

Critical Role of PPAR- α in Perfluorooctanoic Acid- and Perfluorodecanoic Acid-Induced Downregulation of Oatp Uptake Transporters in Mouse Livers

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Perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) have been detected globally in wildlife and humans. Data from a gene array indicate that PFOA decreases organic anion transporting polypeptides (Oatps) in liver. Na⁺-taurocholate cotransporting polypeptide (Ntcp) and Oatp1a1, 1a4, and 1b2 are major transporters responsible for uptake of bile acids (BAs) and other organic compounds into liver. The purpose of the present study was to determine the effects of two perfluorinated fatty acids, PFOA and PFDA, on mRNA and protein expression of hepatic uptake transporters Oatps and Ntcp, and to determine the underlying regulatory mechanisms by using peroxisome proliferator-activated receptor alpha (PPAR- α), constitutive androstane receptor, pregnane-X receptor, NF-E2-related factor 2, and farnesoid X receptor-null mouse models. After 2 days following a single i.p. administration, PFOA did not alter serum BA concentrations, but PFDA increased serum BA concentrations 300%. Furthermore, PFOA decreased mRNA and protein expression of Oatp1a1, 1a4, and 1b2, but not Ntcp in mouse liver. In contrast, PFDA decreased mRNA and protein expression of all four transporters, and decreased the mRNA expression in a dose-dependent manner, with the decrease of Oatp1a4 occurring at lower doses than the other three transporters. Multiple mechanisms are likely involved in the down-regulation of mouse Oatps and Ntcp by PFDA. By using the various transcription factor-null mice, PPAR- α was shown to play a central role in the down-regulation of Oatp1a1, 1a4, 1b2, and Ntcp by PFDA. The current studies provide important insight into understanding the mechanisms by which PFDA regulate the expression of hepatic uptake transporters. In conclusion, PFOA and PFDA decrease mouse liver uptake transporters primarily via activation of PPAR- α .

Key Words: Na⁺-taurocholate cotransporting polypeptide (Ntcp); Organic anion transporting polypeptide (Oatp); perfluorodecanoic acid (PFDA); perfluorooctanoic acid (PFOA); peroxisome proliferator-activated receptor alpha (PPAR- α).

Certain perfluorocarboxylic acids (PFCAs or perfluorinated fatty acids), such as perfluorooctanoic acid (PFOA), perfluorononanoic acid, and perfluorodecanoic acid (PFDA) have been used for decades to make products that resist heat, oil, stains, grease, and water, such as in stain-resistant carpets and fabrics, in the manufacture of non-stick cookware, components of fire-fighting foam, and other industrial uses (the information available through Environmental Protection Agency web site: <http://www.epa.gov/oppt/pfoa/pubs/pfoainfo.htm>). These perfluorinated compounds have been detected globally in wildlife (Falandysz *et al.*, 2006; Gannon *et al.*, 2006; Houde *et al.*, 2006; Kim and Kannan, 2007; Sinclair *et al.*, 2006) and humans (Calafat *et al.*, 2007a, b; Fei *et al.*, 2007; Olsen *et al.*, 2004, 2007; Tao *et al.*, 2008). Perfluorooctanoic acid (PFOA) and certain perfluorochemicals have become a public health concern in recent years as studies have revealed their environmental persistence (De Silva and Mabury, 2004; Oono *et al.*, 2008; Skutlarek *et al.*, 2006; Trudel *et al.*, 2008), and as data have emerged about their toxicity in laboratory animals (George and Andersen, 1986; Harris *et al.*, 1989; Jensen and Leffers, 2008; Langley, 1990; Lau *et al.*, 2004; Son *et al.*, 2008; Starkov and Wallace, 2002; Van Rafelghem *et al.*, 1987; Wei *et al.*, 2008; Wolf *et al.*, 2008). Liver is thought to be a prime target organ of PFOA and related perfluorochemicals, due to efficient hepatic uptake and persistence in liver (George and Andersen, 1986; Van Rafelghem *et al.*, 1987; Vanden Heuvel *et al.*, 1991a, b). In livers of mice and rats, PFOA and/or PFDA caused hepatomegaly, peroxisome proliferation, and alteration in hepatic lipid content (Brewster and Birnbaum, 1989; Harris *et al.*, 1989; Kudo and Kawashima, 1997; Son *et al.*, 2008; Van Rafelghem *et al.*, 1987; Wolf *et al.*, 2008; Yamamoto and Kawashima, 1997). PFOA has been shown to promote hepatocarcinogenesis in rats, but PFDA has not (Abdellatif *et al.*, 1991; Borges *et al.*, 1993). Both PFOA and PFDA are peroxisome proliferator-activated receptor alpha (PPAR- α) activators and capable of triggering oxidative stress (Cai *et al.*, 1995; Langley, 1990; Takagi *et al.*, 1991; Vanden Heuvel *et al.*, 2006). In addition, PFDA, but not PFOA, increased tumor necrosis factor- α concentrations in rat sera (Adinezhadeh and Reo, 1998).

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The Na⁺-taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptides (Oatps) are major transporters responsible for uptake of bile acids (BAs) and other organic compounds into liver. Ntcp is a sodium-dependent hepatic BA uptake transporter (Kullak-Ublick *et al.*, 2000; Meier *et al.*, 1997; Meier and Stieger, 2002). Organic anion transporting polypeptides (rodents: Oatps; human: OATPs) represent a gene superfamily that mediates sodium-independent transport of various amphipathic organic compounds such as BAs, drugs (e.g., rifampicin, digoxin), toxins (e.g., microcystin), steroid conjugates, eicosanoids, and thyroid hormones into cells. In rats and mice, Oatp1a1, 1a4, and 1b2 are highly expressed in liver (Cheng *et al.*, 2005a; Hagenbuch and Meier, 2003; Li *et al.*, 2002; Ogura *et al.*, 2000; Ogura *et al.*, 2001). The effects of some xenobiotics on the regulation of Oatps have been reported previously (Cheng *et al.*, 2005b, 2006; Guo *et al.*, 2002a; Staudinger *et al.*, 2001a). For example, the PXR ligand pregnenolone-16 α -carbonitrile (PCN) markedly induces mouse and rat Oatp1a4 expression (Cheng and Klaassen, 2006; Cheng *et al.*, 2005b; Guo *et al.*, 2002b).

