

# Uptake of Cardiovascular Drugs Into the Human Heart Expression, Regulation, and Function of the Carnitine Transporter OCTN2 (SLC22A5)

Markus Grube, PhD; Henriette E.U. Meyer zu Schwabedissen, MD; Damaris Präger; Jeanette Haney, BSc; Klaus-Uwe Möritz, PhD; Konrad Meissner, MD; Dieter Roskopf, MD; Lothar Eckel, MD; Michael Böhm, MD; Gabriele Jedlitschky, PhD; Heyo K. Kroemer, PhD

**Background**—To date, the uptake of drugs into the human heart by transport proteins is poorly understood. A candidate protein is the organic cation transporter novel type 2 (OCTN2) (SLC22A5), physiologically acting as a sodium-dependent transport protein for carnitine. We investigated expression and localization of OCTN2 in the human heart, uptake of drugs by OCTN2, and functional coupling of OCTN2 with the eliminating ATP-binding cassette (ABC) transporter ABCB1 (P-glycoprotein).

**Methods and Results**—Messenger RNA levels of OCTN2 and ABCB1 were analyzed in heart samples by quantitative polymerase chain reaction. OCTN2 was expressed in all auricular samples that showed a pronounced interindividual variability (35 to 1352 copies per 20 ng of RNA). Although a single-nucleotide polymorphism in OCTN2 (G/C at position -207 of the promoter) had no influence on expression, administration of  $\beta$ -blockers resulted in significantly increased expression. Localization of OCTN2 by in situ hybridization, laser microdissection, and immunofluorescence microscopy revealed expression of OCTN2 mainly in endothelial cells. For functional studies, OCTN2 was expressed in Madin-Darby canine kidney (MDCKII) cells. Using this system, verapamil, spironolactone, and mildronate were characterized both as inhibitors ( $EC_{50}$ =25, 26, and 21  $\mu$ mol/L, respectively) and as substrates. Like OCTN2, ABCB1 was expressed preferentially in endothelial cells. A significant correlation of OCTN2 and ABCB1 expression in the human heart was observed, which suggests functional coupling. Therefore, the interaction of OCTN2 with ABCB1 was tested with double transfectants. This approach resulted in a significantly higher transcellular transport of verapamil, a substrate for both OCTN2 and ABCB1.

**Conclusions**—OCTN2 is expressed in the human heart and can be modulated by drug administration. Moreover, OCTN2 can contribute to the cardiac uptake of cardiovascular drugs. (*Circulation*. 2006;113:1114-1122.)

**Key Words:** pharmacology ■ drug transport ■ organic cation transport proteins ■ carnitine

Successful drug therapy requires a reliable achievement of therapeutic concentrations at the site of the intended drug action. This process was thought to be determined mainly by physicochemical factors of drugs, such as lipophilicity, molecular size, and degree of ionization. It has become increasingly clear, however, that drug concentrations and hence effects at the site of action result from a complex process of transporter-mediated drug uptake, intraorgan processing, and subsequent transporter-mediated elimination. This concept has been thoroughly demonstrated for human liver. Here, transporters of the solute carrier family such as OATP1B1 or OATP1B3 facilitate uptake of many drugs, such as pravastatin and digoxin, from the blood stream.<sup>1,2</sup> Subsequently, drugs like these are metabolized in part by hepatic cytochrome P450 mono-oxygenases, which results in oxidation of

## Clinical Perspective p 1122

the parent compound, or glucuronosyltransferase, to name but a few.<sup>3</sup> The products formed by metabolism are hydrophilic and require active transport to be eliminated from the liver. This elimination process is frequently mediated by ATP-binding cassette (ABC) transporters such as ABCB1 (P-glycoprotein) or ABCC2 (multidrug resistance-related protein 2, MRP2).<sup>4,5</sup> Functional coupling of uptake and elimination processes therefore determines the intrahepatic concentrations of drugs.

In contrast to the case of the liver, very little is known about drug disposition in the human heart. Several studies, however, suggest that the individual components described above for the liver (eg, transport and metabolism) are also present in the human heart.

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From the Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, Department of Pharmacology, Ernst Moritz Arndt University, Greifswald, Germany (M.G., H.E.U.M.z.S., D.P., J.H., K.-U.M., K.M., D.R., G.J., H.K.K.); the Klinikum Karlsburg, Karlsburg, Germany (L.E.); and the Department of Cardiology, Saarland University, Homburg/Saar, Germany (M.B.).

Correspondence to Heyo K. Kroemer, PhD, Department of Pharmacology, Friedrich Loefflerstraße 23d, 17487 Greifswald, Germany. E-mail kroemer@uni-greifswald.de

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With respect to cardiac metabolism, Thum and Borlak<sup>6</sup> reported expression and function of several cytochrome P450 enzymes in the human heart, among them cytochrome P450 2D6, which is involved in the metabolism of many cardiovascular drugs. Cardiac expression and function of several ABC transporters, such as ABCB1, ABCC5, and ABCG2, have been demonstrated by our group.<sup>7-9</sup> However, cardiac expression of transporters from the solute carrier (SLC) superfamily, which would facilitate uptake processes, has not been addressed in detail so far.

An interesting candidate for cardiac drug uptake is the organic cation transporter novel type II (OCTN2/SLC22A5), a high-affinity, sodium-dependent uptake transporter for L-carnitine. With screening approaches, OCTN2 messenger RNA (mRNA) had been detected in kidney, heart, skeletal muscle, and placenta.<sup>10</sup> L-Carnitine plays an important physiological role, particularly in  $\beta$ -oxidation, in which it facilitates long-chain fatty acid transport across the inner mitochondrial membrane. However, only a few organs, such as liver and kidney, have the ability to biosynthesize carnitine. Therefore, many tissues like skeletal and heart muscle are highly dependent on active carnitine uptake from blood.<sup>11</sup> Consequently, the rare syndrome of hereditary systemic carnitine deficiency, which results from several mutations in the *OCTN2* gene, leads, among other symptoms, to severe cardiomyopathy, which indicates an important physiological function of OCTN2 for the human heart.<sup>12</sup>

In addition to these physiological functions, OCTN2 is of potential pharmacological relevance, because several drugs such as  $\beta$ -lactam antibiotics and valproic acid have been described as substrates of OCTN2 or inhibitors of carnitine transport.<sup>13,14</sup> We, therefore, examined cardiac expression, localization, and function of OCTN2, in particular with respect to transport of cardiovascular drugs. We assayed OCTN2 mRNA levels in human heart samples and localized the OCTN2 expression by in situ hybridization and immunofluorescence microscopy. Interindividual variability could be associated with environmental factors (eg, administration of drugs) rather than genetic components. Given the latter results, we studied the interaction of different cardiovascular drugs with OCTN2 using an in vitro expression system for this protein. Proceeding further from the finding of a cardiovascular coexpression of OCTN2 and ABCB1, we established an epithelial model system that overexpressed both transporters to study the role and interaction of both proteins in transcellular transport.

