Rat Organic Anion Transporter 3 and Organic Anion Transporting Polypeptide 1 Mediate Perfluorooctanoic Acid Transport

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The mechanism by which perfluorooctanoic acid (PFOA) is transported in the kidney was studied in rats. We hypothesized that some transporters that are expressed in the basolateral and/or brush border membrane of proximal tubular cells mediate the transport of PFOA. Mannitol infusion, which caused an increase in the urine flow rate, significantly increased the renal clearance (CL_R) of PFOA in both male and female rats. Feeding a low-phosphate diet that causes an increase in the expression of rat type II sodium-dependent phosphate transporter (Npt2) reduced the CL_R in both male and female rats. These suggest that PFOA is reabsorbed in the proximal tubules, and that a phosphate transporter may be responsible for the renal transport of PFOA. The CL_R of PFOA in Eisai hyperbilirubinemic rats that lack multidrug resistance-associated protein 2 (MRP2) was not different from that of the wild type, suggesting that MRP2 is not responsible for the renal transport of PFOA. Three candidate transporters, organic anion-transporting polypeptide 1 (oatp1), Npt2, and organic anion transporter 3 (OAT3) were studied to clarify whether these transporters facilitate [^{14}C]PFOA transport in functional studies in *Xenopus laevis* oocytes. Both oatp1 and OAT3 facilitated [^{14}C]PFOA transport while Npt2 did not. These results suggest that both oatp1 and OAT3 mediate, at least in part, the transport of PFOA in the proximal tubules of rat kidney.

Key words —— perfluorooctanoic acid, renal transport, organic anion-transporting polypeptide 1, organic anion transporter 3

INTRODUCTION

Perfluorooctanoic acid (PFOA), a fully fluorinated octanoic acid, is used as a chemical intermediate, and its salts are used in emulsifier and surfactant applications.[1)] PFOA is persistent in the environment without being degraded because of the high energy of the carbon-fluorine bond. In an early study, PFOA was found in the serum of occupationally exposed workers.[2)] Sub-sequent studies revealed that PFOA was detected in the serum of general populations at low levels.[3)] Since PFOA has been found in some wildlife species as well,[4–6)] contamination with this chemical seems to be widespread. A half-life study on retirees from a PFOA plant was undertaken where the mean serum half-life of the chemical was calculated to be 4.37 years.[7)] PFOA is thought to be metabolically inert,[8)] and therefore elucidation of excretion mechanisms is necessary to estimate the toxicity of this chemical.

Toxicokinetic studies have shown that PFOA is mainly eliminated in urine and that enterohepatic circulation would prolong the half-life of this chemical.[8–10)] In addition, several lines of evidence suggested that PFOA is secreted in the renal proximal tubules *via* a carrier-mediated process; a marked sex-related difference was observed in the renal excretion rate;[8,9)] and urinary elimination of PFOA is inhibited by probenecid, an inhibitor of several organic anion transporters.[9,11)] These led us to consider the possibility that PFOA is secreted in the

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renal proximal tubules *via* a carrier-mediated process in rats. A sex difference was observed in the expression of several transporters including rat organic anion transporting-polypeptide 1 (oatp1) that is present in the renal proximal tubules.[9,12–15] In addition, we suggested that rat organic anion transporter 2 (OAT2) and rat organic anion transporter 3 (OAT3) are responsible for the renal transport of PFOA by comparing the renal clearance (CL_R) of PFOA with mRNA levels of various organic anion transporters in the kidney under various hormonal states.[9] To date, however, no information is available on the molecular mechanism by which PFOA is excreted into urine. In the present study, we examined whether some organic anion transporters that are expressed in renal tubular cells mediate the transport of PFOA.

