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Perfluorooctanoic Acid–Induced Developmental Toxicity in the Mouse is Dependent on Expression of Peroxisome Proliferator–Activated Receptor-alpha

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Perfluorooctanoic acid (PFOA) is a member of a family of perfluorinated chemicals that have a variety of applications. PFOA persists in the environment and is found in wildlife and humans. In mice, PFOA is developmentally toxic producing mortality, delayed eye opening, growth deficits, and altered pubertal maturation. PFOA activates peroxisome proliferators–activated receptor-alpha (PPARα), a pathway critical to the mode of induction of liver tumors in rodents. The present study uses 129S1/SvlmJ wild-type (WT) and PPARα knockout (KO) mice to determine if PPARα mediates PFOA-induced developmental toxicity. Pregnant mice were dosed orally from gestation days 1–17 with water or 0.1, 0.3, 0.6, 1, 3, 5, 10, or 20 mg PFOA/kg. PFOA did not affect maternal weight, embryonic implantation, number, or weight of pups at birth. At 5 mg/kg, the incidence of full litter resorptions increased in both WT and KO mice. In WT, but not KO, neonatal survival was reduced (0.6 mg/kg) and eye opening was delayed (1 mg/kg). There was a trend across dose for reduced pup weight (WT and KO) on several postnatal days (PND), but only WT exposed to 1 mg/kg were significantly different from control (PND7–10 and 22). Maternal factors (e.g., background genetics) did not contribute to differences in postnatal mortality, as PFOA induced postnatal mortality in heterozygous pups born to WT or KO dams. In conclusion, early pregnancy loss was independent of PPARα expression. Delayed eye opening and deficits in postnatal weight gain appeared to depend on PPARα expression, although other mechanisms may contribute. PPARα was required for PFOA-induced postnatal lethality and expression of one copy of the gene was sufficient to mediate this effect.

Key Words: **perfluorooctanoic acid; PFOA; developmental toxicity; peroxisome proliferator activated receptor-alpha—PPAR-α.**

Perfluorooctanoic acid (PFOA), a member of the perfluoroalkyl acid (PFAA) family of compounds, is a strong surfactant that is used primarily as a processing aide in the manufacture of certain fluoropolymers. It is also a possible degradation product of materials used for a variety of commercial and industrial applications, including use as fire-fighting foams, paint additives, surfactants, water, and stain repellants for use on clothing, upholstery, carpets, and as a coating on paper products for food containers. PFOA persists in the environment and is found in both wildlife and humans (Giesy and Kannan, 2002; Hansen *et al.*, 2002; Harada *et al.*, 2004; Hoff *et al.*, 2003; Kannan *et al.*, 2002, 2005; Kubwabo *et al.*, 2004; Olsen *et al.*, 2003a,b). In samples taken from the general population of the United States (2000–2001), the average concentration of PFOA in serum was estimated to be 5 ng/ml with 95th percentile upper bounds of approximately 14 ng/ml (Calafat *et al.*, 2007; Olsen *et al.*, 2003a). Studies of a population residing near a production facility in West Virginia detected levels ranging from 298 to 370 ng/ml and the predominant exposure route for this population was considered to be the community water supply (Emmett *et al.*, 2006). Toxic effects of PFOA observed in laboratory animals include liver hypertrophy, body weight reduction, tumorigenicity, developmental toxicity, reduction in serum cholesterol, triglycerides, and thyroid hormone levels (Kennedy *et al.*, 2004; Kudo and Kawashima, 2003; Lau *et al.*, 2004).

The developmental toxicity of PFOA has been examined in rats and mice (Butenhoff *et al.*, 2004; Hinderliter *et al.*, 2005; Lau *et al.*, 2006). In studies of CD-1 mice, the developmental toxicity induced by exposure to PFOA throughout gestation included dose-related full litter resorptions (FLR), reduced postnatal survival, delayed eye opening, growth deficits, and sex-specific alterations in pubertal maturation (Lau *et al.*, 2006). A cross-foster study indicated that the postnatal effects on survival, eye opening, and weight gain were a consequence of gestational exposure and that exposure via lactation was not a major factor (Wolf *et al.*, 2007).

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The mechanism through which PFOA produces developmental toxicity remains unknown, but activation of peroxisome proliferator activated receptor (PPARα) is considered to be a critical step in the mode of action of PFOA-induced liver tumors in the rat (Biegel *et al.*, 2001; Kennedy *et al.*, 2004; Klaunig *et al.*, 2003). PPARα is a ligand-activated nuclear receptor that regulates gene expression in response to various endogenous and exogenous ligands, presenting a recognizable and characteristic profile of altered gene expression (Desvergne and Wahli, 1999; Mandard *et al.*, 2004). The PPARα pathway plays a major role in maintaining lipid and glucose homeostasis, as well as having critical functions in regulating inflammatory responses, cell proliferation, and differentiation (Escher and Wahli, 2000). There is not much information available regarding the expression of PPARα during embryonic development. PPARα protein was detected immunohistochemically in the mouse embryo as early as gestation day (GD) 5 and on GD11 was found in liver, heart, digestive tract, tongue, and vertebrae (Keller *et al.*, 2000). In the rat, PPARα mRNA was detected on GD13.5 (roughly equivalent to GD11.5 in the mouse) in the central nervous system, liver, heart, digestive tract, tongue, and vertebrae (Braissant and Wahli, 1998; Michalik *et al.*, 2002). Peroxisomal proteins and enzymatic activity were detected in rat fetuses at GD15 or later (Cibelli *et al.*, 1988; Stefanini *et al.*, 1985, 1989; Tsukada *et al.*, 1968; Wilson *et al.*, 1991).

Thus, PPARα is present during embryonic development and disruption of PPAR-regulated gene expression could contribute to the developmental toxicity produced by PFOA exposure. The present study addresses the hypothesis that PFOA-induced developmental toxicity requires expression of PPARα. In this study, 129S1/SvlmJ wild-type (WT) and PPARα knockout (KO) mice were exposed to PFOA throughout gestation and parameters evaluated included maternal weight gain, numbers of embryos implanted, pre- and postnatal mortality, number of pups at birth (live and dead), pup birth weight, day of eye opening, and postnatal weight gain. Any contribution of maternal factors (i.e., possible differences in background genetics) was examined by evaluating heterozygous pups born to WT and KO dams. The study identified responses to PFOA that were dependent on PPARα expression as well as effects that did not require expression of this gene.

