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Perfluoroalkyl acids-induced liver steatosis: Effects on genes controlling lipid homeostasis

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ABSTRACT

Persistent presence of perfluoroalkyl acids (PFAAs) in the environment is due to their extensive use in industrial and consumer products, and their slow decay. Biochemical tests in rodent demonstrated that these chemicals are potent modifiers of lipid metabolism and cause hepatocellular steatosis. However, the molecular mechanism of PFAAs interference with lipid metabolism remains to be elucidated. Currently, two major hypotheses are that PFAAs interfere with mitochondrial beta-oxidation of fatty acids and/or they affect the transcriptional activity of peroxisome proliferator-activated receptor α (PPARα) in liver. To determine the ability of structurally-diverse PFAAs to cause steatosis, as well as to understand the underlying molecular mechanisms, wild-type (WT) and PPARα-null mice were treated with perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), or perfluorohexane sulfonate (PFHxS), by oral gavage for 7 days, and their effects were compared to that of PPARα agonist WY-14643 (WY), which does not cause steatosis. Increases in liver weight and cell size, and decreases in DNA content per mg of liver, were observed for all compounds in WT mice, and were also seen in PPARα-null mice for PFOA, PFNA, and PFHxS, but not for WY. In Oil Red O stained sections, WT liver showed increased lipid accumulation in all treatment groups, whereas in PPARα-null livers, accumulation was observed after PFNA and PFHxS treatment, adding to the burden of steatosis observed in control (untreated) PPARα-null mice. Liver triglyceride (TG) levels were elevated in WT mice by all PFAAs and in PPARα-null mice only by PFNA. *In vitro* β-oxidation of palmitoyl carnitine by isolated rat liver mitochondria was not inhibited by any of the 7 PFAAs tested. Likewise, neither PFOA nor PFOS inhibited palmitate oxidation by HepG2/C3A human liver cell cultures. Microarray analysis of livers from PFAAs-treated mice indicated that the PFAAs induce the expression of the lipid catabolism genes, as well as those involved in fatty acid and triglyceride synthesis, in WT mice and, to a lesser extent, in PPARα-null mice. These results indicate that most of the PFAAs increase liver TG load and promote steatosis in mice We hypothesize that PFAAs increase steatosis because the balance of fatty acid accumulation/synthesis and oxidation is disrupted to favor accumulation.

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1. Introduction

Perfluoroalkyl acids (PFAAs) such as perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorohexane sulfonate (PFHxS) are persistent environmental contaminants (Giesy and Kannan, 2001). Perfluoroalkyl sulfonyl chloride-based intermediates and fluorinated telomer alcohols are probable precursor compounds that may

Abbreviations: PFAAs, Perfluoroalkyl acids; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonate; TG, triglycerides; WY, Wyeth 14,643; WT, wild type; Null, PPARα-null; GEO, Gene Expression Omnibus; PC, palmitoylcarnitine; CPT, acylcarnitine transporter; DNP, 2,4,-dinitrophenol; C-228, surfactant mixture; LPL, lipoprotein lipase; LDL, Low density lipoprotein; VLDL, Very low density lipoprotein; PPAR, peroxisome proliferator-activated receptors.

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undergo transformation reactions in the environment leading to the formation of these bioaccumulative and potentially toxic perfluoroalkyl substances (D'Eon et al., 2006; Dinglasan et al., 2004). Owing to their extraordinary stability, perfluorinated compounds have been used in a variety of industrial and consumer products (Eschauzier et al., 2013; Fromme et al., 2007; Kissa 2001; Renner 2001; Vestergren et al., 2012). These chemicals have been detected ubiquitously and are known to be persistent in the environment. In addition, PFAAs are distributed in human populations worldwide (Calafat et al., 2007; Ode et al., 2013) as well as in a variety of wildlife (Lau et al., 2007). Adverse effects of PFAAs have been reported in laboratory animals and wildlife. These include reproductive and developmental toxicity, immunotoxicity, hepatotoxicity, tumor induction, neurotoxicity, and endocrine disruption (DeWitt et al., 2015; Lau, 2012; Lau et al., 2007). Definitive links between exposure to PFAAs and adverse health outcomes in humans have not been established, although biomonitoring and epidemiological studies in highly exposed as well as general human populations have indicated positive associations between body burdens of PFOS and PFOA and untoward health effects such as increases of serum cholesterol, low density lipoprotein (LDL), and uric acid as well as altered liver enzyme activities. Compounded with the extraordinary persistence of the long-chain PFAAs in humans (with blood serum half-lives in years (Lau, 2012)), considerable public health concerns of these chemicals have been raised.

