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A Species Difference in the Peroxisome Proliferator-Activated Receptor α-Dependent Response to the Developmental Effects of Perfluorooctanoic Acid

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This study examined the effect of prenatal perfluorooctanoic acid (PFOA) administration on pre- and postnatal development using peroxisome proliferator-activated receptor α (*PPARα*)-humanized mice to determine if species differences in receptor activity might influence the developmental effects induced by PFOA. Pregnant mice were treated daily with water or PFOA (3 mg/kg) by po gavage from gestation day 1 (GD1) until GD17 and then either euthanized on GD18 or allowed to give birth and then euthanized on postnatal day 20 (PND20). No changes in average fetal weight, crown-to-rump length, or placental weight were observed on GD18. Expression of mRNA encoding the PPARα target genes acyl CoA oxidase (*Acox1*) and cytochrome P450 4a10 (*Cyp4a10*) in maternal and fetal liver was increased on GD18 in wild-type and *PPARα*-humanized mice but not in *Pparα*-null mice. On PND20, relative liver weight was higher in wild-type mice but not in *Pparα*-null mice or *PPARα*-humanized mice. Hepatic expression of *Acox1* and *Cyp4a10* mRNA was higher in wild-type mice but not in *Pparα*-null mice or *PPARα*-humanized mice on PND20. The percentage of mice surviving postnatally was lower in wild-type litters but not in litters from *Pparα*-null mice or *PPARα*-humanized mice. No changes in pup weight gain, onset of eye opening, or mammary gland development were found in any genotype. Results from these studies demonstrate that the developmental/postnatal effects resulting from prenatal PFOA exposure in mice are differentially mediated by mouse and human PPARα.

Key Words: **development; perfluorooctanoic acid; peroxisome proliferator-activated receptor.**

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated, soluble nuclear receptors. Three PPARs have been identified to date: PPARα, PPARβ/δ (also referred to as PPARβ or PPARδ), and PPARγ. PPARα is expressed at high levels in liver, heart, and muscle and is known to regulate fatty acid transport, catabolism, and energy homeostasis (Peters *et al*. 2005). Hypolipidemic drugs (e.g., fibrates), phthalate monoesters, and some perfluoroalkyl chemicals are known to activate PPARα (Bility *et al*., 2004; Maloney and Waxman, 1999; Vanden Heuvel *et al*., 2006; Wolf *et al*., 2012). Prolonged activation of PPARα can increase hepatocyte proliferation leading to hepatocarcinogenesis in rodents, and these effects require PPARα as they are not found in mice lacking expression of PPARα (Hays *et al*., 2005; Lee *et al*., 1995; Peters *et al*., 1997, 1998). The prevailing evidence indicates that humans are not sensitive to the hepatocarcinogenic effects of PPARα agonists (reviewed in Klaunig *et al*., 2003; Peters *et al*., 2005, 2012). The mechanism underlying this species difference in sensitivity to the effects of PPARα agonists was recently elucidated by studies using *PPARα*-humanized mice. Whereas the mouse PPARα represses oncogenic let-7C miRNA that leads to an increase in c-MYC expression and signaling, the human PPARα does not modulate this signal transduction pathway (Shah *et al*., 2007; Yang *et al*., 2008), thus explaining why *PPARα*-humanized mice are resistant to liver cancer after chronic ligand activation of PPARα (Cheung *et al*., 2004; Morimura *et al*., 2006). This also provides a mechanistic explanation why humans are not sensitive to the hepatocarcinogenic effects of PPARα agonists.

Perfluoroalkyl chemicals have unique surfactant properties accounting for their use in many consumer applications including fire-fighting foam; additives in self-shine floor polishes, cement, lubricants, paint, gasoline, and paper; textile and leather treatments; waterproofing of clothing and carpets; and oil repellant in food packages (Prevedouros *et al*., 2006). Perfluorinated sulfonamide polymers, which are used for stain protection in carpets and textiles, and perfluorinated sulfonamide-based phosphate fluorosurfactants, which are used as leveling and wetting agents and to greaseproof paper food packaging, are two of the more common commercially used perfluoroalkyl chemicals (D'Eon and Mabury, 2011).

The toxicology of perfluorooctanoic acid (PFOA), a perfluoroalkyl chemical, has been studied extensively (Kennedy *et al*., 2004; Steenland *et al*., 2010). Recently, it was shown that PFOA treatment during pregnancy in mice resulted in dose-dependent full-litter resorptions as well as delayed development and postnatal lethality of the pups (Lau *et al*., 2006). The effect of prenatal PFOA exposure on general growth, development, and postnatal weight gain was shown to depend on PPARα expression (Abbott *et al*., 2007). In particular, PPARα was required for PFOA-induced postnatal lethality because this phenotype was not observed in *Pparα*-null mice (Abbott *et al*., 2007). Moreover, prenatal PFOA exposure has been reported to alter mammary gland development in dams and female offspring, effects that could involve PPARα (White *et al*., 2007).

Because there is evidence that the human PPARα can mediate different biological effects compared with the rodent PPARα, this study examined the effects of prenatal PFOA exposure in wild-type, *Pparα*-null, and *PPARα*-humanized mice on pre- and postnatal growth and development. In this study, dams were monitored for weight gain, changes in relative liver weight, and pregnancy outcome. Fetuses (on gestation day 18 [GD18]) and/or pups (on postnatal day 20 [PND20]) were monitored for mortality, growth, development, relative liver weight, and mammary gland development. The hepatic levels of PPARα target genes were determined in the dams, fetuses, and pups.

MATERIALS AND METHODS

Animals and treatments. Wild-type, *Pparα*-null (Akiyama *et al*., 2001; Lee *et al*., 1995), and *PPARα*-humanized (*Pparα*-null mice expressing a human PPARα) (Yang *et al*., 2008) mice on an Sv/129 genetic background were used for this study. Mice were housed in a temperature controlled environment (25°C) with a 12-h light/dark cycle. The protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee. The procedures involving mice were conducted in an International Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Virgin female mice of each genotype were mated overnight with male mice of the same genotype. The presence of a copulatory plug was indicative of successful mating and considered GD0. On GD1, pregnant female mice were treated with either PFOA (3 mg/kg body weight) or water (vehicle control) once per day via po gavage, up to and including GD17. The PFOA used for this study was provided by Dupont Haskell Laboratory for Health and Environmental Health. This concentration and timing of PFOA administration was used to compare with a previous study showing postnatal lethality by gestational PFOA exposure (Abbott *et al*., 2007). However, in contrast to this study (Abbott *et al*., 2007), preliminary analysis in this study indicated no differences in postnatal lethality following gestational PFOA exposure of 0.6 or 1.0 mg/kg in wild-type mice but a modest increase in postnatal lethality following gestational PFOA exposure of 3.0 mg/kg. This could be due to differences in pharmacokinetics of the wild-type mice because the mice used for this study were derived from the congenic Sv/129 line at the National Institutes of Health (Akiyama *et al*., 2001; Lee *et al*., 1995), whereas the wild-type mice used by Abbott and colleagues were obtained from Jackson Laboratories. This is also consistent with differences observed in maternal serum PFOA observed in mice treated with 3.0 mg PFOA/kg during gestation on PND20 in this study which ranged from 2066 to 6812 ng/ml compared with maternal serum PFOA observed in mice treated with 0.3 mg PFOA/kg during gestation on PND22 in the Abbott study which ranged from 2840 ± 387 ng/ml (Abbott *et al*., 2007). Adult female mice, fetuses, and pups were examined on two different time points: GD18 and PND20. For fetuses and litters from *PPARα*-humanized mice, the genotype of fetuses and pups was determined to confirm expression of the human *PPARα* gene as previously described (Yang *et al*., 2008). Data for fetuses and litters from *PPARα*-humanized mice are presented as the mean from entire litters (fetuses and litters including mice that expressed the human PAC *PPARα* gene and those that did not), in addition to the mean data from those fetuses and pups that were only *PPARα*-humanized mice, when available.

On GD18, pregnant female mice were euthanized and livers were carefully dissected. The livers were weighed, cut into sections, and fixed in 10% phosphate-buffered formalin (PBF) for histological analysis or snap frozen in liquid nitrogen for subsequent RNA isolation and analysis for PFOA concentration. Gravid uterine weights were recorded. For each litter, the number of live fetuses, dead fetuses, and resorption sites was counted. The sex of each fetus was determined, crown-to-rump length was measured, and fetal and fetal liver weights were recorded. Fetal livers were cut into sections, fixed in 10% PBF for histological analysis, or snap frozen for RNA analysis. Blood was collected from both the dams and the fetuses and used to isolate serum that was used for analysis of PFOA concentration as described below.

