hOCT1 (●, n = 3–24), or hOCT2 (○, n = 3–29). In the inset, the IC_{50} values calculated from the inhibition curves are shown. (B) Ofloxacin accumulation in wild-type (WT), hOCT1, and hOCT2 expressing HEK293 cells after 10 min incubation at 37 °C (white columns) with 500 μM ofloxacin. Experiments performed at 4 °C are also shown (black columns). Values are mean ± SEM expressed as fmol ofloxacin per cell. * indicates a statistically significant higher ofloxacin accumulation at 37 °C compared with experiments performed at 4 °C ($P < 0.05$, unpaired t test). § indicates a statistically significant higher ofloxacin accumulation in hOCT2- compared with WT- and hOCT1-expressing cells ($P < 0.05$, ANOVA with Tukey post-test). Above the columns are the number of experiments.

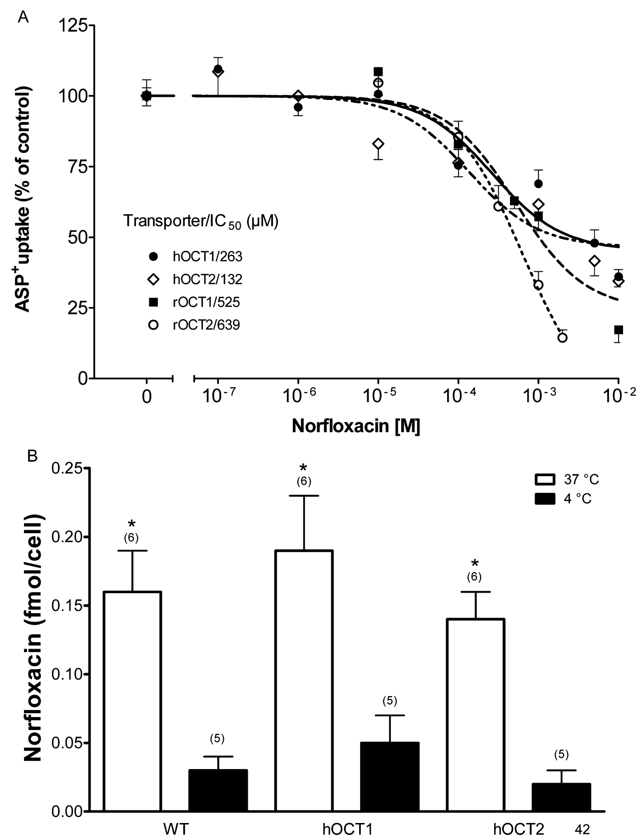

Figure 6. (A) Concentration-dependent inhibition of ASP^+ uptake by norfloxacin in HEK293 cells stably transfected with rOCT1 (◇, n = 3–44), rOCT2 (■, n = 12–32), hOCT1 (●, n = 3–16), or hOCT2 (○, n = 5–44). In the inset, the IC_{50} values calculated from the inhibition curves are shown. (B) Norfloxacin accumulation in wild-type (WT), hOCT1, and hOCT2 expressing HEK293 cells after 10 min incubation at 37 °C (white columns) with 500 μM norfloxacin. Experiments performed at 4 °C are also shown (black columns). Values are mean ± SEM expressed as fmol norfloxacin per cell. * indicates a statistically significant higher norfloxacin accumulation at 37 °C compared with experiments performed at 4 °C ($P < 0.05$, unpaired t test). Above the columns are the number of experiments.

CsA decreased the activity of rOCT1 and hOCT2, it was investigated whether this effect is due to changes in the apparent substrate affinity of the transporters and can also be observed for β-blockers and quinolones. These experiments were performed exemplary for the interaction of pindolol and ofloxacin with the ASP^+ transport by rOCT1. Incubation with 10 μM CsA did not significantly change the apparent affinity of rOCT1 for pindolol (2.6 and 5.5 μM without and with CsA incubation, respectively) but significantly lowered that for ofloxacin (230 and 967 μM without and with CsA incubation, respectively) (Figure 7B).

■ DISCUSSION

It is known that expression and function of OCT are subjected to short-term and also long-term regulation (for a review, see ref 27). Because of the pivotal role of OCT in the renal drug excretion, such a regulation may have important pharmacological consequences. Indeed, changes in the expression levels of renal drug transporters alter the pharmacokinetics of many drugs.[28] In vivo studies have demonstrated that chronic renal failure (5/6 nephrectomized rats) down-regulates the protein expression of rOCT2 but not of rOCT1.[29] Streptozotocin-induced diabetes in male Sprague–Dawley rats was associated with a reduction in kidney gene and protein expression of both rOCT1 and rOCT2.[30] Furthermore, lipopolysaccharide induced endotoxemia[31] and renal ischemia/reperfusion injury[32] down-regulated renal expression of rOCT1 and rOCT2 at the mRNA and protein level. In this work, we demonstrated that the renal expression of rOCT1 is down-regulated by kidney transplantation both at mRNA and protein level. This effect was not due to the rejection process and may probably resemble what was observed in the ischemia/reperfusion injury model.[32] Conversely, the expression of rOCT2 was significantly decreased at both mRNA and protein level only after allogeneic transplantation, indicating an effect induced by the acute rejection process. Therefore, rOCT1, as the majority of genes encoding for transporters with renal relevance,[26] showed decreased expression levels in allogeneic and syngeneic kidney transplantation, while rOCT2 was specifically down-regulated only in the allogeneic transplantation model undergoing acute rejection, as described also for the genes encoding for

