BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Drug and Bile Acid Transporters in Rosuvastatin Hepatic Uptake: Function, Expression, and Pharmacogenetics

RICHARD H. HO,*,‖ ROMMEL G. TIRONA,§,‖ BRENDA F. LEAKE,§,‖ HARTMUT GLAESER,§,‖ WOOIN LEE,§,‖ CHRISTOPHER J. LEMKE,§,‖ YI WANG,¶ and RICHARD B. KIM§,‖

*Department of Pediatrics, §Department of Medicine, and ‖Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee; and ¶Drug Metabolism and Pharmacokinetics, AstraZeneca, Wilmington, Delaware

Background & Aims: **The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, target liver HMG-CoA and are of proven benefit in the prevention of coronary heart disease. Rosuvastatin is an effective statin notable for liver selectivity and lack of significant metabolism. We assessed the extent and relevance of hepatic transporters to rosuvastatin uptake.** ***Methods:*** **Transporters involved in rosuvastatin uptake were determined through heterologous expression of multiple human and rat uptake transporters. Human organic anion transporting polypeptide (OATP) 1B1 and sodium-dependent taurocholate cotransporting polypeptide (NTCP) allelic variants were also assessed. Expression of OATP and NTCP messenger RNA and protein was determined from a bank of human liver samples.** ***Results:*** **Multiple OATP family members, including 1B1, 1B3, 2B1, and 1A2, were capable of rosuvastatin transport. Naturally occurring polymorphisms in *OATP1B1*, including **5*, **9*, **15*, and **18*, were associated with profound loss of activity toward rosuvastatin. Interestingly, the major human hepatic bile acid uptake transporter NTCP, but not rat Ntcp, also transported rosuvastatin. Human hepatocyte studies suggested that NTCP alone accounted for ~35% of rosuvastatin uptake. Remarkably, NTCP*2, a variant known to have a near complete loss of function for bile acids, exhibited a profound gain of function for rosuvastatin. Quantitative messenger RNA analysis revealed marked intersubject variability in expression of OATPs and NTCP.** ***Conclusions:*** **Multiple transporters mediate the overall hepatic uptake of rosuvastatin, and NTCP may be a heretofore unrecognized transporter important to the disposition of rosuvastatin and possibly other drugs/statins in clinical use. Accordingly, transporter expression and polymorphisms may be key determinants of intersubject variability in response to statin therapy in general.**

Coronary heart disease is the single largest cause of death in the United States, responsible for 1 in every 5 deaths, with an estimated cost of $142.1 billion in 2005.[1] Deposition of cholesterol-rich lipoproteins within the arterial wall is a central tenet in the pathogenesis of atherosclerosis.[2] Previous studies have demonstrated a causal relationship between elevated total serum cholesterol levels and coronary heart disease and have shown that cholesterol reduction substantially reduces the incidence of cardiovascular events.[3] 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are first-line pharmacologic therapy for hypercholesterolemia and exert their major therapeutic action in the liver via reduction in low-density lipoprotein (LDL)-C levels.[4] The safety and efficacy of statins for the primary and secondary prevention of coronary heart disease have been established in large clinical trials.[4]

Rosuvastatin is a newer statin with favorable pharmacologic properties including hepatic selectivity, minimal metabolism, and enhanced inhibitory potency of HMG-CoA reductase.[5] Drug disposition studies indicate that 72% of intravenously administered rosuvastatin is eliminated by the liver and that the estimated extraction rato in liver is 0.63,[6] highlighting the importance of this organ in systemic drug clearance and first-pass effects. Because rosuvastatin, like pravastatin, is relatively hydrophilic compared with other statins, active membrane transport mechanisms are thought to be present in the liver to mediate drug accumulation and biliary excretion.[7] Expression of active uptake transporters in hepatocytes with high affinity for organic anions such as rosuvastatin would likely confer a greater degree of hepatic selectivity and decreased access to nonhepatic tissue compartments via passive diffusion. Indeed, in vitro studies reveal rosuvastatin to have markedly lower

Abbreviations used in this paper: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NTCP, sodium-dependent taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; SNP, single nucleotide polymorphism.

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HMG-CoA reductase inhibitory potency in nonliver cells compared with hepatocytes.[7]

Accordingly, carrier-mediated hepatic uptake appears to be important for the disposition and efficacy of many statins, including rosuvastatin. Recent studies have clearly implicated members of the organic anion transporting polypeptide (OATP) superfamily as active transporters of statins such as pravastatin and rosuvastatin.[8,9] Of relevance to statin therapy, certain OATPs appear to be highly expressed in the liver in which they are involved in hepatic clearance of substrates from the portal circulation.[10] The importance of transport processes involved in statin disposition is illustrated by observed interactions with drugs such as gemfibrozil and cyclosporine.[11,12] Indeed, cyclosporine has been shown to increase plasma levels of both pravastatin and rosuvastatin by 6- to 10-fold, despite the fact that these statins are not appreciably metabolized.[12,13] Moreover, inhibition of hepatic OATP1B1-mediated cerivastatin uptake by cyclosporine is a determinant of the observed interaction between these drugs.[14] Although OATP1B1 has been shown to transport several statins including cerivastatin,[14] atorvastatin,[8] pravastatin,[8] and rosuvastatin,[9] the role and relevance of other uptake transporters in hepatic statin clearance and antihyperlipidemic effects have not been reported.

In this report, we demonstrate that multiple OATP family members, including the major hepatic OATPs 1B1, 1B3, and 2B1, in addition to NTCP, the primary bile acid uptake transporter in human liver, are capable of transporting rosuvastatin. Interindividual variability in rosuvastatin disposition may be explained by naturally occurring, functionally significant *0ATP1B1* and *NTCP* single nucleotide polymorphisms (SNPs) that are associated with significantly altered transport kinetics for rosuvastatin, as well as the presence of marked intersubject variability in the expressed levels of these transporters. These findings provide important new mechanistic insights to the disposition of rosuvastatin and have broad implications for the disposition and efficacy profiles of other statins in clinical use.

Materials and Methods

Materials

[^{3}H] rosuvastatin (79 Ci/mmol, 97.1% purity) and unlabeled rosuvastatin were obtained from AstraZeneca (Wilmington, DE). Recombinant vaccinia virus was a gift provided by Dr B. Moss (NIH, Bethesda, MD). Human liver samples were kindly provided by Dr F. P. Guengerich at our institution. All other chemicals and reagents, unless stated otherwise, were obtained from Sigma-Aldrich Research (St. Louis, MO) and were of the highest grade available.

Wild-Type and Variant OATP and NTCP Plasmid Construction

The full open reading frames of human and rat uptake transporter complementary DNA (cDNA) were obtained by polymerase chain reaction (PCR), using AmpliTaq DNA polymerase (PerkinElmer Life Sciences, Boston, MA), from cDNA libraries synthesized from human and rat liver messenger RNA (mRNA), respectively, as described previously.[15] OATP1B1 and NTCP variants used for in vitro functional studies were created as previously described.[16,17]

Transport and Inhibition Studies using Recombinant Vaccinia Virus

HeLa cells grown in 12-well plates ($\sim 0.8 \times 10^6$ cells/well) were infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units (PFU)/cell in serum-free Opti-MEM I medium (Invitrogen, Carlsbad, CA) and allowed to adsorb for 30 minutes at 37°C. Cells in each well were then transfected with 1 μg transporter cDNA packaged in pEF6/V5-His-TOPO vector (Invitrogen), along with Lipofectin (Invitrogen) and incubated at 37°C for 16 hours. The parental plasmid lacking any insert was used as control. Transport was then evaluated using a labeled substrate as outlined previously.[18] For inhibition studies, cells were washed with transport media (Opti-MEM I) and treated with radiolabeled drug in the presence or absence of transport inhibitors cyclosporine and gemfibrozil. At various time intervals, cells were washed 3 times with ice-cold medium then lysed with 1% SDS. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

To measure transport kinetics, [^{3}H] rosuvastatin uptake during the linear phase (first 3 minutes) was assessed in the presence of various concentrations of unlabeled compound. Transporter-dependent uptake was determined in parallel experiments as the difference in drug uptake between transporter and parental plasmid DNA-transfected cells. Michaelis–Menten-type nonlinear curve fitting was used to estimate the maximal uptake rate (V_{max}) and concentration at which half the maximal uptake occurs (K_m). All experiments were carried out in duplicate on at least 2 to 3 experimental days.