For PND20 analysis, pregnant mice were allowed to deliver their litters, and the day of parturition was recorded. The sex of each pup was recorded. Pups were weighed on the day of delivery and on PND7, PND14, and PND20. The pups were observed twice daily to determine postnatal lethality, and the percentage of litters achieving the developmental landmark of eye opening as a measure of postnatal development. Dams and pups were euthanized by overexposure to carbon dioxide on PND20 and livers were obtained by dissection and fixed in 10% PBF for histological analysis or snap frozen for RNA analysis or determining the tissue concentration of PFOA. Blood was collected from both the dams and the pups on PND20 and used to isolate serum that was used for analysis of PFOA concentration as described below.

Quantitative real-time PCR analysis. Total RNA was isolated from snap-frozen liver samples of at least five animals from each group using RiboZol following the manufacturer's recommended protocol (Amresco, Solon, OH). The mRNAs encoding the PPARα target genes, cytochrome P4504a10 (*Cyp4a10*) and acyl-CoA oxidase 1 (*Acox1*), the constitutive androstane receptor (CAR) target gene (*Cyp2b10*), and the pregnane X receptor (PXR) target gene (*Cyp3a11*) were measured using quantitative real-time PCR (qPCR) analysis as previously described (Palkar *et al*., 2010). Relative expression levels of mRNA were normalized to mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and analyzed for statistical significance using ANOVA and *post hoc* testing.

Tissue and serum analysis of PFOA concentration. Serum and liver samples from female mice (GD18 and PND20), GD18 fetuses, and PND20 pups were analyzed for PFOA concentration, using a previously described method (Butenhoff *et al*., 2012). For analysis of samples from dams on GD18 and PND20, five mice from each genotype were used for both liver and serum analysis. For analysis of PFOA concentration in fetuses and pups, representative samples from between 7 and 25 fetuses/pups were analyzed that were obtained from between three and seven litters on GD18 or PND20.

Histopathology. Liver sections were fixed in 10% PBF for 24 h and then transferred to containers with 70% ethanol. Fixed liver sections were embedded in paraffin and 3μM sections obtained. Representative sections from each liver were stained with hematoxylin and eosin and examined by a pathologist using light microscopy. For each genotype, slides were examined by the pathologist without knowledge of the treatment status.

Analysis of mammary glands. The left and right fourth and fifth abdominal mammary glands were carefully dissected from PND20 female pups and spread onto a glass slide, fixed in Carnoy's fixative and then placed in 70% ethanol. Fixed mammary glands were then stained with alum carmine stain. Whole mount preparations were examined using light microscopy. Ductal length and the number of terminal end buds were quantified and are presented as the litter mean ± SEM for each treatment group. Ductal length was measured as the distance from the beginning of the main duct to the end of the mammary

gland tree. Terminal end buds were identified and quantified based on their large bulbous profiles located at the termini of ducts and having a diameter of approximately twice that of their subtending stalk duct (Hovey *et al*., 2011).

Statistical analysis. Data were analyzed for statistical significance using ANOVA and the Bonferroni *post hoc* test (Prism 5.0, GraphPad Software, Inc., San Diego, CA). Significant differences were determined when $p \leq 0.05$. For maternal endpoints, the dam was the statistical unit used for comparisons. For fetal and pup endpoints, the litter was the statistical unit used for comparisons. For qPCR analysis of fetal and pup liver mRNA, at least one to two samples from at least five litters were used and the data were pooled for statistical analysis as described above. For qPCR analysis of maternal liver mRNA, at least five independent samples were used and the data were pooled for statistical analysis as described above.

RESULTS

GD18 Analysis

Pregnant mice were euthanized on GD18 to determine the effect of prenatal PFOA exposure on reproductive outcomes. PFOA administration from GD1 through GD17 had no effect on average maternal weight gain, average gravid uterus weight, the number of implantations per dam, or the number of resorptions per litter in all three genotypes (Table 1). The average number of fetuses per litter, average crown-to-rump length, average body weight, and the number of live or dead fetuses per litter were not influenced by prenatal PFOA exposure in either wild-type, *Pparα*-null, or *PPARα*-humanized mice (Tables 1 and 2). Average relative maternal liver weight was increased by PFOA administration in all three genotypes on GD18 (Table 1). Expression of the PPARα target gene *Acox1* was increased in GD18 maternal liver in response to PFOA administration in wild-type mice but not in *Pparα*-null or *PPARα*-humanized mice (Fig. 1A). Expression of the PPARα target gene *Cyp4a10* was increased in GD18 maternal liver in response to PFOA administration in wild-type and *PPARα*-humanized mice but not in *Pparα*-null mice (Fig. 1B). Expression of the CAR target gene *Cyp2b10* and the PXR target gene *Cyp3a11* was increased in GD18 maternal liver following PFOA administration in all three genotypes (Figs. 1C and 1D). These data are consistent with histopathological analysis. Minimal-to-mild centrilobular hepatocellular hypertrophy was observed in all PFOA-treated groups compared with their respective controls (Fig. 2), but the morphological features of this change differed somewhat across genotypes. In wild-type mice, hypertrophy was characterized primarily by centrilobular hepatocytes with increased amounts of densely eosinophilic and coarsely granular cytoplasm consistent with increased peroxisomes. In *Pparα*-null mice administered PFOA, hypertrophy was generally less prominent than seen in wild-type mice, and affected hepatocytes had pale eosinophilic, finely granular to amorphous cytoplasm. The morphological features of centrilobular hepatocytes in *PPARα*-humanized mice administered PFOA were intermediate between those observed in wild-type and *Pparα*-null mice. That is, in *PPARα*-humanized mice, both the intensity of eosinophilic staining and coarseness of cytoplasm granularity of hypertrophied centrilobular hepatocytes were greater than that observed in *Pparα*-null mice but less than that seen in wild-type mice. Hypertrophy was observed in all mice in the PFOA-treated

TABLE 1
Effect of Prenatal PFOA Exposure on Maternal Weight Gain and Reproductive Outcomes on GD18

Genotype	Wild-type		*Pparα*-null		*hPPARα* (all pups)		*hPPARα* (PAC+)[a]	
Treatment	Control	PFOA	Control	PFOA	Control	PFOA	Control	PFOA
Number of dams	6	5	6	5	8	5	8	5
Maternal weight (g) on GD18	31.2±3.6	33.0±1.6	31.2±3.6	34.9±3.1	29.8±2.4	28.0±1.6	—	—
Maternal weight gain (g) on GD18	9.6±3.1	9.9±1.7	9.2±2.7	12.6±3.2	8.8±1.4	8.0±2.4	—	—
Adjusted maternal weight (g) on GD18[b]	23.3±1.7	25.3±2.3	23.5±1.6	25.5±1.6	22.1±2.0	22.3±2.5	—	—
Maternal liver weight (g)	1.6±0.4	2.1±0.3*	1.4±0.2	2.1±0.1*	1.3±0.1	1.6±0.1*	—	—
Relative maternal liver weight (% body weight)	5.0±1.1	6.4±0.6*	4.4±0.4	6.0±0.4*	4.4±0.4	5.6±0.4*	—	—
Adjusted maternal liver weight[c]	6.6±1.5	8.4±0.5*	5.9±0.9	8.1±2.3*	6.0±0.4	7.1±1.2*	—	—
Gravid uterus weight (g) on GD18	7.9±2.6	7.7±2.0	7.7±2.5	8.6±2.3	7.7±1.2	5.8±2.5	ND	ND
Implants per dam	6.7±2.0	6.0±2.6	6.3±2.3	7.0±2.4	6.4±2.9	5.0±3.3	5.8±2.3	3.3±1.5
Number of live fetuses per litter	6.0±2.0	4.6±1.8	5.0±2.4	6.0±2.5	4.3±1.7	4.0±2.5	3.7±1.9	3.0±1.0
Number of dead fetuses per litter	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.6	0.4±0.7	0.0±0.0	0.0±0.0	0.0±0.0
Number of resorptions per litter	0.7±1.2	1.4±0.9	1.3±1.4	0.6±0.6	1.8±1.8	1.0±1.7	2.2±1.9	0.3±0.6
% Litter loss = [(*D* + *R*)/total × 100]	9.5±17.3	23.3±7.8	22.3±17.8	16.8±12.9	26.6±17.6	15.3±23.7	36.4±21.9	6.7±11.6

Note. Values represent the mean ± SEM. ND, not determined. Gravid uterine weight included uterus, placentas, amniotic sac and fluid, fetuses that were hemizygous for the *hPPARα* transgene, and fetuses that were negative for the *hPPARα* transgene, which precludes practical measurement of this endpoint.

[a] Data presented for fetuses that were hemizygous for the *hPPARα* transgene; fetuses that were negative for the *hPPARα* transgene are excluded.

[b] Maternal weight minus gravid uterine weight.

[c] Liver weight normalized to adjusted maternal liver weight.