## Methods

### Human Samples

After approval was obtained from the local ethics committee, samples from heart auricles were taken from 46 white patients (36 males, 10 females, age  $66.8 \pm 8.1$  years [mean  $\pm$  SD]) undergoing open heart surgery for aortocoronary bypass grafting as described previously.<sup>8</sup> The 15 ventricular samples were taken from excised left heart ventricle during orthotopic heart transplantation; these included samples from nonfailing (NF) hearts obtained from potential donors without any evidence of heart disease on medical history and heart samples from patients with ischemic cardiomyopathy (ICM) and dilated nonischemic cardiomyopathy (DCM;  $n=5$  each). DCM and

ICM samples were obtained from heart transplantations for treatment of end-stage heart failure.<sup>15</sup>

### Cell Culture and Substances

Madin-Darby canine kidney (MDCKII) cells were grown in minimum essential medium (MEM-Earle) containing 2 mmol/L L-glutamine, 1% nonessential amino acids, and 10% fetal calf serum. For double-transfection experiments, ABCB1-transfected pig kidney cells (LLC-PK1), kindly provided by Dr A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, Netherlands), were used. These cells were grown in Dulbecco modified Eagle medium (DMEM) under the same conditions as the MDCKII cells.

Mildronate [3-(2,2,2-trimethylhydrazinium)propionate] and mecamintine were kindly provided by the Grindex company (Riga, Latvia) and the MERZ GmbH (Frankfurt, Germany), respectively. Radiolabeled substances were obtained as indicated in the respective section. All other drugs were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH).

### Genotyping of the SLC22A5 G $\rightarrow$ C Promoter Polymorphism

The G  $\rightarrow$  C promoter polymorphism (National Center for Biotechnology Information single-nucleotide polymorphism [NCBI SNP] database rs2631367) in SLC22A5, originally described by Peltekova et al,<sup>16</sup> was genotyped by restriction fragment length polymorphism analysis. DNA was prepared from tissue specimens with the QiaAmp kit (Qiagen). A DNA fragment harboring the polymorphic site was amplified by polymerase chain reaction (PCR) with the primers 5'-GCCTCGCCAGGTCCCCAGGACAGG-3' and 5'-GCCGCCG-ACCAGGCAAGCCAGGCAG-3'. The amplicon of 272 base pairs (bp) was digested with the restriction endonuclease *Bcn*NI (MBI Fermentas, St. Leon-Rot, Germany), which resulted in fragments of 19, 32, 92, and 129 bp for the G allele and of 19, 32, and 221 bp for the C allele.

### RNA Isolation and Analysis

RNA from the 15 ventricular and 46 auricular samples was isolated and reverse transcribed as described previously.<sup>8</sup> Primer and probe oligonucleotides (forward primer: 5'-AATTTTGAGATGTTTGTC-GTGCTG-3'; reverse primer: 5'-CAAGAATTTCTGTCCC-CAGGAC-3'; probe: 5'-6FAM-TCCTTGTAGGCATGGCCAGAG-TCTCC-3') for the OCTN2 real-time PCR were designed on the basis of the OCTN2 cDNA sequence (accession number AB015050). For real-time PCR quantification with the ABI Prism 7700 Sequence Detector systems, the 18S ribosomal RNA (rRNA),  $\beta_2$ -microglobulin, and CD31 assays, as well as the PCR master mix, were purchased from Applied Biosystems. For quantification, signals were applied to a cloned standard for the respective gene. Auricular mRNA expression of ABCB1 was published by Meissner et al.<sup>8</sup>

### OCTN2 In Situ Hybridization

Five-micrometer sections of human auricular heart samples were fixed for 1 hour with 4% paraformaldehyde in PBS (pH 7.4), washed 3 times with PBS, and incubated for 10 minutes with 0.2 mol/L HCl. After repeated washing steps with PBS, sections were fixed with 4% paraformaldehyde in PBS (5 minutes, on ice) and washed again with PBS (3 times). After acetylation with 0.25% acetic anhydride in triethanolamine (0.1 mol/L, pH 8.0, 15 minutes) and additional washings with PBS and deionized formamide (50% in  $1.5 \times$  SSPE), sections were incubated for 1 hour with the prehybridization buffer (50% formamide, 0.5 ng/ $\mu$ L transfer RNA, 10% blocking solution [Roche] in Denhardt's solution) at 65°C in a humidified atmosphere. Hybridization with digoxigenin (DIG)-labeled riboprobes (8 ng/ $\mu$ L in prehybridization buffer) was performed overnight under the same conditions. Then, sections were washed with prehybridization buffer and the following solutions:  $2 \times$  SSC,  $0.1 \times$  SSC, and PBS. Subsequent to this washing protocol, the DIG-labeled probes were stained with the DIG wash-and-block buffer set (Roche) and an anti-DIG antibody labeled with alkaline phosphatase.

### Laser Capture Microscopy–Assisted Microdissection

Eight-micrometer sections of NF ventricular heart tissue were generated via a CM1900 cryostat (Leica) and placed on membrane slides (MMI AG). Thereafter, rapid hematoxylin and eosin staining was performed (70% ethanol for 15 minutes, 30 seconds of hematoxylin, 15 seconds of water, 1 minute of 70% ethanol, 1 minute of 95% ethanol, 30 seconds of eosin G, 2×1 minute 95% ethanol, 2×1 minute 100% ethanol), followed by incubation in xylene for 10 minutes. Slides were air-dried with an exicator and stored in a vacuum until laser-capture microscopy (LCM) was performed. LCM of blood vessel and control tissue was performed with a laser microdissector SL  $\mu$ Cut (MMI AG). Approximately 250 000  $\mu\text{m}^2$  of vessels or myocytes were cut (for n=4). RNA was isolated immediately after LCM. The RNA isolation, cDNA synthesis, and cDNA amplification were performed according to the method described by Klein et al<sup>17</sup> with the RNA lysis buffer to resuspend the cut tissue pieces from the caps.