MATERIALS AND METHODS

Materials —— PFOA was purchased from Sigma Aldrich, Japan (Tokyo, Japan). [1-^{14}C]-Perfluorooctanoic acid (55 mCi/mmol) was purchased from BlyChem Ltd. (Billingham, U.K.). [Taurine (2-^{3}H)]-Taurocholic acid (50 Ci/mmol) and *p*-[glycyl 1-^{14}C]-amino-hippuric acid (PAH, 40 mCi/mmol) were purchased from ARC (St. Louis, MO, U.S.A.). [^{32}P]-Orthophosphate was from PerkinElmer Life Sciences Inc. (Wellesley, MA, U.S.A.). All other chemicals were of analytical grade.

Animals —— Male and female Wistar rats, Eisai hyperbilirubinemic rats (EHBR), and Sprague-Dawley (SD) rats were obtained from SLC (Hamamatsu, Japan) and subjected to experiments at 9 weeks old after acclimatization for at least 1 week. All animals were acclimatized in a humidity (40–50%)- and temperature (23 ± 2°C)-controlled environment with a 12-hr light-dark cycle. Animals had free access to water and laboratory chow (CE-2, Clea Japan, Tokyo, Japan). Some male and female Wistar rats were fed a low-phosphate diet [AIN-93M diet containing 0.02% (w/w) orthophosphate] for 1 week which was obtained from Oriental Yeast (Tokyo, Japan). All animal studies complied with the regulation of the Institutional Board for Animal Study, Josai University.

Determination of CL_R —— The CL_R of PFOA was determined in three to eight animals in each group. PFOA was added to NaOH solution 1 M at equimolar concentration and dissolved in rat serum obtained from male or female rats (48.63 μmol/ml). Rats were anesthetized with urethane at a dose of 0.9 g/kg body weight and underwent surgical catheter (SP-45) implantation *via* the femoral vein and bladder. One hour after the surgery, these rats were injected with PFOA at a dose of 48.63 μmol/kg body weight *via* the femoral vein, and 0.2 ml of blood was withdrawn at several time points between 0 and 300 min after the injection. Urine samples were collected in polyethylene tubes. The tubes were changed at several time points between 0 and 300 min. Plasma and urine samples were stored at −30°C until the determination of the PFOA concentration as described previously.[16] The area under the curve ($AUC_{0-300\,min}$) was calculated by the trapezoidal rule. The CL_R of PFOA was calculated according to the equation:

$$CL_R\ (\text{ml/h/kg body weight}) = \text{PFOA in urine}_{0-300\,min}\ (\text{nmol})/AUC_{0-300\,min}\ (\text{nmol·h/ml})/\text{body weight (kg)}.$$

For mannitol infusion, 10% (w/v) mannitol/0.9% (w/v) sodium chloride was infused at 0.1 ml/min throughout the experiment. Two hours after the start of mannitol infusion, these rats were injected with PFOA at a dose of 48.63 μmol/kg *via* the femoral vein. Urine and blood samples were collected and the PFOA concentration was determined as described above.

Transporter-mediated Uptake of PFOA —— Total RNA was extracted from the kidneys of adult male rats and cDNA for oatp1, OAT3, and Sodium-dependent phosphate cotransporter (Npt) 2 was synthesized by PCR with a set of degenerated primers (oatp1, sense strand 5′-CAGAAGAACA-CCATGGAGG-3′ and antisense strand TCTTCCCCAGGGAAATCTTA-3′; OAT3, sense strand 5′-CATCCACCTCCAGTCCAACT-3′ and antisense strand 5′-TCCAGAAGGGTACTG-CTTGG-3′; type II sodium-dependent phosphate transporter (Npt2), sense strand 5′-TCAAGGA-CTCATTGTGGGTG-3′ and antisense strand 5′-GGGCTCTGTAAAAATCCCGT). After purification, the PCR product was ligated into pBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.) and transformed into competent *Escherichia coli* (*E. coli*) DH5*α*. The plasmid containing oatp1, OAT3, or Npt2 was purified from *E. coli*, linearized with *Eco*RV and then subjected to synthesis of capped cRNA using the RiboMAX large-scale RNA production system-T7 (Promega, Madison, WI, U.S.A.)