Drugs and other xenobiotics can alter target gene expression through activation of nuclear receptors and other transcription factors including aryl hydrocarbon receptor, constitutive androstane receptors (CAR), pregnane-X receptor (PXR), PPAR- α , and NF-E2-related factor (Nrf2). For example, PCN increases Oatp1a4 expression through activation of PXR in rats and mice (Guo *et al.*, 2002b; Staudinger *et al.*, 2001a), whereas oltipraz and butylated hydroxyanisole activate Nrf2, and induce Mrp2, 3, and 4 expression (Maher *et al.*, 2007).

In a recent gene array of rat liver, it was reported that PFOA down-regulates Oatp1a1, 1a4, and 1b2 mRNA expression (Guruge *et al.*, 2006). Therefore, the purpose of the present study was to determine in mice the effects of two perfluorinated fatty acids, namely PFOA and PFDA, on the gene expression of hepatic uptake transporters, and to determine the underlying regulatory mechanisms by using PPAR- α , CAR-, PXR-, Nrf2-, and FXR-null mouse models.

MATERIALS AND METHODS

Materials. Sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt, HEPES free acid, lithium lauryl sulfate, ethylenediaminetetraacetic acid, and d-(+)-glucose were purchased from Sigma-Aldrich (St Louis, MO). Micro-O-protect was purchased from Roche Diagnostics (Indianapolis, IN) for the bDNA assay. Formaldehyde, 4-morpholinepropane-sulfonic acid, sodium citrate, and NaHCO₃ were purchased from Fischer Chemicals (Fairlawn, NJ). Chloroform, agarose, and ethidium bromide were purchased from AMRESCO, Inc. (Solon, OH). PFOA (free acid form; 96% purity; M.W. 413 g/mol) and PFDA (free acid form; 98% purity; M.W. 513 g/mol) were both purchased from Sigma-Aldrich Co. (St Louis, MO). All other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich Co. Total BA (Colorimetric assay) kit was purchased from Bio-Quant, Inc. (San Diego, CA).

Rabbit anti-rat Ntcp antibody, which has cross-reactivity with mouse Ntcp (Aleksunes *et al.*, 2006; Wolters *et al.*, 2002), was generously provided by

Dr Bruno Stieger (Department of Medicine, University Hospital, Zurich, Switzerland). Polyclonal antibodies of mouse Oatp1a1, 1a4, and 1b2 were developed against mouse Oatp-specific peptide (Oatp1a1-specific peptide, NH₂-CRKFHFPGDIHSPDTE-COOH; Oatp1a4-specific peptide, NH₂-CFPGDIDSSDTP-COOH; Oatp1b2-specific peptide, NH₂-CNPEPVNNNGYSCVPSDEKNSETPL-COOH), and were raised in rabbits by Covance Research Products, Inc. (Berkeley, CA). β -Actin antibody (ab8227) was purchased from Abcam, Inc. (Cambridge, MA). Goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody was purchased from Sigma-Aldrich Co.

Animals and treatment. Eight-week-old adult male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed according to the American Animal Association Laboratory Animal Care (AAALAC) guidance. For the dose-response study of PFDA, adult C57BL/6 male mice ($n = 4$ per treatment) were administered a single i.p. dose of PFDA (0.5, 1.0, 10, 20, 40, or 80 mg/kg of body weight), whereas control mice were treated i.p. with the vehicle, propylene glycol:water (1:1, vol/vol). Two days later, livers were collected and snap-frozen in liquid nitrogen and stored at -80°C .

Breeding pairs of FXR-null mice (Sinal *et al.*, 2000) and PXR-null mice (Staudinger *et al.*, 2001b) in the C57BL/6 background were kindly provided by Dr Frank J. Gonzalez (National Institutes of Health/National Cancer Institute, Bethesda, MD). Breeding pairs of CAR-null mice in the C57BL/6 background were obtained from Dr Ivan Rusyn (University of North Carolina, Chapel Hill, NC), which were engineered by Tularik, Inc. (South San Francisco, CA) as described previously (Ueda *et al.*, 2002). Nrf2-null mice were kindly provided by Dr Jefferson Y. Chan at University of California, Irvine (Leung *et al.*, 2003), and bred to a second generation of C57BL/6 background in our facility. Each mouse genotype ($n = 5$) was administered a single i.p. dose of PFOA (40 mg/kg; or 90 $\mu\text{mol/kg}$) or PFDA (80 mg/kg; or 156 $\mu\text{mol/kg}$), or the vehicle propylene glycol:water (1:1, vol/vol). Two days later, livers were removed and immediately frozen in liquid nitrogen, and stored at -80°C .

The PPAR- α -null mice originally engineered by the laboratory of Dr Frank J. Gonzalez (Lee *et al.*, 1995), were back-crossed to a C57BL/6 background (Akiyama *et al.*, 2001), and bred and housed at an AAALAC-accredited facility at Pennsylvania State University (University Park, PA). The PPAR- α -null mice and their WT controls were treated in the laboratory of Dr. Jeffrey M. Peters (Pennsylvania State University); the dose of PFDA was decreased to 40 mg/kg (or 78 $\mu\text{mol/kg}$) due to increased lethality at 80 mg/kg (or 156 $\mu\text{mol/kg}$). Two days later, livers were collected, frozen, and shipped to the University of Kansas Medical Center (Kansas City, KS) for further studies.