Rosuvastatin and Taurocholate Uptake by Rat and Human Hepatocyte Suspensions

Fresh hepatocytes were obtained from a pool of 3 male Sprague–Dawley rat livers (CellzDirect, Dallas, TX) and from 4 human liver donors (In Vitro Technologies, Baltimore, MD and CellzDirect). Upon arrival, hepatocytes were washed with ice-cold, modified Krebs–Henseleit bicarbonate (KHB) buffer ($MgSO_4$ 1.2 mmol/L, KH_2PO_4 0.96 mmol/L, KCl 4.83 mmol/L, NaCl 118 mmol/L, $CaCl_2$ 1.53 mmol/L, $NaHCO_3$ 23.8 mmol/L, HEPES 12.5 mmol/L, glucose 5 mmol/L, pH 7.4 or pH 6.0) or sodium-free KHB (NaCl and $NaHCO_3$ replaced with choline Cl and choline bicarbonate respectively, pH 7.4 or pH 6.0) then resuspended in a concentration of 2.5×10^6 cells/mL. After a 5-minute preincubation at 37°C and

continuous mixing at 400 rpm to acclimatize rapidly the hepatocytes, drug transport was initiated adding [^{3}H] rosuvastatin (0.1 μmol/L) in KHB or sodium-free KHB at either pH 7.4 or 6.0 with transport inhibitor (gemfibrozil 100 μmol/L) or vehicle (0.1% DMSO final concentration). Drug uptake was terminated after 1 minute by rapid filtration of the hepatocyte suspension through glass microfiber filters (GF/C, Whatman) under vacuum followed by 3 washes with ice-cold KHB or sodium-free KHB. Radioactivity retained on the filter was analyzed by liquid scintillation spectrometry. To account for rapid, nonspecific binding of drug to hepatocytes and the filter, uptake studies were performed on ice with termination of uptake occurring immediately after dose administration (t = 0 uptake). Specific uptake was determined by the difference in retained radioactivity at time = 0 and 1 minute. Uptake was determined in triplicate.

Real-Time mRNA Quantification from Human Liver Samples and Human Hepatocytes

Human liver samples (Nashville Regional Organ Procurement Agency, Nashville, TN) from white donors without liver pathology and human hepatocyte suspensions were used. Total RNA was extracted with a Versagene RNA Tissue Kit (Gentra Systems Inc., Minneapolis, MN) or for hepatocyte suspensions with Trizol reagent (Invitrogen). The integrity of RNA was assessed by 260/280 nm absorbance ratio and agarose gel electrophoresis or by an automated microfluidics-based system (Bioanalyzer 2100; Agilent, Palo Alto, CA). First-strand liver cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using iCycler iQReal-Time Detection System (Bio-Rad). PCR reactions were carried out with iQ SYBR Green Supermix (Bio-Rad) and a specific primer pair in a final concentration of 200 nmol/L. Specific primer pairs are as follows: OATP1B1, forward 5′-TGAACACCGTTGGAATTGC-3′ and reverse 5′-TCTCTATGAGATGTCACTGGAT-3′; OATP1B3, forward 5′-GTCCAGTCATTGGCTTTGCA-3′ and reverse 5′-CAACCCAACGAGAGTCCTTAGG-3′; OATP2B1, forward 5′-CTTCATCTCGGAGCCATACC-3′ and reverse 5′-GCTTGAGCAGTTGCCATTG-3′; NTCP, forward 5′-ACTGGTCCTGGTTCTCATTCC-3′ and reverse 5′-GTGGCAATCAAGAGTGGTGTC-3′; and 18S rRNA, forward 5′-CGGCTACCACATCCAAGGAA-3′ and reverse 5′-GCTGGAATTACCGCGGCT-3′. The cycling conditions were as follows: single cycle at 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute (OATP1B3, OATP2B1, and NTCP) and at 65°C for 1 minute (OATP1B1); single cycle at 95°C for 3 minutes, 34 cycles at 95°C for 15 seconds, and 65°C for 1 minute (18S rRNA).

To confirm amplification of a specific product, melting curve analysis was performed and PCR products directly visualized on 2% low-melting agarose gels. Quantification was calculated using a standard curve plotting log amount of DNA against C_t value. DNA standards were amplified from liver cDNA using iQ SYBR Green Supermix (Bio-Rad). PCR products were purified and DNA concentration was determined using Picogreen assay (Molecular Probes, Eugene, OR). Standard samples with known copy numbers were prepared and gene expression was normalized to 18S rRNA content.

Genotyping of Human Liver Samples for OATP1B1 Polymorphisms

Total genomic DNA was isolated from human liver samples via the DNeasy tissue kit (Qiagen, Valencia, CA). Samples were genotyped for the *OATP1B1*1b* (A388G) and *5 (T521C) alleles using the following primer pairs: exon 4 (**1b*), forward 5′-GCAAATAAAGGGGAATATTTCTC-3′ and reverse 5′-AGAGATGTAATTAAATGTATAC-3′; exon 5 (*5), forward 5′-GTTAAATTTGTAATAGAAATGC-3′ and reverse 5′-GTAGACAAAGGGAAAGTGATCATA-3′. Using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA), PCR was carried out using ~200 ng of human liver genomic DNA consisting of dNTPs (0.25 mmol/L each), the specific primer pair (4 μmol/L each), 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2.5 mmol/L $MgCl_2$, and 2.5 units of AmpliTaq DNA polymerase (PerkinElmer Life Sciences) in a final reaction volume of 50 μL. PCR was carried out at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 20 seconds for 30 cycles. PCR products of expected sizes were completely visualized using ethidium bromide-stained 2% agarose gels and were sequenced with an ABI 3700 DNA analyzer (Applied Biosystems).

Immunoblots of OATP1B1 and 1B3 From Human Liver Samples and Human Hepatocytes

Each liver sample or pelleted hepatocyte was placed in 500 μL HED buffer (25 mmol/L HEPES, 1.5 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.4), homogenized, sonicated for 30 seconds, and placed on ice. Samples were centrifuged at 4000*g* for 5 minutes. Supernatant was saved and total protein quantified using BCA Protein Assay Kit (Pierce, Rockford, IL). Samples were diluted with Laemmli buffer, and either 10 μg (OATP1B3) or 50 μg (OATP1B1) of total protein was loaded onto 10% gels and separated by SDS-PAGE. Following transfer onto nitrocellulose membranes, blots were probed with a polyclonal antibody to the C-terminal peptide of OATP1B1 (1:3000 dilution; ESLNKNKHFVPSAGADSETHC) or OATP1B3 (1:2500 dilution; SKTCNLDMQDNAAAN), custom-made by ResGen (Invitrogen, Huntsville, AL), and appropriate secondary antibody. To normalize sample loading, blots were stripped (Restore; Pierce) and reprobed with a mouse monoclonal anti-actin antibody (Sigma). Bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). To test antibody crossreactivity, lysates from HeLa cells transfected with OATP1B1 or OATP1B3 cDNA were probed with OATP1B1 or OATP1B3 antibody and appropriate secondary antibody. Enzymatic deglycosylation of OATP1B1 and OATP1B3 was performed in an individual human liver sample utilizing a previously de-

scribed method.[17] Densitometric analysis was performed using ImageJ (http://rsb.info.nih.gov/ij/).

