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www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 156(1), 2017, 84–95

doi: 10.1093/toxsci/kfw236
Advance Access Publication Date: December 24, 2016
Research article

Organic Anion Transporting Polypeptides Contribute to the Disposition of Perfluoroalkyl Acids in Humans and Rats

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ABSTRACT

Perfluoroalkyl sulfonates (PFSAs) such as perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonate (PFOS) have very long serum elimination half-lives in humans, and preferentially distribute to serum and liver. The enterohepatic circulation of PFHxS and PFOS likely contributes to their extended elimination half-lives. We previously demonstrated that perfluorobutane sulfonate (PFBS), PFHxS, and PFOS are transported into hepatocytes both in a sodium-dependent and a sodium-independent manner. We identified Na^+/taurocholate cotransporting polypeptide (NTCP) as the responsible sodium-dependent transporter. Furthermore, we demonstrated that the human apical sodium-dependent bile salt transporter (ASBT) contributes to the intestinal reabsorption of PFOS. However, so far no sodium-independent uptake transporters for PFSAs have been identified in human hepatocytes or enterocytes. In addition, perfluoroalkyl carboxylates (PFCAs) with 8 and 9 carbons were shown to preferentially distribute to the liver of rodents; however, no rat or human liver uptake transporters are known to transport these PFCAs. Therefore, we tested whether PFBS, PFHxS, PFOS, and PFCAs with 7–10 carbons are substrates of organic anion transporting polypeptides (OATPs). We used CHO and HEK293 cells to demonstrate that human OATP1B1, OATP1B3, and OATP2B1 can transport PFBS, PFHxS, PFOS, and the 2 PFCAs (C8 and C9). In addition, we show that rat OATP1A1, OATP1A5, OATP1B2, and OATP2B1 transport all 3 PFSAs. In conclusion, our results suggest that besides NTCP and ASBT, OATPs also are capable of contributing to the enterohepatic circulation and extended human serum elimination half-lives of the tested perfluoroalkyl acids.

Key words: perfluoroalkyl sulfonates, perfluorobutane sulfonate, perfluorohexane sulfonate, perfluorooctane sulfonate, perfluoroalkyl carboxylates.

Perfluoroalkyl acids (PFAAs), including perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs), are fluorinated fatty acid-like chemicals, which are widely used in commerce since the 1950s (Buck *et al.*, 2011; Prevedouros *et al.*, 2006). Certain PFSAs and PFCAs such as perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), respectively, have received attention in recent years, because they are resistant to environmental degradation (Kissa 2001; Prevedouros *et al.*, 2006) and frequently detectable in blood specimens of humans (Fromme *et al.*, 2009; Kato *et al.*, 2011; Zhao *et al.*, 2012). The presence of certain PFAAs in human blood may, in part, be due to their retention in the body through reabsorptive processes of

renal proximal tubule reabsorption and enterohepatic circulation after exposures from various environmental sources (Andersen *et al.*, 2008).

There are several differences in the elimination kinetics of PFAAs depending on species, sex within species, and number of carbons in the perfluoroalkyl chain. In particular, the PFSAs, PFOS, and perfluorohexane sulfonate (PFHxS) as well as the PFCA, PFOA, have serum elimination half-lives in humans of several years (Bartell *et al.*, 2010; Olsen *et al.*, 2007). Pharmacokinetic studies on these 3 PFAAs in nonhumans have demonstrated that serum elimination half-lives can vary considerably between species (Chang *et al.*, 2012; Hundley *et al.*, 2006; Sundstrom *et al.*, 2012) and, in some cases, between genders within species (Hundley *et al.*, 2006; Sundstrom *et al.*, 2012), but are generally much shorter than the reported human serum elimination half-lives. For example, for PFOS, the reported serum elimination half-lives in rats and mice were on the order of 1–2 months and, in monkeys, approximated 4 months (Chang *et al.*, 2012), as opposed to several years in humans (Olsen *et al.*, 2007). In another example, the serum elimination half-life of PFOA in female rats is a few hours versus several days in male rats (Hundley *et al.*, 2006; Kennedy *et al.*, 2004).

In addition to these species and sex differences in elimination for certain PFAAs, it has also been noted that PFAAs with fewer carbons in the perfluoroalkyl chain tend to have shorter serum elimination half-lives than their longer homologs (Lau, 2015). For example, the serum elimination half-lives in humans for perfluorobutane sulfonate (PFBS) and perfluorohexanoate (PFHxA) are approximately 1 month (Olsen *et al.*, 2009; Russell *et al.*, 2013) versus several years for PFOS and PFOA (Bartell *et al.*, 2010; Olsen *et al.*, 2007).

In attempting to explain these kinetic differences, attention has focused on the role of membrane transporters. Kudo *et al.* (2002) first provided experimental support for sex hormone-mediated expression of organic anion transporters (OATs) in rat kidneys to explain male and female differences in clearance of PFOA, and suggested a possible role for organic anion transporting polypeptide 1A1 (OATP1A1) in proximal tubule reabsorption of PFOA in male rats. Subsequent experimental and modelling studies have provided substantial further support for a role of transporter-mediated renal proximal tubule reabsorption in the differential retention of PFOA (Andersen *et al.*, 2006; Han *et al.*, 2003; Katakura *et al.*, 2007; Loccisano *et al.*, 2011; Weaver *et al.*, 2010; Yang *et al.*, 2009). At this point in time, it is fair to conclude that renal transporters play a key role in defining the kinetic characteristics of PFAAs.

Whereas there has been a fair amount of effort to investigate renal proximal tubule transporters, there have been fewer studies focused on the role of enterohepatic circulation, which likely is important based on the observation that PFAAs tend to be preferentially distributed to liver and serum (Bogdanska *et al.*, 2011; Chang *et al.*, 2012; Kemper, 2003; Kudo *et al.*, 2007; Sundstrom *et al.*, 2012). The available experimental evidence supports the role of enterohepatic circulation as a possible contributing factor to the retention of PFHxS and PFOS in humans (Genuis *et al.*, 2010, 2013), and PFOS and PFOA in rats (Johnson *et al.*, 1984). These studies investigated the effect of the binding agent, cholestyramine, in facilitating the gastrointestinal clearance of PFAAs.

We have previously demonstrated that the uptake of PFBS, PFHxS, and PFOS into freshly isolated human and rat hepatocytes was sodium-dependent and Na^+/taurocholate cotransporting polypeptide (NTCP), a sodium-dependent bile salt transporter expressed at the sinusoidal membrane of hepatocytes, was the responsible transporter (Zhao *et al.*, 2015). We have also shown that bromosulfophthalein could inhibit the sodium-independent portion of the uptake of PFBS and PFHxS into hepatocytes, indicating the involvement of sodium-independent transporters. At the hepatocyte sinusoidal membrane (Figure 1), there are several organic anion transporting polypeptides (OATPs) expressed, including hOATP1B1, hOATP1B3, and hOATP2B1 in humans (Hsiang *et al.*, 1999; König *et al.*, 2000; Roth *et al.*, 2012; Tamai *et al.*, 2000) and rOATP1A1, rOATP1A4, rOATP1B2, and rOATP2B1 in rats (Bergwerk *et al.*, 1996; Cattori *et al.*, 2001; Kakyo *et al.*, 1999; Nishio *et al.*, 2000; Noé *et al.*, 1997). These are sodium-independent transporters that mediate the uptake of endogenous and exogenous compounds, such as taurocholate, estrone-3-sulfate, bilirubin, and numerous drugs including statins (Hagenbuch and Stieger, 2013).