Ovaries of *Xenopus laevis* (*X. laevis*) were

purchased from Copacetic (Aomori, Japan). The oocytes at stage V and stage VI were prepared from the ovaries according to the method of Yao *et al.*[17] Then, the oocytes were injected with 30 nl of cRNA solution (30 ng of cRNA) or RNase-free water. Two or 3 days after the microinjection with cRNA, the oocytes were divided into several experimental groups (three oocytes each) and kept at 18°C until use. Oocytes were preincubated in the uptake buffer [NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, Hepes/Tris 10 mM (pH 7.5)] for 5 min at 25°C followed by 1-hr incubation with [^{3}H]-taurocholate 10 μM, [glycyl 1-^{14}C]-PAH 10 μM, [^{32}P]-orthophosphate 100 μM or [^{14}C]-PFOA 10 μM, respectively, in the uptake buffer. At the end of the uptake periods, oocytes were washed three times with 1 ml of ice-cold uptake buffer and then solubilized in 10% (w/v) sodium dodecylsulfate solution. To an aliquot of the cell lysate was added 8 ml of scintillation cocktail, and the radioactivity was determined with a liquid scintillation counter (Aloka 5100, Tokyo, Japan).

Statistical Analyses —— Analysis of variance was used to test the significance of the difference among the four experimental groups of rats. When differences were significant, the statistical significance between any two means was determined using Shèffe's multiple range tests. Statistically significant differences between male and female rats were analyzed using Student's *t*-test.

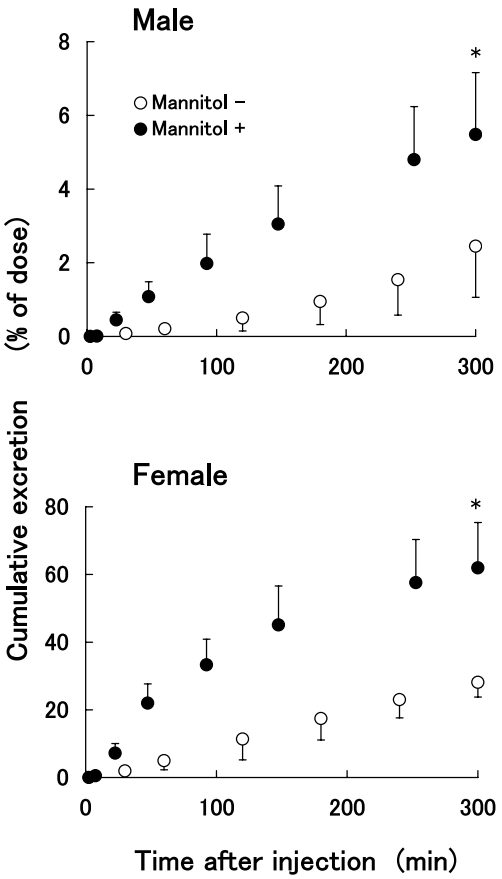

Fig. 1. Effect of Mannitol Infusion on Cumulative Excretion of PFOA into Urine

Cumulative excretion of PFOA into urine after an iv injection (48.63 mmol/kg) was determined in male and female rats. Values are means ± S.D. for three or four rats. Differences from the values of mannitol infusion are statistically significant ($^*p < 0.05$).

RESULTS

Effects of Mannitol Infusion on CL_R of PFOA

To determine whether PFOA is reabsorbed in the renal tubules, the effects of mannitol infusion on urinary excretion of PFOA were examined. When mannitol was not infused, the urine flow rate was approximately 0.006 ml/min. Infusion of 10% mannitol/0.9% NaCl solution at a rate of 0.1 ml/min significantly increased the urine flow rate to approximately 0.1 ml/min. Mannitol infusion significantly increased PFOA excretion into urine in both male and female rats (Fig. 1). The urinary excretion rate of PFOA was much higher in female rats compared with male rats, irrespective of mannitol infusion. Mannitol infusion increased the CL_R of PFOA 2-fold and 3.5-fold in male and female rats, respectively (Table 1).