Total BA quantification in mouse serum. Adult C57BL/6 male mice ($n = 5$ per treatment) were administered a single i.p. dose of PFOA (40 mg/kg; or 90 $\mu\text{mol/kg}$) or PFDA (80 mg/kg; or 156 $\mu\text{mol/kg}$), and control mice were injected i.p. with the vehicle, propylene glycol:water (1:1, vol/vol). Two days later, mice were euthanized with an overdose of pentobarbital. Blood was collected by cardiac puncture, allowed to coagulate, and centrifuged at $10,000 \times g$ for 15 min. The resulting supernatant (serum) was collected for analysis. Serum BA concentrations were quantified according to the manufacturer's protocol (Total BA [Colorimetric assay] kit, Bio-Quant, Inc.).

Total RNA isolation. Total RNA was isolated using RNA Bee reagent (Tel-Test, Inc., Friendswood, TX) per the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm.

Branched DNA signal amplification assay. The mRNA levels for mouse Oatps and Ntcp were quantified using the branched DNA (bDNA) assay (Quantigene bDNA signal amplification kit; Panomics, Inc., Fremont, CA). The strategy of multiple oligonucleotide probe set design and the probe set of mouse Ntcp and Oatps have been described previously (Cheng and Klaassen, 2006; Cheng *et al.*, 2007; Hartley and Klaassen, 2000). The luminescence for each gene is reported as relative light units (RLUs) per 10 μg of total RNA.

Membrane protein preparation. Crude plasma membrane samples were prepared from mouse livers as described previously (Johnson *et al.*, 2002). Briefly, 200–300 mg liver was minced in 10 ml of ice-cold homogenizing

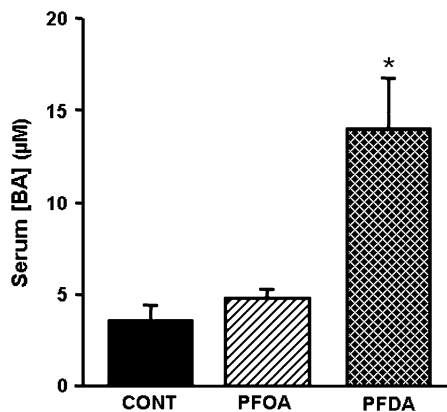


FIG. 1. Effects of PFOA and PFDA on total BA concentrations in mouse serum. BA concentration in serum from adult male C57BL/6 WT mice 2 days after a single i.p. administration of control (propylene glycol:water; vol/vol, 1:1), PFOA (40 mg/kg), or PFDA (80 mg/kg). The data are presented as mean \pm SEM ($n = 5$ mice). Asterisks (*) represent a statistical difference ($p < 0.05$) between control and treatment group.

buffer (0.25M sucrose, 10mM Tris-HCl at pH 7.5, containing 25 μ g/ml leupeptin, 50 μ g/ml aprotinin, 40 μ g/ml phenylmethanesulphonylfluoride, 0.5 μ g/ml pepstatin, and 50 μ g/ml antipain). The minced tissue was poured into a Dounce homogenizer (Kontes, Vineland, NJ) and homogenized on ice for 10 strokes. The homogenate was filtered through one layer of gauze sponges (Tyco Healthcare Group LP, Mansfield, MA), and then centrifuged at $100,000 \times g$ for 1 h at 4°C. The resulting pellet was mixed with resuspension buffer (0.25M sucrose, 10mM HEPES, and 40 μ g/ml phenylmethanesulphonylfluoride). Protein concentration of each sample was determined with a Bradford protein assay kit from Sigma.

Western blots. Membrane protein samples mixed with sample loading buffer (75 μ g protein/lane) were loaded after heating onto a 10% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins in the gel were electrotransferred to a nitrocellulose membrane for 4.5 h at 34 V at 4°C. Membranes were blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Blots were then incubated overnight with each polyclonal antibody of mouse Oatps and rat Ntcp at 4°C. β -Actin antibody was used as a loading control. After thorough washing (three 20-min washes with excess TBS-T), blots were incubated with goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:5000 dilution with 5% non-fat milk in TBS-T) for 1 h. Blots were washed again. Immunoreactive bands were detected with an enhanced chemical luminescence kit (Pierce Biotechnology Inc., Rockford, IL). Oatp1a1, 1a4, 1b2, and Ntcp protein bands were visualized by exposure to Fuji Medical X-Ray film. The protein band intensity on the films was quantified with Gel-Pro 3.1 image analysis software (MediaCybernetics, Silver Spring, MD).

Statistical analysis. Data are represented as mean \pm SEM. Data were analyzed by one-way ANOVA, followed by Duncan's *post hoc* test. Statistical significance was set at $p < 0.05$. When only the difference between control and PFDA treatment was determined, data were analyzed by student's *t*-test, and statistical significance was considered at $p < 0.05$.

RESULTS

Effect of PFOA and PFDA on Total BA Concentrations in Adult Male C57BL/6 WT Mouse Serum

As shown in Figure 1, 2 days after treatment, PFOA did not increase serum BA concentrations. In contrast, PFDA increased serum BA concentrations 300%.