MATERIALS AND METHODS

Animals. Male and female 129S1/SvlmJ WT (stock #002448) and PPARα KO (Ppara-tm1Gonz/J, stock #003580) mice were obtained from Jackson Laboratories (Bar Harbor, MA). The PPARα KO mice were produced by targeted mutation of exon 8, encoding the ligand-binding domain, in the laboratory of F. J. Gonzalez and are a viable, fertile strain (Lee *et al.*, 1995). Strain 129S1/SvImJ is recommended as the best approximate match to the 129S4/SvJae background of the KO strain. Breeding colonies of WT and KO mice were established in the EPA Reproductive Toxicology Facility in Research Triangle Park, NC. Genotypes of the mice were confirmed by PCR analysis (method provided by Jackson Laboratories) using genomic DNA prepared from tail biopsies taken from at least one pup from every litter. Mice were housed in ventilated Tecniplast cages (Tecniplast USA, Exton, PA) and provided pellet chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and tap water *ad libitum.* Animal facilities were controlled for temperature (20°C–24°C) and relative humidity (40–60%), and kept under a 12-h light–dark cycle. All animal studies were conducted in accordance with guidelines established by the U.S. Environmental Protection Agency Office of Research and Development/National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee. Procedures and facilities were consistent with the recommendations of the 1996 National Research Council "Guide for the Care and Use of Laboratory Animals," the Animal Welfare Act, and Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Study protocol. Male and female mice of the same strain were bred overnight and the next morning females were examined for presence of a mating plug and this was designated GD0. For some of the studies, heterozygous litters (HET) were produced by mating KO male × WT female and WT male × KO female. In addition, some litters were produced by mating HET male × KO or WT females to produce litters in KO dams with both KO and HET pups and litters in WT dams to have both WT and HET pups. Plug positive female mice were weighed, randomly assigned to treatment groups, and housed individually in polypropylene cages. PFOA (ammonium salt; >98% pure) was purchased from Fluka Chemical (Steinhiem, Switzerland). PFOA was dissolved in deionized water and all dosing solutions were prepared fresh daily. On GD1–17 mice were weighed and dosed by gavage with either deionized water or PFOA at 0.1, 0.3, 0.6, 1, 3, 5, 10, or 20 mg/kg/day (10 ml/kg).

At parturition, the number of live and dead pups was recorded, male and female pups were weighed (all female pups in the litter weighed as a group, all male pups weighed as a group). The number of live pups in each litter was recorded and pups were weighed by sex on PND1–10, 14, 17, and 22. Eye opening was monitored beginning on PND12. On PND22, pups were weighed, weaned and males and females housed separately. Weaned pups were held for further study and weighed monthly. At weaning, one pup (selected randomly from each litter), and all adult females were weighed and killed. Blood samples were collected and serum prepared and stored frozen at −80°C for later PFOA analysis. Uteri were removed and stained with 2% ammonium sulfide and implantation sites counted (Narotsky and Kavlock, 1995).

Serum PFOA determination. Analysis of PFOA in serum was performed using a modification of a method originally developed by Hansen *et al.* (2001). Briefly, 25 μl of serum was combined with 1 ml of 0.5M tetrabutylammonium hydrogen sulfate (pH 10) and 2 ml of 0.25M sodium carbonate and then vortexed for 20 min a 15-ml polypropylene tube. Three hundred microliters of this mixture was then transferred to a fresh 15-ml polypropylene tube and 25 μl of a 1 ng/μl solution of ^{13}C2-PFOA (Perkin–Elmer, Wellesley, MA) was added as an internal standard. Five milliliters of methyl *tert*-butyl ether (MTBE) was then added and vortexed again for 20 min. The tube was centrifuged to separate the aqueous and organic phases, and 1 ml of the MTBE layer was extracted and transferred to a 5-ml polypropylene tube where it was evaporated to dryness at 45°C under a gentle stream of dry nitrogen. The residue was then redissolved in 400 μl of a 2mM ammonium acetate/acetonitrile (1:1) solution and transferred to a polypropylene autosampler vial. Extracts were analyzed using an Agilent 1100 high-performance liquid chromatograph (Agilent Technology, Palo Alto, CA) coupled with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) (liquid chromatography tandem mass spectrometry). Ten microliters of the extract was injected onto a Luna C18(2) 3 × 50 mm, 5-μm column (Phenomenex, Torrance, CA) using a mobile phase consisting of 30% 2mM ammonium acetate solution and 70% acetonitrile at a flow rate of 200 μl/min. PFOA and $^{13}C_2$-PFOA were monitored using parent and daughter ion transitions of 413 > 369 and 415 > 370, respectively. Peak integrations and areas were determined using Analyst software (Applied Biosystems Version 1.4.1). For each analytical batch, matrix-matched calibration curves were prepared using mouse serum spiked with varying levels of PFOA (Fluka Chemical, Steinhiem, Switzerland) as described above. For quality control, check standards were prepared by spiking large volumes of mouse serum at several arbitrary levels.

These check standards were stored frozen and aliquots analyzed with each analytical set. In addition, control mouse serum samples were fortified at two or three levels in duplicate with known quantities of PFOA during the preparation of each analytical set. Duplicate fortified and several check standards were run in each analytical batch to assess precision and accuracy. The limit of quantitation (LOQ) was set as the lowest calibration point on the standard curve. Analytical batches were considered to be acceptable if matrix and reagent blanks had no significant PFOA peaks approaching the LOQ, the standard curve had a correlation coefficient > 0.98, and all standard curve points, fortified, and check samples were within 70–130% of the theoretical and previously determined values, respectively.

Statistical analysis. Means and standard errors were calculated by SAS Proc Means and tests of differences among groups were performed within analyses of variance using SAS Proc GLM (SAS/STAT User's Guide, Version 9, Cary, NC, SAS Institute, Inc., 2003). Pairwise tests were run with adjustments for multiple comparisons using Dunnett's or Bonferroni tests where differences from the control group were of interest and a Tukey–Kramer adjustment when multiple group differences were screened. Fisher's exact test was used to compare the number of dams with and without FLR. Linear regression models were also run to test for trends across dose. For reproductive outcomes, body weight, liver weight, pup survival, development, and growth, analyses were done separately by strain (KO and WT), and pup data were analyzed on a litter basis. Pup weights at birth and PND1–22 were analyzed separately for male and female. Pup body weight, liver weight, and liver-to-body ratios at weaning were calculated after combining data from male and female pups killed on PND22. Analyses of serum PFOA were performed using log10-transformed data and the analysis of variance models included strain (WT, KO), dose group, and adult female status with respect to pregnancy and presence of pups at wean. Adult females were grouped for analysis into four categories: dams with live pups at weaning, dams whose pups all died on or before PND5 (D), females with FLR early in pregnancy, and females that were not pregnant (NP; no uterine implantation sites). For some comparisons, the adult females were placed into two groups: females with or without live pups at weaning (the latter group including NP, FLR, and D). PFOA levels in serum were compared between pups and dams. Regression models were run to detect an association between the total number of pups at birth and PFOA serum levels in dams at weaning, allowing for separate intercepts for each dose group, and calculating an average slope for all groups or a separate slope for each group.