CL_R of PFOA in EHBR

The possibility that multidrug resistance-associated protein 2 (MRP2) is responsible for the renal transport of PFOA was examined. Urinary excretion rates of PFOA in male and female EHBR that lack MRP2 were comparable with those in their respective controls (SD rats, Fig. 2). No difference in the CL_R of PFOA was observed between EHBR and SD rats (Table 1). This suggests that MRP2 does not participate in renal PFOA transport. The CL_R of PFOA in female EHBR was higher than that in male EHBR (Table 1). SD rats and EHBR showed a higher CL_R of PFOA compared with Wistar rats irrespective of sex.

CL_R of PFOA in the Rats Fed a Low-phosphate Diet

Npt2 is expressed in brush the border membrane of renal proximal tubules and play a crucial role in inorganic phosphate reabsorption.[18, 19]

Table 1. Effects of Mannitol Infusion, Defect of MRP2 or Low-Phosphate Diet on Renal Clearance of PFOA in Male and Female Rats

	Male (ml/h/kg body weight)	Female (ml/h/kg body weight)
Mannitol (−)	1.02 ± 0.59	12.82 ± 4.15
Mannitol (+)	2.11 ± 0.93	44.42 ± 13.94*
SDR	8.13 ± 4.85	92.46 ± 23.30
EHBR	5.81 ± 1.69	75.49 ± 21.54
Normal diet	2.11 ± 0.93	44.42 ± 13.94
Low-phosphate diet	1.14 ± 0.78	22.36 ± 4.44*

Renal clearance was determined after anintravenous injection of PFOA in Wistar rats, S.D. rats, or EHBR. All rats except for mannitol (−) were infused 10% mannitol solution throuout the experiment. Values are means ± S.D. for three to eight rats. Differences from the values of mannitol infusion are statistically significant (* $p < 0.05$).

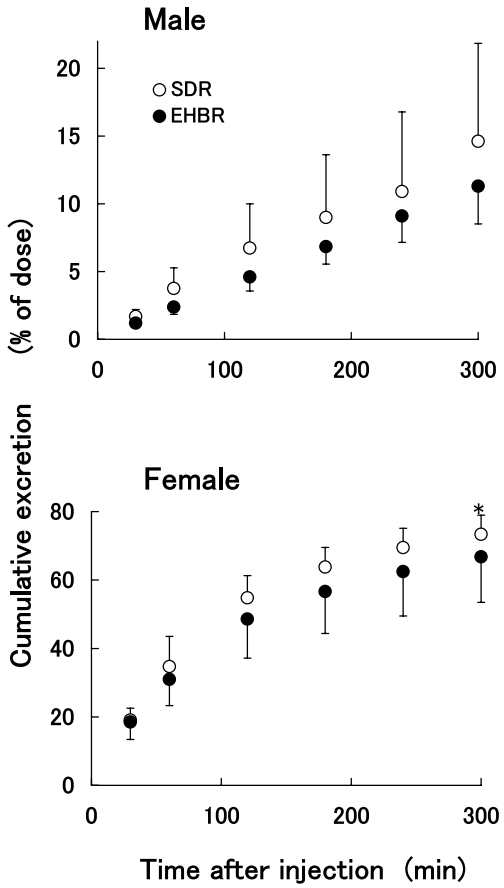

Fig. 2. Cumulative Excretion of PFOA into Urine in EHBR

Cumulative excretion of PFOA into urine after an injection (48.63 mmol/kg, i.v.) was determined in male and female EHBR rats. Values are means ± S.D. for three or four rats. Differences from the values of mannitol infusion are statistically significant (* $p < 0.05$).