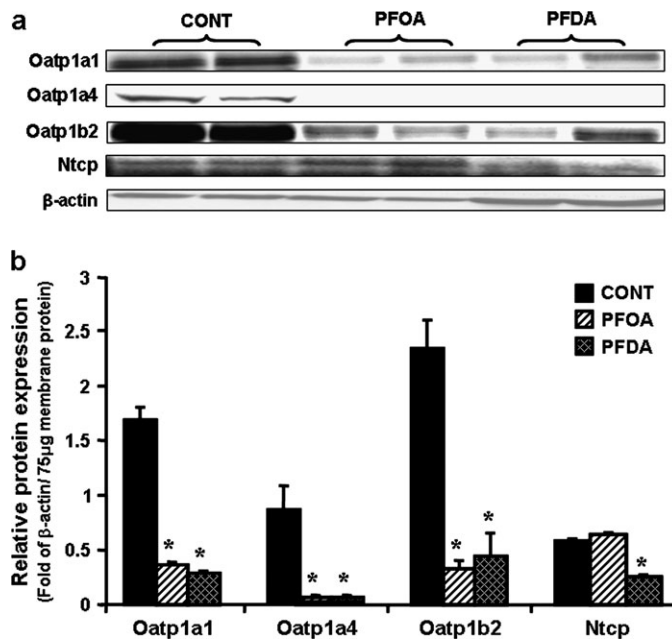


FIG. 2. Effects of PFOA and PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp protein expression in mouse livers. Adult male C57BL/6 WT mice ($n = 5$ per treatment) were given a single i.p. administration of control (propylene glycol:water; vol/vol, 1:1), PFOA (40 mg/kg), or PFDA (80 mg/kg). Two days after, mouse livers were collected. Total membrane protein was isolated from mouse livers. Two of five protein samples from each treatment were analyzed by Western blots. (a) Protein levels of Oatp1a1, 1a4, and 1b2, as well as Ntcp in mouse liver membranes were analyzed by Western blotting. (b) Protein levels of Oatp1a1, 1a4, and 1b2, as well as Ntcp in mouse liver membranes are expressed as ratio of each transporter to β -actin protein levels per 75 μ g total membrane protein. Data are presented as mean \pm SEM. Asterisks indicate statistically significant differences between control and PFOA or PFDA-treated male mice ($p < 0.05$).

Effects of PFOA and PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp Protein Expression in Adult Male C57BL/6 WT Mouse Liver

The effects of PFOA and PFDA on the four hepatic uptake transporters in mouse livers are shown in Figure 2. Two days after treatment, PFOA decreased protein levels of Oatp1a1, 1a4, and 1b2 about 79, 97, and 87%, respectively. However, PFOA did not alter Ntcp protein expression. Similar to PFOA, 2 days of PFDA treatment decreased Oatp1a1, 1a4, and 1b2 by 84, 99, and 82%, respectively. In contrast to PFOA, PFDA decreased Ntcp protein levels approximately 65%.

Effects of PFOA and PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA Expression in Adult Male C57BL/6 WT Mouse Liver

To determine whether the decrease in mouse Oatp and Ntcp protein expression by PFOA and PFDA corresponds to a decrease in mRNA, mRNA levels of each transporter were quantified. As depicted in Figure 3, similar to the alterations at the protein level, PFOA and PFDA markedly decreased mRNA

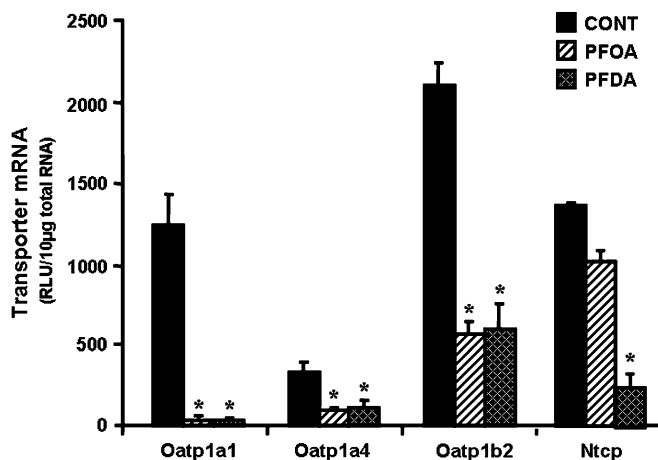


FIG. 3. Effects of PFOA and PFDA on mRNA expression of Oatp1a1, 1a4, and 1b2, as well as Ntcp in mouse livers. Adult male C57BL/6 WT mice ($n = 5$ per treatment) were given a single i.p. administration of control (propylene glycol:water; vol/vol, 1:1), PFOA (40 mg/kg), or PFDA (80 mg/kg). Two days later, mouse livers were collected. Total RNA from mouse livers ($n = 5$ per treatment) was analyzed by the bDNA assay for the mRNA expression of each transporter. The data are reported as RLUs per 10 μ g of total RNA, and are presented as mean \pm SEM. Asterisks indicate statistically significant differences between control and PFOA or PFDA-treated male mice ($p < 0.05$).

levels of Oatp1a1, 1a4, and 1b2. PFOA tended to decrease mRNA expression of Ntcp (25%), but this was not statistically significant, whereas PFDA decreased Ntcp mRNA levels 82%. Therefore, the downregulation of Oatp protein expression by both PFOA and PFDA, and Ntcp by PFDA correlates with the decrease in the mRNA.

Due to the apparent similarity in regulation of mouse Oatps by PFOA and PFDA and the fact that Ntcp was only altered by PFDA, further mechanistic studies focused mainly on PFDA.

Effects of Various Doses of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA Expression in Adult Male C57BL/6 WT Mouse Liver

As shown in Figure 4, the lowest doses of PFDA used that decreased Oatp1a1 (75%), 1a4 (50%), 1b2 (45%), and Ntcp (43%) mRNA expression are 40, 0.5, 40, and 10 mg/kg, respectively. When doses increased, the mRNA expression of each transporter decreased further. With 80 mg/kg of PFDA, the mRNA of Oatp1a1, 1a4, 1b2, and Ntcp decreased approximately 94, 97, 75, and 75%, respectively.