### Immunofluorescence Microscopy

Protein localization was investigated by confocal laser scanning immunofluorescence microscopy. For OCTN2, a polyclonal antibody described previously was used (rabbit, dilution 1:200).<sup>18</sup> ABCB1 was detected with the monoclonal antibody C494 (mouse, dilution 1:25; Alexis Biochemicals, San Diego, Calif).

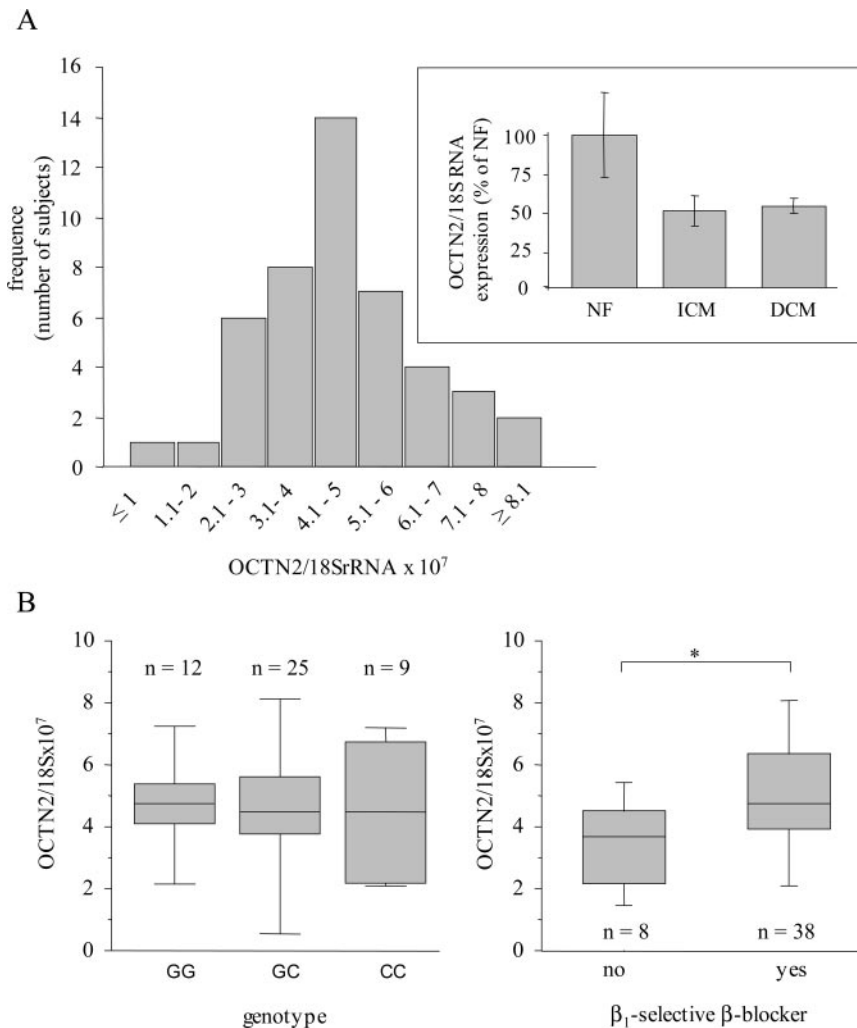
Paraffin sections of 2  $\mu\text{m}$  were prepared by standard methods. Incubation with primary antibodies was performed at 4°C overnight. For the peptide competition, the OCTN2 antiserum was preincubated

with 400 ng of the peptide at 4°C overnight. After being washed with TBS, the sections were incubated for 1 hour with Alexa Fluor 488- or 568-labeled IgG (anti-rabbit or anti-mouse IgG). Staining of nuclei was performed with a 1:2000 dilution of TOTO-3 iodide with DAKO fluorescent mounting medium (DakoCytomation). All secondary antibodies and the TOTO dye were purchased from Molecular Probes (Invitrogen).

### Cloning and Transfection

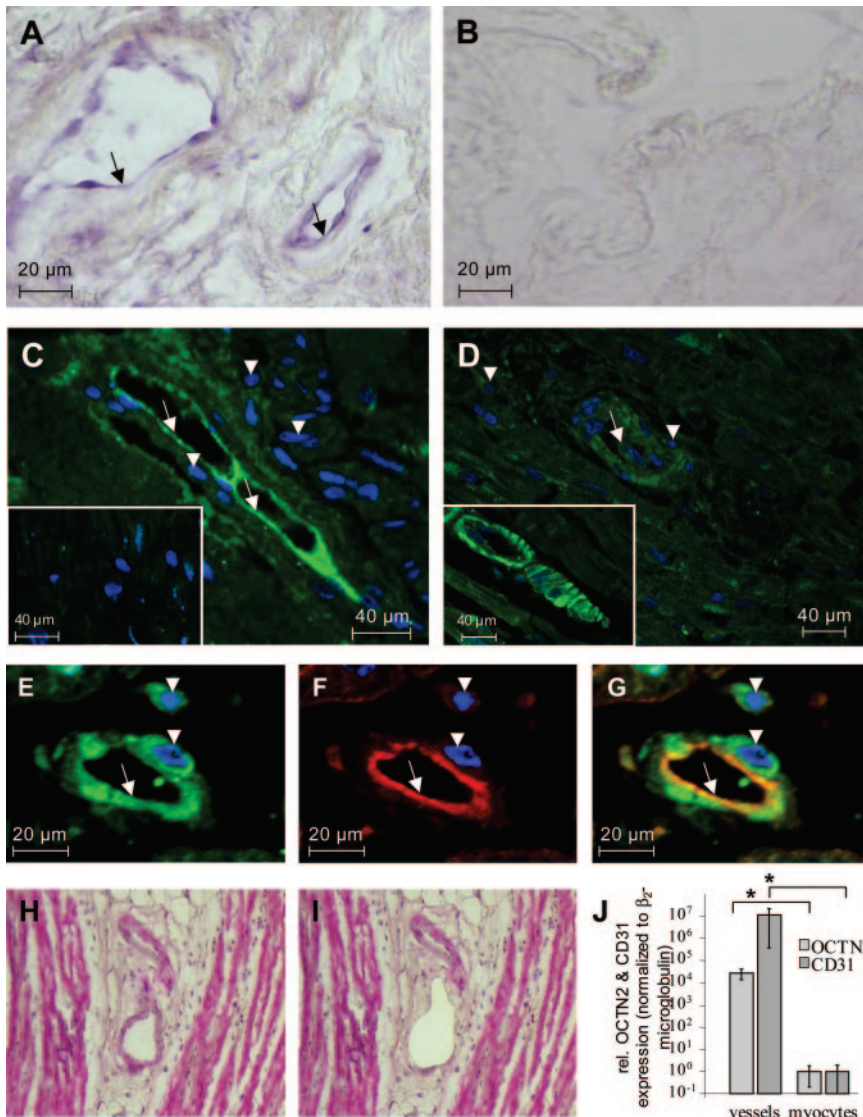
The OCTN2 cDNA was amplified with reverse-transcribed RNA from placenta (forward primer: 5'-CGCTCTGTGGCCTCTGA-3'; reverse primer: 5'-ACTGGAAGCGATGTTAGAAGGC-3') and cloned into the expression vector pcDNA3.1/hygro (Invitrogen). After verifying the sequence by cycle sequencing and matching against the OCTN2 reference sequence (accession number AB015050), MDCKII, LLC-PK1, and LLC-ABCB1 cells were transfected with this construct using the FuGENE 6 transfection reagent (Roche). Cells were selected for antibiotic resistance with 0.6 mg/mL hygromycin B (Invitrogen).