* Significantly different than respective control, $p \leq 0.05$.

TABLE 2
Effect of Prenatal PFOA Exposure on Fetal Outcomes on GD18

Genotype	Treatment group	Number of fetuses per litter	Crown-to-rump length (mm)	Body weight (g)
Wild-type	Control	6.0 ± 2.0	20.2 ± 1.5	0.97 ± 0.08
	PFOA	4.6 ± 1.8	20.2 ± 2.3	1.12 ± 0.22
Pparα-null	Control	5.0 ± 2.4	21.2 ± 1.5	1.11 ± 0.08
	PFOA	6.0 ± 2.5	20.0 ± 1.0	1.09 ± 0.13
hPPARα (all pups)	Control	4.3 ± 1.7	19.4 ± 4.1	1.04 ± 0.12
	PFOA	4.0 ± 2.5	20.0 ± 2.3	1.14 ± 0.19
hPPARα (PAC+)[a]	Control	3.7 ± 1.9	18.6 ± 4.9	1.02 ± 0.14
	PFOA	3.0 ± 1.0	19.8 ± 2.7	1.03 ± 0.19

Note. Values represent the litter mean ± *SD*.
[a]Data presented for fetuses that were hemizygous for the *hPPARα* transgene; fetuses that were negative for the *hPPARα* transgene are excluded.

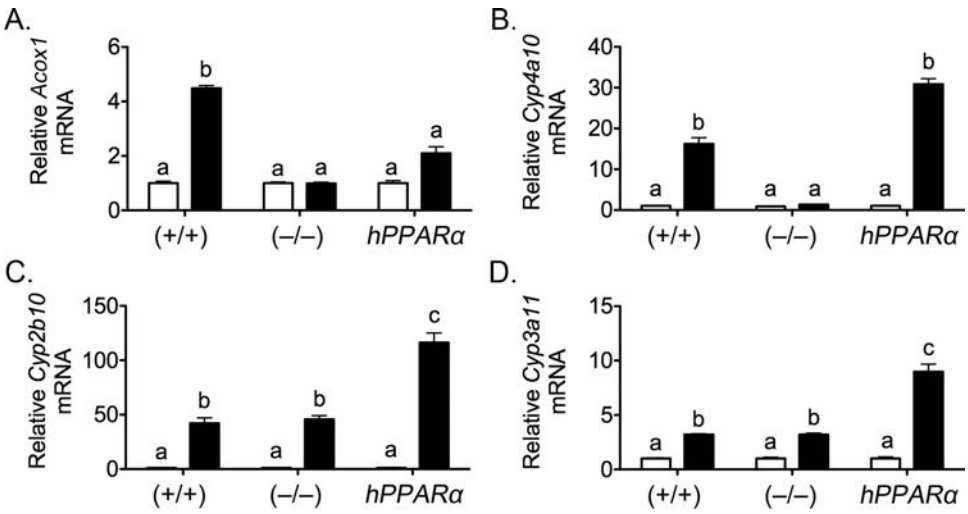

FIG. 1. Effect of prenatal PFOA exposure on hepatic expression of PPARα, CAR, and PXR target genes in maternal liver on GD18. Pregnant wild-type (+/+), *Pparα*-null (–/–) and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Maternal liver was isolated on GD18 for analysis. Relative expression of (A) *Acox1*, (B) *Cyp4a10*, (C) *Cyp2b10*, or (D) *Cyp3a11* mRNA was determined by qPCR. Values represent the mean ± SEM. Values with different letters are significantly different, $p \leq 0.05$.

groups and was graded as mild in wild-type mice, minimal in *Pparα*-null mice, and minimal or mild in *PPARα*-humanized mice. An additional finding in PFOA-treated *Pparα*-null and *PPARα*-humanized mice, but not in wild-type mice, was the presence of few clear, discrete vacuoles within the cytoplasm of centrilobular hepatocytes (Fig. 2).

Average relative fetal liver weight was also increased by PFOA treatment in wild-type and *PPARα*-humanized mouse fetuses, and not in fetuses from *Pparα*-null mice (Fig. 3A). Similarly, hepatic expression of the PPARα target genes *Acox1* and *Cyp4a11* was increased by PFOA administration in wild-type and *PPARα*-humanized mouse fetuses, but not in *Pparα*-null mouse fetuses (Figs. 3B and 3C). Hepatic expression of *Cyp2b10* mRNA in GD18 fetal liver was unchanged in all three genotypes (Fig. 3D). Whereas hepatic expression of *Cyp3a11* mRNA in liver was unchanged by PFOA administration in GD18 wild-type and *Pparα*-null fetuses, it was higher *PPARα*-humanized fetal liver (Fig. 3E). Histopathological analysis revealed evidence of peroxisome proliferation in the liver of 2/5 PFOA-treated GD18 wild-type mice which had diffuse hepatocellular hypertrophy characterized by hepatocytes with increased amounts of coarsely granular eosinophilic cytoplasm (Fig. 4) Although liver glycogen content was variable across control and PFOA-treated groups in all genotypes (based on hepatocellular cytoplasmic clearing on routine histology), liver glycogen content in wild-type mice administered PFOA was generally less compared with wild-type controls. No definitive microscopic changes were observed in the livers of *Pparα*-null or *PPARα*-humanized fetuses (Fig. 4).

PND20 Analysis

Separate cohorts of mice from all three genotype were allowed to deliver their offspring to determine the postnatal effects of prenatal PFOA exposure. The day of parturition was

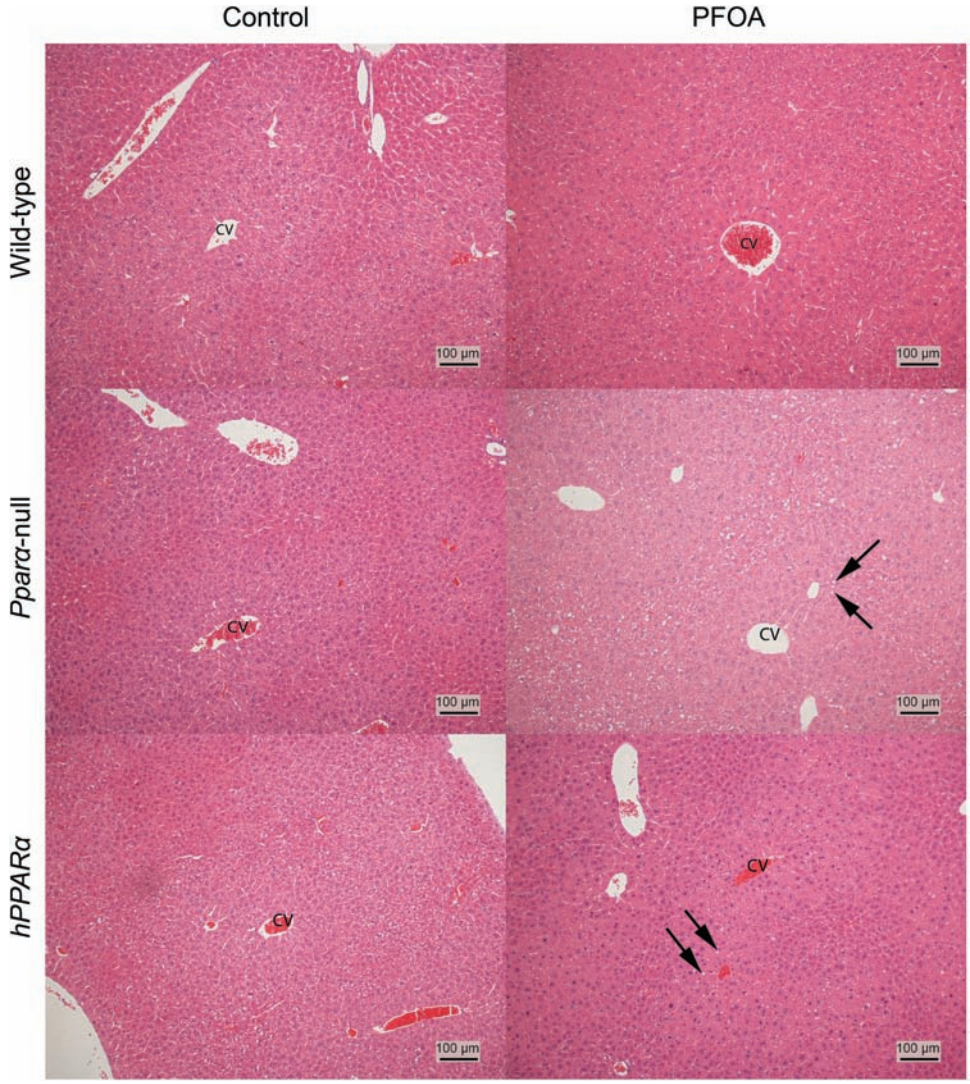

FIG. 2. Effect of prenatal PFOA exposure on maternal liver histopathology on GD18. Pregnant wild-type (+/+), *Pparα*-null (–/–) and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Maternal liver was isolated on GD18 for histopathological analysis. Normal centrilobular architecture seen in controls is accentuated due to hepatocellular hypertrophy in PFOA-treated mice. Hypertrophied centrilobular hepatocytes have coarsely granular, bright eosinophilic cytoplasm in PFOA-treated wild-type mice, but have pale staining, finely granular cytoplasm in PFOA-treated *Pparα*-null mice. The cytoplasmic staining and granularity of hypertrophied hepatocytes in PFOA-treated *PPARα*-humanized mice is intermediated between wild-type and *Pparα*-null mice. Vacuoles (arrows) are present in centrilobular hepatocytes of PFOA-treated *Pparα*-null and *PPARα*-humanized mice, but not in wild-type mice. CV = central vein; bar = 100 µm.