Effects of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA Expression in WT and PPAR- α -Null Mice

PFDA is a well-known peroxisome proliferator and a potent PPAR- α agonist (Cai *et al.*, 1995; Cimini *et al.*, 2000; Johnson and Klaassen, 2002). Therefore, the wild-type and PPAR- α -null mice were first used to determine whether the PFDA-induced down-regulation of Oatp1a1, 1a4, 1b2, and Ntcp is via PPAR- α . As shown in Figure 5, disruption of PPAR- α

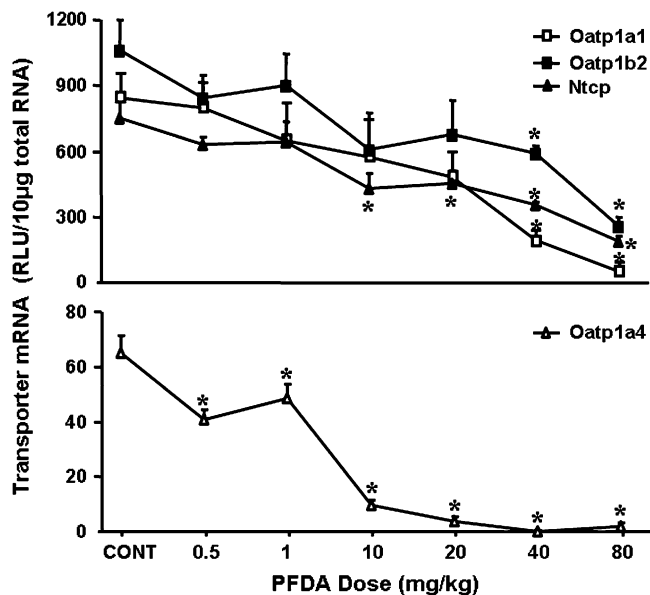


FIG. 4. Effects of various doses of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in mouse livers. Adult male C57BL/6 WT mice were administered PFDA at a single i.p. dose (0.5, 1.0, 10, 20, 40, or 80 mg/kg of body weight), whereas control mice were treated i.p. with the vehicle, propylene glycol:water (1:1, vol/vol). After 2 days, livers were collected. Total RNA samples from treated male mouse livers ($n = 4$) were analyzed by the bDNA assay. The data are reported as RLUs per 10 μ g of total RNA, and are presented as mean \pm SEM. Asterisks indicate statistically significant differences between control and PFDA-treated male mice ($p < 0.05$).

increased the constitutive expression of Oatp1a1 mRNA 65%. PFDA decreased Oatp1a1 mRNA 76% in wild-type mice, and 56% in PPAR- α -null mice. Constitutive mRNA levels of Oatp1a4 increased 170% in PPAR- α -null mice. PFDA decreased mRNA levels of Oatp1a4 in wild-type mice 75%, but not in PPAR- α -null mice, suggesting a PPAR- α -dependent mechanism. Removal of functional PPAR- α protein, as illustrated in the PPAR- α -null mice, increased constitutive mRNA levels of Oatp1b2 (40%). PFDA decreased Oatp1b2 mRNA expression 73% in wild-type mice and only 27% in PPAR- α -null mice, which is not statistically significant, indicating downregulation of Oatp1b2 mRNA expression by PFDA is PPAR- α dependent. Disruption of PPAR- α increased constitutive Ntcp mRNA 54%. PFDA decreased Ntcp mRNA 54% in wild-type mice, but not in PPAR- α -null mice, indicating that downregulation of Ntcp by PFDA is also PPAR- α dependent.

Effects of PFDA on Oatp1a1, 1a4, 1b2, and Ntcp mRNA Expression in WT and CAR-Null Mice

PFOA treatment markedly increased the mRNA expression of Cyp2B15, which is a CAR responsive gene in rats (Guruge *et al.*, 2006; Slatter *et al.*, 2006). Therefore, the wild-type and CAR-null mice were used to determine whether the PFDA-induced down-regulation of Oatp1a1, 1a4, 1b2, and Ntcp is via

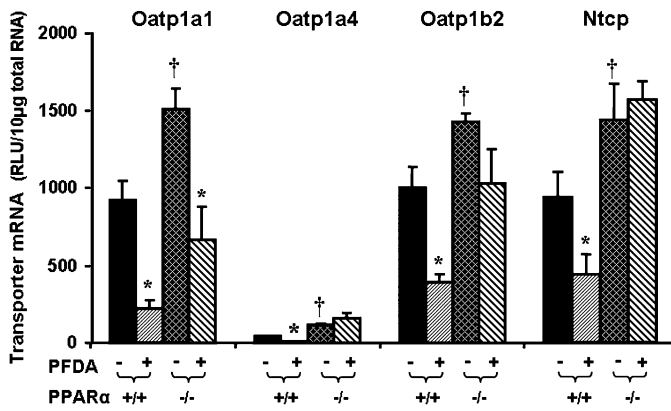


FIG. 5. Effects of PFDA on mouse Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in wild-type and PPAR- α -null mice. The mouse treatments are separated into 4 groups: PPAR $^{+/+}$ + CONT, wild-type mice treated with the vehicle, propylene glycerol:water (1:1, vol/vol); PPAR $^{+/+}$ + PFDA, wild-type mice treated with PFDA at dose 40 mg/kg of body weight; PPAR $^{-/-}$ + CONT, PPAR- α -null mice treated with the vehicle; PPAR $^{-/-}$ + PFDA, PPAR- α -null mice treated with PFDA at dose 40 mg/kg of body weight. The mice were given a single i.p. administration. After 2 days, livers were removed. Total RNA from treated mouse livers was analyzed by the bDNA assay. All data are expressed as mean \pm SEM of four to five mice for each treatment. Asterisk indicates statistically significant difference between treated and control mice ($p < 0.05$). Single dagger (†) represents statistically significant differences ($p < 0.05$) between the vehicle-treated wild-type male mice and the vehicle-treated PPAR- α -null male mice.