A growing number of studies in animals have linked PFAA exposure to fatty liver disease, also called hepatic steatosis, which is characterized by excessive triacylglycerol accumulation within hepatocytes. Fatty liver disease is the most common liver disease in humans, encompassing a spectrum of hepatic steatosis that can progress to an inflammatory state (steatohepatitis), sometimes leading to cirrhosis and hepatocellular carcinoma (Kawano and Cohen, 2013). Fatty liver disease is often the result of a complex combination of increased energy uptake, increased hepatic lipogenesis, decreased energy combustion, and decreased hepatic secretion of liver triglycerides (TG) (Al-Eryani et al., 2015). The importance of steatosis as an endpoint of regulatory significance is highlighted by the fact that the steatosis endpoint has been used as the critical effect in EPA's Integrated Risk Information System (IRIS) assessments to determine exposure limits for a number of chemicals (Kaiser et al., 2012).

Previous studies have shown that PFOS (Bijland et al., 2011; Martin et al., 2007; Wan et al., 2012; Wang et al., 2014) and PFOA (Haughom and Spydevold 1992; Kudo and Kawashima 1997; Martin et al., 2007; Tan et al., 2013; Wang et al., 2013) cause extensive micro- and macro-vesicular steatosis in hepatocytes and an attendant, accumulation of TG in the mouse liver. One suggested mechanism of chemical-induced steatosis is an inhibition of mitochondrial fatty acid β-oxidation in which fatty acids are diverted to TG synthesis (Begriche et al., 2013). PFOS has been shown to inhibit mitochondrial fatty acid β-oxidation in mouse liver (Wan et al., 2012). Although additional studies indicate that other PFAAs cause increases in liver TG (Bijland et al., 2011; Wang et al., 2014), more studies are needed to establish the relationships between alterations in lipid homeostasis genes including mitochondrial fatty acid β-oxidation, increases in TG, and steatosis for structurally-diverse PFAAs.

The primary targets of PFAAs include peroxisome proliferator-activated receptors (PPARs). PPARs are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily. Three subtypes designated as α, β/δ, and γ exist, all of which are involved in lipid homeostasis (Desvergne and Wahli, 1999). The PPARα subtype is activated by peroxisome proliferator chemicals (PPCs), a large class of structurally heterogeneous pharmaceutical and industrial chemicals originally identified as inducers of the size and number of peroxisomes in rodent livers. PPARα plays a dominant role in mediating the effects of hypolipidemic and xenobiotic PPCs in the liver (Corton et al., 2014). Activation of PPARα results in a predictable set of phenotypic responses in the livers of rats and mice, including short-term responses of hepatocyte peroxisome proliferation, hepatomegaly, and hepatocyte hyperplasia. PPARα regulates a large battery of genes critical for the therapeutic hypolipidemic effects of PPARα targeted drugs. These include those encoding enzymes involved in fatty acid oxidation in the peroxisome, mitochondria, and microsomes (Kersten, 2014). Administration of PPARα agonists, such as the widely prescribed fibrate drugs clofibrate, gemfibrozil, and fenofibrate, ameliorate hyperlipidemia in humans (Staels et al., 1998) and hepatic steatosis in mice (Harano et al., 2006). Examination of phenotypic and transcriptional effects of PFAAs in wild-type and PPARα-null mice have clarified the role of PPARα in mediating many of the effects of PFAAs. PPARα is required for the majority of transcriptional effects after exposure to PFOA, PFOS, PFNA, and PFHxS (Oshida et al., 2015b; Ren et al., 2009; Rosen et al., 2008b, 2010). An examination of the PPARα-independent genes altered by PFAAs indicated their regulation by a number of other nuclear receptors including other PPAR family members as well as the xenobiotic-activated receptor CAR (Bjork et al., 2011; Oshida et al., 2015a; Rosen et al., 2008a).

The present study was designed to determine whether structurally-diverse PFAAs increase liver TG levels and cause steatosis as well as to identify the underlying mechanisms, we evaluated liver histology and biochemistry after a subchronic PFAA exposure. We capitalized on the availability of a large number of microarray comparisons between WT and PPARα-null mice exposed to PFAAs or hypolipidemic agents allowing a global analysis of the pathways that regulate TG levels in the hepatocyte. In addition, the effects of a number of PFAAs on mitochondrial fatty acid β-oxidation were measured to determine if inhibition was a common event shared by the PFAAs. Our work demonstrated that all PFAAs tested caused steatosis, which may be mediated in part by increased expression of fatty acid and triglyceride synthesis genes but not by inhibition of mitochondrial fatty acid transport or β-oxidation.