similar for all three genotypes and was not affected by PFOA administration except in *PPARα*-humanized mice where the average day of parturition was slightly later compared with the other treatment groups (Table 3). The average weight gain per dam from the day of parturition until PND20 was not different between any treatment group in all three genotypes (Fig. 5A). Average relative maternal liver weight on PND20 was higher in wild-type mice treated prenatally with PFOA compared with controls, and this effect was not observed in either *Pparα*-null or *PPARα*-humanized mice (Fig. 5B). Expression of the PPARα target gene *Acox1* was also higher in PND20 maternal liver from wild-type mice treated prenatally with PFOA compared with controls, and this effect was not observed in either *Pparα*-null or *PPARα*-humanized mice (Fig. 5C). Hepatic expression of *Cyp2b10* mRNA was unchanged on PND20 in maternal liver in all three genotypes (Fig. 5D). Expression of *Cyp3a11* mRNA was higher on PND20 in maternal liver in response to PFOA administration in all three genotypes (Fig. 5E). For all three genotypes, histopathological changes in the livers of PND20 dams were similar to those seen in the respective GD18 dams (Fig. 6) but were decreased in incidence, as well as in severity in wild-type and *PPARα*-humanized mice (all changes were graded as minimal). Incidences of hepatocellular hypertrophy were 3/5, 2/5, and 1/5, respectively, in the wild-type, *Pparα*-null,

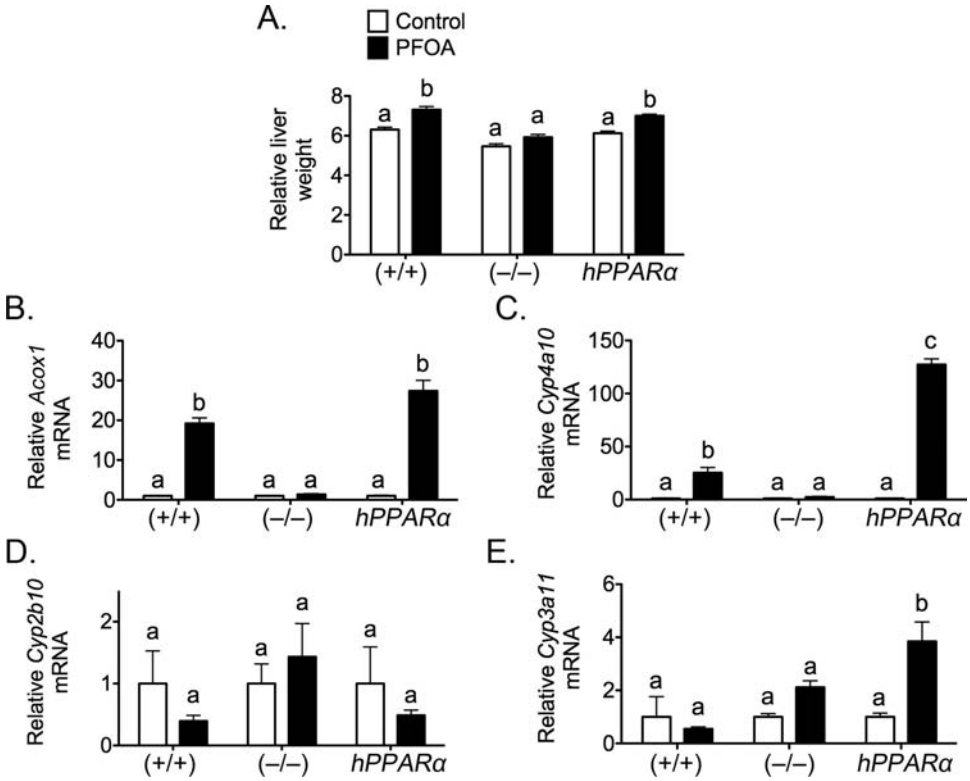

FIG. 3. Effect of prenatal PFOA exposure on fetal relative liver weight and expression of hepatic PPARα, CAR, and PXR target genes on GD18. Pregnant wild-type (+/+), *Pparα*-null (–/–) and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Fetal liver was obtained on GD18 for analysis. (A) Average relative fetal liver weight on GD18. Relative expression of (B) *Acox1*, (C) *Cyp4a10*, (D) *Cyp2b10*, or (E) *Cyp3a11* mRNA was determined by qPCR. Values represent the mean ± SEM. Values with different letters are significantly different, $p \leq 0.05$.

TABLE 3
Effect of Prenatal PFOA Exposure on Maternal and Pup Endpoints During Postnatal Development

				Average number of pups per litter		Sex ratio	
Genotype	Treatment group	Day of parturition	Number of litters	PND0	PND20	Female	Male
Wild-type	Control	19.4 ± 0.3	8	5.6 ± 0.6	5.4 ± 0.7	55.1 ± 11.4	44.9 ± 11.4
	PFOA	19.4 ± 0.2	9	5.3 ± 0.8	3.7 ± 0.7*	41.5 ± 8.0	58.5 ± 8.0
Pparα-null	Control	19.2 ± 0.2	9	6.2 ± 0.5	5.9 ± 0.5	52.5 ± 8.0	47.5 ± 8.0
	PFOA	19.6 ± 0.2	9	5.2 ± 0.8	5.2 ± 0.8	33.9 ± 11.1	66.1 ± 11.1
hPPARα (all pups)	Control	19.1 ± 0.1	14	4.6 ± 0.6	3.9 ± 0.5	66.5 ± 7.3	33.5 ± 7.3
	PFOA	19.7 ± 0.1*	8	5.1 ± 0.7	4.4 ± 0.5	43.5 ± 7.3*	56.5 ± 7.3*
hPPARα (PAC+)[a]	Control	19.1 ± 0.1	14	3.4 ± 0.5	2.6 ± 0.4	62.2 ± 12.0	37.8 ± 12.0
	PFOA	19.7 ± 0.1*	8	3.9 ± 0.7	3.3 ± 0.7	30.8 ± 14.2*	69.2 ± 14.2*

Note. Values represent the mean ± SEM.
[a]Data presented for fetuses that were hemizygous for the *hPPARα* transgene; fetuses that were negative for the *hPPARα* transgene are excluded.
*Significantly different than respective control, $p \leq 0.05$.

and *PPARα*-humanized mice. These histopathological changes were consistent with partial, but not complete reversibility of effects seen in dams dosed through GD18.

The average number of pups born per litter was not different between treatment groups of any genotype (Table 3). Interestingly, although the ratio of female to male pups in each litter was not different between treatment groups in wild-type or *Pparα*-null mice, there were on average more male pups than female pups in litters from PFOA-treated *PPARα*-humanized mice compared with controls (Table 3). The average weight per pup per litter and average weight gain from PND0 through PND20 were not different between treatment groups of any genotype (Fig. 7A, Table 4). Previous studies have shown that smaller litter size can cause greater postnatal weight gain