For the in situ hybridization, a 294-bp PCR fragment of the OCTN2 cDNA (forward primer: 5'-TGTCCACCATTGTGACCG-AGT-3'; reverse primer: 5'-CAAGAATTCTGTCCCCAGGAC-3') was cloned in the pDrive vector (Qiagen). The orientation of the insert and its correct sequence were proofed by cycle sequencing. Before reactions were labeled with the DIG RNA labeling kit (SP6/T7; Roche), the vector was linearized with the endonucleases *Bam*HI or *Hind*III depending on the labeling reaction with the SP6 or T7 polymerase.



**Figure 1.** OCTN2 mRNA expression in human heart. A, Distribution of OCTN2 mRNA levels in auricular heart samples normalized to 18S rRNA (n=46). Inset, OCTN2 mRNA expression in human ventricular heart samples with ICM or DCM compared with NF hearts. Expression is presented as percent of NF control samples (mean±SEM; n=5). B, Correlation between OCTN2 mRNA expression in auricular heart samples and C(-207)G genotype (left) or medication with  $\beta_1$ -specific  $\beta$ -blockers (right; \*P<0.05).





**Figure 2.** Localization of OCTN2 in human heart. A and B, OCTN2 in situ hybridization (A, antisense probe; B, sense probe). C through G, Confocal immunofluorescence microscopy. C through E, Staining with an OCTN2 anti-serum (green). C, Inset, preimmune serum control. D, Peptide competition; inset, noncompetitive antiserum control. F, Staining with anti-ABCB1 antibody (C494; red). G, Merger of E and F. Nuclei were stained with TOTO-3-iodide (blue). All pictures were taken with a 40 $\times$  objective. Arrows indicate vascular endothelium; arrowheads, nuclei. H through J, Laser microdissection. H and I, hematoxylin and eosin staining of NF human heart before (H) and after (I) dissection of blood vessels. J, OCTN2 and CD31 real-time PCR analysis of dissected endothelium and myocytes. Expression was normalized to expression of  $\beta_2$ -microglobulin; data represent mean $\pm$ SEM for n=4; \* $P$ <0.05.

## Transport Studies

For characterization of OCTN2-transfection, the respective cells were incubated with [ $^3$ H]carnitine (specific activity 2.96 TBq/mmol; Hartmann Analytic) for 5 minutes, washed 3 times with ice-cold PBS, and lysed with 0.2% SDS. An aliquot was dissolved in a 5-mL scintillation cocktail (Rotiszint, Roth) and measured in a scintillation  $\beta$ -counter (type 1409, LKB-Wallac, Turku, Finland). For characterization of ABCB1 transfectants, cells were incubated with 10  $\mu$ mol/L rhodamine-123 for the indicated times, washed thrice with ice-cold PBS, and lysed with 0.2% SDS. Fluorescence was determined with the 1420 Victor 2 multilabel counter (LKB-Wallac; excitation wavelength 530 nm, emission wavelength 485 nm).

For carnitine uptake inhibition and for transport studies with [ $^3$ H]verapamil (60 to 85 Ci/mmol; Biotrend) and [ $^3$ H]spironolactone (50 Ci/mmol; Biotrend), cells were seeded in 6-well dishes, cultured to confluence, and incubated for the indicated times with the respective substance at 37 $^{\circ}$ C. Rates of carnitine transport were given in picomoles of carnitine per milligram of protein $^{-1}$  per min $^{-1}$  or as ratio of control.

The transepithelial transport of [ $^3$ H]verapamil was determined with the transwell systems as described previously with minor modifications.<sup>19</sup> In brief, 0.75 and 0.5 mL of incubation buffer with [ $^3$ H]verapamil (2  $\mu$ Ci/mL) were added to the basal or apical compartment, respectively, whereas the opposite compartment was

filled with incubation buffer alone. At the indicated times, aliquots of 20  $\mu$ L were taken from the opposite compartment and dissolved in 5 mL of scintillation cocktail, and radioactivity was measured as described above. The leakage of this system was tested by basal to apical transport of [ $^{14}$ C]inulin (0.4  $\mu$ Ci/mL; 2.5 mCi/mmol, Biotrend Chemicals), which is not transported through the cells. In all cases, leakage was  $\leq$ 1%/h. All studies were performed with an incubation buffer containing 140 mmol/L NaCl<sub>2</sub>, 5 mmol/L KCl, 1 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1.5 mmol/L CaCl<sub>2</sub>, 5 mmol/L glucose, and 12.5 mmol/L HEPES (pH 7.3).