Immunohistochemistry of OATP1B1 and 1B3 in Normal Human Liver Sections

Paraffin-embedded sections from normal human liver tissue (5 μm) were obtained from Human Tissue Acquisition Shared Resources at Vanderbilt-Ingram Cancer Center. Analyses were performed on H&E-stained sections. Sections were deparaffinized using EZ-Dewax (Biogenex), followed by antigen retrieval procedure Retrieve-It (pH 8, Biogenex) and incubated with peroxidase blocking reagent (Biogenex) for 10 minutes at room temperature. After rinsing with phosphate-buffered saline (PBS), sections were incubated with blocking buffer (Powerblock, Biogenex) for 60 minutes and incubated with OATP1B1 or OATP1B3 antisera diluted in blocking buffer (1:1000 dilutions) for 2 hours at room temperature. After 3 washes with PBS containing 0.4% Triton X-100, sections were incubated with biotinylated anti-rabbit IgG (Biogenex) for 20 minutes and streptavidin-horseradish peroxidase (HRP) conjugate (Biogenex) for 20 minutes. After washes, the immune reaction was visualized using 3-amino, 9-ethyl-carbazole (AEC, Biogenex) and nuclei counterstained with hematoxylin (Biogenex). Specificity of reactive signals for OATP1B1 and OATP1B3 was verified by negative controls, incubated with rabbit preimmune serum or blocking buffer instead of polyclonal antisera as well as polyclonal antiserum that had been neutralized by preincubation with the antigenic peptide at 37°C for 2 hours.

Data Fitting and Statistical Analysis

Parameters for saturation kinetics (V_{max} and K_m) and IC_{50} values for inhibition were estimated by nonlinear curve fitting using Prism (GraphPad Software, Inc, San Diego, CA). Determination of statistical differences between group parameters was determined using Student *t* test, Mann–Whitney *U* test, 1-way ANOVA, analysis of variance (using Tukey–Kramer multiple comparison test), or Fisher exact test, as appropriate. A *P* value of $<.05$ was taken to be the minimum level of statistical significance.

Results

Hepatic Uptake of Rosuvastatin Uptake Is Mediated by Human OATP and NTCP Transporters

A panel of hepatic transporter expression plasmids was assessed for rosuvastatin transport in HeLa cells using a recombinant vaccinia system. This screen identified several members of the human OATP family (OATP1B1, OATP1B3, OATP2B1, and OATP1A2) as rosuvastatin transporters (Figure 1*A*). OATP-specific rosuvastatin uptake was saturable with K_m values of 2.6 μmol/L for OATP1A2, 2.4 μmol/L for OATP2B1, 9.8 μmol/L for OATP1B3 (Figure 1*B*), and 4.0–7.3 μmol/L for OATP1B1 (Figure 2*B*). Similarly, rat Oatp family members exhibited highly efficient uptake of rosuvastatin. Interestingly, human, but not rat, NTCP mediated rosuvastatin transport. NTCP-specific rosuvastatin uptake was saturable with a K_m value of 65 μmol/L (Figure 5*A*). Neither OCT1 nor apical sodium-dependent bile acid transporter showed demonstrable uptake.

Naturally Occurring OATP1B1 Variants and Rosuvastatin Uptake

We previously reported that *OATP1B1* polymorphisms were associated with markedly decreased transport of prototypical substrates in vitro.[16] Analysis of rosuvastatin uptake by 18 OATP1B1 variants revealed markedly decreased transport activity by the *2 (T217C), *3 (T245C, A467G), *5 T521C), *9 (G1463C), *12 (T217C, A1964G), *13 (T245C, A467G, A2000G), *15 (A388G, T521C), and *18 (A388G, G1463C) variants (Figure 2*A*). Kinetic analysis of the commonly occurring variants for rosuvastatin uptake demonstrated no significant differences for OATP1B1*1a (A388) vs *1b (G388) (Figure 2*B*).

Real-Time Quantification of OATP/NTCP mRNA in Human Liver Samples and Human Hepatocytes

Real-time PCR quantification was performed on cDNA samples from a bank of 20 human livers (12 male, 8 female). When normalized to 18S rRNA, the relative mean mRNA expression of rosuvastatin transporters was OATP1B3>OATP2B1>OATP1B1>NTCP (Figure 3*A*). There was significant individual variability in expression with all transporters examined. Sex analysis revealed OATP1B3 levels to be highest in both males and females compared with OATP2B1, OATP1B1, or NTCP (data not shown). There were no significant sex differences for levels of each individual gene when mRNA expression was compared between males and females. Moreover, no differences in terms of mRNA levels were seen among those with the *OATP1B1*1a* vs **1b* genotypes.

Real-time PCR quantification was performed with cDNA synthesized from total RNA extracted from human hepatocyte suspensions utilized for rosuvastatin and taurocholate transport studies and compared side by side with real-time PCR quantification of a representative human liver sample with mRNA copy numbers approximately the average for the human liver bank for OATP1B3, OATP2B1, OATP1B1, and NTCP. There was significant variability in copy number for individual transporter genes between hepatocyte suspensions, but,

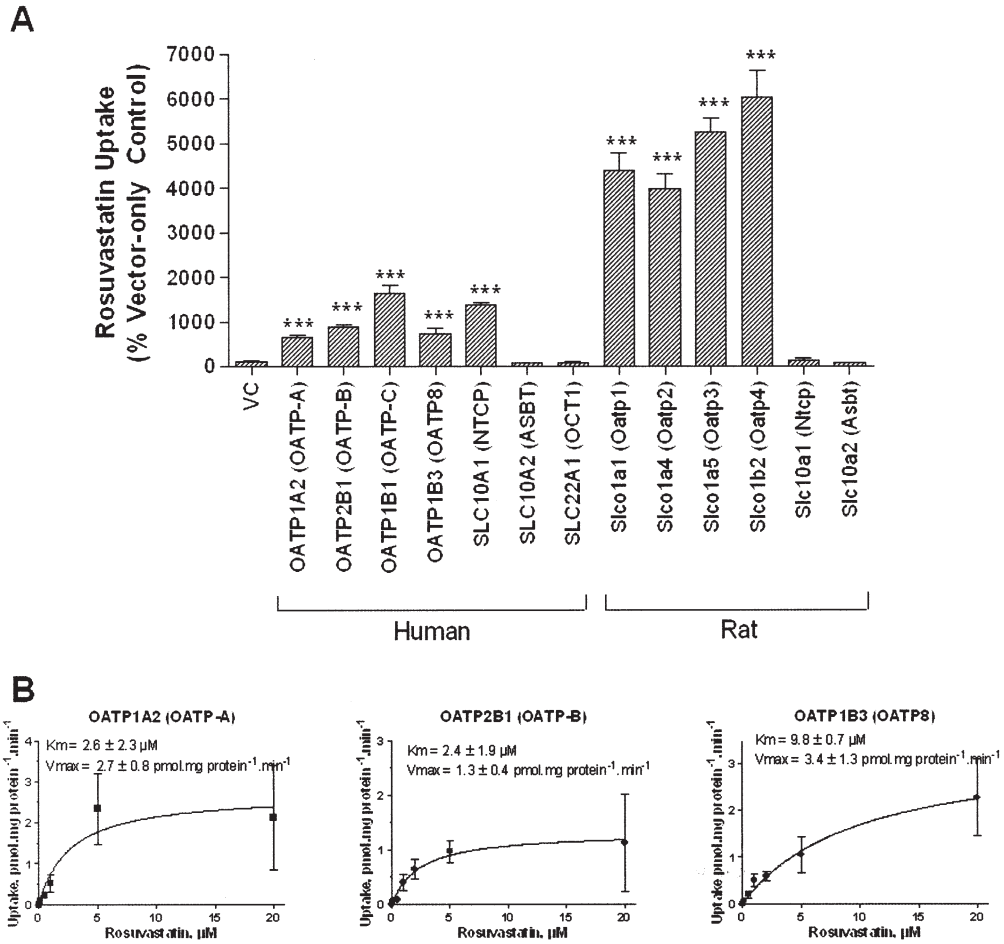

Figure 1. Rosuvastatin transport by uptake transporters and OATP1A2, 2B1, and 1B3 transport kinetics. (*A*) Rosuvastatin uptake (0.1 μmol/L) at 5 minutes expressed as percentage of vector control. (*B*) Concentration-dependent rosuvastatin uptake by OATP1A2, 2B1, and 1B3. Data shown as mean ± SE (n = 4 experiments). ***$P < .001$ vs vector control.

