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Perfluorooctane sulfonate-induced changes in fetal rat liver gene expression

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ABSTRACT

In utero exposure of laboratory rats to perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$), a chemically stable surfactant that is widely disseminated in the environment and present in serum samples from wildlife and humans, is associated with decreased neonatal survival, and growth deficits as well as hepatomegaly. This hepatomegaly in newborn rats exposed to PFOS *in utero* resembles that observed in adults and is characterized by peroxisome proliferation and decreased liver triglycerides, both of which are suspected to be manifested through PPARα-mediated transcriptional regulation. The purpose of the present investigation was to determine whether these changes in metabolic status are a reflection of transcriptional changes in fetal rat liver using global gene expression array analyses. Gravid Sprague–Dawley rats were administered 3 mg/kg PFOS by gavage daily from gestational day 2–20 and terminated on day 21. Although there was no treatment-related frank terata, there was a substantial effect of PFOS on the perinatal hepatic transcriptome—225 unique transcripts were identified as statistically increased and 220 decreased by PFOS exposure; few transcripts were changed by more than two-fold. Although the PPARα transcript (Ppara) itself was not affected, there was a significant increase in expression of gene transcripts associated with hepatic peroxisomal proliferation as well as those responsible for fatty acid activation, transport and oxidation pathways (both mitochondrial and peroxisomal). Additional metabolic pathways altered by *in utero* PFOS exposure were a stimulation of fetal hepatic fatty acid biosynthesis and a net reduction of Cyp7a1 transcript, which is required for bile acid synthesis. There were minimal effects on the expression of thyroid-related gene transcripts. In conclusion, gene expression analysis provides strong evidence indicating transcriptional control of the altered metabolic status of neonates following PFOS exposure *in utero*, much of which appears to be under the influence of a functional perinatal PPARα regulatory pathway.

1. Introduction

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$), which has been used for its strong surface tension reducing characteristics and chemical stability, is also the final metabolic and environmental degradation product of materials made from perfluorooctane sulfonyl fluoride that were used widely in applications requiring resistance to soil, grease, and water, e.g., food packaging and fabric stain-repellants as well as flame retardant foams (Tomy et al., 2004; Xu et al., 2004; Moriwaki et al., 2005). In addition to being exceptionally stable to environmental and metabolic degradation, PFOS is not easily cleared from the body and has serum elimination half-lives of months in rats and monkeys (Seacat et al., 2002, 2003) to several years in humans (Olsen et al., 2007). In 2000, 3M Company, the primary manufacturer of PFOS and materials that can degrade to PFOS, made the decision to discontinue manufacture of these materials based on confirmation of the wide-spread environmental distribution and persistence of PFOS (Renner, 2001). PFOS has been detected in samples from wildlife and humans throughout the world, and many of these studies have been reviewed in Butenhoff et al. (2006), Houde et al. (2006), and Lau et al. (2007). Evidence for a significant decline in serum PFOS concentrations in the United States general population since 3M's decision to cease manufacturing has been reported by Calafat et al. (2007a,b) and Olsen et al. (2008).

The toxicity and hazard profile for PFOS has been reviewed (OECD, 2002; Seacat et al., 2002, 2003; Lau et al., 2004, 2007). At sufficiently high concentrations, subchronic dietary exposure of adult rats or monkeys to PFOS results in a multitude of changes associated with disruption of carbohydrate and lipid metabolism (Ikeda et al., 1985; Haughom and Spydevold, 1992; Seacat et al., 2002, 2003). In rats, PFOS intoxication exhibits a steep, threshold-limited

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dose-response that is most characteristically associated with arrest of weight gain accompanied by centrilobular hepatocellular hypertrophy with cytoplasmic vacuolation, mild-to-moderate proliferation of hepatic peroxisomal bodies and stimulation of peroxisomal acylCoA oxidase activity, hypocholesterolemia, and increased incidence of hepatocellular adenomas that is believed to result from a nongenotoxic mechanism.