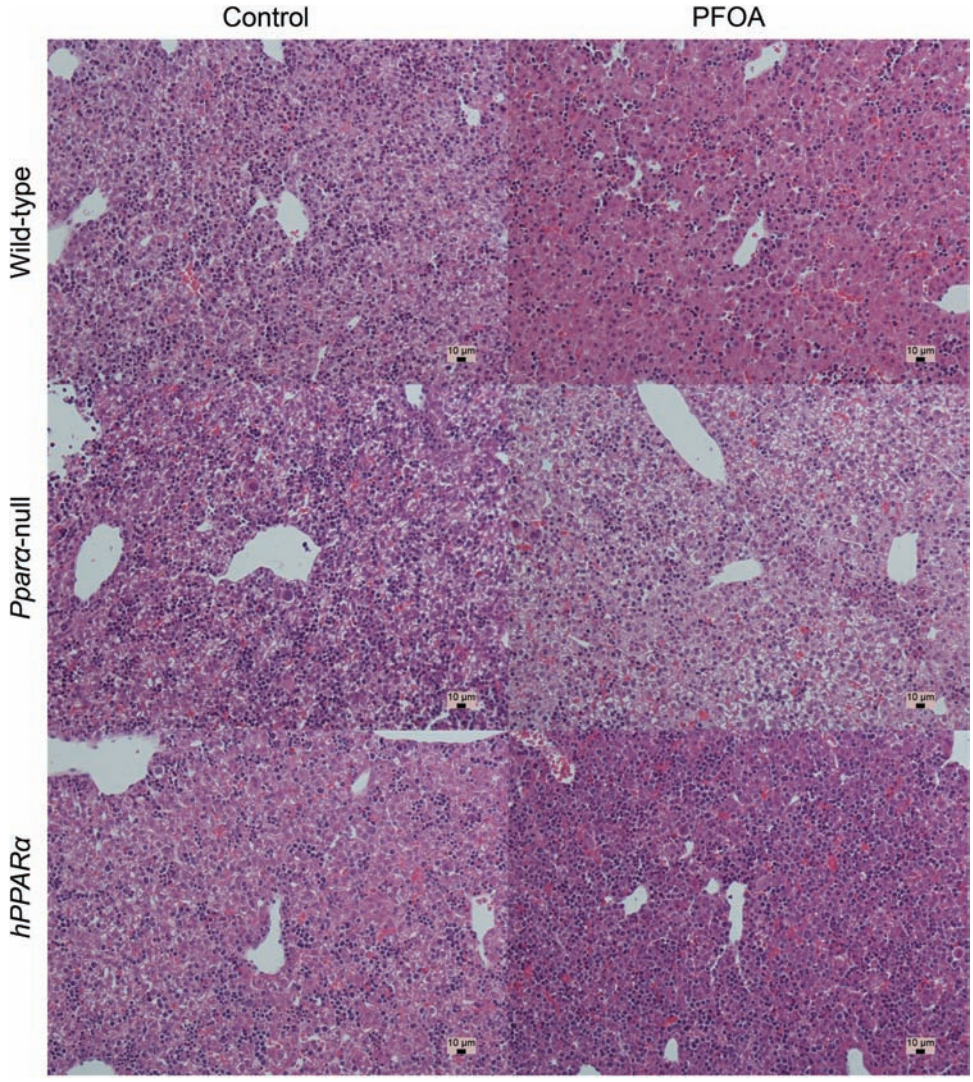

FIG. 4. Effect of prenatal PFOA exposure on fetal liver histopathology on GD18. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Fetal liver was isolated on GD18 for histopathological analysis. Control fetal livers have hepatocytes with eosinophilic- to clear-staining cytoplasm, and diffuse aggregates of hematopoietic cells. Fetal liver from a PFOA-treated wild-type mouse has diffuse hypertrophy of hepatocytes, which have coarsely granular, eosinophilic cytoplasm. In a PFOA-treated *PPARα*-humanized fetus, diffusely decreased cytoplasmic clearing, suggestive of decreased liver glycogen liver glycogen content, was present compared with control. Livers of PFOA-treated and control *Pparα*-null fetuses were similar microscopically. Bar = 10 µm.

compared with that from larger litters (Nelson and Robison, 1976) and thus the increased lethality observed in the PFOA-treated wild-type pups could potentially offset the lack of change in weight gain observed in wild-type mice. However, given the relative small difference in litter size (~5–6 vs. ~4–5), this does not seem likely to explain the lack of change in this endpoint. The onset of eye opening was not different between treatment groups of any genotype (Fig. 7B). A higher incidence of postnatal lethality was observed in wild-type litters from dams treated with PFOA than was found in control wild-type litters (Fig. 7C, Table 3). By contrast, postnatal lethality was not different between control and PFOA-treated litters from *Pparα*-null or *PPARα*-humanized mice (Fig. 7C, Table 3). Average liver weight on PND20 of wild-type pups from PFOA-treated dams was higher compared with controls, and this effect was not observed in *Pparα*-null or *PPARα*-humanized mice (Fig. 8A). Hepatic expression of *Acox1* and *Cyp4a10* mRNA was higher on PND20 in wild-type pups from dams administered PFOA prenatally, and this effect was either lacking or largely diminished in similarly treated *Pparα*-null or *PPARα*-humanized mice compared with controls (Figs. 8B and 8C). Hepatic expression of *Cyp2b10* mRNA in PND20 pup liver was increased by PFOA treatment in all three genotypes (Fig. 8D). Expression of *Cyp3a11* mRNA was increased by PFOA treatment in wild-type and *PPARα*-humanized pup liver but unchanged in *Pparα*-null pup liver on PND20. In wild-type

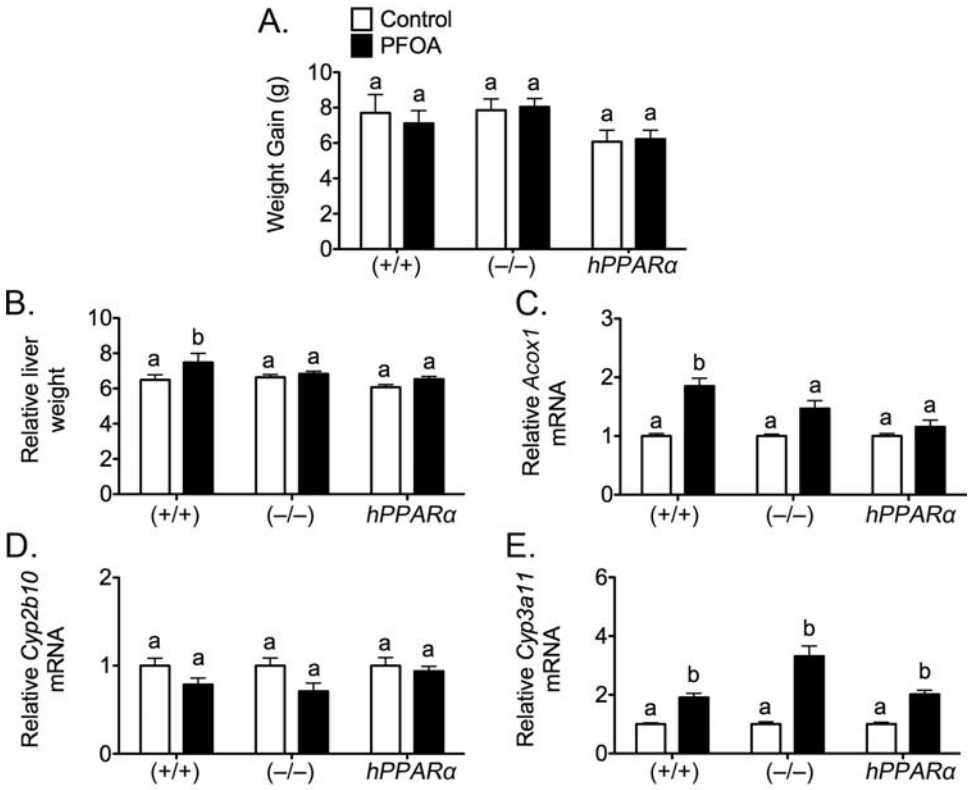

FIG. 5. Effect of prenatal PFOA exposure on maternal endpoints on PND20. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Maternal liver was isolated on PND20. (A) Average maternal weight gain from GD0 until the day of parturition. (B) Average relative maternal liver weight on PND20. Relative expression of (C) *Acox1*, (D) *Cyp2b10*, or (E) *Cyp3a11* mRNA was determined by qPCR. Values represent the mean ± SEM. Values with different letters are significantly different, $p \leq 0.05$.

TABLE 4
Effect of Prenatal PFOA Exposure on Average Pup Weights in Female and Male Offspring

Wild-type control				Wild-type PFOA			
Female		Male		Female		Male	
14 days	20 days	14 days	20 days	14 days	20 days	14 days	20 days
5.8 ± 0.7	6.7 ± 1.1	5.7 ± 1.0	6.9 ± 1.3	6.5 ± 0.7	7.5 ± 1.0	6.2 ± 0.9	7.5 ± 1.1
Pparα-null control				*Pparα*-null PFOA			
Female		Male		Female		Male	
14 days	20 days	14 days	20 days	14 days	20 days	14 days	20 days
6.1 ± 0.8	7.5 ± 0.9	5.9 ± 0.8	7.4 ± 0.9	6.0 ± 0.6	7.2 ± 0.8	6.0 ± 1.1	7.1 ± 1.3
hPPARα control				*hPPARα* PFOA			
Female		Male		Female		Male	
14 days	20 days	14 days	20 days	14 days	20 days	14 days	20 days
7.0 ± 0.8	8.3 ± 1.1	6.9 ± 0.7	8.3 ± 1.0	7.4 ± 0.6	8.3 ± 0.6	7.7 ± 0.9	8.8 ± 0.9

Note. Values represent the mean ± SEM.