in general, these were within 10-fold of the copy number from the human liver sample with the following expressed fractions: OATP1B3, 3%–38%; OATP2B1, 21%–103%; OATP1B1, 9%–125%; NTCP, 5%–280% and a relative rank order OATP1B3>OATP2B1>NTCP>OATP1B1 when normalized to 18S rRNA (data not shown).

OATP1B1 and OATP1B3 Protein Expression in Human Liver Samples

Human liver samples were analyzed for OATP1B1 and OATP1B3 protein expression to assess interindividual variability. There was modest intersubject variation in OATP1B3 expression among liver samples (Figure 3*D*) with ~6.7-fold variation in protein expression determined by densitometric analysis. On the other hand, there was significant intersubject variability in OATP1B1 expression (Figure 3*F*). OATP1B1 migrated as a double band (~100 kilodaltons and ~75 kilodaltons). Enzymatic deglycosylation experiments suggested the higher molecular-weight band to correspond to glycosylated cell surface-expressed OATP1B1 (Figure 3*G*). Taking the total protein fractions (lower band) and glycosylated fractions (upper band) into account, densitometric analysis revealed an ~21-fold variability in OATP1B1 protein expression. There was no significant correlation between mRNA copy number and protein expression for either OATP1B3 (Figure 3*B*) or OATP1B1 (Figure 3*C*) from human liver samples or human hepatocyte suspensions (data not shown). Furthermore, there was no significant relationship between OATP1B1 genotype, *OATP1B1*1b* (A388G) or *OATP1B1*5* (T521C), and protein expression in the human liver bank.

OATP1B1 and OATP1B3 Immunohistochemistry in Human Liver Sections

As expected, we demonstrated that OATP1B1 and OATP1B3 are highly expressed in normal human

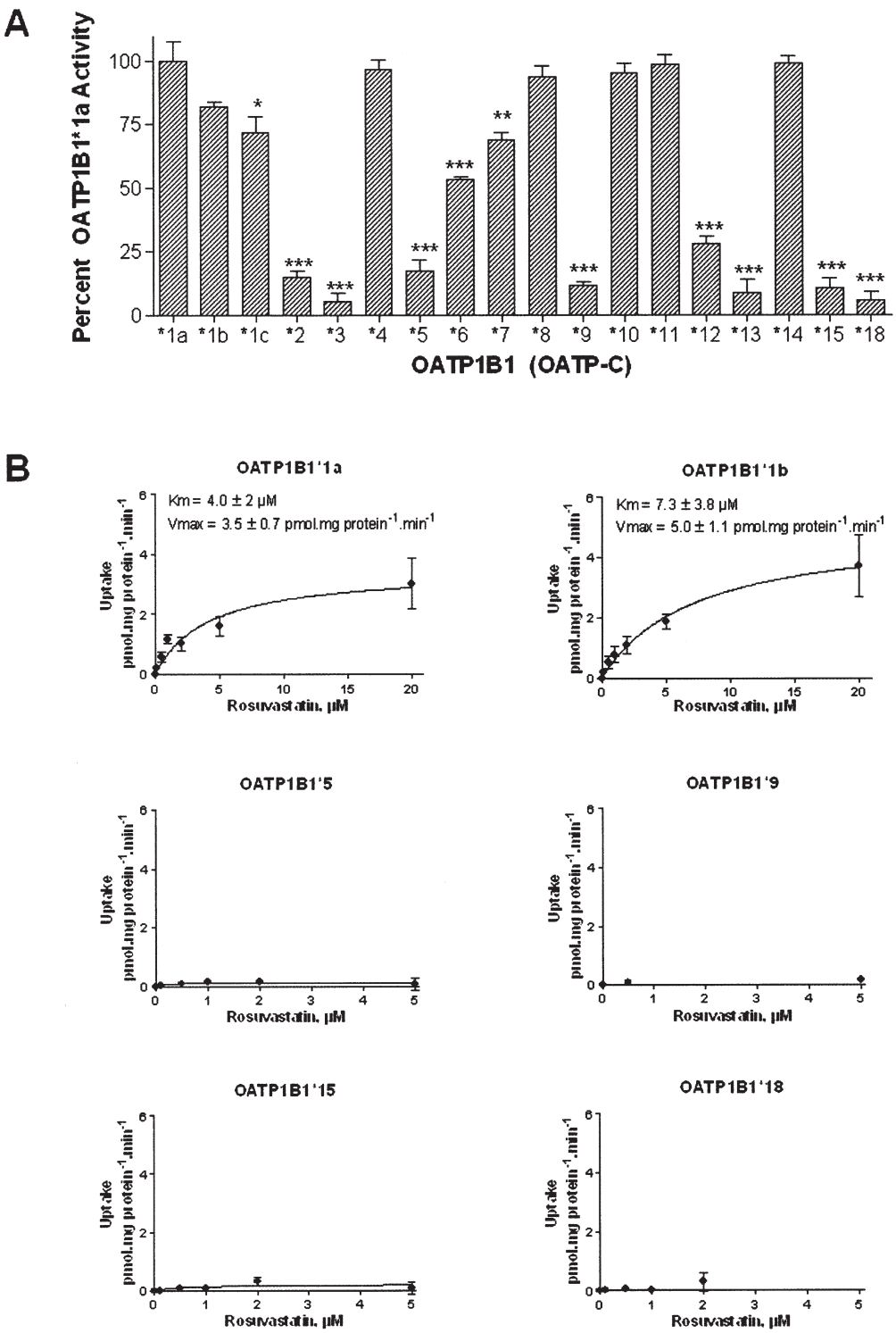

Figure 2. Rosuvastatin uptake and transport kinetics by OATP1B1 variants. (*A*) Rosuvastatin uptake at 5 minutes expressed as percentage of OATP1B1*1a activity. (*B*) Concentration-dependent rosuvastatin uptake of rosuvastatin by wild-type OATP1B1 (*1a, *1b) and variants (*5, *9, *15, *18). Data shown as mean ± SE (n = 4 experiments). $*P < .05$; $**P < .01$; $***P < .001$.

liver, although the pattern of expression differed between the 2 proteins. OATP1B1 exhibited a diffuse staining pattern throughout liver sections, whereas OATP1B3 demonstrated a more restricted staining pattern, with the greatest degree of expression being confined to perivenous distribution (Figure 4). Because HMG-CoA reductase is exclusively expressed in periportal hepatocytes,[19] it appears that colocalized uptake transporters