RESULTS

Maternal Weight, Reproductive Outcomes, and Pup Birth Weight

Maternal weight and weight gain from GD1–17 (excluding NP and females with FLR early in gestation) were unaffected by PFOA exposure at any dose in both WT and KO dams (Table 1). The number of embryonic implantation sites in the uteri and the total number of pups at birth (live and dead pups) was also unaffected by PFOA exposure in both strains. However, the incidence of FLR was significantly increased at doses of 5 mg/kg/day or higher in both WT and KO dams (Table 1). It is concluded that the FLR occurred early in gestation, as the dams had implantation sites but failed to gain weight from GD1–17 and at necropsy the sites in the uteri were

TABLE 1
Maternal Weights and Reproductive Outcomes in PPARα KO and WT 129S1/SvlmJ Mice after Exposure to PFOA on GD1–17

Dose (mg/kg/day)	Pregnant females (#)[a]	Maternal weight (g) GD17 (#)[b]	Maternal weight gain (g) GD17[b]	Implants (# per litter)	Total # pups per litter[c]	% Litter loss: all litters[d]	% Litter loss (exclude FLR)[e]	% of Dams with FLR[f]
WT 129S1/SvlmJ								
0	22	31.9 ± 0.6 (21)	9.1 ± 0.4	8.5 ± 0.4	6.0 ± 0.4	43 ± 6	34 ± 4	5
0.1	12	30.9 ± 0.7 (11)	8.2 ± 0.9	7.4 ± 0.9	5.4 ± 0.8	44 ± 11	33 ± 9	8
0.3	8	30.9 ± 0.7 (7)	9.4 ± 0.8	7.9 ± 0.7	6.1 ± 0.6	48 ± 10	41 ± 8	13
0.6	16	31.7 ± 0.8 (14)	8.7 ± 0.7	8.4 ± 0.5	5.4 ± 0.7	71 ± 8*	48 ± 8	13
1	18	32.4 ± 0.5 (13)	9.7 ± 0.5	8.4 ± 0.4	5.6 ± 0.5	70 ± 6*	58 ± 6*	28
5	6	35.6 ± 0 (1)	12.1 ± 0	6.8 ± 1.0	0	100 ± 0**	—	83***
10	5	34.2 ± 0 (1)	11.0 ± 0	7.2 ± 1.4	0	100 ± 0**	—	80**
20	7	— (0)	—	6.0 ± 0.7	0	100 ± 0**	—	100***
PPARα KO								
0	23	31.2 ± 0.5 (19)	7.8 ± 0.5	7.0 ± 0.5	5.1 ± 0.5	42 ± 7	29 ± 5	17
0.1	14	32.4 ± 0.6 (11)	8.9 ± 0.7	7.0 ± 0.7	6.3 ± 0.6	36 ± 11	18 ± 6	21
0.3	10	32.2 ± 0.9 (9)	9.1 ± 0.8	7.3 ± 0.8	5.9 ± 0.6	30 ± 9	22 ± 6	10
1	10	31.6 ± 1.0 (10)	8.3 ± 0.9	7.4 ± 1.0	5.5 ± 0.9	28 ± 8	28 ± 8	0
3	20	32.7 ± 0.6 (17)	9.8 ± 0.6	7.4 ± 0.5	6.3 ± 0.5	31 ± 8	19 ± 5	15
5	6	32.8 ± 0 (1)	11.7 ± 0	6.2 ± 1.1	7 ± 0	86 ± 14*	14 ± 0	83**
10	5	31.8 ± 0 (1)	10.6 ± 0	7.6 ± 0.7	6 ± 0	85 ± 15*	25 ± 0	80**
20	4	35.6 ± 0 (1)	12.3 ± 0	8.3 ± 0.5	4 ± 0	100 ± 0**	—	75*

Note. Means (dams) or litter means (pups) ± SEM. GD, gestational day. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to control.

[a]Pregnancy verified by presence of uterine implantation sites.

[b]Excludes NP and dams with FLR early in gestation.

[c]Total number of pups per litter at parturition, live + dead combined; exclude litters with early FLR.

[d]Percent litter loss (all litters) = (#implants − #live pups)/#implants × 100; includes litters with early FLR.

[e]Percent litter loss (exclude litters with early FLR) = (#implants − #live pups)/#implants × 100.

[f]Percent of dams with FLR = (# dams with early FLR)/# dams with implants × 100. Fisher's Exact Test was used for comparison of # dams with and without FLR.

small and only faintly stained, compared to implantation sites from dams with full term litters. When dams with FLR were excluded from the analysis of % litter loss, the survival from implantation to birth was similar for all dose groups in the KO, indicating that PFOA did not affect embryonic survival later in gestation or at exposures less than 5 mg/kg/day. The percent litter loss (excluding FLR) increased with dose in WT dams and was significantly different from the control after exposure to 1 mg/kg/day ($p < 0.05$). Pup birth weights were not significantly affected by PFOA in either WT or KO litters (Table 2). After exposure to 5 or 10 mg/kg/day, only one litter (KO) survived in each of these dose groups, but the male and female pup weights appeared similar to those in litters exposed to lower doses (Table 2).

Effects of PFOA on Relative Liver Weight in Adult Females and Weaned Pups

The relative liver weights (Fig. 1) are calculated from individual body weight and liver weight measured at weaning on PND22 (data not shown). PFOA significantly increased the relative liver weight (liver weight/body weight × 100) of WT and KO adult females and weaned pups (sexes combined for calculation of relative liver weight of pups). There was a dose-related trend for increased relative liver weight in both WT and KO adults and weaned pups. The lowest dose at which relative liver weight was significantly increased was 0.1 mg/kg/day in WT pups or 1 mg/kg/day in WT adult females and 3 mg/kg/day in the KO (adults and pups). The PFOA-exposed adult female body weights on PND22 were not significantly different from control, but there was a dose-related trend for increased absolute liver weights. Absolute liver weight was significantly increased in the WT exposed to 1 mg/kg/day or higher and in KO exposed to 3 mg/kg/day or higher (data not shown). Whether or not the females were pregnant or carried litters to term or nursed pups up to weaning did not influence the outcomes for body weight or liver weight on PND22.