The reproductive and developmental toxicology of PFOS has also been reviewed in recent years (Lau et al., 2004, 2007). Notably, *in utero* exposure to PFOS has been reported to cause neonatal mortality in rats and mice, in a dose-dependent manner (Grasty et al., 2003a,b; Luebker et al., 2005a,b; Lau et al., 2003). Decreased postnatal growth, hypothyroxinemia (Lau et al., 2003; Thibodeaux et al., 2003; Luebker et al., 2005b), and decreased liver triglycerides (Luebker et al., 2005b) were observed in survivors. These effects occur without remarkable frank terata at doses below those that cause maternal toxicity (Lau et al., 2004; Case et al., 2001). The mode of action for the observed neonatal mortality continues to be investigated but may involve respiratory distress at birth (Grasty et al., 2005).

The accumulation of body burden of PFOS on repeated exposure of rats is reflected in the fact that morbidity and mortality is a function of cumulative dose (Luebker et al., 2005b; Grasty et al., 2003a,b). Grasty et al. (2003a,b) explored critical windows of prenatal development in rats and found that exposure during late gestation produced a high incidence of pup mortality within the first 5 days after birth. Moving the 4-day window of exposure to earlier in gestation was associated with progressively less neonatal mortality. Of particular note was that maternal and pup serum concentrations at birth that were associated with postnatal morbidity were on the order of 100 μg/mL. This is consistent with the data from of Luebker et al. (2005b) that indicate that rat maternal serum PFOS concentrations greater than 100 μg/mL in the perinatal period are associated with decreased neonatal survival. Of significance is that the Luebker et al. study design dosed maternal rats for six weeks prior to mating, and through mating, gestation, and lactation; whereas the Grasty et al. study only treated the pregnant rats for 4-day intervals. This abrupt departure from linearity in dose–response corresponds to the same dose-dependence observed in adult rats and monkeys (Seacat et al., 2003, 2002) and suggests that cumulative dose during critical windows of development, irrespective of the dosing regimen, is the definitive determinant of PFOS toxicity.

Although prenatal exposure to PFOS in rats can cause serious detriment to viability, hepatocellular hypertrophy persists in both rats and mice that survive the first month of post natal life following *in utero* exposure to high doses of PFOS (Lau et al., 2003). One prevailing theory to explain the metabolic effects associated with PFOS intoxication in laboratory animals is that it is a ligand for the peroxisome proliferator activated receptor alpha (PPARα), a nuclear receptor that regulates the expression of genes involved in lipid and carbohydrate metabolism, peroxisome proliferation and cell replication (Corton et al., 2000). PFOS has been shown to activate both mouse and human PPARα in a Cos-1 cell-based luciferase reporter trans-activation assay (Shipley et al., 2004). In addition, Takacs and Abbott (2007) demonstrated that PFOS also transactivates PPARβ/δ, but not PPARγ in both mouse and human transfected Cos-1 cells. Using a nuclear receptor ligand-binding domain/Gal4 DNA-binding domain chimeric reporter system, Vanden Heuval et al. (2006) compared activation of PPAR isoforms from humans, rats, and mice by PFOS to activation by natural ligands. PFOS activated PPARα and γ from all three species in this reporter system but to a much lesser extent than natural ligands. PPARβ/δ was not activated by PFOS. Activation of PPARα and expression of the corresponding gene targets by PFOS is consistent with the observed induction of PPARα–controlled enzyme activities, including peroxisomal bifunctional enzyme, 3-ketoacyl-CoA thiolase, and acyl-CoA oxidase activities, as well as the stimulation of peroxisome proliferation that has been observed both *in vitro* and *in vivo* in rodents (Ikeda et al., 1985; Berthiaume and Wallace, 2002; Shipley et al., 2004). Accordingly, PPARα is considered to be an important mediator of PFOS-induced disruption of metabolic homeostasis in adult experimental animals.