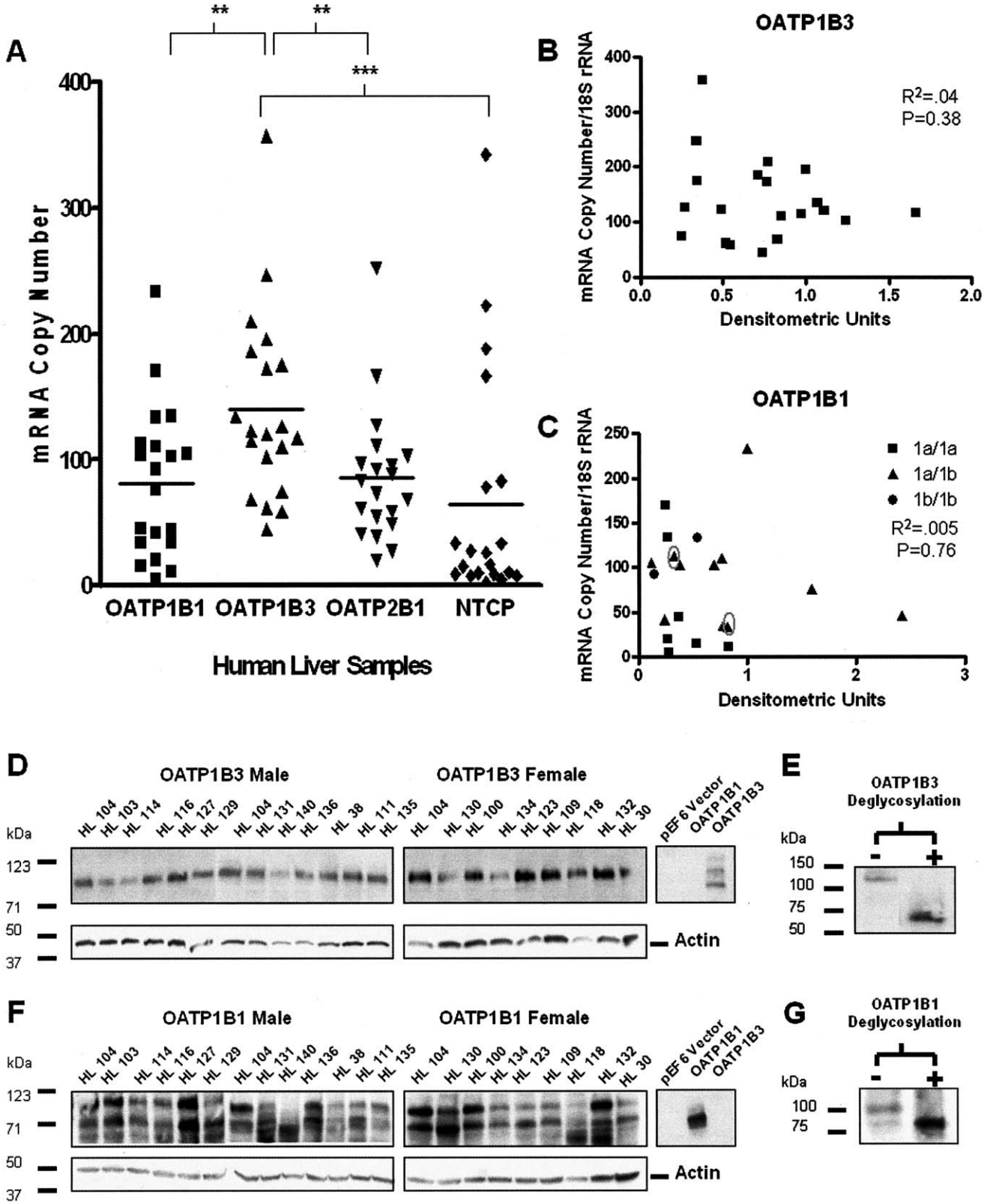

Figure 3. mRNA analysis of OATPs (1B1, 1B3, 2B1) and NTCP and protein analysis of OATP1B1 and 1B3 from human liver samples. (*A*) mRNA copy number for OATP1B1, 1B3, 2B1, and NTCP, normalized to 18S rRNA. Lines represent mean mRNA copy number. $*P < .05$; $**P < .01$; $***P < 001$. Scatter plot of mRNA copy number vs densitometric units for OATP1B3 (*B*) and OATP1B1 (*C*) (grouped by G388A [*1a/*1b] genotype; *circles* indicate subjects heterozygous at T521C position). OATP1B3 (*D*) and OATP1B1 (*F*) protein expression (*top panels*) from the same liver samples and crossreactivity blots of OATP1B1- and OATP1B3-transfected HeLa cells. Samples were normalized for loading using antiactin antibody (*bottom panels*). Enzymatic deglycosylation demonstrates both OATP1B3 (*E*) and OATP1B1 (*G*) to be glycosylated plasma membrane proteins.