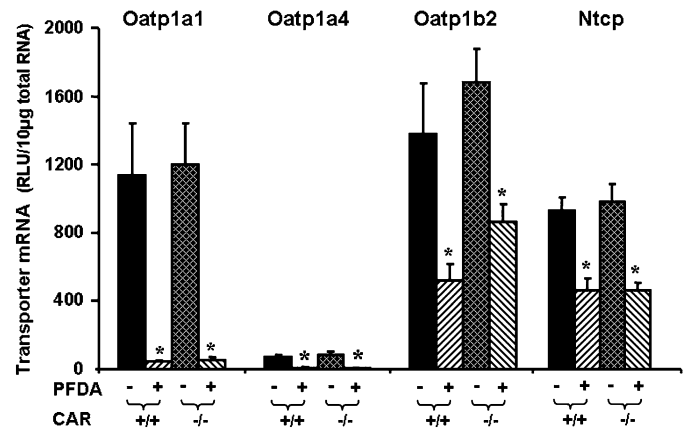


FIG. 6. Effects of PFDA on mouse Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in wild-type and CAR-null mice. The mouse treatments are separated into four groups: CAR $^{+/+}$ + CONT, wild-type mice treated with the vehicle, propylene glycerol:water (1:1, vol/vol); CAR $^{+/+}$ + PFDA, wild-type mice treated with PFDA at dose 80 mg/kg of body weight; CAR $^{-/-}$ + CONT, CAR-null mice treated with the vehicle; CAR $^{-/-}$ + PFDA, CAR-null mice treated with PFDA at dose 80 mg/kg. The mice were given a single i.p. administration. After 2 days, livers were removed. Total RNA from treated mouse livers was analyzed by the bDNA assay. All data are expressed as mean \pm SEM of five mice for each treatment. Asterisk indicates statistically significant difference between treated and control mice ($p < 0.05$).

CAR. As depicted in Figure 6, PFDA decreased Oatp1a1, 1a4, 1b2, and Ntcp mRNA levels in the livers of wild-type mice. PFDA produced similar down-regulation of each transporter in the CAR-null mice, indicating CAR-independent mechanisms.

Effects of PFDA on Oatp1a1, 1a4, 1b2, and Ntcp mRNA Expression in WT and PXR-Null Mice

Oatp1a4 is a well-known PXR target gene in mice and rats (Cheng and Klaassen, 2006; Guo *et al.*, 2002b). Therefore, the wild-type and PXR-null mice were used to determine whether the PFDA-induced down-regulation of Oatp1a4 and other Oatps (Oatp1a1 and 1b2), as well as Ntcp is via PXR. As shown in Figure 7, disruption of PXR, as demonstrated in the PXR-null mice, decreased the constitutive expression of Oatp1a4 mRNA, but did not alter the constitutive expression of Oatp1a1, 1b2, or Ntcp mRNA. PFDA treatment decreased mRNA expression of Oatp1a1, 1b2, and Ntcp in both wild-type and PXR-null mice. PFDA decreased Oatp1a4 mRNA expression in the wild-type mice, and tended to decrease Oatp1a4 mRNA in the PXR-null mice, but this was not statistically significant.

Effects of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA Expression in WT and Nrf2-Null Mice

PFCAs cause oxidative stress, which can be sensed by the transcription factor Nrf2 (Cai *et al.*, 1995; Itoh *et al.*, 1997; Maher *et al.*, 2007). Therefore, the wild-type and Nrf2-null

mice were used to determine whether the PFDA-induced down-regulation of Oatp1a1, 1a4, 1b2, and Ntcp is via Nrf2. As shown in Figure 8, PFDA decreased Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA levels in wild-type mouse livers.

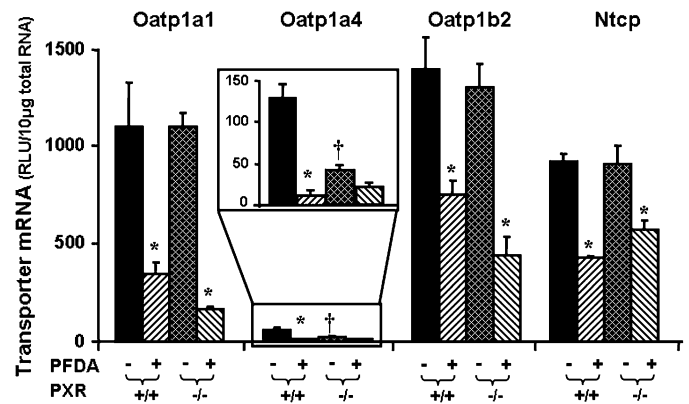


FIG. 7. Effects of PFDA on mouse Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in wild-type and PXR-null mice. The mouse treatments are separated into four groups: PXR $^{+/+}$ + CONT, wild-type mice treated with the vehicle, propylene glycerol:water (1:1, vol/vol); PXR $^{+/+}$ + PFDA, wild-type mice treated with PFDA at dose 80 mg/kg of body weight; PXR $^{-/-}$ + CONT, PXR-null mice treated with the vehicle; PXR $^{-/-}$ + PFDA, PXR-null mice treated with PFDA at dose 80 mg/kg. The mice were given a single i.p. administration. After 2 days, livers were removed. Total RNA from untreated or treated mouse livers was analyzed by the bDNA assay. All data are expressed as mean \pm SEM of five mice for each treatment. Asterisk indicates statistically significant difference between PFDA-treated and control mice ($p < 0.05$); single dagger (†) represents statistically significant differences ($p < 0.05$) between the vehicle-treated wild-type male mice and the vehicle-treated PXR-null male mice.