2. Materials and methods

All chemicals used in this experiment were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Perfluorononanoic acid (PFNA, 97% pure) and perfluorooctanoic acid (PFOA-NH_4^+, 97% pure) purchased from Sigma (St. Louis, MO), and perfluorohexane sulfonate (PFHxS-K^+, 97% pure) from 3 M (St. Paul, MN), were dissolved in deionized water to prepare stock solutions that were then diluted as per requirements of the experiment. For all studies, both stock and diluted solutions were prepared fresh daily.

2.1. Animal treatments

Adult male SV129 wild-type (WT) and peroxisome proliferator-activated receptor alpha (PPARα)-null (Null) male mice (Jackson Lab) were maintained as colonies in-house at US EPA. All animal studies were conducted in accordance with the Institutional Animal Care and Use Committee guidelines established by the National Health and Environmental Effects Research Laboratory U. S. Environmental Protection Agency Office of Research and Development. Procedures and facilities were consistent with the recommendations of the 1996 National Research Council's "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. Animal facilities were controlled for temperature (20–24 °C), relative humidity (40–60° C) and 12-h

light-dark cycle. Four animals per group were given PFOA, PFNA, or PFHxS, once daily for 7 days by oral gavage at 10 mg/kg. Wyeth 14,643 (WY) (Sigma Aldrich, St. Louis, MO) at 50 mg/kg was used as positive control. The weight of each animal was recorded daily during 7-day treatment. Mice from each dosage group, along with concurrent controls, were sacrificed 24 h after the last treatment. At necropsy, serum and liver samples were processed accordingly for biochemical assays.

2.2. Histology and morphometric analysis

Samples from the large lobe of each liver were collected and flash-frozen in liquid nitrogen, and stored at −80° C. Six μm thick frozen sections were prepared, thaw-mounted onto glass slides, stained with either Oil Red O or Mayer's Hematoxylin (Sigma-Aldrich), and two regions of each section were photographed. Morphometric analysis was performed using ImagePro Plus (Media Cybernetics, Inc., Rockville, MD) to quantify the sum of the areas of the sections that were stained red (total lipid area in μm^2) and the total area occupied by the cells (total area of the image minus the total area without cells) from the Oil Red O stained sections. Percent lipid for each section was calculated as the total area occupied by lipid divided by the total area occupied by cells. The Hematoxylin stained sections were used to calculate the average cell size in μm^2 by determining the total number of cells in the image and the total area occupied by cells (as above). The average cell size was calculated as total area occupied by cells divided by total number of cells in that image. Data were generated from 4 animals of each genotype and treatment group. GraphPad Prism version 4 (GraphPad Software, Inc, San Diego, CA) was used to analyze and graph the data. The data from the two regions of each liver were combined to obtain the mean for further calculations or statistical analysis. The data were analyzed with 2-way ANOVA and Bonferroni post test to determine strain and treatment effects and interactions, and also one-way ANOVA and Dunnett's multiple comparison test to evaluate treatment effects within the strain.

2.3. Liver triglyceride assays

Liver samples from the large lobe from each animal were collected and stored at −80 °C for the triglyceride assay. Liver TG was measured using a Triglyceride Assay Kit from Zen-bio Inc. (Research Triangle Park, NC). Briefly, liver tissue and wash buffer (1:5) was sonicated for 15 s in cold. Diluted homogenate (150 μl) was added into each well of a 96-well costar plate for TG assay, and remaining homogenate was used to assay DNA concentration. Twenty μl of Reagent B (supplied in the kit) was added to each well and the manufacturer's recommended procedures were used to assay TG in the plate which was incubated overnight at room temperature. The morning, plates were read on a plate reader (FluroStar Omega, BMG Lab tech) at 540 nm. TG concentrations were determined by regression analysis of the standards and interpolation of a value for each well. Three biological replicates on each independent plate were averaged and linear regression analysis was performed to determine the trends across treatment for triglyceride concentration. One-way ANOVA with Dunnett's multiple comparison test was used to determine significance for each treatment relative to control.

2.4. Liver DNA assay

Assay for DNA content was performed according to (Labarca and Paigen, 1980). Briefly, 5 μl of a (1:10) homogenate sample (described in the TG assay) was added into each well of a 96-well plate containing 188 μl of high salt buffer (2.2 M NaCl, 10 mM Tris, 1.5 mM EDTA; pH 7.6). The plate was covered and sealed with parafilm and incubated at 4 °C for overnight. After incubation, 7 μl of a 3 μg/ml Hoechst 33258 dye stock (Sigma Chemical) was added into each well and mixed. The samples and calf thymus DNA (Sigma Chemical) standards, with a range of 50 ng to 3 μg, were read on a plate reader (FluroStar Omega, BMG Lab tech) with an excitation of 355 nm and an emission of 456 nm. Total DNA content per well was determined by regression analysis of the standard curve and interpolation of a value for each well. Three biological replicates on each independent plate were averaged and linear regression analysis was performed to determine trends across treatment and determine significance for each treatment relative to control by one-way ANOVA with Dunnett's multiple comparison test.