PND20 pups, histopathological changes in the liver were morphologically similar to those described for GD18 wild-type dams and were consistent with peroxisome proliferation (Fig. 9). Equivocal evidence of centrilobular hypertrophy was present in 1/5 *Pparα*-null PND20 pups, and no definitive liver changes were observed in *PPARα*-humanized mice (Fig. 9).

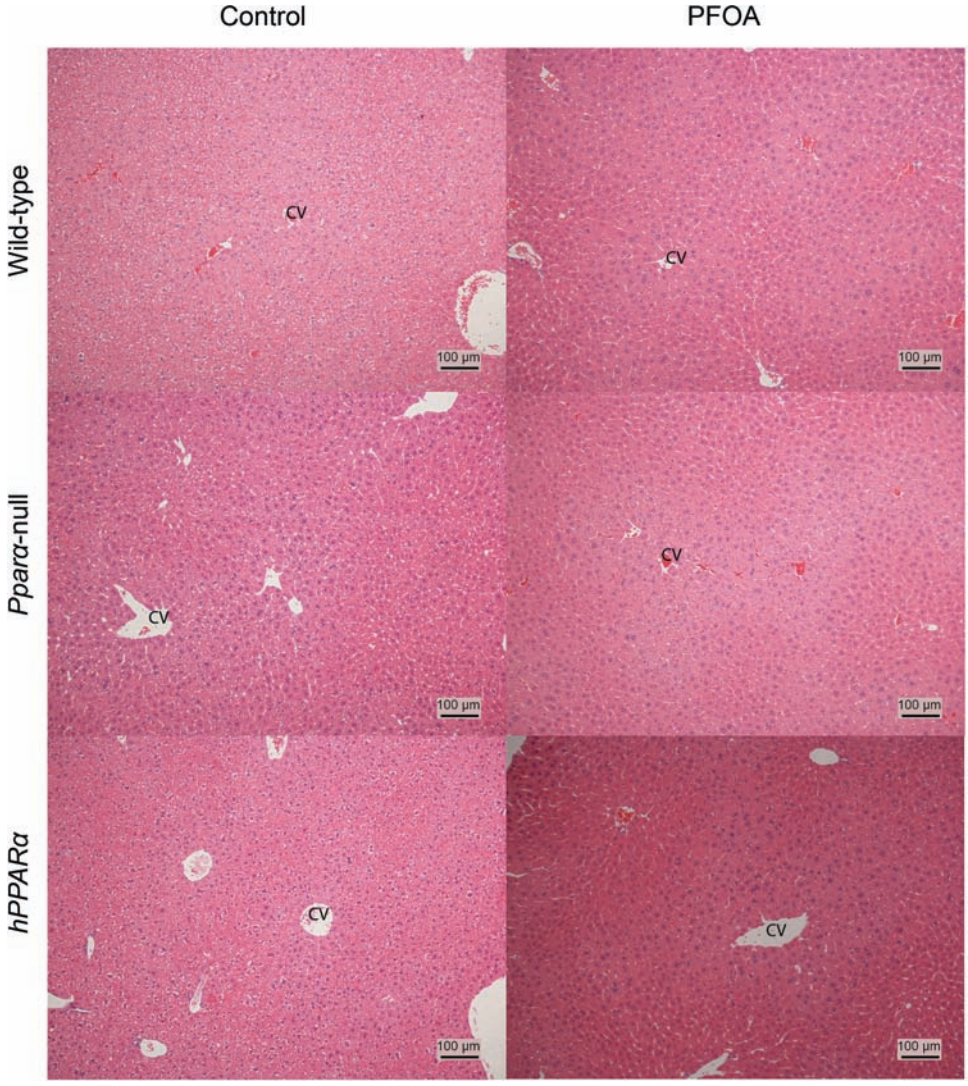

FIG. 6. Effect of prenatal PFOA exposure on maternal liver histopathology on PND20. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Maternal liver was isolated on PND20 for histopathological analysis. Microscopic changes were similar to those described for GD18 dams but were less severe and/or occurred in lower incidences. Bar = 100 µm.

The average length of mammary gland ducts and the average number of terminal end buds per mammary gland per litter were not different between treatment groups in a cohort of samples from all genotypes (Table 5).

Dosimetry of PFOA Exposure in Maternal, Fetal, and Pup Tissues

The concentration of PFOA was quantified in liver and serum from PFOA-treated groups of mice to determine relative exposure. The background concentration of PFOA in liver samples from dams, fetuses, and pups not treated with PFOA ranged between < 5 and 1060 ng/ml. The background concentration of PFOA in serum samples from dams, fetuses, and pups not treated with PFOA ranged between < 5 and 1370 ng/ml. The concentration of PFOA from the control samples typically was below the level of detection (< 5 ng/ml). The reason why a small cohort of samples exhibited PFOA at detectable levels in the control samples cannot be determined from these studies but could be due to contamination resulting from being housed in the same animal room. In samples obtained from mice treated with PFOA, the concentration of PFOA in maternal serum was somewhat lower in pregnant dams on GD18 compared with nonpregnant females (Fig. 10A). The concentration of PFOA in serum of PND20 dams was markedly lower compared with that found in GD18 pregnant dams (Fig. 10A). In samples obtained from mice treated with PFOA, the concentration of PFOA in maternal liver was similar in wild-type and *PPARα*-humanized pregnant dams on GD18 compared with nonpregnant females of all three genotypes (Fig. 10B). The concentration of PFOA in maternal liver in

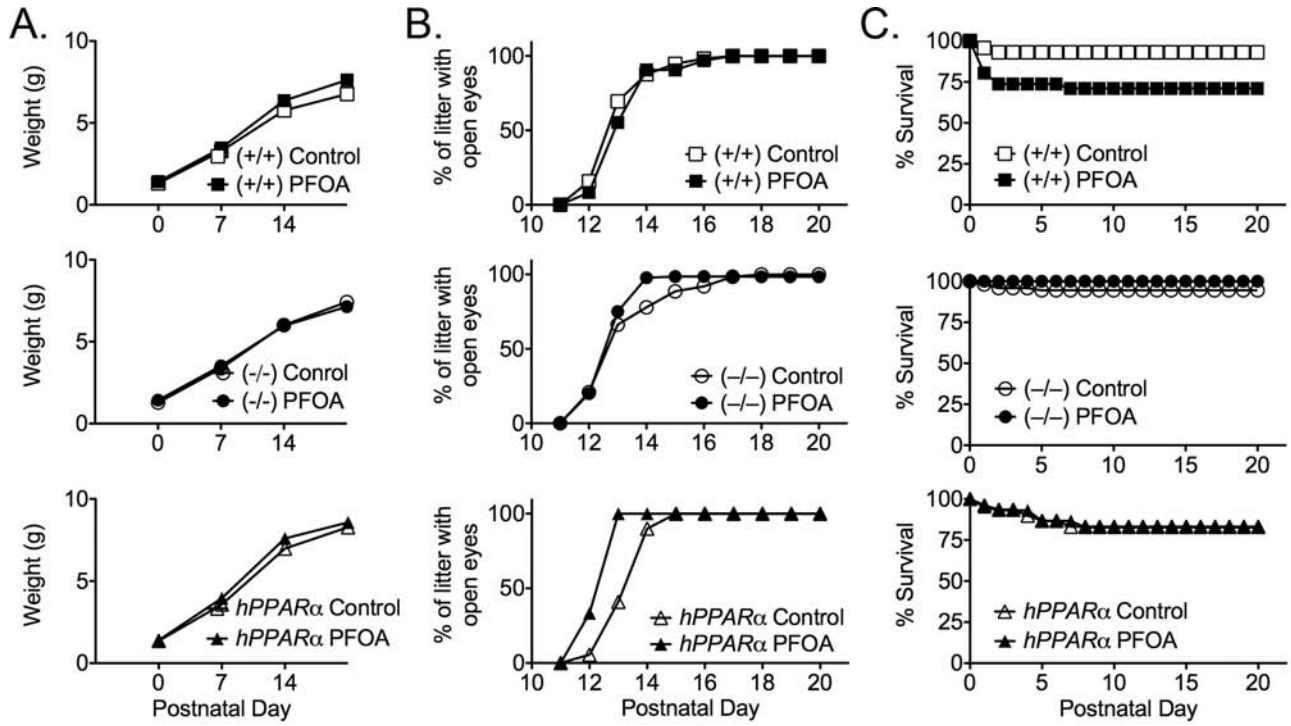

FIG. 7. Effect of prenatal PFOA exposure on postnatal development. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. (A) Average litter weight was measured at parturition and once per week up until PND20. Values represent the mean ± SEM. (B) The timing of eye opening was assessed twice daily until PND20. Values represent the average number of pups with open eyes per litter. (C) The number of surviving pups was monitored daily until PND20. Values for the *PPARα*-humanized (*hPPARα*) mice include those from pups that were hemizygous for the *hPPARα* transgene and pups that were negative for the *hPPARα* transgene.