In addition to the liver, we also demonstrated that human apical sodium-dependent bile salt transporter (ASBT), but not rat ASBT, expressed at the apical membrane of the small intestine can transport PFOS, contributing to the intestinal reabsorption of PFOS in humans (Zhao *et al.*, 2015). However, transporters involved in the enterohepatic circulation of PFHxS and PFOS in rats and of PFHxS in humans have not been identified. Candidate transporters in the human intestine are hOATP1A2 and hOATP2B1, but, based on recent proteomics work, only hOATP2B1 should be considered as an intestinal transporter (Figure 1) (Drozdzik *et al.*, 2014). In rats, the corresponding transporters are rOATP1A5 and rOATP2B1 (Figure 1). All 3 transporters are involved in the intestinal absorption of several drugs including pravastatin, pitavastatin, and fexofenadine (Kikuchi *et al.*, 2006; Kobayashi *et al.*, 2003; Shirasaka *et al.*, 2010).

PFOA is a substrate of OATPs and OATs. In rats, rOATP1A1, rOAT1, rOAT3 and in humans, hOAT1, hOAT3, hOAT4 mediate the transport of PFOA (Katakura *et al.*, 2007; Nakagawa *et al.*, 2008, 2009; Yang *et al.*, 2009). Consistently, our laboratory previously has shown chain-length dependent transport of PFCAs with 7–10 carbons by rOAT1, rOAT3, and rOATP1A1 (Weaver *et al.*, 2010). Because rOATP1A1 is expressed at a higher level at the apical membrane of proximal tubules in male rats than in female rats (Lu *et al.*, 1996) and seems to be involved in the reabsorption of PFOA, rOATP1A1-mediated reabsorption of PFOA is most likely the main mechanism for the longer half-life observed in male rats as opposed to females, as first speculated by Kudo et al. (Kudo *et al.*, 2002).

Based on these combined previous findings, we hypothesized that OATPs expressed in hepatocytes and in enterocytes can transport PFAAs and can contribute to the enterohepatic circulation and hepatic accumulation in humans and rats. Therefore, in the present study, we characterized the transport of PFBS, PFHxS, and PFOS and PFCAs with 7 to 10 carbons by the 3 human transporters hOATP1B1, hOATP1B3, hOATP2B1, and the transport of PFBS, PFHxS, and PFOS by the rat transporters rOATP1A1, rOATP1A5, and rOATP1B2.

MATERIALS AND METHODS

Materials. Potassium PFBS (K^+PFBS, 98.2% pure), potassium perfluorohexane sulfonate (K^+PFHxS, >99% pure), and potassium PFOS (K^+PFOS, 86.9% pure) were received from the 3M Company (St Paul, Minnesota). Perfluoroalkyl carboxylates (C7–C10) were purchased from Sigma Aldrich (St Louis, Missouri).

Plasmid construction. The open reading frames of rOATP1B2 and rOATP2B1 were PCR amplified to include a C-terminal 6 His-tag using the primers containing the restriction sites listed in Table 1

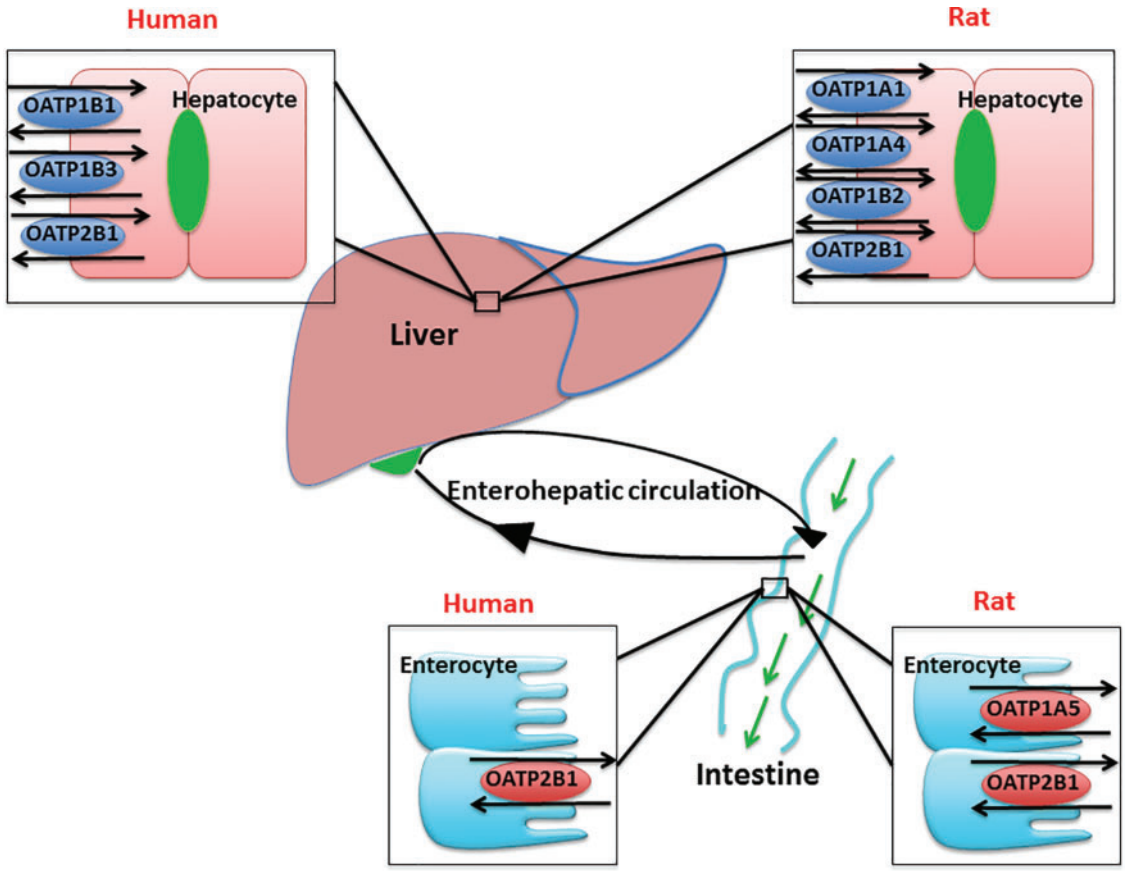

FIG. 1. Expression of human and rat OATPs in hepatocytes or enterocytes.

from a pSport1 vector containing the respective cDNAs. The open reading frames of rOATP1A1 and rOATP1A5 were subcloned using restriction enzymes from a pSport1 vector containing the cDNA of rOATP1A1 or rOATP1A5 into a pExpress1 or a pCMV-pSport6 vector, respectively. Correctness of all sequences was verified by DNA sequencing.

Tissue culture and transporter expression. The generation and culture condition of Chinese Hamster Ovary (CHO) cells stably expressing human OATP1B1 and OATP1B3 as well as the CHO Flp-In cells expressing hOATP2B1 were described previously (Gui *et al.*, 2008; Pacyniak *et al.*, 2010). Culture conditions for CHO Flp-in and Human Embryonic Kidney 293 (HEK293) cells were as described previously by our laboratory (Zhao *et al.*, 2015).