TABLE 2
Birth Weights of PPARα KO and WT 129S1/SvlmJ Pups after exposure to PFOA on GD1–17

		Male pups		Female pups	
	Dose group	# Litters	Birth weight (g)	# Litters	Birth weight (g)
WT	0	17	1.31 ± 0.03	18	1.28 ± 0.04
	0.1	9	1.36 ± 0.04	9	1.34 ± 0.04
	0.3	7	1.35 ± 0.06	7	1.29 ± 0.05
	0.6	8	1.27 ± 0.05	8	1.29 ± 0.05
	1	12	1.28 ± 0.05	11	1.30 ± 0.04
	5	0	—	0	—
	10	0	—	0	—
	20	0	—	0	—
PPARα KO	0	17	1.35 ± 0.03	18	1.35 ± 0.05
	0.1	11	1.35 ± 0.05	11	1.33 ± 0.05
	0.3	9	1.38 ± 0.04	9	1.32 ± 0.04
	1	10	1.41 ± 0.06	10	1.40 ± 0.05
	3	15	1.32 ± 0.03	17	1.30 ± 0.05
	5	1	1.33 ± 0	1	1.37 ± 0
	10	1	1.27 ± 0	1	1.37 ± 0
	20	0	—	0	—

Pup Survival, Development and Growth

Survival of pups from birth to weaning (PND1–22) was significantly ($p < 0.001$) reduced in WT litters (Fig. 2A), but was not affected by PFOA in the KO (Fig. 2B). WT litters exposed to 0.6 or 1 mg/kg/day had only 42.5 ± 16% ($n = 7$) or 43 ± 14% ($n = 10$) of pups alive on PND22 compared to 78.9 ± 10% ($n = 10$) in WT control litters. KO litters exposed to 1 mg/kg/day had 96 ± 4% of pups in each litter survive to PND22 ($n = 8$). A dose of 3 mg/kg/day was selected to further challenge KO postnatal survival (using higher doses was not feasible as 5 mg/kg/day results in FLR). KO litters exposed to 3 mg/kg/day had 87 ± 7% ($n = 16$) survival to PND22, and this was not significantly different from 92 ± 6% ($n = 7$) in the KO control litters.

To eliminate the possibility that maternal strain (i.e., potential variability in WT and KO genetic background) was a factor in the differences in survival of WT and KO pups, heterozygous (HET) litters were produced in WT and KO dams and exposed to PFOA during gestation and survival was monitored up to PND15 (Fig. 3). Survival was significantly ($p < 0.001$) decreased for WT pups ($n = 10$ litters) and HET pups ($n = 8$ litters) born to WT dams dosed at 1 mg/kg/day. HET pups of KO dams exposed to 3 mg/kg died within the first week after birth ($p < 0.001$ compared to control HET pups). On PND15, only 5% of PFOA-exposed HET pups from KO dams were alive ($n = 7$ litters), compared with 76% of the HET pups in control KO dams ($n = 8$ litters). In addition, several litters were produced by mating HET males and KO females and these dams had both HET and KO pups. In control mixed litters, survival of the HET and KO pups was 100% and 89%, respectively. In PFOA-dosed mixed litters (3 mg/kg from GD1–17), none of the HET pups survived. As expected, PFOA reduced survival of both WT and HET pups born to WT dams exposed to 1 mg/kg/day. The survival of KO pups and deaths of HET pups born to KO dams (all HET or mixed HET and KO litters) indicated that expression of PPARα is required for induction of postnatal lethality by PFOA and that one functional copy of the gene is sufficient to significantly increase postnatal pup deaths.

Postnatal development was evaluated by recording the age at which both eyes were fully opened and calculating percent of pups per litter with open eyes and the mean day of eye opening. The percent of pups per litter with both eyes open increased from PND12–17 for all groups, WT and KO (Fig. 4). The WT pups exhibited a dose-related trend for delayed eye opening ($p < 0.05$) and at the 1 mg/kg/day exposure the mean day of

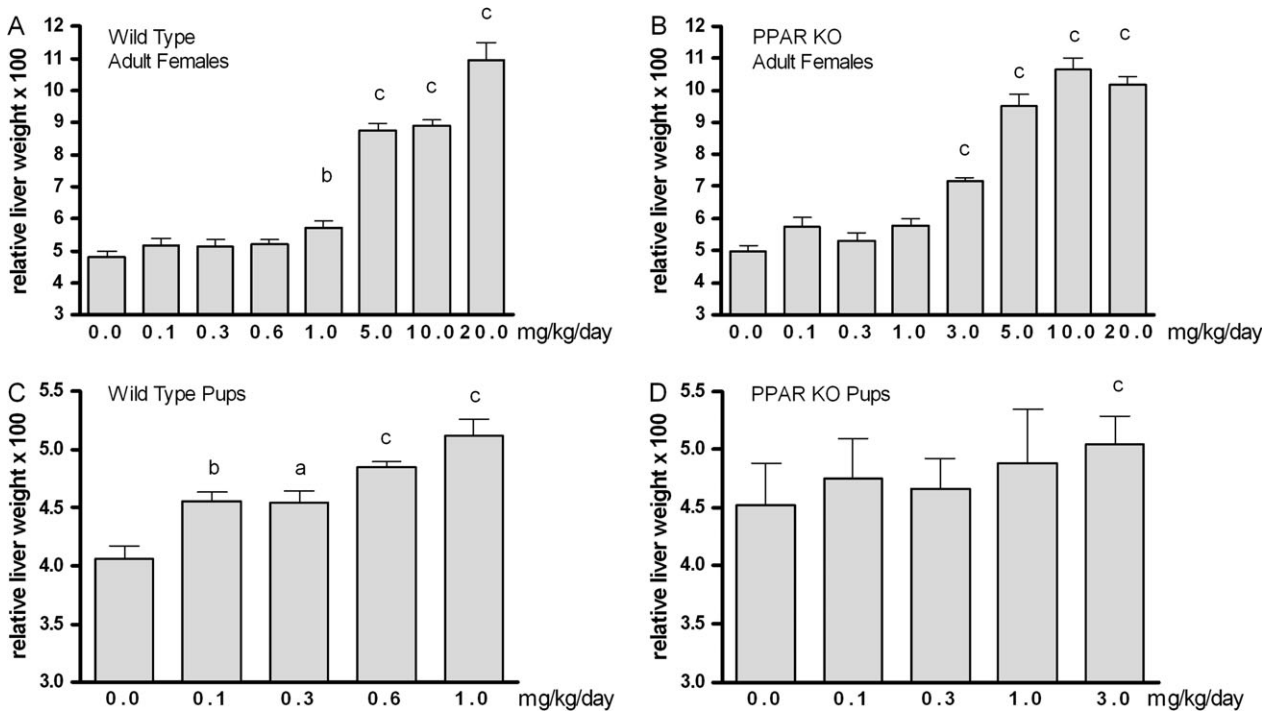

FIG. 1. Relative liver weight of WT or PPARα KO adult females (A, B) and pups (C, D; relative liver weight for pups included both sexes) was calculated from the absolute liver weight and body weight measured on PND22 at necropsy. Mated, adult, plug positive females were dosed from GD1–17 with water or PFOA at levels shown. Data included all females, as pregnancy status did not significantly alter relative liver weight within treatment groups. In A and B, the number of adult females in each group was 36, 15, 16, 26, 25, 12, 11, 16, and 42, 19, 20, 20, 43, 17, 17, and 17 (0–20 mg/kg/day, respectively). In C and D, the number of litters (one pup per litter) in each group was 9, 8, 6, 4, 6, and 8, 11, 9, 8, 14 (control to highest dose, respectively).