TABLE 5
Effect of Prenatal PFOA Exposure on Mammary Gland Development in PND20 Female Offspring

Genotype	Treatment	TEB/gland	Ductal length (mm)	*N*
Wild-type	Control	2.1 ± 0.1	2.4 ± 0.3	3
	PFOA	2.2 ± 0.2	2.4 ± 0.4	4
Pparα-null	Control	2.1 ± 0.2	2.5 ± 0.1	9
	PFOA	2.6 ± 0.6	1.7 ± 0.4	3
hPPARα	Control	2.1 ± 0.3	2.7 ± 0.3	4
	PFOA	1.5 ± 0.3	2.6 ± 0.2	5

Note. Values represent the litter mean ± SEM.

GD18 *Pparα*-null dams was lower compared with nonpregnant dams and wild-type and *PPARα*-humanized pregnant dams treated with PFOA (Fig. 10B). The concentration of PFOA in liver of PND20 dams was markedly lower compared with that found in GD18 pregnant dams of all three genotypes (Fig. 10B). In samples obtained from mice treated with PFOA, the concentration of PFOA in serum and liver of GD18 fetuses was generally higher compared with the concentration in these tissues observed in pups on PND20 (Figs. 10C and 10D). The concentration of PFOA in GD18 fetal liver was relatively lower in *Pparα*-null fetuses compared with GD18 fetal liver from wild-type and *PPARα*-humanized fetuses (Fig. 10D).

DISCUSSION

Results from this study confirm previous experiments showing PPARα - dependent postnatal lethality in offspring from dams exposed prenatally to PFOA (Abbott *et al*., 2007). This study extends these experiments by demonstrating that when the human PPARα is expressed in mice, PFOA-induced postnatal lethality resulting from prenatal exposure is not observed. This suggests that there could be a species difference in the developmental response to PFOA modulated by PPARα. Neonatal lethality occurred within the first day or two of postnatal development in wild-type mice but not in either *Pparα*-null

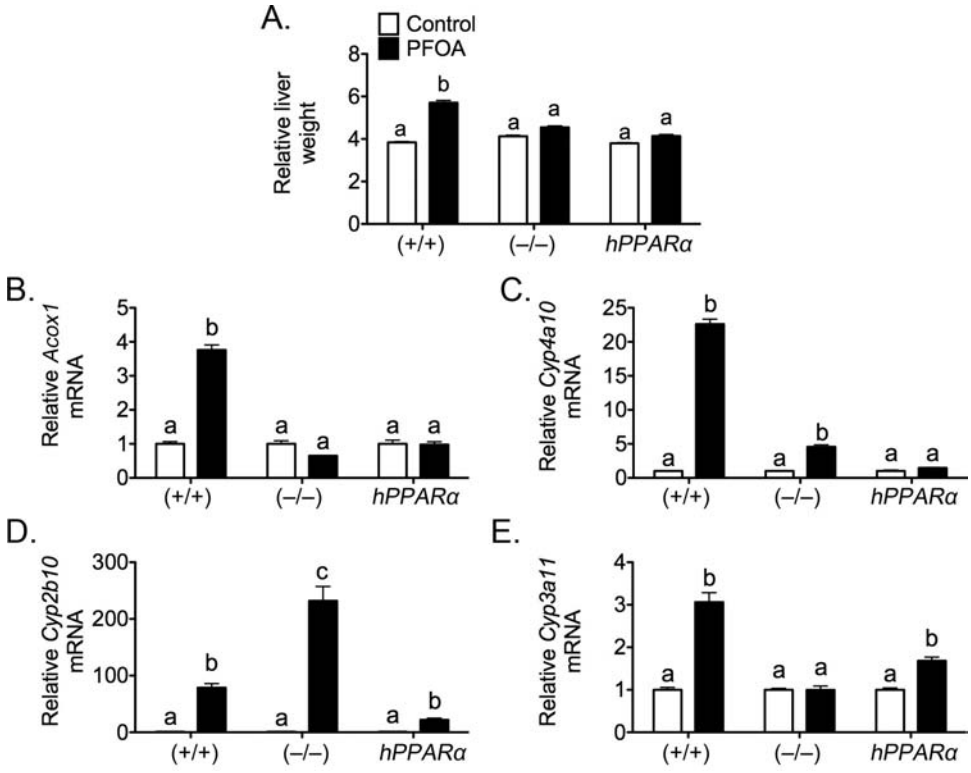

FIG. 8. Effect of prenatal PFOA exposure on pup liver effects on PND20. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Pup liver was isolated on PND20. Average fetal relative liver weight on PND20. Relative expression of (B) *Acox1*, (C) *Cyp4a10*, (D) *Cyp2b10*, or (E) *Cyp3a11* mRNA was determined by qPCR. Values represent the mean ± SEM. Values with different letters are significantly different, $p \leq 0.05$.

or *PPARα*-humanized mice. It is unlikely that differences in pharmacokinetics are responsible for the differences in neonatal mortality observed between genotypes. The average concentration of PFOA in maternal and fetal liver in *Pparα*-null mice on GD18 was lower than those found in wild-type and *PPARα*-humanized maternal and fetal liver on GD18. However, the average concentration of PFOA in maternal and fetal serum between all three genotypes on GD18 was similar. Moreover, the average concentration of PFOA in maternal and fetal liver on GD18 in wild-type and *PPARα*-humanized mice was similar. These observations collectively suggest that the lack of a PFOA-mediated increase in neonatal mortality in the *PPARα*-humanized pups compared with wild-type pups indicates that the mouse PPARα is involved in the etiology of PFOA-induced neonatal mortality, and that a fundamental difference in the human PPARα may protect against neonatal mortality in mice following gestational exposure to PFOA. Expression of PPARα target genes that modulate lipid metabolism was increased by both the mouse and human PPARα on GD18 in both maternal and fetal liver just prior to lethality, suggesting that the molecular mechanisms underlying the difference in early postnatal lethality observed between wild-type and *PPARα*-humanized mice are likely dependent on other PPARα target genes that may not regulate lipid metabolism. This could be similar to the effects observed in adult liver following treatment with PPARα agonists where increased expression of lipid metabolizing enzymes is found in both wild-type and *PPARα*-humanized mice but expression of genes that regulate cell cycle progression is increased only in wild-type mice (Cheung *et al.*, 2004; Shah *et al.*, 2007). This differential response mediated by either the mouse or human PPARα explains why *PPARα*-humanized mice are refractory to the hepatocarcinogenic effects of PPARα agonists (Morimura *et al.*, 2006). Whether mouse PPARα mediates similar effects in PFOA-treated maternal, fetal, or pup target tissue(s) is one possibility that could explain why wild-type mice exhibit postnatal lethality in response to perinatal PFOA exposure, while this effect is not found in *PPARα*-humanized mice. Further studies are needed to examine this hypothesis and identify the target tissue or tissues that could underlie this effect.

In addition to the possibility that mouse PPARα differentially regulates target genes compared with the human PPARα, differences in relative potency and/or receptor activities could also explain the difference in postnatal lethality observed between wild-type and *PPARα*-humanized mice. PFOA is not a potent activator of PPARα, and it is known that human PPARα requires a higher concentration of many different ligands to be activated compared with the concentration of ligand required