## Statistical Methods

Values are mean $\pm$ SD or SEM as indicated at the respective figures. Mann-Whitney  $U$  and Student  $t$  tests were used for comparison of the respective uptake experiments and OCTN2 expression in the different heart samples. The correlation between OCTN2 and ABCB1 mRNA expression was tested with the Pearson test. The differences were considered significant at  $P$ <0.05. The EC<sub>50</sub> values were calculated with Graph-Pad Prism software 3.0 (Graph-Pad Software). The  $K_m$  and  $K_i$  values were calculated from double-reciprocal Lineweaver-Burk plots.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

## Results

### mRNA Expression Data

Ventricular samples showed a significantly higher OCTN2 mRNA expression than the auricular samples ( $1194 \pm 244$  versus  $555 \pm 42$  copies per 20 ng of reverse-transcribed RNA [mean  $\pm$  SEM]; ventricular samples  $n=15$ , auricular samples  $n=46$ ). Among the ventricular samples, OCTN2 mRNA expression was reduced in the DCM and ICM samples, with expression of 45% and 43% compared with expression in NF hearts, respectively (Figure 1A, inset). The mRNA level of CD31 as a vascular marker was unchanged between the NF ( $5.9 \pm 0.2 \times 10^{-6}$  CD31/18S RNA expression [mean  $\pm$  SEM]), DCM ( $6.8 \pm 4.1 \times 10^{-6}$ ; 116% of NF), and ICM ( $5.3 \pm 0.1 \times 10^{-6}$ ; 90% of NF) samples. As shown in Figure 1A, the OCTN2 expression of the auricular samples exhibited a broad interindividual variation, with expression levels ranging from 0.55 to  $8.95 \times 10^7$  OCTN2/18S rRNA (or 35 to 1352 copies/20 ng reverse-transcribed RNA).

Genotyping analysis of the G/C promoter polymorphism for the 46 auricular specimens revealed 12 GG, 25 GC, and 9 CC genotypes. This genotype distribution was in accordance with a Hardy-Weinberg equilibrium. However, no association between SLC22A5 genotypes and transcript levels of this transporter was found, either alone or with adjustment for sex or medication (Figure 1B, left panel).

OCTN2 mRNA expression was not influenced by population characteristics such as age, body mass index, sex, or size, but changes in OCTN2 mRNA expression according to the individual drug treatment could be observed. Patients treated with ADP inhibitors (clopidogrel and ticlopidine) showed significantly lower OCTN2 mRNA expression than nontreated patients (OCTN2/18S rRNA  $3.96 \pm 0.42 \times 10^7$  versus  $5.05 \pm 0.3 \times 10^7$ ,  $P < 0.05$  [mean  $\pm$  SEM]). In addition, OCTN2 mRNA levels in patients taking  $\beta_1$ -selective  $\beta$ -blockers (bisoprolol [26 patients], metoprolol [11], and talinolol [1]) were 40% higher than in the control group (OCTN2/18S rRNA  $4.86 \pm 0.28 \times 10^7$  versus  $3.50 \pm 0.50 \times 10^7$ ;  $P < 0.05$  [mean  $\pm$  SEM]; Figure 1B, right panel). All other drugs used (aspirin, ACE inhibitors, dihydropyridine calcium channel blockers, digitalis, nitrates, proton pump inhibitors, and diuretics) had no significant effect. Finally, the auricular OCTN2 expression exhibited a significant positive correlation with the mRNA expression of the ABCB1 transporter (correlation 0.332;  $P = 0.017$ ).

### Localization of OCTN2

The localization of OCTN2 in human heart was assessed on the mRNA and protein levels. On the transcript level, an *in situ* hybridization visualizing the OCTN2 mRNA was performed that demonstrated a predominant signal in the vascular endothelium (Figures 2A and 2B). As a second approach, LCM of blood vessels and myocytes was performed, followed by quantitative real-time PCR. OCTN2 and CD31 mRNA levels were significantly higher in vessels than in myocytes (Figures 2H through 2J). On the protein level, immunofluorescence staining was performed for OCTN2 and ABCB1 with the polyclonal antiserum against human OCTN2 and a monoclonal antibody against ABCB1. Here, a

### Inhibition of Carnitine Uptake Into OCTN2-Transfected MDCKII Cells by Various Drugs

| Substance    | Concentration, $\mu\text{mol/L}$ | % of Control $\pm$ SD |
|--------------|----------------------------------|-----------------------|
| Control      | ...                              | 100 $\pm$ 3           |
| Acebutolol   | 50                               | 102 $\pm$ 2           |
| Memantine    | 50                               | 102 $\pm$ 2           |
| Canrenoate   | 100                              | 97 $\pm$ 1            |
| Captopril    | 100                              | 118 $\pm$ 1           |
| Carvedilol   | 100                              | 90 $\pm$ 23           |
| Cortisone    | 50                               | 64 $\pm$ 3*           |
| Digoxin      | 100                              | 102 $\pm$ 8           |
| Enalapril    | 50                               | 71 $\pm$ 4*           |
| Lidocaine    | 50                               | 69 $\pm$ 7*           |
| Metoprolol   | 50                               | 108 $\pm$ 9           |
| Mildronate   | 50                               | 42 $\pm$ 7*           |
| Propranolol  | 50                               | 105 $\pm$ 6           |
| Spirolactone | 50                               | 48 $\pm$ 3*           |
| Verapamil    | 50                               | 58 $\pm$ 2*           |

Cells were incubated with [ $^3\text{H}$ ]carnitine (1  $\mu\text{mol/L}$ ; 0.5  $\mu\text{Ci/mL}$ ) in the presence of the listed compounds or the respective solvent, and carnitine uptake within 5 minutes was calculated as described in Methods (\* $P < 0.05$ ).