eye opening was significantly later than the control ($p < 0.05$, 13.8 ± 0.3 and 14.6 ± 0.3, control $n = 9$ and treated $n = 6$, respectively). The KO pups did not show delayed opening of the eyes and although the 3 mg/kg/day group did not have open eyes on PND13, there was not a significant difference in the mean day of eye opening (14.1 ± 0.2 and 14.3 ± 0.2, control $n = 8$ and treated $n = 14$, respectively).

Postnatal male and female pup body weights were monitored from PND1–22 (Fig. 5). WT pups exposed to 1 mg/kg/day had significant ($p < 0.05$) differences from control on specific postnatal days (WT males on PND9, 10, and 22; females on PND7–10, and 22, as shown on Figs. 5A and 5B). KO pups did not show significant differences in body weight between control and PFOA-exposed groups (males or females) on any of the PNDs (Figs. 5C and 5D). Dose-related trends ($p < 0.05$) were detected for WT and KO body weight on specific days (KO male PND2 through 10, 14, 17, and 22; KO female PND2 through 4, 6 through 10, 14, 17 and 22; WT male PND2, 3, 7 through 10 and 22; WT female PND6 through 10, 14, and 22). There was also a significant trend ($p < 0.01$) across dose for reduced weight gain in male and female pups of WT and KO groups (difference in weight on PND1 and PND22). However, reduced weight gain was significantly different from control only in WT pups exposed to 1 mg/kg/day ($p < 0.05$) (10.0 ± 0.4 vs. 8.1 ± 1.2 and 9.4 ± 0.4 vs. 7.8 ± 0.6 g, control and treated, male and female respectively; data not shown for other groups). After weaning, body weights of WT and KO mice were not significantly different between controls and PFOA-exposed male (monitored up to 28 weeks of age) or female mice (52 weeks of age at the time of publication) (data not shown).

Serum Levels of PFOA in Dam and Pups

The level of PFOA in serum of adult females 23 days after the last dose and in pups at weaning increased with dose in both WT and KO mice (Table 3). WT and KO mice receiving the same dose had comparable PFOA levels (i.e., there was no effect of strain or PPARα expression on the serum PFOA levels). The levels of PFOA in dams and their pups at weaning were very similar (comparing dams and pups of the same dose group). At the higher doses of PFOA, the serum levels appear to plateau as there were no significant differences between WT females exposed to 5 mg or higher or between KO groups exposed to 3 mg/kg or higher (additional comparisons between dose groups are indicated on Table 3).

In both WT and KO, there was a dramatic difference in the PFOA levels in females without pups at weaning compared to the dams that nursed pups up to weaning. The level of PFOA in the WT was 2.8 to 3.7 times higher and KO females without pups was 3–5.4 times higher than in the females with pups at

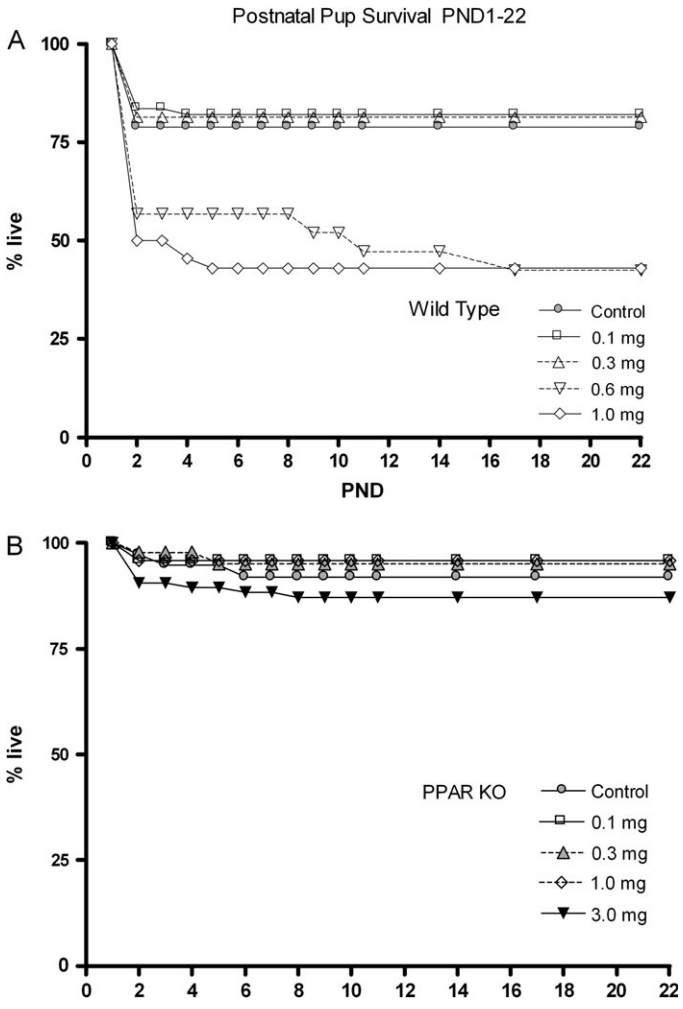

FIG. 2. The postnatal survival of pups is shown as the percent of the litter alive on PND1–10, 14, 17, and 22 for WT (A) and PPARα KO (B) strains. A significant decrease ($p < 0.001$) compared to control occurred only in the WT litters of dams exposed to 0.6 or 1 mg/kg on GD1–17. Data shown are the mean of litter means for 10, 9, 6, 7, and 10 WT and 7, 11, 9, 8, and 16 KO litters, in the order listed in the legends, respectively.