dx.doi.org/10.1021/mp4000234 | Mol. Pharmaceutics 2013, 10, 2370–2380

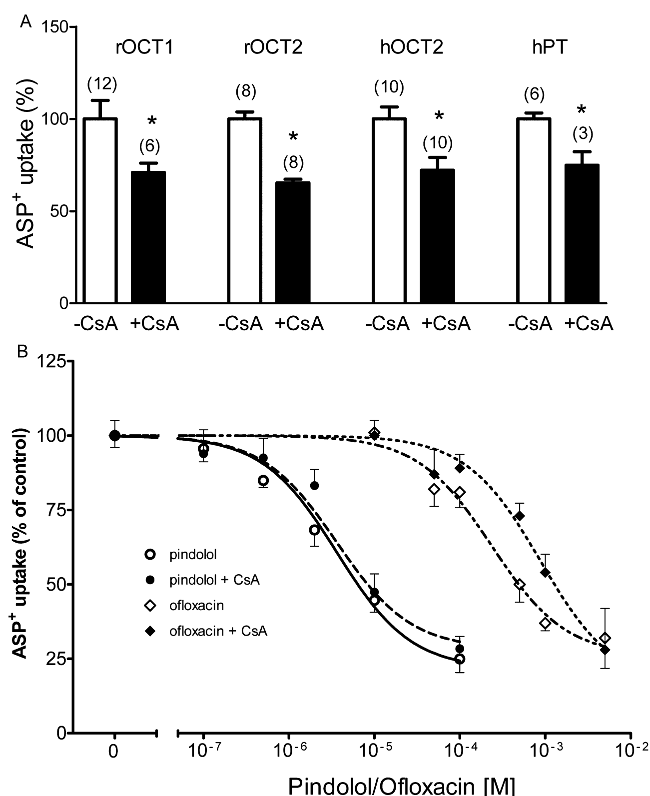

Figure 7. (A) Influence of 10 min incubation with 10 μM CsA on the ASP^+ uptake in HEK293 cells stably transfected with rOCT1, rOCT2, or hOCT2 and also in freshly isolated human proximal tubules cells. * Indicates a statistically significant difference compared to control experiments, where the cells or tubules were incubated for 10 min without CsA ($P < 0.05$, unpaired t test). Above the columns are the number of experiments. (B) Concentration-dependent inhibition of ASP^+ uptake by pindolol (○, n = 6−10) or ofloxacin alone (◇, n = 3−10) and in the presence of 10 μM CsA (●, n = 5−10 and ◆, n = 3−10, for pindolol and ofloxacin, respectively). All incubations with CsA were performed for 10 min. Pindolol alone inhibited the ASP^+ uptake with an IC_{50} of 3 μM and in combination with CsA with an IC_{50} of 5 μM. The difference between the curves is not statistically significant (GraphPad Prism). Ofloxacin alone inhibited the ASP^+ uptake with an IC_{50} of 230 μM and in combination with CsA with an IC_{50} of 967 μM. The difference between the curves is statistically significant (GraphPad Prism).

aquaporin 2 (AQP2) and the epithelial sodium channel (ENaC).[26] However, there are also examples of genes whose expression is up-regulated by acute rejection.[26] It should be mentioned that in the rat acute renal transplantation rejection model the renal blood flow can be reduced,[33] contributing to reduced extraction of drugs from the circulation.

The translational importance of these findings is underlined by the comparison of existing microarray data from kidney samples of normal donors and of patients with acute kidney rejection,[34] which shows also in humans a down-regulation of hOCT2 expression under an acute kidney rejection reaction.

Renal transplantation is followed by hypertension in up to 80% of human transplant recipients with poor graft survival, which leads to reduced life expectancy, largely due to increased cardiovascular mortality.[35−38] Renal transplantation patients are all treated with immunosuppressive drugs to prevent rejection and often in addition with antihypertensives and antibiotics to cope with hypertension and infections. Because OCT play an important role in renal drug excretion and because these transporters are down-regulated after transplantation, we have further investigated whether the immunosuppressive drug CsA, the antihypertensive β-blockers atenolol and pindolol, and the fluoroquinolone antibiotics ofloxacin and norfloxacin, which all are used in transplanted patients, interact specifically with OCT. Because we had to use rat transplantation models for this controlled study, interactions of these drugs were initially examined for rOCT1 and rOCT2. Moreover, to allow translation of these findings to the human situation expression of the relevant renal hOCT isoform, hOCT2, the interaction of these drugs with hOCT1 and hOCT2 was studied.