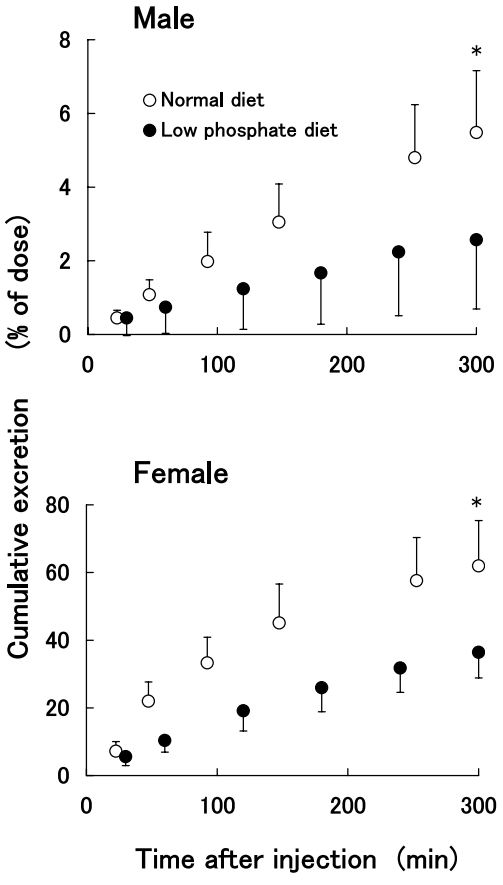

Fig. 3. Effects of Low-Phosphate Diet on Cumulative Excretion of PFOA into Urine

Cumulative excretion of PFOA into urine after an injection (48.63 mmol/kg, i.v.) was determined in male and female Wistar rats that were fed low-phosphate diet. Values are means ± S.D. for three or four rats. Differences from the values of mannitol infusion are statistically significant (* $p < 0.05$).

To determine whether Npt2 is responsible for renal PFOA transport, the CL_R of PFOA was examined in rats fed a low-phosphate diet since that upregulates Npt2 expression in the proximal tubules.[18] The low-phosphate diet decreased the urinary excretion rate of PFOA (Fig. 3), and the CL_R of PFOA was reduced by 50% in both male and female rats.

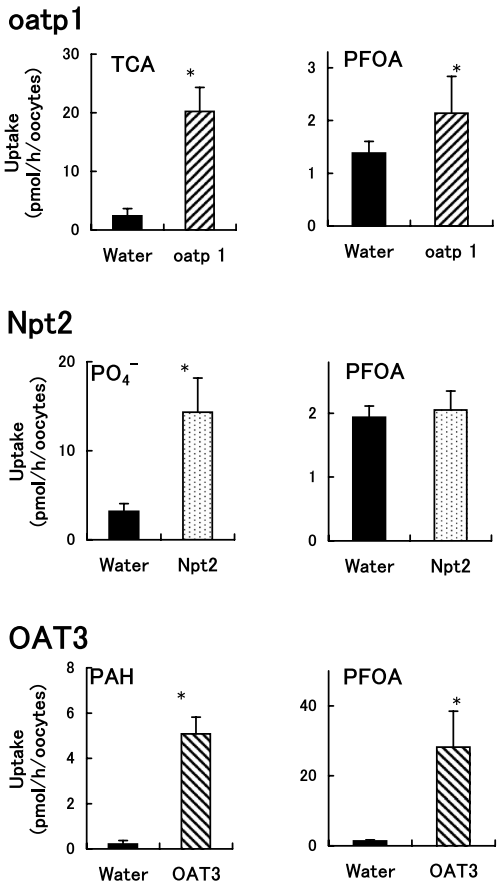

Fig. 4. PFOA Uptake by *X. laevis* Oocytes Expressing oatp1, Npt2, or OAT3

Oocytes were injected with cRNA of oatp1, Npt2, OAT3, or water and then incubated in the buffer containing [^{3}H]-taurocholate 10 mM, [^{32}P]-PO_4^{3-} 100 mM, [glycyl 1-^{14}C]-PAH 10 mM or [^{14}C]-PFOA 10 mM for 1 hr. Values are mean ± S.D. for 10 oocytes. Significant differences from water-injected oocytes ($^*p < 0.01$).