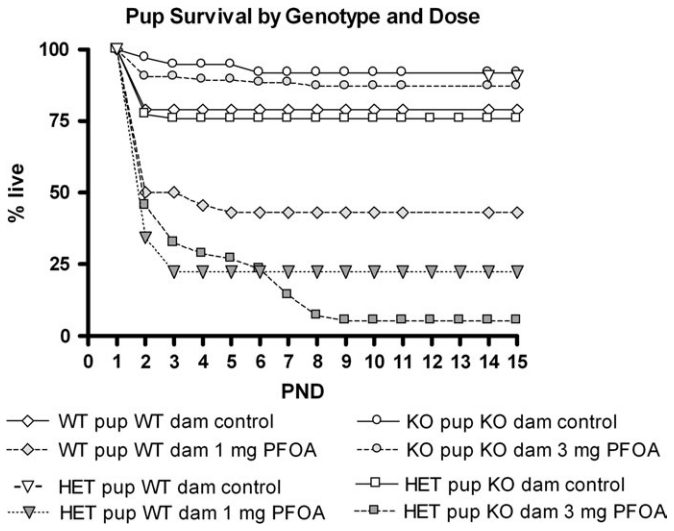

FIG. 3. The postnatal survival of heterozygous (HET) pups born to either WT or KO dams is shown. HET pups born to either WT or KO dams and exposed to PFOA *in utero* have significantly decreased survival compared to control pups ($p < 0.001$). Survival of KO pups born to KO dams and WT pups born to WT dams (control and PFOA-exposed) are shown for comparison with HET pups. Data shown are the mean of litter means for 7, 16, 8, and 7 KO and 10, 10, 26, and 8 WT dams, as listed in the legend, respectively.

weaning. For further comparisons, the adult females were separated into categories based on pregnancy and lactation status: NP, FLR, D, and P (see Methods, statistical analysis). In the WT strain, a significant difference was found between D and NP/FLR females, such that females nursing pups at least up to PND5 had lower PFOA levels than females that did not deliver pups. The D females had significantly higher serum levels relative to the P females, resulting in a serum PFOA ranking of NP and FLR > D > P (data not shown). In the KO, serum PFOA levels were not different among the NP, FLR, and D females and these groups were significantly higher than the levels in the P females (data not shown). The difference between D and NP/FLR females was not detected in the KO, probably due to the smaller numbers of dams in the D group for that strain. A correlation was detected between the number of pups in the litter and serum levels in the dam at weaning, with more pups being associated with lower PFOA levels in the dam (data not shown). This significant association was detected in the 0.1, 1, and 3 mg/kg PFOA-treated KO dams, and was significantly different from control (where no such association existed). However, this correlation could not be detected in the WT, and this is probably due to the large number of dams that lost all pups before weaning among the PFOA-treated WT dams. A few KO females in the 5, 10, and 20 mg/kg/day dose groups were evaluated on PND4 and the mean serum levels were 123,952, 149,764, and 109,495 ng/ml, respectively. The ratio of the PND23 to the PND4 levels was 0.5 to 0.6 (comparing data for females without pups), consistent with the reported half-life of 16–19 days in mice (Lau *et al.*, 2005).

DISCUSSION

Our study of the responses of 129S1 WT and PPARα KO mice to PFOA exposure demonstrates that PPARα expression is critical to the mode of action for PFOA-induced postnatal pup lethality. Survival of WT pups is significantly decreased after exposure to 0.6 mg/kg/day, while at a higher exposure (3 mg/kg/day), KO pups have survival rates equivalent to that of controls. The expression of one copy of the PPARα gene is sufficient to mediate this response, as almost all of the PFOA-exposed HET pups died within days of birth. That HET pups died in litters born to either WT or KO dams also eliminated the possibility that the outcome was a result of undefined maternal factors (i.e., possible differences in background genetics in the

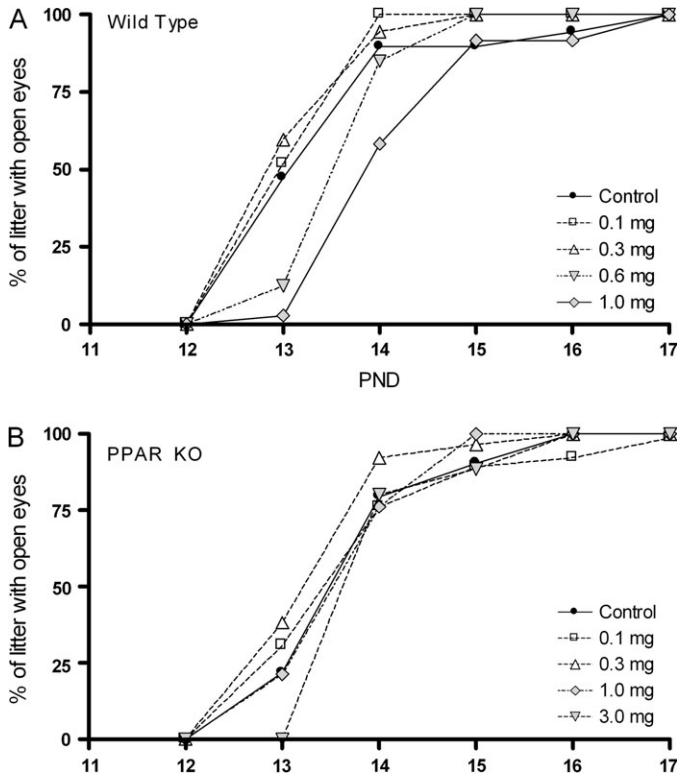

FIG. 4. The percentage of pups with both eyes fully open on PND12–17 is shown for WT (A) and KO (B) litters. The progress in achieving open eyes appeared delayed in the WT litters exposed to 0.6 or 1 mg/kg on GD1–17. Although none of the KO litters exposed to 3 mg/kg had open eyes on PND13, there was no difference between treated and controls by PND14. By PND17, 100% of pups in all groups had both eyes open. Data shown are the mean of litter means for 9, 8, 6, 5, and 6 WT and 8, 10, 9, 8, and 14 KO litters, in the order listed in the legends, respectively.

WT and KO strains or dam-related PPARα-mediated effects that would occur only in WT).