For CHO cells, 40 000 cells/well were plated in 24-well plates. Cells were induced with 5 mM sodium butyrate 48 h after plating and uptake assays were performed 24 h later.

For transporter expression in HEK293 cells, 250 000 cells/well were plated in 24-well plates coated with 0.1 mg/ml poly-D-lysine. Twenty-four hours later, cells were transfected with 0.5 μg plasmid DNA and 1.5 μl Fugene HD (Promega, Madison, Wisconsin) per well. Forty-eight hours after transfection, cells were used for uptake assays. Medium was changed when needed.

For kinetics, the initial linear range of uptake was determined at low (10 μM) and high (200–400 μM) substrate concentrations between 15 seconds and 5 minutes (Supplementary Figs. 1–5). The maximal initial linear range was determined visually based on net substrate uptake and kinetic experiments were performed at the shortest possible time point yielding a clear uptake signal.

Cell-based transport assay and LC–MS/MS analysis were as previously described (Weaver *et al.*, 2010; Zhao *et al.*, 2015).

Statistical analysis. Data were analyzed for significant differences using one-way ANOVA followed by Tukey's multiple comparison test. Student's *t*-test was used when comparing just 2 groups. $P < .05$ was considered significant.

TABLE 1. Primers Used to Subclone rOATP1B2 and rOATP2B1

		Restriction Site
rOATP1B2	Forward (5' → 3'): AGAGGCTAGCACCATGGACCACACTCAGCAGTCAAG	NheI
	Reverse (5' → 3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGAAGAGGTGTTTCATTG	NotI
rOATP2B1	Forward (5' → 3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGAAGAGGTGTTTCATTG	KpnI
	Reverse (5' → 3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGCAGTTCTTGTAGGTCTGA	NotI

RESULTS

Uptake of PFSAs by Human OATP1B1 and OATP1B3

To determine whether PFSAs are substrates of the liver-specific transporters hOATP1B1 and hOATP1B3, uptake of 10 μM PFBS, PFHxS, and PFOS was measured for 1 min using CHO cells stably expressing hOATP1B1 (Figure 2A) or hOATP1B3 (Figure 2B). The results demonstrate that PFBS, PFHxS and PFOS are substrates of both hOATP1B1 and hOATP1B3. Net OATP1B1-mediated PFHxS uptake was higher than uptake of PFBS, whereas the signal-to-noise ratio for PFOS was lower than for the other 2.

To further characterize the transport by hOATP1B1 and hOATP1B3, time-dependent uptake of PFBS, PFHxS, and PFOS was measured at low (10 μM) and high (400 μM for PFBS and PFHxS, 200 μM for PFOS) substrate concentrations (Table 2). The initial linear range of hOATP1B1-mediated uptake of PFBS, PFHxS, and PFOS was 2 min at low concentrations and 1 min at high concentrations. At low concentrations, hOATP1B3-mediated uptake of PFBS, PFHxS, and PFOS was linear up to 1, 5, and 2 min, respectively. At high concentrations, the initial linear range was 1, 2, and 2 min, respectively (Table 2). Based on these time-dependent uptake results, kinetics were performed at 40 s for PFBS and PFHxS, and at 1.5 min for PFOS and the results are shown in Figure 3A–F. The kinetic parameters were calculated based on the Michaelis–Menten equation and are summarized in Table 3. PFOS was transported with higher affinity (23 μM for hOATP1B1 and 32 μM for hOATP1B3) than PFHxS (101 μM for hOATP1B1 and 86 μM for hOATP1B3). In addition, OATP1B3-mediated PFBS uptake had a higher affinity than PFHxS (63 μM vs 86 μM). In terms of capacity (V_{max} values) PFHxS was transported with the highest capacity (2.10 nmol/mg protein min^{-1} for hOATP1B1 and 2.43 nmol/mg protein min^{-1} for hOATP1B3). Dividing the V_{max} by the K_m value yields the transport efficiency (or transporter clearance) and suggested that PFHxS and PFOS are transported about 10-fold more efficiently than PFBS (Table 3), correlating with the longer serum elimination half-lives of PFHxS and PFOS as compared to PFBS.

Uptake of PFSAs by human OATP2B1

In addition to hOATP1B1 and hOATP1B3, hOATP2B1 is also expressed at the basolateral membrane of hepatocytes and appears to be the major multi-specific OATP expressed at the apical membrane of human enterocytes. To determine whether hOATP2B1 can transport PFSAs, uptake of 10 μM PFBS, PFHxS, and PFOS was measured at 2 min using CHO Flp-in cells stably expressing hOATP2B1 (Figure 4A). Net transporter-mediated uptake of PFOS and PFHxS was higher than that of PFBS.

Next, time-dependent uptake of PFBS, PFHxS, and PFOS was measured at low (10 μM) and high (400 μM) substrate concentrations to determine the initial linear range (Table 2). At low substrate concentrations, hOATP2B1-mediated uptake of PFBS, PFHxS, and PFOS was linear up to 2, 1, and 5 min, respectively. At high substrate concentrations, uptake for all 3 substrates was linear only for 1 min. Therefore, concentration-dependent uptake of all 3 PFSAs was measured at 1 min, and the results are summarized in Figure 4B–D. After correcting for empty-vector control cells, the data were fitted using nonlinear regression analysis to the Michaelis–Menten equation, and the calculated kinetic parameters (K_m and V_{max}) are shown in Table 3. All 3 substrates were transported by hOATP2B1 with similar affinities. However, differences were seen for the respective V_{max} values. Transport of PFOS had the largest capacity (4.73 nmol/mg protein min^{-1}) followed by PFHxS (1.99 nmol/mg protein min^{-1}) and PFBS (0.56 nmol/mg protein min^{-1}). As a consequence, transport efficiency for PFOS was twice as high as for PFHxS and even 20 times higher than for PFBS (Table 3). Again, these numbers correlated well with the longer serum elimination half-lives for PFHxS and PFOS as compared to PFBS.

Previous studies demonstrated that transport by hOATP2B1 is pH-dependent (Nozawa *et al.*, 2004). However, no significant difference was observed for the transport of the 3 PFSAs by hOATP2B1 when uptake was measured at pH 7.4 or pH 5.5 (data not shown).

TABLE 2. Summary of Initial Linear Ranges of Human and Rat OATP-mediated Uptake of PFBS, PFHxS, and PFOS

Transporter	Low Concentration			High Concentration		
	Initial Linear Range (min)			Initial Linear Range (min)		
	PFBS 10 μM	PFHxS 10 μM	PFOS 10 μM	PFBS 400 μM	PFHxS 400 μM	PFOS 400 μM
hOATP1B1	2	2	2	1	1	1[a]
hOATP1B3	1	5	2	1	2	2[a]
hOATP2B1	2	1	5	1	1	1
rOATP1A1	nd	5	5	nd	5	5
rOATP1A5	1	10	10	2	1	5

nd, not determined.
[a]PFOS concentration was 200 μM for this transporter.