2.5. Palmitate oxidation by human liver cells in culture

HepG2/C3A human liver cells were purchased from American Tissue Culture Collection (ATCC; Manassas, VA) and grown in 75 cm^2 flasks with Eagle's Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5%CO_2 for several passages. Two days prior to the experiment, cells were plated at 40 K per well on Seahorse Bioscience® XF96 cell culture plates and allowed to attach overnight in EMEM growth media. On the day prior to the assay, the media was switched to a substrate limited media of Dulbecco's Modified Eagle Medium (DMEM) with1% FBS, 0.5 mM glucose, 1 mM glutamine and 0.5 mM carnitine to which dilutions of 0–50 μM PFOA or PFOS were added. On the day of the assay, the cells were equilibrated in a non-CO_2, 37 °C incubator for 45 min in the assay media consisting of Krebs-Hensleit buffer (pH 7.4) with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES. 40 μM etomoxir, an inhibitor of mitochondrial fatty acid oxidation, was added to control wells for an additional 15 min after which either 0.17 mM palmitate-0.03 mM BSA or 0.03 mM fatty acid free BSA was added such that samples consisting of +/−palmitate, and +/−etomoxir were measured at all PFAA concentrations. The assay itself consisted of sequential additions of 1 μM oligomycin, 2 μM FCCP and 0.5 μM rotenone/antimycin A (final concentrations), recording the rate of oxygen consumption between intervals of addition. As described in the manufacturers' instructions, oligomycin inhibited the basal rate of cell respiration whereas as the uncoupler FCCP stimulated cell respiration which was blocked by adding the combination of rotenone and antimycin A. In all cases, the rate of cell respiration was suppressed either in the absence of palmitate or in the presence of etomoxir, a potent and irreversible inhibitor of carnitine palmitoyltransferase (1 CPT1).

2.6. Isolation of mitochondria

Mitochondria were isolated from liver of adult male Sprague-Dawley rats (~200 g body weight) by a conventional differential centrifugation procedure (Henry and Wallace, 1995). Animals were killed by decapitation. Liver was excised, weighed and cooled in 40 ml of isolation medium (210 mM mannitol, 10 mM sucrose, 5 mM HEPES-KOH (pH 7.4), 1 mM EGTA). Cooled liver was minced with scissors and washed twice with 20 ml of isolation medium, then suspended in the same medium (1 g/8 ml) and homogenized for 1 min with a motor-driven Potter homogenizer (Teflon pestle, glass beaker). The homogenate was filtered through gauze and centrifuged for 10 min at 700 *g* and 4 °C, and the mitochondrial pellet recovered from the supernatant by centrifugation at 10,000*g* × 10 min. The pellet was resuspended in 10 ml of washing medium (210 mM mannitol, 10 mM sucrose, 5 mM HEPES-KOH, pH 7.4) supplemented with fatty acid free bovine serum albumin (BSA,

1 mg/ml). The suspension of mitochondria was diluted to 35 ml with the same medium without BSA, and centrifuged at 10,000g × 10 min. The final mitochondrial pellet was resuspended in washing medium to a protein concentration of 70–80 mg/ml and stored on ice until used that same day. Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a calibration standard.

2.7. Mitochondrial respiration

Mitochondrial respiration was determined polarographically using a Clark-type oxygen electrode (Model 53; Yellow Springs Instrument Co., Yellow Springs, OH) connected to a continuous strip chart recorder as described originally by Solem et al. (Solem et al., 1994). The reaction medium consisted of 0.8–1.0 mg