Transporter-mediated Uptake of PFOA

Next, three transporters, oatp1, Npt2, or OAT3, were expressed in *X. laevis* oocytes and PFOA transport was estimated (Fig. 4). The renal level of oatp1 mRNA was altered by changing sex-hormonal states.[9] Considering that oatp1 is expressed in the brush border membrane of renal proximal tubules[20] and is responsible for reabsorption of several drugs,[21] oatp1 is a candidate PFOA transporter. Expression of oatp1 in the cells significantly increased the uptake of both [^{3}H]-taurocholate, a known substrate for oatp1, and [^{14}C]-PFOA (Fig. 4). Expression of Npt2 facilitated [^{32}P]-phosphate transport while it did not facilitate PFOA transport (Fig. 4). OAT3 is a candidate PFOA transporter, as demonstrated in our previous study.[9] Injection of OAT3 cRNA facilitated uptake of [^{14}C]-PFOA by oocytes as well as that of [^{14}C]-PAH, a known substrate for OAT3 (Fig. 4).

DISCUSSION

The present study showed that the CL_R of PFOA was increased upon mannitol infusion, suggesting that PFOA is extensively reabsorbed in the proximal tubules, since mannitol infusion increased urine flow rate. These results suggest that sex-related differences in CL_R of PFOA are due to the difference in the expression of the transporter responsible for reabsorption of PFOA. Oatp1 is a plausible candidate for the transporter that mediates reabsorption of PFOA in the proximal tubules, since this transporter was demonstrated to be expressed in the brush border membrane of proximal tubular cells in rats.[20] In addition, there was a significant sex-related difference in the expression of oatp1 mRNA and its protein in the kidney, where it is expressed in male rats but is hardly detected in female rats.[15,21] The present results suggest that oatp1 mediates PFOA transport. It is possible that oatp1 is responsible for reabsorption of PFOA and that the sex-related difference in CL_R is, in part, due to the difference in oatp1 expression between male and female rats *in vivo*.

PFOA appears to undergo little glomerular filtration since most of PFOA in blood binds to serum proteins.[10] Instead, the renal tubular excretion system is thought to be responsible for urinary elimination of this chemical. The first step of the renal excretion of PFOA is uptake from blood. OAT3 is the transporter expressed in the basolateral membrane of proximal tubular cells in rats[22] and functions in the renal uptake of several organic anions.[23]

OAT3 was shown to transport PFOA (Fig. 4) and therefore thought to be responsible for the uptake of PFOA from blood into the proximal tubular cells. For OAT3, there was no sex-related difference in the level of renal mRNA, but it was increased by ovariectomy in female rats.[9] The increased level of mRNA was accompanied by increased protein expression in ovariectomized female rats.[?] Considering the fact that the CL_R of PFOA was also increased by ovariectomy of female rats,[9] OAT3 would be responsible for the renal uptake of PFOA from the circulation even though OAT3 may not be responsible for sex-related difference in CL_R of PFOA. In contrast to OAT3, the physiologic significance of OAT2 and its localization in the kidney has not been elucidated yet. In the present study therefore, we did not determine whether OAT2 transports PFOA although its mRNA level was differed between various sex hormonal states.[9]

In the present study, the expression of Npt2 did not facilitate PFOA transport in *X. laevis* oocytes (Fig. 4) although feeding a low-phosphate diet that causes Npt2 upregulation reduced the CL_R of PFOA (Table 1). A low-phoaphate diet may alter the expression of some other transporters that mediate PFOA transport in the rat kidney, although the transporter that undergoes a regulation by the dietary phosphate level is not well characterized.

In conclusion, both oatp1 and OAT3 mediate PFOA transport *in vitro* and would be responsible for, at least in part, the renal reabsorption and uptake of PFOA, respectively, in rats *in vivo*. This is the first report showing that organic anion transporters mediate the transport of PFOA.

In our previous study, biological half-lives of perfluorcarboxylic acids depended on their urinary excretion rates.[10] Therefore elucidation of the renal PFOA transport mechanism could lead to detoxification of PFOA. The present study did not exclude the contribution of other transporters in renal transport of PFOA. The entire mechanism of renal PFOA transport remains to be elucidated.

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