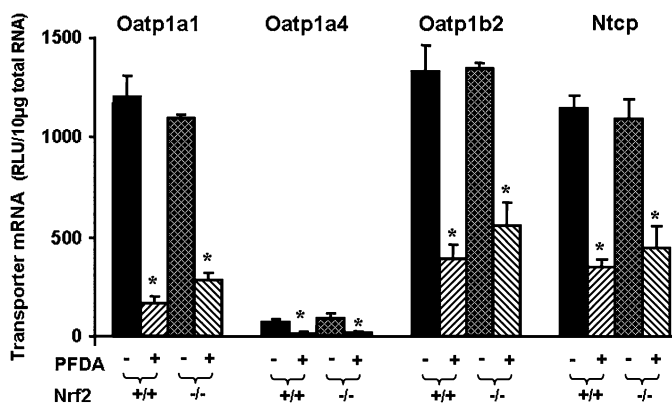


FIG. 8. Effects of PFDA on mouse Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in wild-type and Nrf2-null mice. The mouse treatments are separated into four groups: Nrf2^(+/+) + CONT, wild-type mice treated with the vehicle, propylene glycol:water (1:1, vol/vol); Nrf2^(+/+) + PFDA, wild-type mice treated with PFDA at dose 80 mg/kg of body weight; Nrf2^(-/-) + CONT, Nrf2-null mice treated with the vehicle; Nrf2^(-/-) + PFDA, Nrf2-null mice treated with PFDA at dose 80 mg/kg. The mice were given a single i.p. administration. After 2 days, livers were removed. Total RNA from treated mouse livers was analyzed by the bDNA assay. All data are expressed as mean \pm SEM of five mice for each treatment. Asterisk indicates statistically significant difference between treated and control mice ($p < 0.05$).

Similar down-regulation of Oatp1a1, 1a4, 1b2, and Ntcp was observed in the Nrf2-null mice.

Effects of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA Expression in WT and FXR-Null Mice

PFDA is a perfluorinated fatty acid. Fatty acids have been shown to be ligands of FXR (Zhao *et al.*, 2004). Therefore, it was determined whether down-regulation of hepatic Oatps and Ntcp by PFDA is via FXR activation. As shown in Figure 9, disruption of FXR, as demonstrated in the FXR-null mice, decreased the constitutive expression of Oatp1a4 (45%) and Ntcp (50%) mRNA, but did not alter the constitutive expression of Oatp1a1 and 1b2 mRNA. PFDA treatment decreased mRNA expression of Oatp1a1 (64%), 1a4 (88%), 1b2 (60%), and Ntcp (55%) in the wild-type mice. In comparison, PFDA decreased mRNA expression of Oatp1a1 (88%), 1a4 (70%), 1b2 (72%), and Ntcp (68%) in the FXR-null mice. Therefore, in FXR-null mice, PFDA decreased the mRNA expression of Oatps and Ntcp even more than that in wild-type mice.

DISCUSSION

The present study demonstrates that PFOA did not alter serum BA concentrations, but PFDA increased serum BA concentrations 300% 2 days after a single i.p. administration. Both PFOA and PFDA markedly decreased the expression of liver-predominant Oatp1a1, 1a4, and 1b2 at both protein and mRNA levels (Figs. 2 and 3). PFDA, but not PFOA decreased

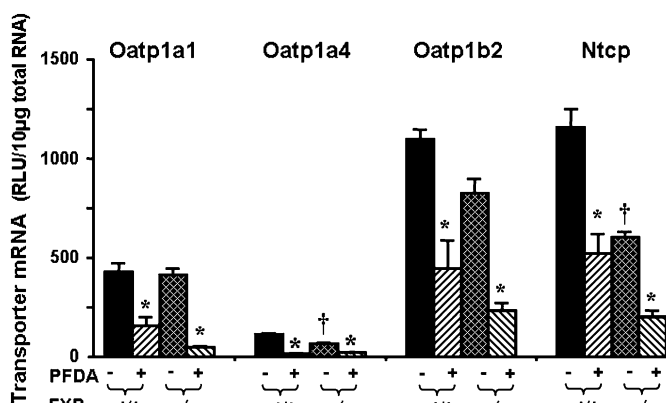


FIG. 9. Effects of PFDA on Oatp1a1, 1a4, and 1b2, as well as Ntcp mRNA expression in wild-type and FXR-null mice. The black and the dark-latticed bars represent regulation of each transporter by vehicle treatment; the striated bars depict regulation of each transporter by PFDA treatment. Adult C57BL/6 wild-type or FXR-null male mice were treated with the vehicle, propylene glycol:water (1:1, vol/vol) or PFDA at dose 80 mg/kg of body weight. The mice were treated for 2 days. Total RNA from untreated or treated mouse livers was analyzed by the bDNA assay. All data are expressed as mean \pm SEM of five mice for each treatment. Asterisk indicates statistically significant difference between PFDA-treated and control mice ($p < 0.05$). Single dagger (\dagger) represents statistically significant differences ($p < 0.05$) between the vehicle-treated wild-type male mice and the vehicle-treated FXR-null male mice.

Ntcp mRNA and protein expression in mouse liver. Furthermore, PFDA treatment caused a dose-dependent down-regulation of mRNA expression of mouse Oatp1a1, 1a4, 1b2, and Ntcp (Fig. 4), with a decrease of Oatp1a4 being observed at lower doses than the other three transporters.