CsA alone had no effect on OCT expression in control kidneys. There is little knowledge of how CsA is taken up by cells and transported across or through them. In this work, we were not able to demonstrate a direct interaction of CsA with OCT as substrate. However, short CsA incubation of cells stably transfected with rOCT1, rOCT2, or hOCT2 decreased significantly the transport of organic cations and led to changes in the apparent affinity for some substrates (ofloxacin but not for pindolol). Interestingly, down-regulation of organic cation transport by CsA incubation was also observed in freshly isolated human proximal tubules, confirming what was found in hOCT2 expressing cells. Because it is known that the activity of OCT is subjected to short-term regulation by several kinases[27] and that CsA regulates the activity of such kinases in renal cells,[39] it is plausible that CsA influences the function of OCT indirectly by activation/inhibition of specific protein kinases. The regulation of OCT by CsA could be explained considering that CsA acts as a calmodulin antagonist[40] and rOCT1,[19] rOCT2,[19] and hOCT2[24] are all stimulated by calmodulin. Interestingly this CsA-modulated regulation seems to affect differently the apparent affinity of pindolol and ofloxacin for rOCT1. This finding can possibly be explained taking into account that OCT have a big substrate binding pocket, containing partially overlapping interaction domains for different substrates.[41] Such a CsA-modulated regulation of OCT may influence renal excretion of other cationic drugs as well and thus alter pharmacokinetics of these drugs.

The tested antibiotics as well as the antihypertensives reduced the transport of the model OCT substrate ASP^+ on all four tested OCT isoforms. Comparing the apparent affinities of atenolol, pindolol, ofloxacin and norfloxacin for rOCT1, rOCT2, hOCT1, and hOCT2, it is evident that there are subtype and species dependent differences. For example, ofloxacin has a drastically lower apparent affinity for hOCT1 than for rOCT1. Pindolol shows a relatively high affinity for rOCT1 and also for rOCT2, while human transporters have a lower one. Again, hOCT2 has a higher affinity for atenolol than hOCT1. Of the two human OCT subtypes, only the renal hOCT2 was able to actually transport atenolol, pindolol, and ofloxacin, while norfloxacin was not transported and the liver specific hOCT1 did not transport any of these drugs. Because the apparent affinities of hOCT2 for these substances are lower than their measured plasma concentration in patients, OCT may probably represent a low-affinity high-capacity renal excretion system. Measured maximal plasma drug concentration (C_{max}) and area under the curve (AUC) values corrected for protein binding are: 30 μM and 75 μM·h for atenolol,[42] 0.3 μM and 1.5 μM·h for pindolol,[43] 4 μM and 40 μM·h for ofloxacin,[44] and 4 μM and 18 μM·h for norfloxacin.[45] However, these values can change according to the therapeutic protocol and in pathological conditions: for example, the AUC for atenolol in patients with terminal renal failure increases to 500

μM·h.[46] Down-regulation of OCT in renal failure, as experimentally demonstrated here and elsewhere,[27,29,32] may be the reason for increased steady state plasma concentrations and/or elimination half-life observed for atenolol,[47] pindolol,[48] and ofloxacin[49] in patients with renal disease.

For β-blockers an interaction with OCT had been already demonstrated.[50,51] There exist also numerous reports indicating an interaction of fluoroquinolones with renal transport systems such as OCT.[52−54] However, we have for the first time directly demonstrated that the interaction of atenolol, pindolol, and ofloxacin with hOCT2 is linked to a transport of these molecules by the transporter.

Unfortunately, the limited availability of human renal tissue for the isolation and study of functionally intact proximal tubules renders such studies in this ex vivo preparation impossible. For the renal excretion of these drugs, a complementary transport system on the apical membrane of tubular cells is necessary. The interaction of β-blockers[55] and fluoroquinolones[54,56] with apical transport systems has also been studied, but the molecular entities responsible for this transport process have not yet been clearly identified. It can be speculated that at least the fluoroquinolones are transported by the multidrug and toxin extrusion protein 1 (MATE1).[57]

In conclusion, we have demonstrated that the renal expression of OCT is specifically decreased by renal transplantation and graft rejection. Moreover, their activity is acutely down-regulated by CsA. These facts, together with the demonstration that some drugs commonly used in the treatment of transplant recipients are transported by the human renal hOCT2 (atenolol, pindolol, and ofloxacin), underline the importance of OCT in renal drug excretion, especially in situations of reduced renal function such as, e.g., kidney transplantation. A detailed knowledge of the mechanisms governing renal drug excretion and drug−drug interactions is important to establish rational therapeutic protocols aimed to optimize treatment effects in patients suffering from various diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure showing that CsA does not exert acute effects on ASP^+ transport mediated by rOCT1, rOCT2, hOCT2, and also in freshly isolated human proximal tubules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG CI107/4-2 to G.C. and E.S.) and by the Ministry of Science, Sports and Education, Republic of Croatia (grant no. 022-0222148-2146 to I.S.).

■ ABBREVIATIONS USED

ASP^+, 4-(4-(dimethyl-amino)styryl)-*N*-methylpyridinium iodide; HEK293, human embryonic kidney cells; rOCT1−2, rat organic cation transporter type 1 or 2; CsA, cyclosporine A; hOCT1−2, human organic cation transporter type 1 or 2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase

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