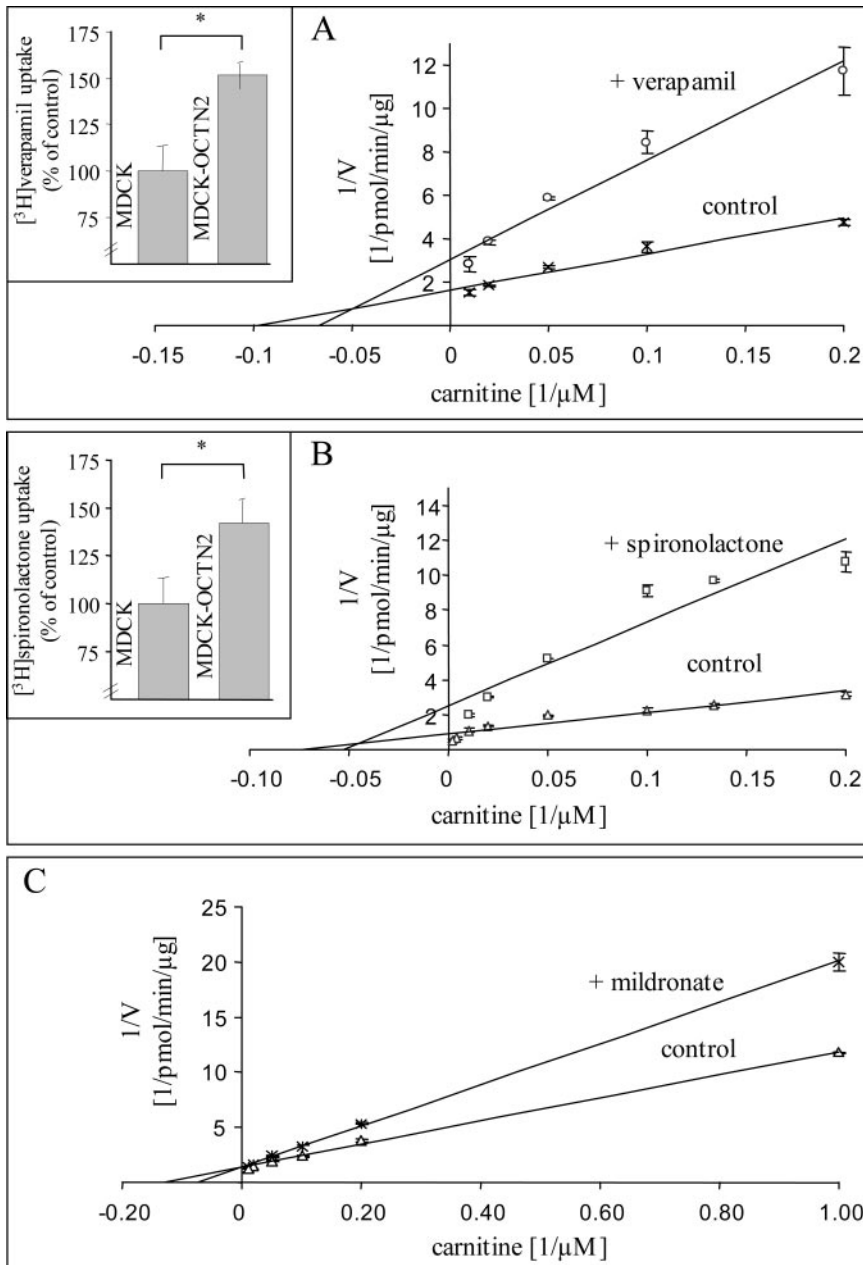
positive signal for both proteins could be detected in the vascular endothelium (Figures 2C through 2G).

### Uptake Studies

OCTN2-transfected MDCKII and LLC-PK1 cells were used to determine the interaction of various drugs with this transporter. First, OCTN2-transfected cells were studied with respect to their carnitine transport characteristics. Carnitine uptake was time- and sodium-dependent, with a  $K_m$  value of 7.7  $\mu\text{mol/L}$  for carnitine, as described for recombinant OCTN2 (data not shown).<sup>10</sup> Inhibition studies showed a significantly lower carnitine uptake in transfected MDCKII cells in the presence of cortisone (64% of control), enalapril (71%), lidocaine (69%), mildronate (42%), spironolactone (48%), and verapamil (58%; Table). The effects of the most potent inhibitors (mildronate, spironolactone, and verapamil) were analyzed further. The  $EC_{50}$  data of the mildronate, verapamil, and spironolactone effect were calculated at 21, 25, and 26  $\mu\text{mol/L}$ , respectively. Moreover, mildronate showed a competitive inhibition kinetic, with a  $K_i$  value of  $\approx 26.1$   $\mu\text{mol/L}$ . In contrast, for verapamil and spironolactone, no distinct inhibition type could be detected (Figure 3). However, radiolabeled verapamil and spironolactone showed a significantly higher accumulation in OCTN2-transfected cells (150% and 145% of control, respectively; Figure 3 insets).

### Transcellular Transport Studies

To show both the involvement of OCTN2 and the interaction of OCTN2 and ABCB1 in transepithelial transport, the transport of verapamil as a model substance was characterized in OCTN2 and ABCB1 double-transfected epithelial cells in a transwell system. As shown in Figure 4, these



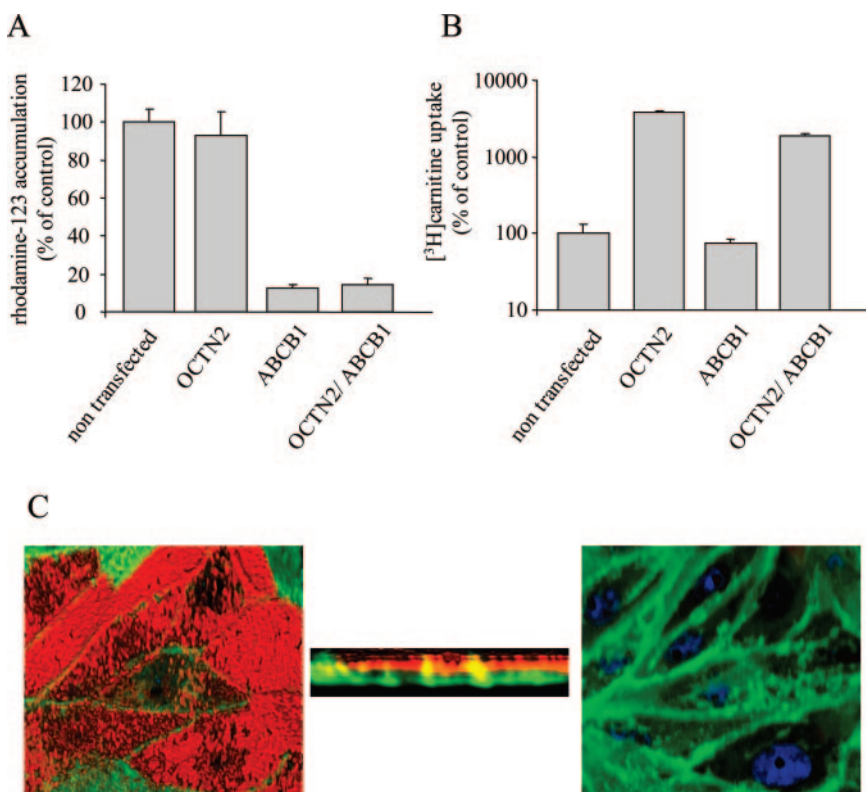
**Figure 3.** Inhibition of OCTN2-mediated carnitine transport by cardiovascular drugs. Rates of OCTN2-mediated [ $^3$ H]carnitine transport in OCTN2-transfected MDCKII cells was determined at different substrate concentrations in the presence or absence of A, verapamil (50  $\mu$ mol/L); B, spironolactone (25  $\mu$ mol/L); and C, mildronate (20  $\mu$ mol/L). Points represent mean  $\pm$  SD (n=3). Insets: Uptake of verapamil (top) and spironolactone (middle) into OCTN2-transfected MDCKII cells vs control cells. Cells were incubated for 10 minutes with 10 nmol/L [ $^3$ H]verapamil and 8 nmol/L [ $^3$ H]spironolactone (n=3; \* $P$ <0.05).