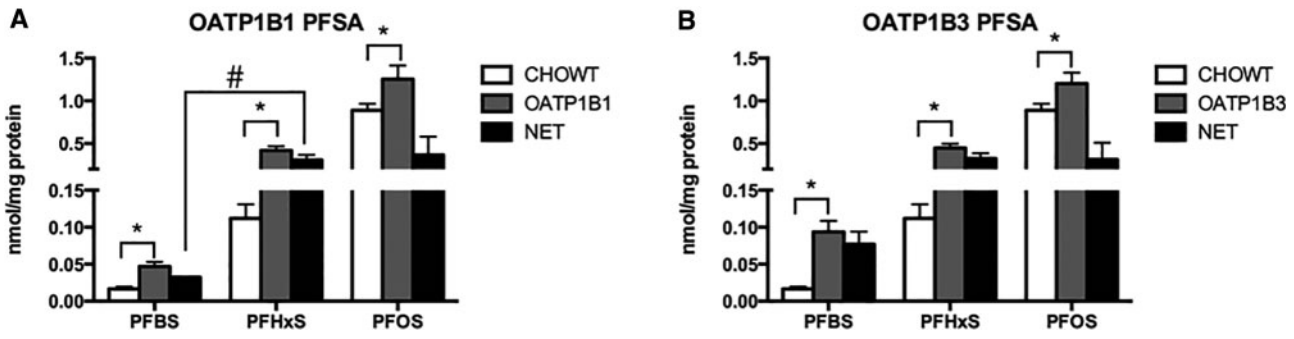

FIG. 2. Uptake of PFSAs by hOATP1B1 (A) and hOATP1B3 (B). CHO wild-type (white bars) or CHO cells stably expressing hOATP1B1 or hOATP1B3 (gray bars) were used to measure the uptake of 10 μM PFBS, PFHxS, and PFOS for 1 min at 37 °C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by wild-type cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD from 3 independent experiments each performed with triplicate determinations. The results were corrected for the total protein concentration in each well. $^{*,\#}P < .05$.

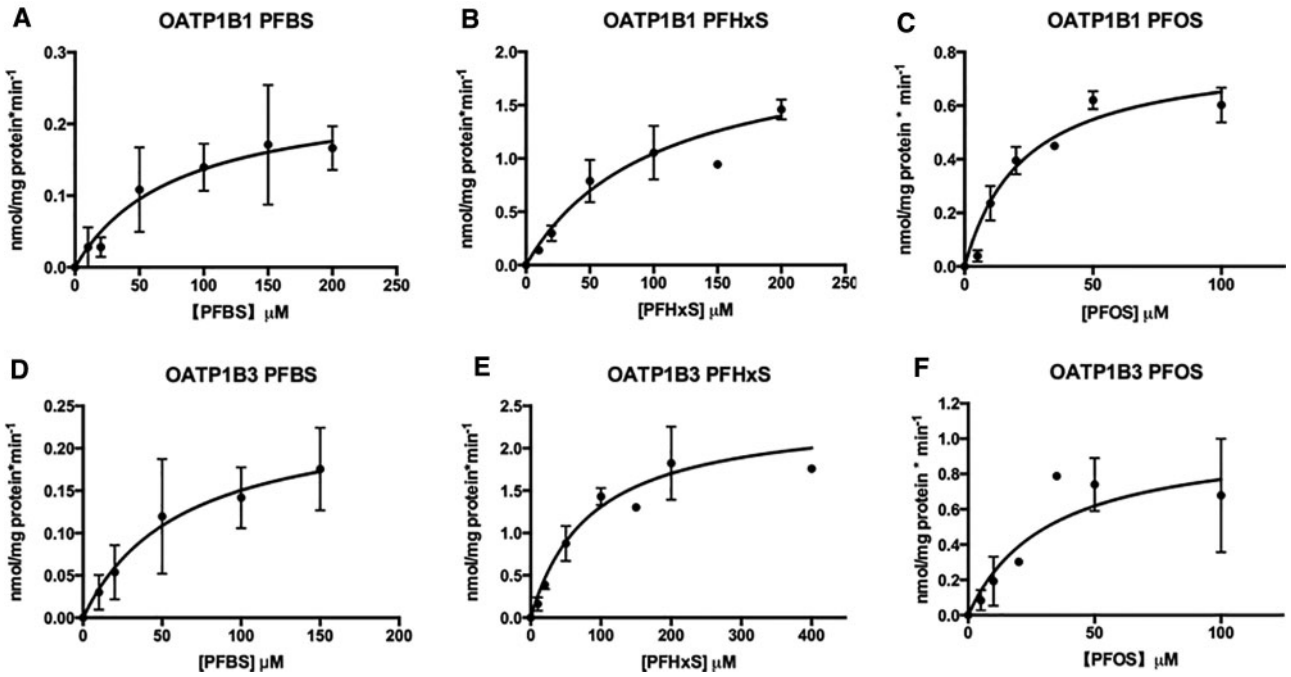

FIG. 3. Kinetics of hOATP1B1- (A–C) and hOATP1B3- (D–F) mediated transport of PFBS (A, D), PFHxS (B, E), and PFOS (C, F). CHO wild-type, CHO-hOATP1B1 and hOATP1B3 cells were used to measure the uptake (at 40 s for PFBS and PFHxS, and 90 s for PFOS) of increasing concentrations of PFBS, PFHxS, and PFOS. Net uptake was calculated by subtracting the values of uptake mediated by wild-type cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. The results were corrected for the total protein concentration in each well. Each point represents the mean ± SD from 3 independent experiments performed in triplicate.

TABLE 3. Kinetic Parameters of PFBS, PFHxS, and PFOS Transport Mediated by Human OATP1B1, OATP1B3, and OATP2B1

Transporter	PFSA	K_m (μM)	V_{max} (nmol/mg protein min^{-1})	V_{max}/K_m (μl/mg protein min^{-1})
hOATP1B1	PFBS	80 ± 47	0.25 ± 0.06[b]	2.5
	PFHxS	101 ± 32	2.10 ± 0.31	21
	PFOS	23 ± 6[a]	0.80 ± 0.07[a]	35
hOATP1B3	PFBS	63 ± 37[b]	0.24 ± 0.07[b]	3.2
	PFHxS	86 ± 24	2.43 ± 0.27	28
	PFOS	32 ± 19[a]	1.02 ± 0.24[a]	31
hOATP2B1	PFBS	122 ± 57	0.56 ± 0.11[c]	4.9
	PFHxS	53 ± 19	1.99 ± 0.22	38
	PFOS	48 ± 19	4.73 ± 0.65[a]	99

[a]$P < .05$ PFOS versus PFHxS.
[b]$P < .05$ PFBS versus PFHxS.
[c]$P < .05$ PFBS versus PFOS.