The mode of action for PFOA-induced postnatal mortality is unknown, but it is possible that more than one factor is contributing to the deaths, as some pups died within hours of birth while others survived up to 10 days. Some of the newborn pups were observed to be cyanotic and gasping for breath and this was consistent with finding dead pups in that litter later in the day or on the next day. Similar observations were reported for newborn Sprague–Dawley rat or CD1 mouse pups exposed to another PFAA, perfluorooctane sulfonate (PFOS) throughout gestation, or during the last few days of gestation (Grasty *et al.*, 2003; Lau *et al.*, 2003). In the rat pups exposed to PFOS, effects on lung maturation were suspected as the cause of death, but subsequent investigations were unable to correlate the apparent morphological immaturity of the lung with changes in composition or secretion of pulmonary surfactant, phospholipids concentrations, or markers of alveolar differentiation and an explanation for the mortality remained elusive (Grasty *et al.*, 2003, 2005). Luebker *et al.* (2005) in a two-generation and cross-foster study of PFOS in rats also examined neonatal lungs and did not find changes in alveolar cell lamellar bodies. *In vitro* investigations of the direct effects of PFOS, PFOA, and other perfluorinated compounds on surfactant surface properties (Xie *et al.*, 2007) suggest that an alternative mode of action could be direct interference with lung surfactant function. If this were the mode of action that resulted in early postnatal lethality after *in utero* exposure to PFOA, then it would be expected to occur regardless of the status of PPARα gene expression (i.e., KO pups should be affected). However, that was not the case in our studies and thus it seems unlikely that there was sufficient interference by PFOA with the function of lung surfactant to cause the deaths of the newborn mice. This does not eliminate the possibility that PFOS could be acting through such a mechanism, as the physical and chemical properties of PFOS and PFOA differ considerably. A study of PFOS in the PPARα KO could provide additional insight into the possibility that PFOS, but not necessarily PFOA, was producing early postnatal lethality via direct effects on lung surfactant properties.

While our results show that PPARα was required for mediating postnatal mortality, the role of the pathway in the induction of developmental delay and weight gain deficits was more difficult to assess. A PPARα-dependent mode of action for these endpoints is suggested as in the WT both eye opening and body weight gain were significantly affected by exposure to 1 mg/kg/day, but exposure of KO pups to 1 or 3 mg/kg/day produced no significant effects on the mean day of eye opening or on body weight (compared to control on each PND). However, the interpretation is complicated by the observations that: (1) there was a trend across dose for decreased KO pup weight on some PNDs; and (2) that none of the KO pups from dams exposed to PFOA at 3 mg/kg/day had open eyes on PND13, although some KO control pups had open eyes at that age. Although the observations in WT and KO do suggest that PPARα is involved in mediation of the effects of PFOA on eye opening and weight gain, there is some possibility that PPARα-independent mechanisms could contribute to producing these responses. Similarly, (2-ethylhexyl)phthalate (DEHP) (a peroxisome proliferator which activates the PPARα pathway), was shown to have both PPAR-dependent and independent toxic responses in the mouse (Ward *et al.*, 1998). In WT mice, DEHP produced mortality, weight loss, and lesions of the liver, kidney, and testes. PPARα KO mice did not have excess mortality or liver lesions, but did develop toxic lesions of the kidney and testes, indicating that PPARα-independent pathways were involved in mediating the renal and testicular toxicity.

The analysis of PFOA levels in the serum of the dams and pups demonstrated that the expression of PPARα did not affect elimination of the chemical as both WT and KO strains of mice had similar serum levels at 23 days after the last dose. However, the data clearly demonstrated that parturition and/or lactation have a major impact on the elimination of PFOA in the dams. Females without pups had considerably more PFOA in their serum than those females that nursed litters up to weaning.

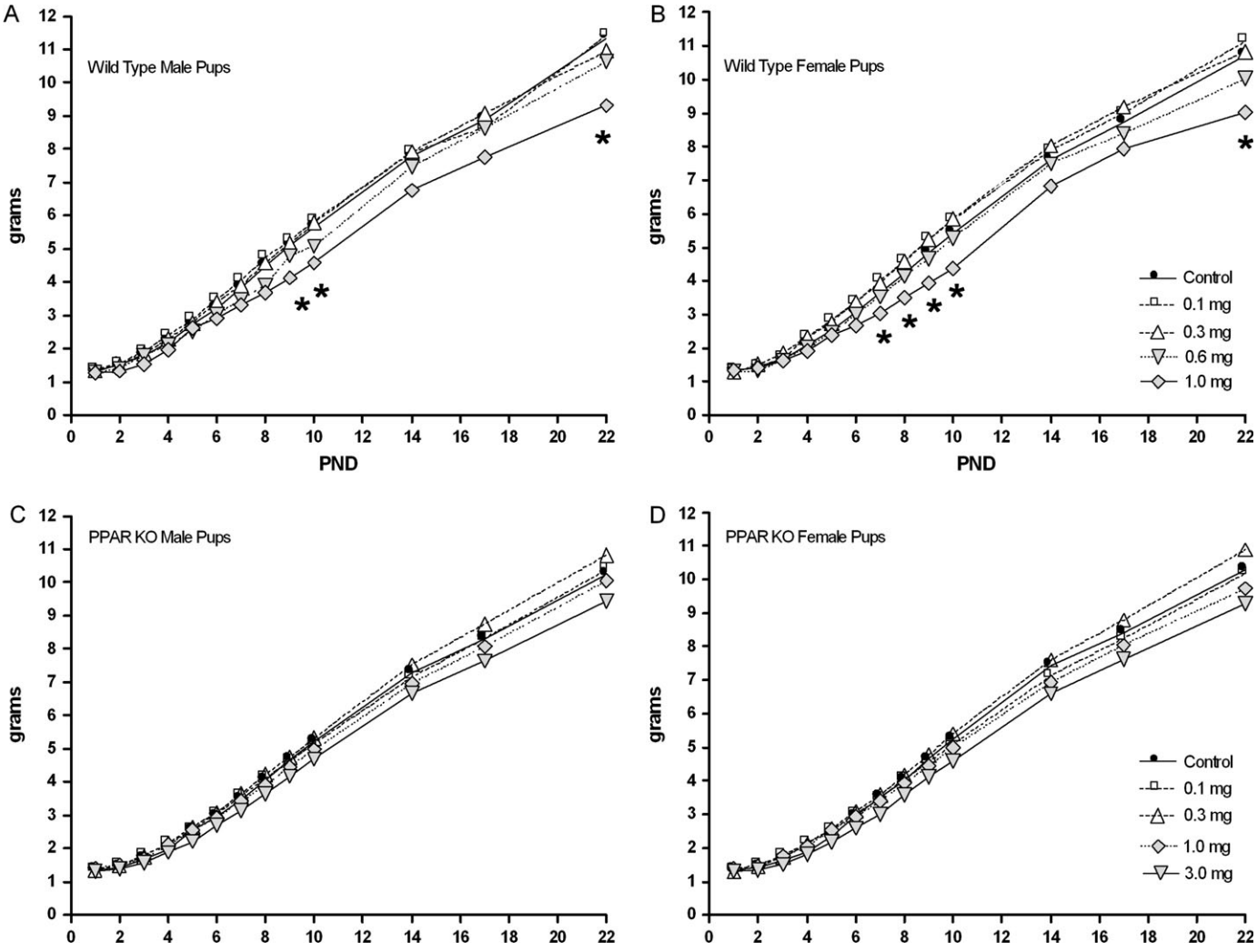

FIG. 5. The mean body weights of WT male (A) and female (B) and PPARα KO male (C) and female (D) pups are shown for PND1–10, 14, 17, and 22. Body weight of WT pups was significantly decreased ($p < 0.05$) on PND9, 10, and 22 (males) and PND7, 8, 9, 10, and 22 (females). Data shown are the means of 11, 9, 7, 8, and 12 WT litters and 9, 11, 9, 10, and 17 KO litters, in the order shown in the legends, respectively. * Indicates significant difference ($p < 0.05$) between control and 1 mg/kg dose group for that PND.