double-transfected cells were characterized and compared with the single-transfected cells with respect to ABCB1 (rhodamine-123 accumulation, Figure 4A) and OCTN2 function (carnitine uptake, Figure 4B). Furthermore, localization of the 2 proteins was analyzed by confocal immunofluorescence microscopy (Figure 4C), which revealed (baso)lateral expression of OCTN2 and apical expression of ABCB1. Using this expression system, transepithelial basal to apical verapamil transport was measured over 4 hours. Although there was no difference in verapamil transport across untransfected LLC-PK1 cells and OCTN2-transfected cells, ABCB1- and OCTN2/ABCB1-transfected cells demonstrated a significantly increased transepithelial transport. When we compared ABCB1-transfected and ABCB1/OCTN2-double-transfected cells, there was substantially enhanced transport in double-transfected cells (Figure 5).

## Discussion

In this study, we demonstrate a new uptake mechanism of drugs into endothelial cells of human heart. Physiologically, OCTN2/SLC22A5 contributes to cellular uptake of carnitine. Aside from its role in energy metabolism, OCTN2 appears to be capable of facilitating the uptake of various cardiovascular drugs. OCTN2 is coexpressed with the eliminating transport protein ABCB1 in the vascular endothelium, and as suggested by our in vitro model, coordinated action of both transporters can modify cardiac concentrations of drugs. The population investigated in the present study exhibited a wide interindividual variability in expression of OCTN2, and it remains unclear whether this variability originates from inherited or environmental factors. In terms of genetics, rare mutations in the *OCTN2* gene have been identified as being responsible for the primary systemic carnitine deficiency (SCD; OMIM





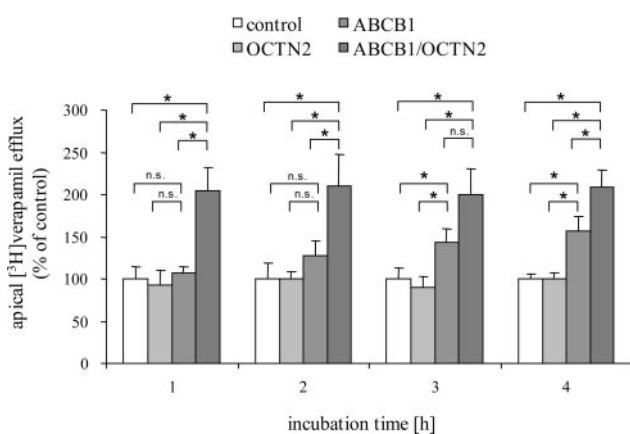
**Figure 4.** Characterization of OCTN2-, ABCB1-, and double-transfected LLC-PK1 cells. A, ABCB1 function was studied by rhodamine-123 accumulation. The respective cells were incubated with rhodamine (10  $\mu$ mol/L) for 30 minutes (data represent rhodamine-123 accumulation in relation to untransfected cells; mean  $\pm$ SD, n=3). B, OCTN2 function was determined by carnitine uptake measurement. The respective cells were incubated with [ $^3$ H]carnitine for 5 minutes (data represent mean  $\pm$ SD, n=3). C, Confocal laser scanning immunofluorescence microscopy of OCTN2 (green) and ABCB1 (red) in double-transfected LLC-PK1 cells. Left, Top view of the cell monolayer; middle, cross section of the cells; right, bottom view of cell monolayer. Nuclei were stained with TOTO-3-iodide (blue).

212140), which is associated with myopathy and hypertrophic cardiomyopathy.<sup>12</sup> Moreover, a promoter polymorphism (rs 2631367) has been identified recently that is associated with an enhanced risk for Crohn's disease and that has been shown to influence OCTN2 promoter activity.<sup>16</sup> For human auricular cardiac tissue, we did not observe an effect of this polymorphism on transcript levels.

In contrast, medication had a significant influence on OCTN2 expression. Use of  $\beta_1$ -adrenergic receptor blockers

resulted in increased expression of OCTN2. On the basis of our in vitro data, we can exclude a direct interaction of the  $\beta_1$ -selective metoprolol and the nonselective carvedilol with OCTN2 (Table). However, adrenergic stimulation leads to an increased ATP production in heart by preferentially increasing glucose metabolism.<sup>20</sup> Therefore, an inhibition of this mechanism by administration of adrenergic receptor blockers may require a switch to additional energy sources. A possible physiological reaction may consist of enhanced expression of OCTN2, because the resulting increase in carnitine uptake would enhance transfer of fatty acids as a prerequisite of  $\beta$ -oxidation.