Uptake of PFSAs by Rat Liver OATPs

In rats, rOATP1A1, rOATP1A4, rOATP1B2, and rOATP2B1 are all expressed at the basolateral membrane of hepatocytes. To determine which of these OATPs are involved in the liver accumulation of PFSAs in rats, uptake of 10 μM PFBS, PFHxS, and PFOS was measured in HEK293 cells transiently expressing these transporters individually. As shown in Figure 5A, rOATP1A1 could transport all 3 PFSAs. Because the signals were well above the empty-vector transfected cells for PFHxS and PFOS, kinetics were performed at a 5-min uptake point (within the initial linear range, Table 2) and the results are summarized in Figure 5B and C and in Table 4. The capacity for PFHxS and PFOS transport was similar (1.01 nmol/mg protein min^{-1} for PFHxS and 0.66 nmol/mg protein min^{-1} for PFOS), and although the affinity for PFOS ($K_m = 37$ μM) seemed to be about 7-fold higher than for PFHxS ($K_m = 256$ μM) (Table 4) the difference was not statistically significant.

Results for the uptake of 10 μM PFBS, PFHxS, and PFOS at 1 and 5 min by rOATP1B2 and rOATP2B1 are shown in Figure 6. All 3 PFAAs are transported by both transporters, and the signals were comparable between rOATP1B2 and rOATP2B1. For both OATPs, the net transporter-mediated uptake of PFBS was about 10-fold lower than uptake of PFHxS and PFOS. However, signal-to-background ratios were not high enough to perform kinetic analyses. For the fourth rat OATP, rOATP1A4, no net transporter-mediated uptake could be detected at either 1 or 5 min for any of the PFAAs (data not shown).

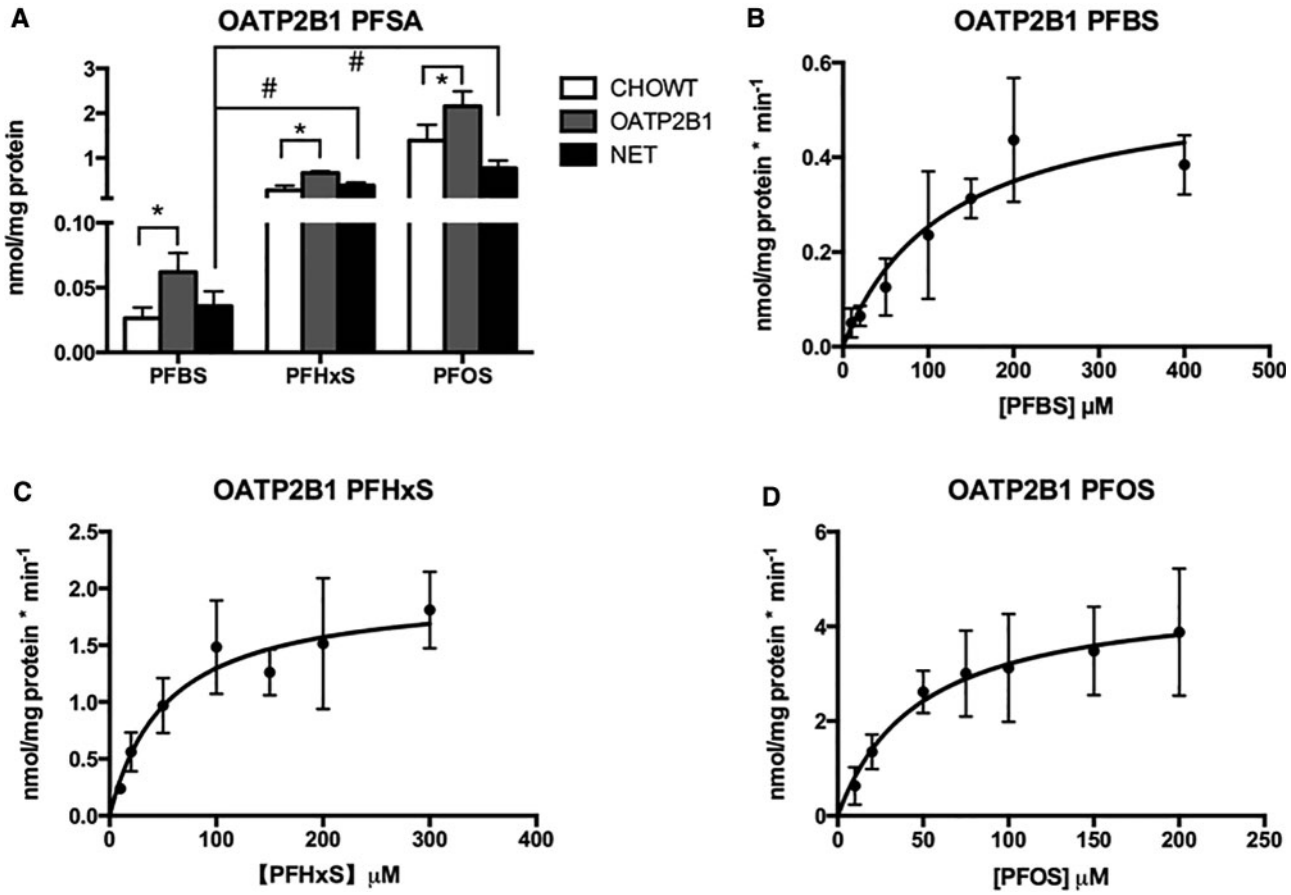

FIG. 4. Uptake of PFSAs by hOATP2B1. CHO Flp-in cells transfected with pcDNA5/FRT (EV) or stably expressing hOATP2B1 were used to measure the uptake of PFBS, PFHxS, and PFOS (A). Uptake of 10 μM PFBS, PFHxS and PFOS was determined at 37 °C for 2 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by CHO Flp-In EV cells from uptake mediated by transporter-expressing cells. B–D, Uptake of increasing concentrations of PFBS, PFHxS, and PFOS was determined at 37 °C for 1 min. Net uptake was calculated by subtracting the values of uptake mediated by Flp-In EV cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Each point represents the mean ± SD from 3 independent experiments performed in triplicate. The results were corrected for total protein concentration in each well. $^{*,\#}P < .05$.

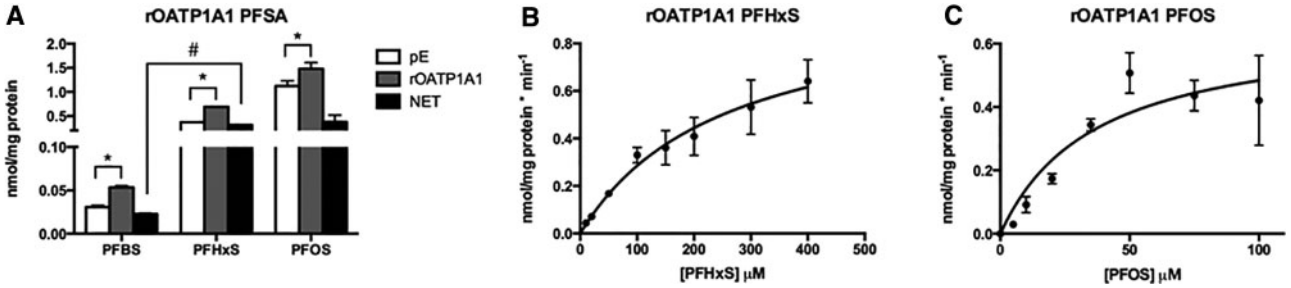

FIG. 5. Uptake of PFSAs by rOATP1A1. HEK293 cells transiently transfected with empty vector pExpress-1 (pE) or rOATP1A1 were used to measure the uptake of PFBS, PFHxS, and PFOS (A). Uptake of 10 μM PFBS, PFHxS and PFOS was measured at 37 °C for 1 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by pE transfected HEK293 cells from uptake mediated by transporter-expressing cells. B, C, Uptake of increasing concentrations of PFHxS and PFOS was determined at 37 °C for 5 min. Net uptake was calculated by subtracting the values of uptake mediated by cells transfected with pE from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Each point represents the mean ± SD from 3 independent experiments performed in triplicate. The results were corrected for total protein concentration in each well. $^{*,\#}P < .05$.