The present data indicate that activation of the nuclear receptor PPAR- α is the main mechanism by which PFDA decreases the expression of all hepatic uptake transporters examined at the doses evaluated in this study, namely Oatp1a1, 1a4, 1b2, and Ntcp (Fig. 5). PPAR- α is a nuclear receptor that is critical in mediating both peroxisome proliferation and tumorigenesis in livers of mice exposed to PPAR- α agonists. PPARs, including PPAR- α , can influence gene expression indirectly, and usually negatively, through competition with other transcription factors (Semple *et al.*, 2006). PPAR- α can be activated by endogenous ligands, such as polyunsaturated fatty acids, and by synthetic agonists such as the fibrate drugs. PPAR- α is expressed mainly in liver, kidney, and skeletal muscle and is involved in fatty acid oxidation (Gouni-Berthold and Krone, 2005). PFCA structurally mimic fatty acids, thus they bind and subsequently activate PPAR- α (Vanden Heuvel *et al.*, 2006). The present data are consistent with previous reports that activation of PPAR- α decreases mouse Oatp1a1 mRNA and protein expression, and Ntcp protein levels (Cheng *et al.*, 2005b; Kok *et al.*, 2003).

The present study indicates that PPAR- α is not only involved in chemical regulation of mouse Oatps and Ntcp, but also the constitutive expression of Oatps and Ntcp. As

shown in Figure 5, elimination of PPAR- α increased the basal expression of *Oatp1a1*, *1a4*, *1b2*, and *Ntcp*. Previous studies showed a tendency for increase in *Oatp1a1* and *Ntcp* mRNA expression in PPAR- α -null mice, but the increases were not statistically significant (Kok *et al.*, 2003).

The current data suggest that PPAR- α activation plays an important role in PFDA-induced down-regulation of *Oatp* and *Ntcp* expression. However, the theory does not apply to the regulation of *Ntcp* expression by PFOA, another PPAR- α agonist (Takacs and Abbott, 2007). Unlike down-regulation of *Ntcp* by PFDA, PFOA did not alter *Ntcp* expression (Figs. 2 and 3). Moreover, previous reports showed that some other PPAR- α ligands (ciprofibrate, clofibrate, and diethylhexylphthalate) did not decrease *Oatps* and *Ntcp* mRNA expression as did PFDA (Cheng *et al.*, 2005b, 2007). Our recent unpublished data showed that (1) compared to ciprofibrate, clofibrate, and diethylhexylphthalate, PFDA is a much more effective PPAR- α activator; and (2) another potent PPAR- α activator, Wy-14643, decreased *Oatp1a1*, *1a4*, *1b2*, and *Ntcp* mRNA expression in mouse liver. Therefore, diverse PPAR- α ligands differently regulate the expression of *Oatps* and *Ntcp*, possibly due to variation of intensity of PPAR- α activation.

In addition to activation of PPAR- α , other mechanisms may contribute to regulation of individual hepatic uptake transporters by PFDA. For example, PFDA decreased *Oatp1a1* mRNA 76% in wild-type mice, and somewhat less (56%) in PPAR- α -null mice. In the promoter of mouse *Oatp1a1*, there is a DR-1 DNA fragment "TGACCTaTGATCT," which is a putative PPAR- α response element. Therefore, down-regulation of *Oatp1a1* by PFDA is only partially PPAR- α dependent (Fig. 5). It has been shown that PFDA causes testicular atrophy in rats (Olson and Andersen, 1983), and interferes with testosterone biotransformation and secretion, leading to lower levels of serum androgens in rats (Bookstaff *et al.*, 1990; Boujrad *et al.*, 2000). *Oatp1a1* expression is androgen-dependent in mouse liver and kidney (Cheng *et al.*, 2006; Isern *et al.*, 2001). Thus, in addition to PPAR- α , PFDA-induced decrease in serum androgen concentrations might also contribute to decreased *Oatp1a1* expression.

In addition to PPAR- α -null mice, numerous transcription factor-null mouse models including CAR-, PXR-, Nrf2-, and FXR-null mice were used to characterize the underlying regulatory mechanisms for the downregulation of *Ntcp* and *Oatps* by PFDA. However, except for PPAR- α , downregulation of *Ntcp* and *Oatps* by PFDA are not dependent on CAR, PXR, Nrf2, and FXR activity (Figs. 6–9). PFOA has been recently suggested to activate CAR (Rosen *et al.*, 2008). However, CAR activation is not necessary for regulation of *Ntcp* and *Oatps* by PFDA (Fig. 6).

Alteration in the regulation of hepatic uptake transporters can have physiological effects. For example, in the present study, PFDA increased BA concentrations in mouse serum about 300% 2 days after a single i.p. administration, but PFOA did not alter BA concentrations in mouse serum. Recent

unpublished data from this laboratory also showed that after 2 weeks following a single i.p. administration, 100 $\mu\text{mol/kg}$ of PFDA increased serum BA concentrations more than 20-fold in mice, but 100 $\mu\text{mol/kg}$ of PFOA did not. BAs can be taken up into liver by *Ntcp* and *Oatps*, but mainly by *Ntcp*. The decrease in *Ntcp* by PFDA most likely is responsible for the increased BA concentrations in mouse serum by PFDA.

Taken together, PFDA clearly decreases expression of *Ntcp* and three liver-predominant *Oatps*, namely *Oatp1a1*, *1a4*, and *1b2* in mouse liver, but PFOA only decreases *Oatps* in mouse liver. PPAR- α plays a central role in regulating these uptake transporters by PFDA. The current studies provide important insight into the underlying regulatory mechanisms of drug transporters by these PFCAs.

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