The purpose of the current investigation was to explore the transcriptional profile associated with the perinatal hepatotoxicity of PFOS using global gene expression analysis. The expectation was that rat pups exposed *in utero* to doses of PFOS known from prior studies to produce responses would exhibit altered hepatic gene expression, which is concordant with the altered metabolic state that has been reported in surviving neonate and adult animals. Of particular focus was the effect of prenatal exposure to PFOS on PPARα-regulated fatty acid metabolism, cholesterol homeostasis and thyroid hormone status.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats were bred for 4 h in the afternoon on gestational day 0 (GD0) at Charles River Laboratories (Raleigh, NC). Sperm positive animals were delivered to the U.S. EPA facilities on GD1 and the dams were treated from GD2 through GD20 with 3 mg/kg PFOS prepared fresh daily in 0.5% Tween-20 by oral gavage. This same dose administered throughout gestation (GD2–GD20) causes a 60% mortality of pups within the first 3 days post-partum, with newborn serum PFOS concentrations of ca., 85 ppm (Lau et al., 2003). Surviving pups exhibit hepatocellular toxicity similar to that observed in adults and believed to be mediated in large part via activation of PPARα. This dosing regimen was chosen to determine whether the metabolic, and perhaps pathogenomic, consequences of PFOS exposure *in utero* are subject to transcriptional regulation. Control dams received an equivalent volume (1 mL/kg) of 0.5% Tween-20 vehicle daily. On GD 21, 24 h after the final PFOS treatment, dams were weighed then sacrificed by decapitation. Fetal livers were quickly removed and weighed. Livers from all the fetuses within each litter were pooled, flash-frozen, and stored at −80 °C.

For age comparison purposes, adult male Sprague–Dawley rats were administered a single intraperitoneal (IP) injection of PFOS (100 mg/kg in DMSO); controls received DMSO vehicle (1 mL/kg). On day three post-exposure, the rats were sacrificed by decapitation and the livers were quickly excised, flash frozen in liquid nitrogen, and stored at −80 °C until RT-PCR analysis. All animal husbandry procedures were in accordance with and closely monitored by the respective institutional animal care committee. Studies were performed in laboratories accredited by International Association for the Accreditation of Laboratory Animal Care. All procedures involving rats were reviewed and approved by Institutional Animal Care and Use Committees. Animals care and procedures followed the US Department of Health and Human Services Guide for the Care and the Use of Laboratory Animals Guidelines (Institute of Laboratory Animal Resources, 1996).

2.2. RNA isolation

Total liver RNA was isolated from 6 control (Ctl) and 6 PFOS-treated (Tx) litters using RNAwiz (Ambion, TX). 30–60 mg of frozen tissue was pulverized with mortar and pestle in liquid nitrogen, immediately homogenized in 10 volumes RNAwiz using a ground glass Duall tissue grinder, and processed according to the manufacturer's instructions. The RNA was further purified using RNeasy Mini Kit (Qiagen, CA) as described by the manufacturer's RNA clean-up protocol. RNA pellet from the organic extraction was resuspended in 100 μl of water and loaded onto RNeasy columns. RNA was eluted from the columns with 30 μl water and then quantitated spectrophotometrically. A ratio of absorbance at 260 nm/280 nm greater than 1.6 was considered acceptable for use in the microarray experiment. The quality of the RNA from samples that met the absorbance criterion was further assessed using a Bioanalyzer 2000 (Agilent, CA). Clearly defined 28s and 18s ribosomal RNA peaks with a 28s/18s ratio greater than 1.5, and no peaks greater than half the 18s peak height was considered acceptable for gene array analysis.

2.3. cRNA preparation

Purified RNA samples were processed according to the manufacturer's instructions (Affymetrix, 701021 Rev. 4, CA). 8 μg of total RNA was used in a reverse transcription and second strand cDNA synthesis reaction using the SuperScript Choice System (Life Technologies, MD) with the GeneChip T7-oligo(dT) promoter primer kit (Affymetrix, CA). The temperature adjustment after primer hybridiza-