Uptake of PFSAs by Rat OATP1A5

Besides rOATP2B1, rOATP1A5 is also expressed on the apical membrane of enterocytes (Walters *et al.*, 2000). To test whether rOATP1A5 could also contribute to the disposition (absorption) of PFSAs, HEK293 cells transiently expressing rOATP1A5 were used to measure the uptake of 10 μM PFBS, PFHxS, or PFOS. As summarized in Figure 7A, rOATP1A5 transports all 3 PFSAs. Net rOATP1A5-mediated uptake was highest for PFOS, followed by PFHxS and PFBS.

In order to perform kinetics analyses, first, time-dependent uptake was measured at low (10 μM) and high (400 μM) substrate concentrations (Table 2). Based on these experiments, the concentration-dependent uptake of PFBS and PFHxS was measured at 1 min, whereas for PFOS, 5 min were used. As shown in

TABLE 4. Kinetic Parameters of PFBS, PFHxS, and PFOS Transport Mediated by Rat OATP1A1 and OATP1A5

Transporter	PFSA	K_m (μM)	V_{max} (nmol/mg protein min^{-1})	V_{max}/K_m (μl/mg protein min^{-1})
rOATP1A1	PFHxS	256 ± 69	1.01 ± 0.14	3.9
	PFOS	37 ± 19	0.66 ± 0.14	19
rOATP1A5	PFBS	117 ± 41	0.23 ± 0.04[a]	1.7
	PFHxS	160 ± 122	1.24 ± 0.47	7.5
	PFOS	55 ± 18	1.18 ± 0.13	22

[a]$P < .05$ PFBS versus PFOS.

Table 4, all 3 substrates had similar affinities. In terms of capacity, PFHxS and PFOS were transported with the same V_{max} values (1.24 and 1.18 nmol/mg protein min^{-1}), whereas PFBS was transported with a 6-fold lower capacity. As a consequence, transporter efficiency for PFOS was 3-fold higher than for PFHxS and 13-fold higher than for PFBS.

Uptake of Perfluoroalkyl Carboxylates by Human OATP1B1, OATP1B3, and OATP2B1

To test which OATPs can contribute to the transport of perfluoroheptanoate (C7), PFOA (C8), perfluorononanoate (C9), and perfluorodecanoate (C10) into human hepatocytes, 1 min uptake of 10 μM of C7–C10 was measured in HEK293 cells transiently expressing hOATP1B1, hOATP1B3, or hOATP2B1. The results are summarized in Figure 8 and demonstrate that C8 and C9 are substrates of all 3 human OATPs that are expressed in hepatocytes. The signals for transporter-mediated uptake of C7 and C10 did not reach significance and thus these 2 compounds are not substrates of any of the 3 human OATPs. Transport for C9 was higher than that for C8 for OATP1B1 indicating a chain-length dependent increase.

DISCUSSION

In the present study, we have demonstrated that PFAAs, including PFBS, PFHxS, and PFOS are substrates of human hOATP1B1, hOATP1B3, hOATP2B1, and rat rOATP1A1, rOATP1B2, rOATP2B1, and rOATP1A5. Furthermore, we could show that hOATP1B1, hOATP1B3, and hOATP2B1 can transport PFCAs with 8 or 9 carbons.

In our previous study, we demonstrated that bromosulfophthalein could inhibit sodium-independent uptake of PFBS and PFHxS into human and rat hepatocytes, whereas sodium-independent uptake of PFOS was not inhibited (Zhao *et al.*, 2015). Those results suggested that, besides the sodium-dependent portion mediated by hNTCP or rNTCP, there would also be a sodium-independent portion of uptake at least for PFHxS and PFBS. Our findings reported in this study clearly demonstrate that the human hOATP1B1, hOATP1B3, and hOATP2B1, as well as the rat rOATP1A1, rOATP1B2, and rOATP2B1, can transport PFBS and PFHxS and therefore likely contribute to the sodium-independent uptake into human and rat hepatocytes. Although there was no inhibition of sodium-independent PFOS uptake by bromosulfophthalein, direct uptake measurements showed that all 3 human OATPs were able to transport PFOS. Comparing the calculated transport efficiencies (V_{max}/K_m values) for the individual OATPs with respect to the 3 different PFSAs, our data clearly indicated that PFBS was transported with the lowest efficiency by all 3 human OATPs (Table 3). Transport efficiencies for PFHxS and PFOS were almost 10-fold higher than for PFBS for all 3 human OATPs, correlating with the much longer serum elimination half-lives of PFHxS and PFOS. Thus, in addition to hNTCP, hOATP1B1, hOATP1B3, and hOATP2B1 can contribute to the enterohepatic circulation of PFHxS and PFOS and seem to contribute much less to the enterohepatic circulation of PFBS.

Human OATP2B1 is expressed in hepatocytes (Peng *et al.*, 2015; Prasad *et al.*, 2014), and also at the apical membrane of human enterocytes (Drozdzik *et al.*, 2014). It can mediate the uptake of steroid hormones, drugs, and other xenobiotics (Roth *et al.*, 2012) and hence facilitate their enterohepatic circulation. Our findings that hOATP2B1 can transport all 3 PFSAs suggest that, in addition to the previously reported hASBT (Zhao *et al.*, 2015), hOATP2B1 also mediates the uptake of PFOS into enterocytes and thus contributes to the enterohepatic circulation of PFOS. Furthermore, because hASBT cannot transport PFHxS (Zhao *et al.*, 2015), hOATP2B1 is the first human transporter expressed in the intestine that is reported to mediate the reabsorption of PFHxS.

In addition to these human liver OATPs, we also identified 3 OATPs expressed in rat livers, rOATP1A1, rOATP1B2, and rOATP2B1 that are able to mediate the uptake of PFBS, PFHxS, and PFOS. Rat OATP1A1 transported PFHxS with a similar affinity ($K_m = 256$ μM) as rNTCP ($K_m = 294$ μM). Its affinity to PFOS ($K_m = 37$ μM) was much higher and close to the affinities of the human liver OATPs. Rat OATP1A1 is also expressed in the apical membrane of renal proximal tubular cells (Bergwerk *et al.*, 1996), where it can mediate the reabsorption of organic anions in exchange for intracellular reduced glutathione (Li *et al.*, 1998). In addition, rOATP1A1 is expressed in a male-dominant manner (Kato *et al.*, 2002). We previously demonstrated that rOATP1A1 transports several PFCAs, such as PFOA, and hence, at least partly, contributes to the longer serum elimination half-life of PFOA in male rats (Weaver *et al.*, 2010). As for PFHxS, pharmacokinetic studies revealed that the elimination half-life is much slower in male rats ($t_{1/2} = 29.1$ days) than in female rats ($t_{1/2} = 1.6$ days) (Sundstrom *et al.*, 2012). Increased reabsorption of PFHxS in male rats via rOATP1A1 could be part of the mechanism for this gender-specific difference in elimination. Although rOATP1A1 can also transport PFOS, the elimination half-life of PFOS in male rats is somewhat shorter than in female rats (Chang *et al.*, 2012), indicating that rOATP1A1 is not the major transporter for the renal elimination of PFOS. Rat OAT1 and OAT3 that are expressed in a male dominant manner in the kidneys (Ljubojevic *et al.*, 2004) could potentially be responsible, but experiments using the respective knockouts or selective inhibitors will be required to prove this hypothesis.