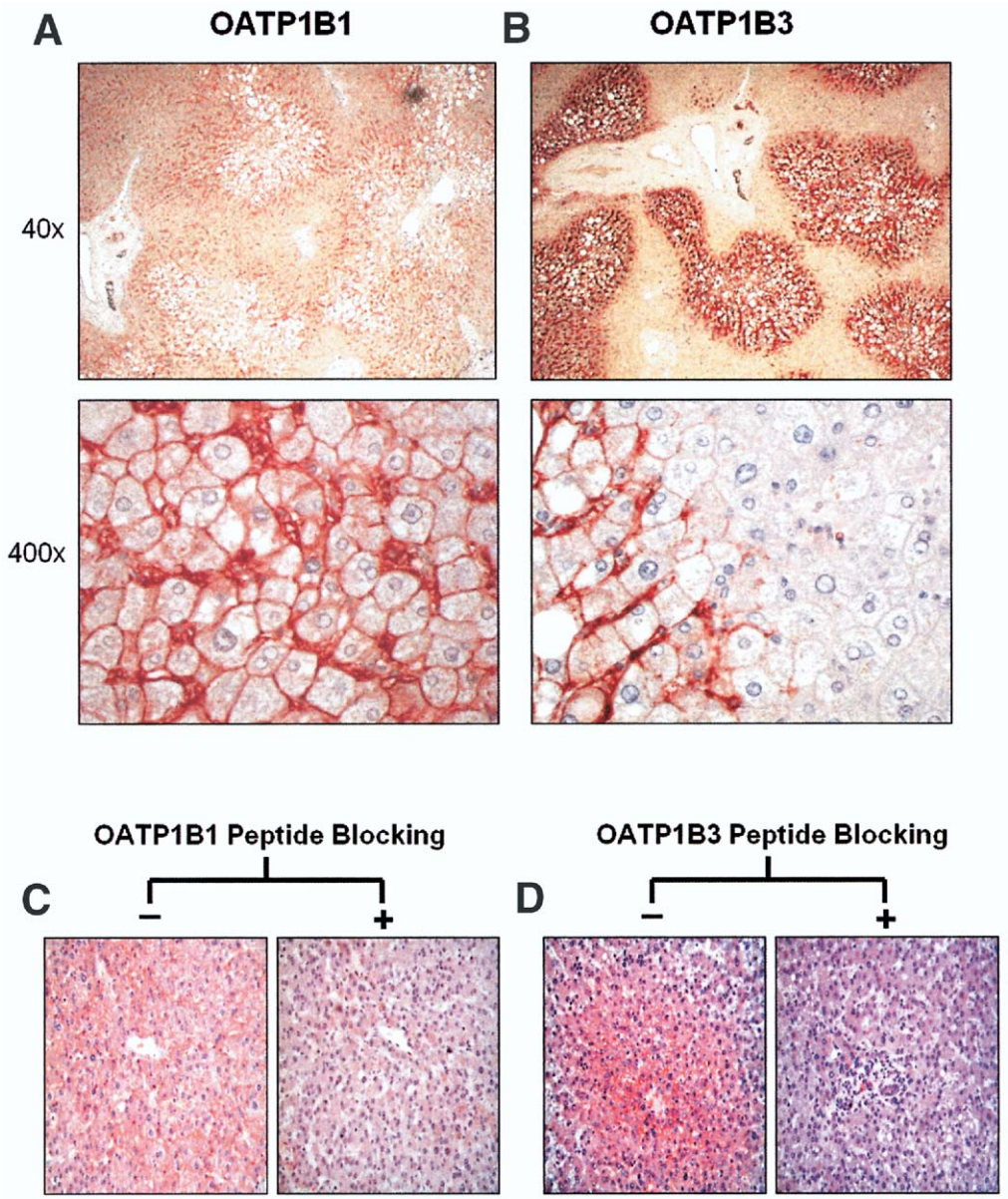

Figure 4. OATP1B1 and OATP1B3 localization in human liver. Paraffin-embedded liver sections (5 μm) stained (*red signal*) with OATP1B1 (*A*) and OATP1B3 (*B*) antibody, detected by avidin-biotin-peroxidase complex, demonstrate diffuse staining for OATP1B1 and more limited perivenous distribution for OATP1B3 staining. Immunohistochemical staining in the absence and presence of specific antigenic peptides for OATP1B1 (*C*) and OATP1B3 (*D*), respectively, demonstrate specificity of staining.

such as OATP1B1 and NTCP[20] are responsible for presenting statins to their target in liver. Peri-venously expressed OATP1B3 would be expected to contribute along with OATP1B1 and NTCP to the hepatic elimination of statins as a result of colocalization with biliary excretory proteins including multidrug resistance–associated protein 2[21,22] and breast cancer–related protein.[22,23] OATP1B1 and OATP1B3 antibody specificity was demonstrated by antigen peptide blocking (Figure 4*C* and 4*D*).

Naturally Occurring NTCP Variants and Rosuvastatin Uptake

We have shown that functional polymorphisms exist in *NTCP*.[17] *NTCP**2* (C800T) and *3* (T668C) variants were seen in Asian Americans (~7.5% allele frequency) and African Americans (~5.5%), respectively, and were associated with significantly impaired transport activity for bile acids in vitro.[17] Remarkably, for rosuvastatin transport, NTCP*2 demonstrated marked gain of function transport

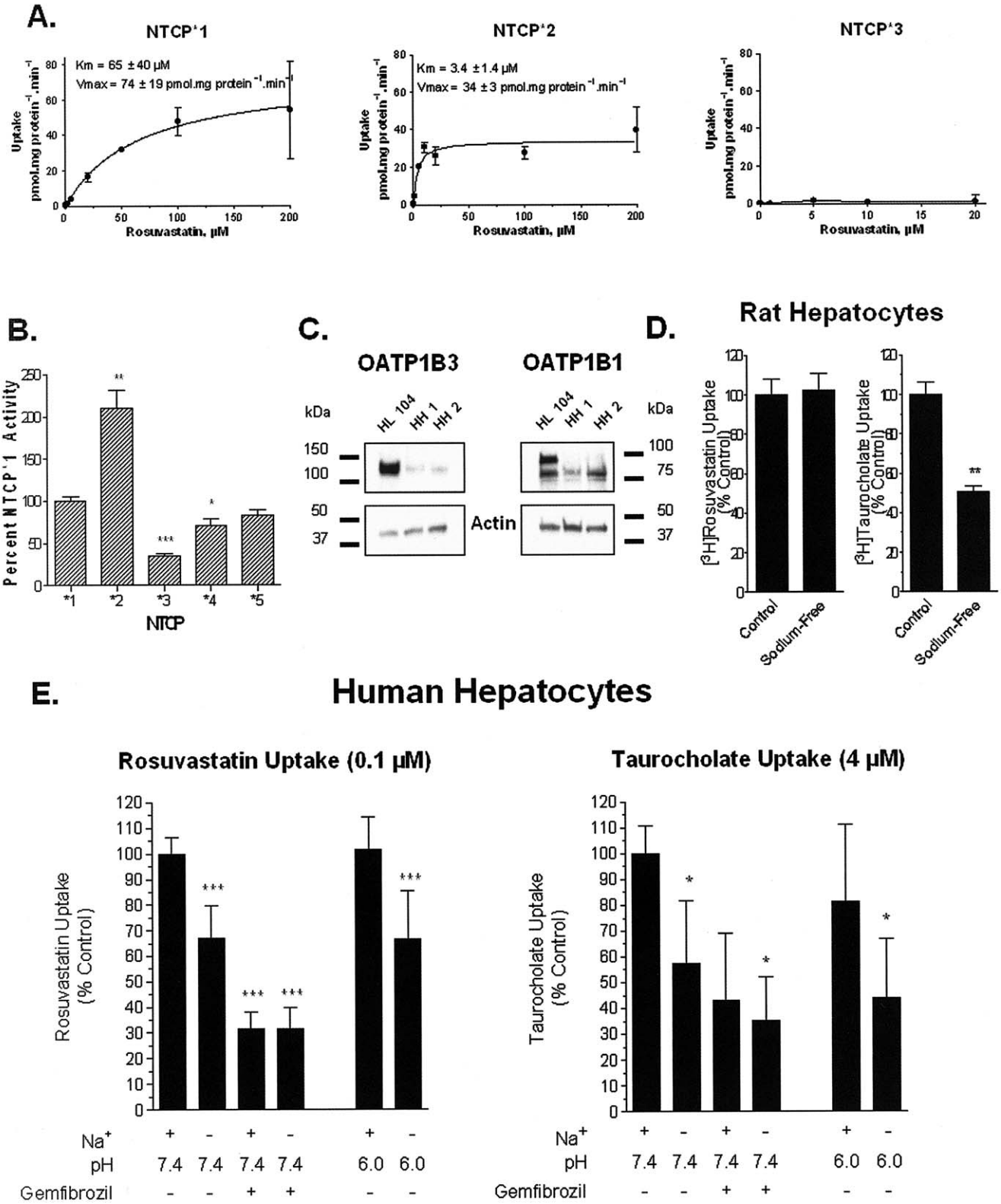

Figure 5. Rosuvastatin uptake by NTCP and transport studies in rat and human hepatocytes. (*A*) Concentration-dependent rosuvastatin uptake by wild-type NTCP*1, NTCP*2, and NTCP*3. (*B*) Rosuvastatin uptake at 5 minutes expressed as percentage of NTCP*1 activity. (*C*) Comparison of OATP1B1 and OATP1B3 protein expression by Western blot analysis (*top panels*) in a human liver sample (HL 104) and human hepatocytes (HH1, HH2), normalized to actin expression (*bottom panels*). (*D*) Rosuvastatin and taurocholate uptake in rat hepatocytes in sodium-containing (control) or sodium-free buffer after 60 seconds. (*E*) Rosuvastatin and taurocholate uptake in human hepatocytes in sodium-containing (control), sodium-free buffer, pH 7.4 vs 6.0, with transport inhibitor (gemfibrozil) or vehicle after 60 seconds. Data shown as mean ± SE (n = 4 experiments). $*P < 05$; $**P < .01$; $***P < .001$ compared with control.

activity compared with NTCP*1 (Figure 5*B*). Kinetic analysis of NTCP*2-mediated rosuvastatin transport showed a significantly lower K_m value (3.4 μmol/L) resulting in an ~10-fold higher intrinsic clearance compared with NTCP*1 (Figure 5*A*). Impaired NTCP*3 activity was consistent with our previous observation that this SNP results in mistrafficking of NTCP to the cell surface.