The number of pups in a litter and the duration of nursing also impact the elimination of PFOA in serum of the dam. Having more pups was associated with a lower serum PFOA and dams that nursed pups to weaning had lower levels than dams that nursed for shorter periods. The females with FLR or that were NP had the highest levels of serum PFOA. Lactation may be a major route of elimination for the dam, particularly if there are large numbers of pups nursing during the maximal milk-producing stage (which occurs during the second week postpartum in mice). Even though the amount of PFOA in milk is reported to be only a tenth or less of the amount in maternal serum (human or rat milk (Hinderliter *et al.*, 2005; Kuklenyik *et al.*, 2004), the impact of elimination of PFOA in milk may well be related to the total volume of milk produced. In the case of the rat or mouse, that would be related to duration of the lactational period as well as to the number of pups in a litter. It is also of interest that the levels of PFOA at weaning were very similar in dams and pups (dam and pup levels were significantly correlated, Pearson coefficient $R = 0.98$, $p < 0.0001$). Although this study was not designed to evaluate the pharmacokinetics of elimination in the dam and pups, these observations provide further information regarding what is likely a complex interaction between dam and pup in the postnatal pharmacokinetics of PFOA.

The effects of PFOA that lead to FLR are independent of PPARα gene expression. FLR occurred in both WT and KO dams at equivalent levels and few litters survived to parturition in either strain after exposure to 5 mg/kg/day or above. The incidence of FLR was similar in groups dosed at 5, 10, or 20 mg/kg/day and these groups also had similar serum PFOA levels. It is concluded that the embryonic deaths occurred very early in gestation, as the dams did not gain weight from

TABLE 3
Serum Levels of PFOA in Adult Females and Pups on PND22

		Adult females: no pups at wean[a]		Adult females: pups at wean[b]		Pup at weaning (sexes combined)	
	Dose group	*N*	PFOA (ng/ml)	*N*	PFOA (ng/ml)	*N*	PFOA (ng/ml)
WT	0	17	131 ± 27.1 A	18	33.2 ± 6.02 F	9	17.3 ± 2.51 F
	0.1	7	4400 ± 725 B	8	1600 ± 214 G	8	798 ± 96.0 G
	0.3	10	10,400 ± 781 C	6	2840 ± 387 G,H	6	2150 ± 324 H
	0.6	21	17,400 ± 1240 C,D	5	5170 ± 913 H,I	4	3810 ± 562 H
	1	19	26,300 ± 1520 D	6	9290 ± 611 I	6	9860 ± 1420 I
	5	12	76,600 ± 3650 E		ND		ND
	10	11	72,400 ± 3230 E		ND		ND
	20	11	68,200 ± 5900 E		ND		ND
PPARα KO	0	25	92.9 ± 9.67 A	15	27.7 ± 3.56 F	8	29.1 ± 3.99 F
	0.1	8	4220 ± 647 B	11	1020 ± 167 G	11	1160 ± 257 G
	0.3	11	9730 ± 649 C	9	2850 ± 282 H	9	2540 ± 383 H
	1	12	24,700 ± 1830 D	9	8260 ± 937 I	8	7730 ± 1900 I
	3	28	61,100 ± 2360 E	16	18,400 ± 1430 J	15	10,600 ± 1010 I
	5	12	81,800 ± 3430 E	1	15,100 I,J		ND
	10	13	78,500 ± 4340 E		ND		ND
	20	12	73,100 ± 1870 E		ND		ND

Note. Means ± SEM. Data were rounded to 3 significant figures. ND = no data.

[a]Adult females dosed from GD1–17, with no pups surviving past PND5. Includes females with no implantations, FLR, only dead pups at birth, or all pups died by PND5 (values were NS different between these categories and data were combined as a group).

[b]Adult females dosed GD1–17 with live pups at weaning. A–E: Groups in the "adult females: no pups at wean" column with the same letter were not significantly different. F–I: Groups in the "adult females: pups at wean" and "pup at weaning" columns with the same letter were not significantly different.

GD1–17, even though the numbers of embryos implanted in the uteri were not different from controls. This conclusion is further supported by a study in our laboratory that examined the uterine contents of PFOA-exposed CD-1 dams on GD7 and GD8. CD-1 dams were exposed to PFOA at a dose known to produce FLR in that strain (20 mg/kg/day starting on GD1) and we found that 45% of the embryos died by GD7 and 66% were dead by GD8 (unpublished data). It is also clear that there is a difference between strains of mice in the sensitivity to PFOA. Lau *et al.* (2006) studied the effects of PFOA in CD-1 mice and observed 26% of dams exposed to 5 mg/kg/day throughout gestation had FLR and that 100% of the dams exposed to 40 mg/kg/day had FLR. In the 129S1/SvlmJ (WT) strain of mice, exposure to 5–20 mg/kg/day throughout gestation resulted in 83–100% of dams with FLR. In CD-1 mice, PFOA reduced postnatal survival after exposure to 5 mg/kg/day or higher, but no significant effect was observed at 1 or 3 mg/kg/day. The survival of the 129S1/SvlmJ (WT) neonates was significantly reduced after exposure to 0.6 mg/kg/day. An analysis of the PFOA dose–response with survival from conception to weaning as the endpoint, suggests that the 129S1/SvlmJ WT mice are approximately 9.5 times more sensitive than the CD-1 strain (based on Lau *et al.*, 2006; Wolf *et al.*, 2007, and the present data).

In summary, the mode of action for PFOA-induced postnatal mortality requires PPARα and expression of one copy of that gene is sufficient to mediate this effect. The early prenatal lethality produced by PFOA exposure is independent of PPARα expression. The effects of PFOA eye opening and postnatal weight gain also appear to depend on PPARα expression, but there is a possibility that at higher exposures PPARα-independent mechanisms are involved and the mode of action for these parameters may be multifactorial. There is a strain-dependent difference in the sensitivity to PFOA-induced developmental toxicity between the 129S1/SvlmJ WT mice used in the present study and the CD-1 mice of previous studies. PPARα activation clearly plays an important role in the response to PFOA in the developing mouse and there is a need for further investigations into the mechanisms through which PPARα mediates PFOA-induced developmental toxicity.

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