Enterohepatic circulation of PFOS was observed not only in humans, but also in rats (Johnson *et al.*, 1984). So far the mechanism for intestinal reabsorption of PFOS, and also of PFBS and PFHxS, in rats could not be explained because, unlike in humans, rASBT does not transport these PFSAs (Zhao *et al.*,

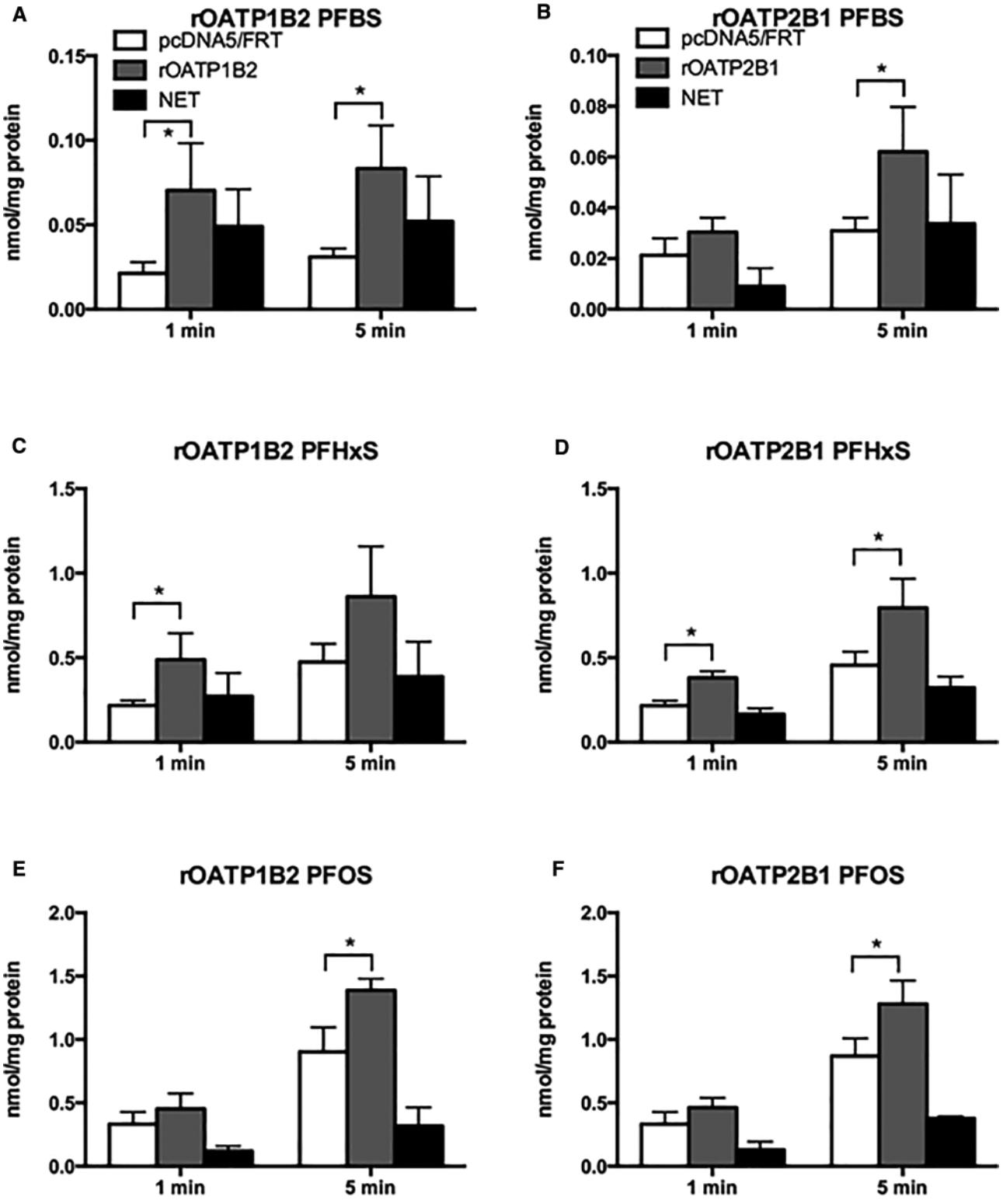

FIG. 6. Uptake of PFSAs by rOATP1B2 (A, C, E) and rOATP2B1 (B, D, F). HEK293 cells transiently transfected with empty vector pcDNA5/FRT (white bars), and rOATP1B2 or rOATP2B1 (grey bars) were used to measure the uptake of 10 μM PFBS, PFHxS or PFOS for 1 and 5 min at 37 °C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by empty vector transfected cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD from 3 independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. *$P < .05$.

2015). In the present study we report, for the first time, that rOATP1A5 and rOATP2B1, OATPs expressed in rat intestine, transport all 3 PFSAs. Thus, reabsorption of PFOS via rOATP1A5 and rOATP2B1 offers an explanation as to how PFOS can be kept in the enterohepatic circulation in the absence of rASBT-mediated transport.