Rosuvastatin Uptake in Rat and Human Hepatocytes Suggests an Important Role for Human but not Rat NTCP

To assess the relative contribution of sodium-independent (OATP-mediated) and sodium-dependent (NTCP-mediated) pathways to rosuvastatin uptake, transport studies were conducted in rat and human hepatocytes using sodium-containing and sodium-free buffer. A significant component of net uptake of the conjugated bile acid taurocholate into hepatocytes has been shown to be sodium dependent.[24] In rat hepatocytes, taurocholate transport was reduced ~50% in the sodium-free buffer, whereas there was no significant difference in rosuvastatin uptake at 1 minute in sodium-containing or sodium-free buffer (Figure 5*D*), consistent with in vitro data demonstrating rat Oatps, but not rat Ntcp, are highly efficient at rosuvastatin transport.

By contrast, in human hepatocytes, we observed comparable reductions in taurocholate (~45%) and rosuvastatin (~35%) transport under sodium-free conditions compared with sodium-containing conditions, suggesting a significant role for NTCP in hepatic uptake of rosuvastatin (Figure 5*E*). Furthermore, the effect of pH gradients was utilized to determine whether the observed sodium dependence of taurocholate and rosuvastatin transport was due to a double ion exchange mechanism such as Na^+-H^+ exchange and OH^--taurocholate/rosuvastatin exchange.[25,26] There were no significant differences in rosuvastatin or taurocholate transport at pH 6.0 vs 7.4, suggesting that ion exchange does not contribute to rosuvastatin or taurocholate transport in human hepatocytes. When rosuvastatin was preincubated with gemfibrozil in the presence or absence of sodium, rosuvastatin transport was further inhibited to a level beyond that observed for sodium-free conditions, suggesting that transporter-mediated inhibition may explain the observed drug interactions between gemfibrozil and statins.

Inhibition of Transporter-Mediated Rosuvastatin Uptake by Cyclosporine and Gemfibrozil

Inhibition studies using cyclosporine revealed potent inhibition of transporter-mediated rosuvastatin transport in a concentration-dependent manner with the following rank order potencies: OATP1B3>OATP2B1>OATP1B1>NTCP (Figure 6*B*). Because peak cyclosporine blood levels approach 1 μmol/L and given the inhibitory potencies toward the transporters (IC_{50}'s 0.07 to 0.37 μmol/L), the observed significant drug interactions with rosuvastatin can be explained by a suppression of hepatocyte statin uptake mechanism. Gemfibrozil was far less potent (~100-fold) as an inhibitor of OATP1B1-, OATP2B1-, and NTCP-specific rosuvastatin transport (Figure 6*A*). Even at a concentration of 100 μmol/L, gemfibrozil was unable to inhibit more than 40% OATP1B3-mediated transport of rosuvastatin. Thus, even among OATPs, there appear to be marked differences in gemfibrozil inhibitory potency. It is notable that, although gemfibrozil has relatively poor inhibitory potency against the rosuvastatin transporters, plasma concentrations of gemfibrozil reach 250 μmol/L, and, therefore, hepatic uptake inhibition can be considered a relevant mechanism for the observed drug-drug interaction.

Discussion

Lipid-lowering drugs are among the most widely prescribed medications worldwide, with more than 20 million people prescribed this class of drugs; since being first approved in the 1980s, statins have become the most widely used lipid-lowering agents.[27] Statins have proven benefit in the treatment of dyslipidemia through inhibition of the rate-limiting step in hepatic cholesterol biosynthesis. Increasingly, both the efficacy as well as the disposition profiles of a number of statins in clinical use have been linked to hepatic drug uptake transporters. Indeed, members of the OATP family appear to be particularly relevant in this regard because a number of statins are known substrates for OATP1B1. In fact, expression and function of OATP1B1 has been thought to be the rate-limiting step in the hepatic clearance of statins such as pravastatin. Of potential clinical relevance are naturally occurring SNPs in *OATP1B1* associated with significant loss of function in vitro[16] as well as elevated pravastatin plasma levels in vivo when assessed in subjects with defined genotypes.[28–30] Rosuvastatin, like pravastatin, has already been noted to be a substrate for OATP1B1. However, no linkage between OATP1B1 polymorphisms and rosuvastatin pharmacokinetic profiles has been reported.

In this report, we carried out a systematic study to define relevant hepatic transporters responsible for rosuvastatin uptake. Remarkably, and unlike other statins studied to date, rosuvastatin hepatic uptake appears to

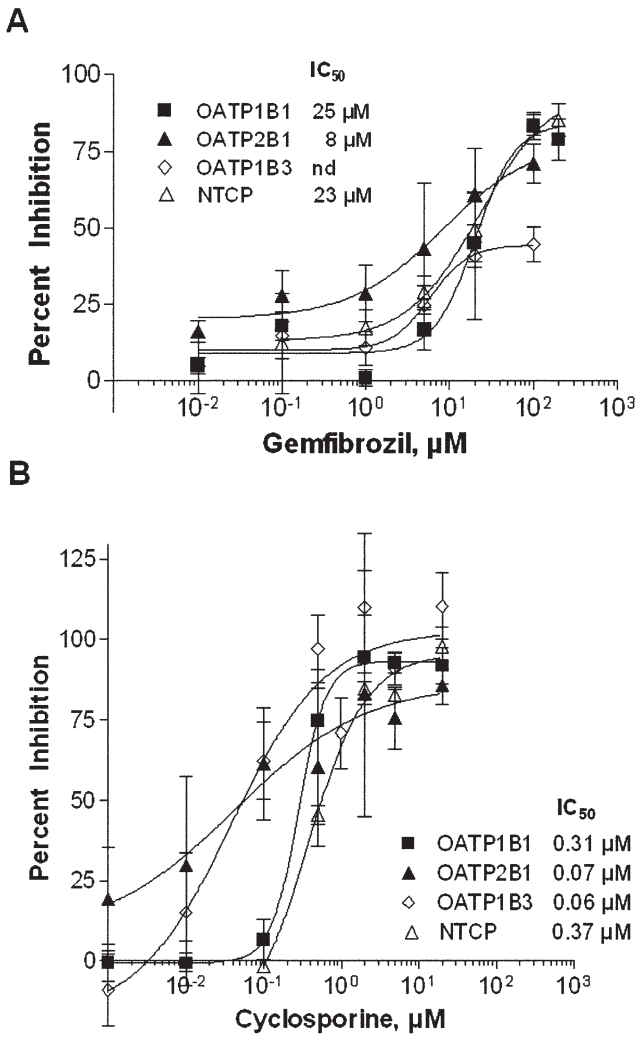

Figure 6. Inhibition of OATP- and NTCP-mediated rosuvastatin transport by gemfibrozil and cyclosporine. OATP 1B1, 1B3, and 2B1 and NTCP and concentration-dependent inhibition of rosuvastatin uptake by gemfibrozil (*A*) and cyclosporine (*B*). Data shown as mean ± SE. Lines and kinetic parameters were obtained by nonlinear regression analysis. nd, not determined.

involve multiple OATP transporters including OATP1B1, 1B3, and 2B1, suggesting that multiple pathways are involved in the hepatic extraction of rosuvastatin in vivo. Kinetic analysis of rosuvastatin transport revealed similar kinetic parameters among OATPs. Comparison of rosuvastatin intrinsic clearance values for OATP transporters in terms of V_{max}/K_m to a known endogenous substrate of these transporters, estrone sulfate, would suggest estrone sulfate to be more efficiently cleared.[16,17,31] However, in the absence of direct side-by-side assessment of hepatic uptake of estrone sulfate vs rosuvastatin uptake, possibly through the use of intravenous administration of such compounds, it would be difficult to know whether the predicted difference would manifest in vivo. RNA analysis from a bank of human livers suggested that the relative expression levels among OATP1B1, 1B3, and 2B1 differ, with OATP1B3 showing the highest mRNA levels. Western blot analysis suggested modest interindividual OATP1B3 variation (6.7-fold), whereas OATP1B1 demonstrated an ~21-fold variation. Comparison of OATP1B3 or OATP1B1 mRNA levels with protein expression from our human liver bank and human hepatocyte suspensions revealed no significant correlation, suggesting that hepatic OATP expression may be significantly controlled by post-transcriptional or posttranslational mechanisms.