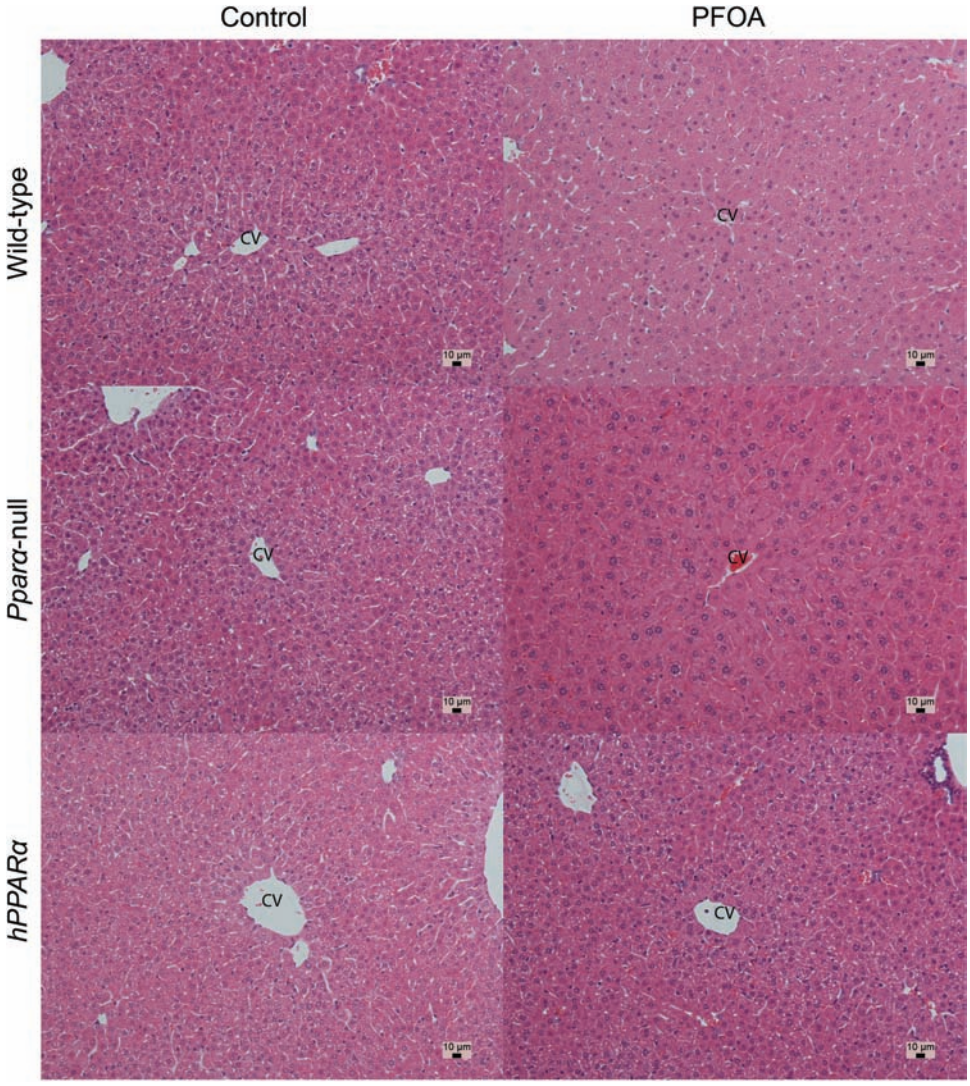

FIG. 9. Effect of prenatal PFOA exposure on fetal liver histopathology on PND20. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Pup liver was isolated on PND20 for histopathological analysis. The livers of PFOA-treated wild-type pups had hepatocellular hypertrophy similar to, but in some animals diffuse than, that observed in dams. In PFOA-treated *Pparα*-null pups, centrilobular hepatocellular hypertrophy similar to that described in GD18 dams was observed in only 1/5 pups. Livers of the remaining PFOA-treated *Pparα*-null and control *Pparα*-null pups were similar to their respective controls microscopically. Bar = 10 µm.

to activate mouse PPARα (Bility *et al*., 2004; Maloney and Waxman, 1999; Vanden Heuvel *et al*., 2006). On GD18, expression of PPARα target genes in maternal and fetal liver and modestly higher liver weight were found in both wild-type and *PPARα*-humanized mice, whereas on PND20 expression of PPARα target genes in maternal and pup liver and modestly higher liver weight were only observed in wild-type mice but not in *PPARα*-humanized mice. Because the concentration of hepatic PFOA was decreased on PND20 compared with that found on GD18, it remains possible that the concentration of PFOA in wild-type mice was sufficient to activate mouse PPARα and mediate changes that lead to postnatal lethality, whereas this similar concentration of PFOA in *PPARα*-humanized mice was insufficient to activate human PPARα. Thus, differences in the relative potency of PFOA to activate the human PPARα versus the mouse PPARα may explain why perinatal PFOA exposure causes postnatal lethality in wild-type mice but not in *PPARα*-humanized mice. Alternatively, it remains possible that the human PPARα expressed in a mouse model does not function identically to the mouse PPARα. For example, coactivators and/or corepressors required by PPARα for chromatin remodeling could be different between species; thus, in a mouse model, the human PPARα cannot modulate the same molecular targets as the mouse PPARα because of differences in accessible chromatin. Because both mouse and human PPARα activate similar genes in a mouse model (Cheung *et al*., 2004; Yang *et al*., 2008), this possibility does not appear to be likely. Still, the specific PPARα - dependent

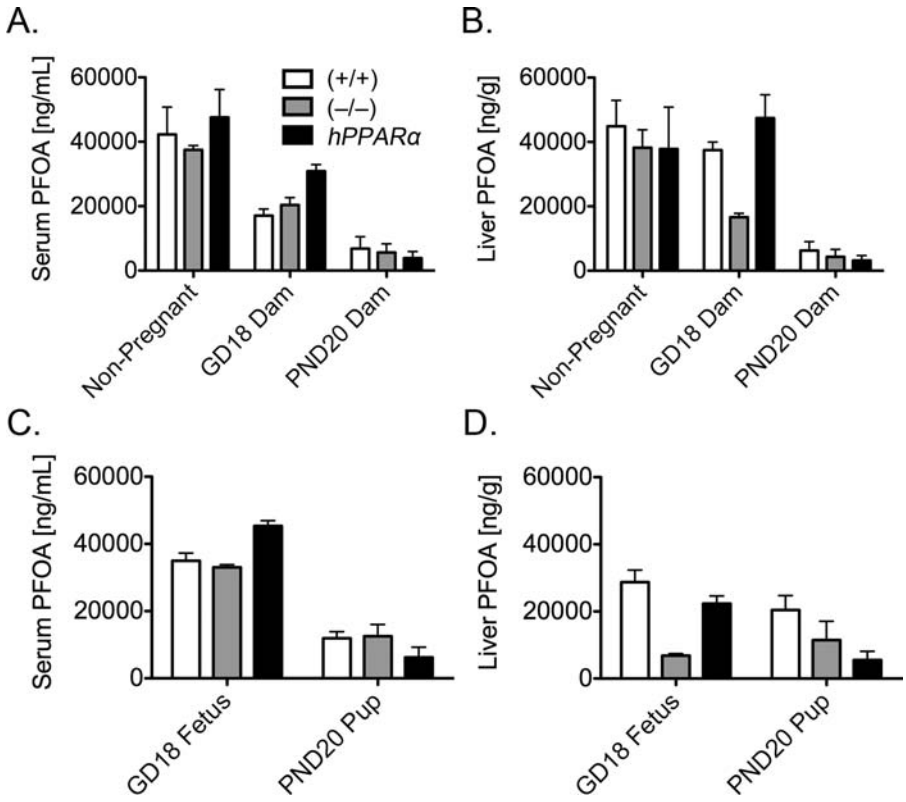

FIG. 10. Tissue concentrations of PFOA in maternal, fetal, and pup tissues from mice treated prenatally with PFOA. Pregnant wild-type (+/+), *Pparα*-null (–/–), and *PPARα*-humanized (*hPPARα*) mice were treated with water or 3 mg/kg PFOA from GD1 to GD17. Maternal serum (A) and liver (B) samples, and fetal or pup serum (C) and liver (D) were obtained on GD18 and PND20 for quantification of PFOA concentration. Values represent the mean ± SEM.

changes that mediate PFOA-induced postnatal lethality remain to be identified.

Prenatal PFOA exposure had no influence on postnatal mammary gland development in pups in these models. This effect has been found in some, but not in all studies (White *et al*., 2007; Yang *et al*., 2009; Zhao *et al*., 2010). The fact that this effect is not observed in all mouse models indicates that significant studies are needed to determine the biological relevance of this phenotype.

Although PPARα is required to mediate the biological effects of PFOA, including postnatal lethality and hepatic effects (Abbott *et al*., 2007; Nakamura *et al*., 2009), there is also evidence from other studies suggesting that CAR and/or PXR may be required (Bjork *et al*., 2011; Cheng and Klaassen, 2008; Elcombe *et al*., 2010; Ren *et al*., 2009; Rosen *et al*., 2008). Results from this study indicate that perinatal PFOA administration activates CAR and/or PXR in both the maternal and fetal/neonatal liver. Because evidence of enhanced CAR/PXR activity was noted in all three genotypes of mice exposed to PFOA, PFOA-induced postnatal lethality was only observed in wild-type mice, thus suggesting that CAR and PXR are not likely involved in mediating this effect. Interestingly, expression of *Cyp2b10* or *Cyp3a11* mRNA was higher in maternal liver and/or fetal/pup liver in response to PFOA in *PPARα*-humanized mice compared with wild-type and *Pparα*-null mice. The mechanism underlying this difference cannot be determined from these studies but deserves further investigation.

In summary, results from these studies confirm that the mouse PPARα can mediate neonatal lethality in mice exposed to PFOA during gestation, but when the human PPARα is expressed in mice, this effect is not observed. Although the results suggest that a species difference could exist in the developmental effects induced by gestational exposure to PFOA, further studies are needed to identify the specific mechanisms that could underlie this apparent species difference.

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