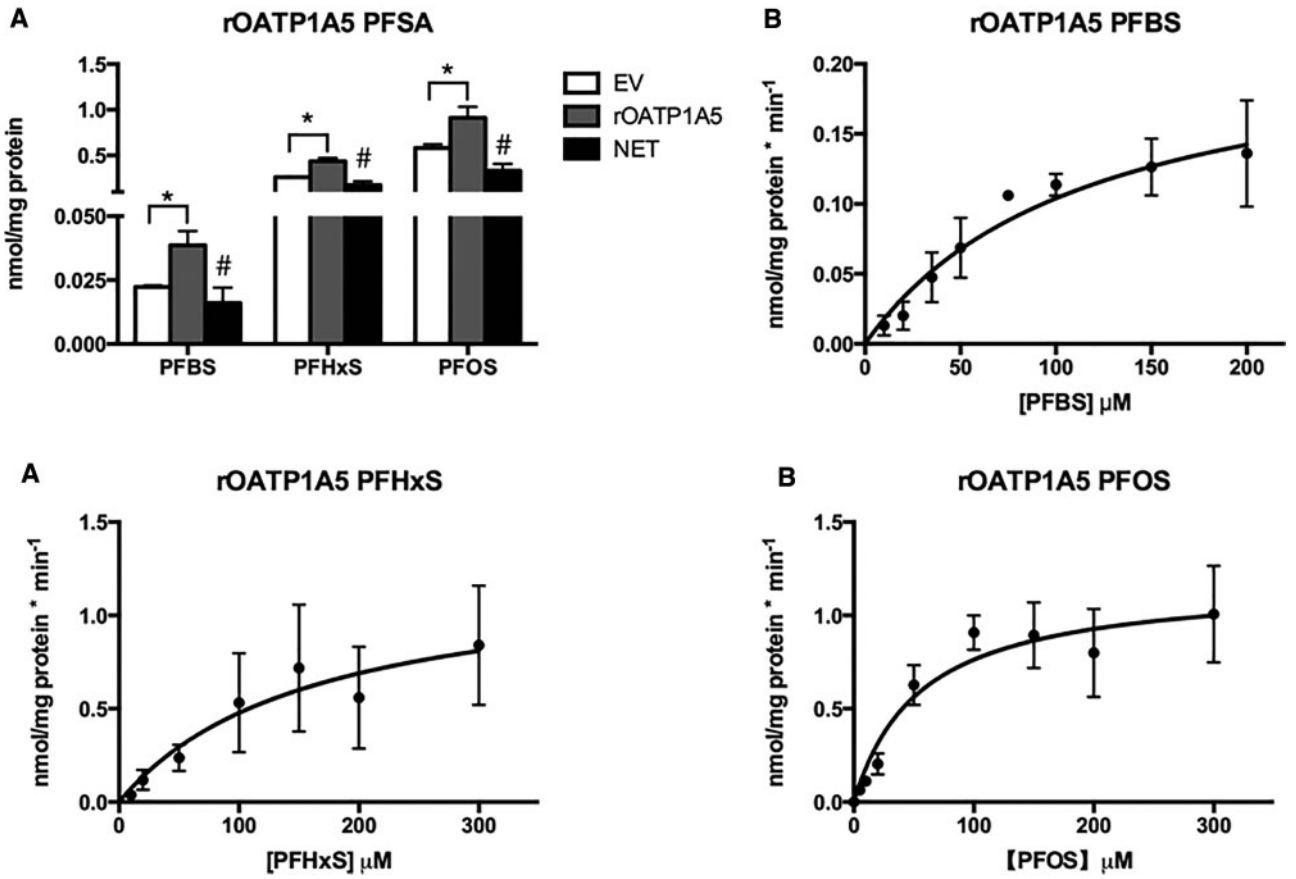

FIG. 7. Uptake of PFSAs by rOATP1A5. HEK293 cells transiently transfected with empty pSport6 vector (EV) or rOATP1A5 were used to measure the uptake of PFBS, PFHxS, and PFOS (A). Uptake of 10 μM PFBS, PFHxS and PFOS was measured at 37 °C for 1 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by EV transfected HEK293 cells from uptake mediated by transporter-expressing cells. B–D Uptake of increasing concentrations of PFBS, PFHxS, and PFOS was measured at 37 °C for 1 (PFBS and PFHxS) or 5 min (PFOS). Net uptake was calculated by subtracting the values of uptake mediated by EV transfected cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Each point represents the mean ± SD from 3 independent experiments performed in triplicates. The results were corrected for total protein concentration in each well. $^{*,\#}P < .05$.

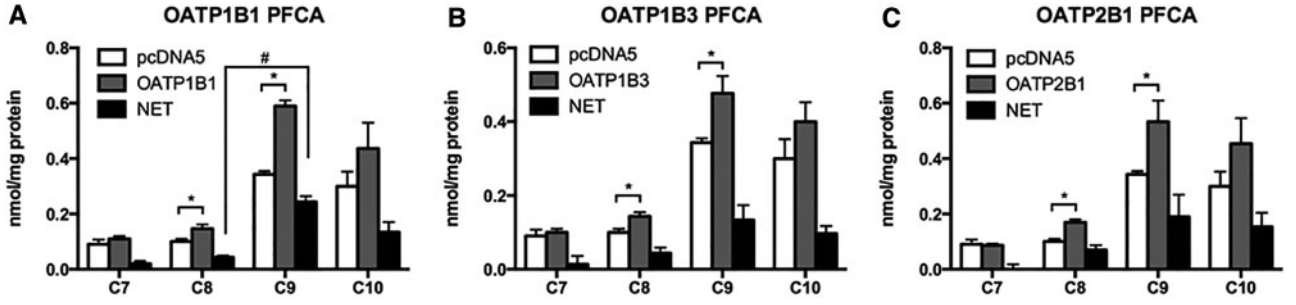

FIG. 8. Uptake of PFCAs by hOATP1B1 (A), hOATP1B3 (B) and hOATP2B1 (C). HEK293 cells transfected with empty vector (pcDNA5 white bars) or cells transiently expressing hOATP1B1, hOATP1B3 or hOATP2B1 (gray bars) were used to measure the uptake of 10 μM C7-C10 PFCA for 1 min at 37 °C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by empty vector transfected cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD from 3 independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. $^{*,\#}P < .05$.

Differential renal clearance was observed for PFCA and PFSA analogs with different chain lengths according to pharmacokinetic studies in animal models. The transport of PFSAs was also chain-length dependent. As we discussed previously, NTCP transported PFSAs with decreasing affinity but increasing capacity as the chain length increased. The opposite trend was seen for OATP-mediated uptake. For all 5 of the OATPs where kinetic analysis was possible, PFOS was transported with the highest affinity, followed by PFBS and PFHxS that were transported with similar but clearly lower affinities. Nevertheless, transport efficiency generally increased with the increase in chain length because V_{max} values were always higher for PFHxS and PFOS. As indicated before, this higher transport efficiency could well be, at least in part, the distinguishing mechanism for the much shorter half-life of PFBS compared to the longer half-lives of PFHxS and PFOS.

We have previously shown that rOATP1A1 can transport the PFCAs C7–C10 (Weaver *et al.*, 2010) and contributes to the gender-specific renal elimination of C8–C10. Given that rOATP1A1 is also expressed in the liver, we tested in the current study whether any of the OATPs expressed in human hepatocytes would transport these carboxylates and thus could contribute to the long half-lives of these chemicals (Olsen *et al.*, 2007) by helping to keep them within the enterohepatic circulation in humans. The fact that all 3 OATPs expressed in human hepatocytes can transport the longer chain C8 and C9, but not the shorter chain C7, suggests a potential role of these OATPs in the longer serum elimination half-lives of these 2 PFCAs. The longer chain C10 might be a transported into hepatocytes with the help of fatty acid transporters that handle long-chain fatty acids such as oleate and palmitate.

In conclusion, our studies demonstrate that PFBS, PFHxS, and PFOS are substrates of several OATPs (hOATP1B1, hOATP1B3, hOATP2B1, rOATP1A1, rOATP1B2, rOATP2B1, and rOATP1A5) expressed in hepatocytes and enterocytes in humans and rats. Furthermore, PFCAs with 8 and 9 carbons are substrates of the 3 human OATPs. Thus, the sodium-independent transporters of the OATP family could serve as additional mediators to facilitate the enterohepatic circulation of PFHxS and PFOS in humans and rats and rOATP1A1 might contribute to the gender-specific renal elimination of PFHxS in rats. In future studies pharmacokinetic studies will have to be performed using animal models where the individual transporters are inactivated either by selective inhibitors or by genetic means in order to proof that these transporters indeed play a role in the long half-lives of PFAAs.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

FUNDING

This work was supported by National Institute of Health grants RR021940 and GM077336, and by an unrestricted research grant from the 3M Company.

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