In the ventricular samples, reduced OCTN2 expression was observed for both groups of diseased hearts but not for the endothelial marker CD31. A similar effect has been observed for ABCB1<sup>18</sup> but not for ABCC5, which is upregulated in ICM samples and remains unaffected between NF and DCM hearts.<sup>7</sup> Because of the small sample size, these data were not statistically significant. To confirm this trend of decreased OCTN2 expression in cardiomyopathic heart, we accessed data from the public domain of the comparative transcription profiling database of diseased and normal human myocardium of the CardioGenomics project (Genomics of Cardiovascular Development, Adaptation, and Remodeling, National Heart, Lung, and Blood Institute Program for Genomic Applications, Harvard Medical School; available at <http://www.cardiogenomics.org>, accessed October, 2005). Here, ventricular gene-expression profiles were obtained from explanted hearts of patients with ischemic (n=32) or idiopathic cardiomyopathic heart failure (n=26), as well as from healthy hearts (n=14). The OCTN2 expression in these samples was significantly reduced from  $1434 \pm 144$  arbitrary units in con-



**Figure 5.** Transcellular transport of [ $^3$ H]verapamil. LLC-PK1 cells (control), OCTN2-transfected LLC-PK1 cells (OCTN2), ABCB1-transfected LLC-PK1 cells (ABCB1), and OCTN2/ABCB1 double-transfected LLC-PK1 cells (OCTN2/ABCB1) were grown in transwell culture dishes, and [ $^3$ H]verapamil (1  $\mu$ Ci/mL, 8 nmol/L) was added to the basal compartment. After indicated incubation time at 37°C, [ $^3$ H]verapamil accumulation in the apical compartment was determined (data represent mean  $\pm$ SD in relation to the transcellular transport in control LLC-PK1 cells for n=3; \* $P$ <0.05).

trols to  $1158 \pm 57$  arbitrary units ( $P=0.036$ , Student *t* test) in ischemic cardiomyopathy and  $1146 \pm 53$  arbitrary units ( $P=0.028$ ) in idiopathic cardiomyopathy, respectively (probe set 205074\_at; mean  $\pm$  SE). Taken together, these data point to a reduced expression of OCTN2 in heart failure; however, this effect needs to be confirmed in larger trials.

Next, we studied the localization of OCTN2 in human heart. All methods applied—in situ hybridization, immunofluorescence microscopy, and laser microdissection followed by reverse transcription-PCR—indicated OCTN2 expression mainly in the cardiovascular endothelium. This endothelial expression is in line with its function as a carnitine uptake transporter, particularly given the cardiac dependency on active carnitine uptake from blood.<sup>21</sup> The carnitine concentration in cardiac muscle cells is  $\approx 50$ -fold higher than serum levels. For this gradient, the endothelial secondary active carnitine uptake transport mediated by OCTN2 is essential. If this gradient is altered as a result of decreased expression of OCTN2, myocyte carnitine concentration may be insufficient.

Moreover, cardiac expression of OCTN2 may also play a role in local drug delivery. Using an OCTN2-overexpressing MDCKII cell line, we were able to show that drugs like cortisone, enalapril, mildronate, and spironolactone significantly inhibited OCTN2-mediated carnitine uptake. In addition, we could also demonstrate a significantly increased uptake of tritium-labeled verapamil and spironolactone into OCTN2-transfected cells (Figure 3). Further studies with verapamil, spironolactone, and mildronate showed EC<sub>50</sub> values for carnitine uptake inhibition of  $\approx 25$   $\mu\text{mol/L}$  for verapamil and spironolactone and 21  $\mu\text{mol/L}$  for mildronate. In the case of spironolactone and verapamil, a mixed inhibition type was observed, whereas mildronate inhibited carnitine uptake in a competitive manner, with a  $K_i$  value of 26  $\mu\text{mol/L}$  (Figure 3). These results extend previous findings on OCTN2-mediated verapamil transport and identify spironolactone and mildronate as new inhibitors and potential substrates for OCTN2.<sup>14,22</sup> Spironolactone has been characterized as an important drug for treatment of congestive heart failure, and uptake via OCTN2 could modify its effects.<sup>23</sup> Previous studies demonstrated that mildronate treatment had positive effects in patients with congestive heart failure and coronary artery disease.<sup>24,25</sup> Mildronate was designed as a carnitine analog inhibiting its biosynthesis by interaction with the  $\gamma$ -butyrobetaine hydroxylase, which in turn leads to reduced  $\beta$ -oxidation.<sup>26,27</sup> Our results indicate OCTN2 as another molecular target of mildronate.

Uptake of drugs via OCTN2 results in intracellular accumulation, which can be modified by elimination transporters. We have identified several of these proteins in human heart, among them ABCB1, a protein involved in transport of many cardiovascular drugs (eg, verapamil and digoxin), and we were able to show a positive correlation between the mRNA expression of both transporters. Moreover, we could demonstrate that both proteins are coexpressed in the vascular endothelium (Figure 2). On the basis of this finding, we developed OCTN2/ABCB1 double-transfected LLC-PK1 cells to study a potential interaction of both transporters. These cells exhibited an apical expression of ABCB1 and a basolateral expression of OCTN2 (Figure

4). When grown in a transwell system, these cells allow the simultaneous study of uptake and elimination by measurement of the substrate concentration in the basal, cellular, and apical compartment. Using this system, we found a higher basal to apical verapamil transport (as a model substrate for both transporters) in the OCTN2/ABCB1- and ABCB1-transfected cells, respectively, than in nontransfected or OCTN2-transfected controls. Moreover, transepithelial transport in the double-transfected cells was significantly higher than in the ABCB1-overexpressing cells. In the cardiovascular endothelium, where both ABCB1 and OCTN2 are present in the apical membrane, compounds such as verapamil may be taken up by OCTN2 from the blood and simultaneously be eliminated back into the lumen by ABCB1. Taken together, we were able to show expression, localization, and function of the carnitine uptake transporter OCTN2 in human heart.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Variable response to drugs is a major problem in clinical practice. Patients treated according to standardized guidelines show a substantial variation in response that ranges from no effect to unwanted side effects. The underlying problem is, in part, our lack of understanding of the mechanisms by which drugs reach their site of action. Although other organs such as the liver have been thoroughly investigated in terms of uptake, metabolism, and elimination of drugs, such data are not available for the human heart. The present report characterizes the expression and function of an uptake transport protein named OCTN2 (organic cation transporter novel type 2) in the human heart. OCTN2 is a high-affinity carnitine transporter, and rare genetic defects in this protein lead to cardiomyopathy. The role of OCTN2, however, is not restricted to carnitine transport; expression studies and in vitro transport assays suggest that OCTN2, confined to cardiac endothelial cells, is involved in the transport of drugs such as verapamil and spironolactone to cardiac tissue. Moreover, drugs (eg,  $\beta$ -blockers) and disease states (eg, idiopathic cardiomyopathy) appear to modulate the expression of OCTN2. Taken together, these data may lead to new concepts on how an orchestrated action of drug transporters influences localized tissue-specific pharmacology and, vice versa, how the interaction of drugs and cardiac diseases modulates drug responses by effects on organ-specific drug handling.