We also explored the potential roles of SNPs in rosuvastatin disposition. We have shown that 2 SNPs in *OATP1B1*, T521C (*OATP1B1*5*) and G1463C (*OATP1B1*9*), were associated with significantly impaired transport of prototypical substrates in vitro.[16] Subsequent in vivo studies utilizing pravastatin as a probe for OATP1B1 activity demonstrated that individuals with the T521C allele in combination with either of 2 reference alleles, A388 (*OATP1B1*1a*) or G388 (*OATP1B1*1b*), have higher plasma exposure of pravastatin, in line with in vitro data.[28–30] Our data demonstrate that naturally occurring SNPs in *OATP1B1*, including **5*, **9*, **15*, and **18*, are associated with marked loss of activity for rosuvastatin uptake in vitro. Of these, recent studies would suggest that *OATP1B1*15* (**1b*+**5*) is a commonly occurring polymorphism in Asian (~10% allele frequency) and white (~15%–18%) populations, whereas the **5* and **18* variants are less common in these populations, and **9* has been noted only in African Americans.[30,31] Note that, in our study, we failed to see significant differences in terms of *OATP1B1*1a* mRNA or protein levels in human liver samples, relative to *OATP1B1*1b*. Therefore, our findings would suggest that at least in the European-American population, observed differences between OATP1B1*5 vs *15 is likely due to T521C polymorphism and not due to the presence of G388 (*OATP1B1*1b*).

Recently published reports in human subjects illustrate the potential clinical relevance of such OATP1B1 polymorphisms to statin disposition. Tachibana-Iiomori et al suggested that individuals with a 521C allele demonstrate attenuated lipid-lowering response when administered statins such as pravastatin, atorvastatin, or simvastatin.[32] Furthermore, a significant association was noted between *OATP1B1*15* and the development of pravastatin- or atorvastatin-induced myopathy.[33] The mechanism of statin-induced myopathy has not been clearly elucidated, but it remains possible that loss of function predicted with the *OATP1B1*15* genotype would result in increased plasma exposure or higher peak plasma levels of statins, potentially permitting greater drug access to other tissue compart-

ments such as muscle. However, the presence of multiple hepatic transport pathways for rosuvastatin may serve to attenuate the predicted in vivo effects of polymorphism-influenced loss of activity with any given transporter, such as OATP1B1.

Unexpectedly, we identified human NTCP, the major bile acid uptake transporter in liver,[34,35] to be capable of rosuvastatin uptake. To our knowledge, this is the first report of a drug in clinical use found to be transported by NTCP. In addition, we noted a marked species dependency because rat Ntcp was unable to transport rosuvastatin. Human hepatocyte experiments indicate that ~35% of rosuvastatin hepatic uptake is sodium dependent, thus suggesting an important role for NTCP in rosuvastatin disposition in vivo. Interestingly, supportive of in vitro findings, rat hepatocyte studies failed to show significant differences in rosuvastatin transport under sodium-free buffer conditions. One of the recognized limitations of utilizing human hepatocytes for drug metabolism and transport studies is rapid down-regulation of gene expression after isolation.[36,37] It has been previously estimated that ~80% of taurocholate uptake in mammalian liver is sodium dependent,[24] although subsequent studies have demonstrated variability in the amount of sodium dependence.[38] Nonetheless, our results suggest that rosuvastatin sodium-dependent uptake in human liver is significant and comparable with taurocholate sodium dependence (~45%), and it is likely that our in vitro analysis underestimates the in vivo contribution of NTCP to rosuvastatin hepatic uptake.

To our surprise, *NTCP*2* (C800T), a variant that we showed to have near complete loss of capacity for bile acids and seen only in Asians,[17] demonstrated a profound gain of transport activity for rosuvastatin. Kinetic analysis of NTCP variants revealed a significantly lower K_m value of 3.4 μmol/L and ~10-fold higher intrinsic clearance for NTCP*2 compared with NTCP*1, strongly suggesting that the associated amino acid at position 267 in the fourth extracellular loop of NTCP is critical for substrate recognition/affinity. Because this is an SNP only seen in Asian populations, the clinical relevance of this polymorphism to the therapeutic efficacy or disposition of rosuvastatin will require a dedicated genotype-phenotype study in these populations, although, analogous to OATP1B1 polymorphisms, multiple pathways for hepatic rosuvastatin uptake may lessen the in vivo functional impact of NTCP polymorphisms because redundancy of hepatic transport capacity for rosuvastatin may compensate for loss of function associated with any individual transporter protein such as OATP1B1 or NTCP.

One concern relating to statin therapy is drug-drug interactions. Because rosuvastatin is not subject to significant metabolism,[7] it is not surprising that there is no significant interaction with known CYP3A inhibitors such as ketoconazole.[39] However, drug-drug interactions involving rosuvastatin have been documented for gemfibrozil and cyclosporine. Concomitant administration of rosuvastatin and gemfibrozil resulted in a modest increase in rosuvastatin plasma area under the curve.[11] When rosuvastatin and cyclosporine were coadministered, rosuvastatin plasma levels were ~7 times higher in patients on cyclosporine.[12] Our findings would suggest that cyclosporine is a potent inhibitor of both OATP- and NTCP-mediated rosuvastatin uptake, whereas gemfibrozil was ~100-fold less potent in inhibiting OATP- and NTCP-mediated uptake. Moreover, OATP1B3 appeared to be particularly resistant to inhibition by gemfibrozil. Thus, our in vitro findings correlate well with observed clinical data, suggesting transporter-mediated inhibition as the mechanism for clinically observed rosuvastatin drug interactions.

There has been increasing interest concerning the pleiotropic effects of statins, with focus on their roles in mediating processes such as vascular dysfunction, inflammation, and cell proliferation/apoptosis. It is plausible that transporter proteins such as OATPs may aid in statin access to various tissues. For example, significant relative reduction in risk for developing colorectal cancer was specifically linked to statin use.[40] OATP2B1 expression in intestinal enterocytes[10] may facilitate intracellular accumulation of statin for mediation of antiproliferative effects. Beneficial effects of statins have also been reported in inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis.[41] OATP1A2 expression in vascular endothelium and capillary endothelial cells at the blood/brain barrier[10] may serve to enhance tissue-specific concentration of statin drugs for subsequent therapeutic benefit.

In conclusion, in this report, we describe the expression, function, and relevance of hepatic uptake transporters in the disposition of the HMG-CoA reductase inhibitor rosuvastatin. Interestingly, we note the involvement of multiple OATP transporters and, to our surprise, an important involvement of human, but not rat, NTCP in the hepatic uptake of rosuvastatin. In human livers, marked intersubject variability in expressed levels of OATP transporters was ob-

served. Moreover, a number of naturally occurring SNPs in *OATP1B1* were associated with impaired rosuvastatin uptake, and an SNP in *NTCP* associated with loss of function for bile acids was shown to have a profound gain of function for rosuvastatin uptake. Moreover, the clinically observed rosuvastatin-cyclosporine and gemfibrozil interaction may be explained in relation to potency of inhibition for OATPs and NTCP. Accordingly, findings outlined in the current report reveal important new insights into the relevance of OATPs and NTCP, as well as known SNPs in such transporters, to rosuvastatin disposition in particular and to statin therapy in general.

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Address requests for reprints to: Richard B. Kim, MD, Professor of Medicine and Pharmacology, 572 Robinson Research Bldg, 23rd Ave. at Pierce Ave, Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232-6602. e-mail: richard.kim@vanderbilt.edu; fax: (615) 343-7605.

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