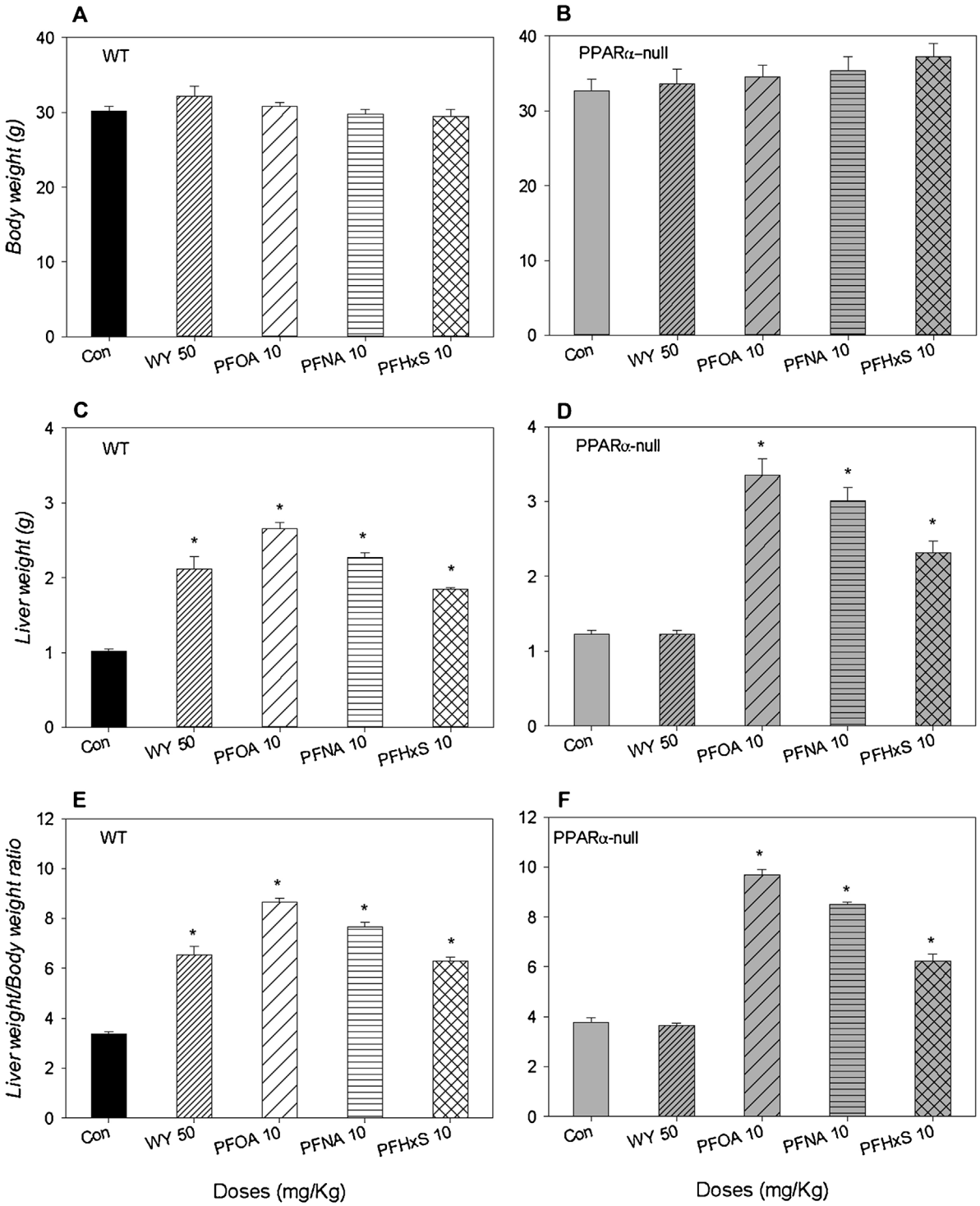

Fig. 1. Effect of PFOA, PFNA, and PFHxS on body weight and liver weight.
A and B. Body weights. C and D. Absolute liver weights. E and F. Liver to body weights ratio. One-way ANOVA with Dunnett's multiple comparison test was used to determine significance for each treatment relative to control. Data are mean ± SE, where n = 4, * represents $p < 0.0001$.

mitochondrial protein per ml of a medium comprising 200 mM mannitol, 10 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, 2 μM oligomycin and 10 mM KH_2PO_4. Mitochondria were energized by adding palmitoylcarnitine (PC) to a final concentration of 40 μM. Glutamate + malate (5 mM each) and succinate (5 mM) + rotenone (2 μM) were premixed and injected as single additions where indicated. The final concentration of 2,4-dinitrophenol (DNP) was 40 μM. Perfluorinated compounds were dissolved in absolute ethanol and added to mitochondria as 1.8 μl of a 50 mM stock solution in ethanol to a total volume of 1.8 ml (final concentration, 50 μM). At this concentration (0.1%), ethanol by itself did not interfere with mitochondrial respiration. "C-228" represents a mix of surfactants of different chemistries that was used as a positive control for a generalized mitochondrial membrane solubilizing activity. Experiments were carried out in triplicate for the following seven perfluoroalkyl compounds; perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorooctane sulfonamide (PFOSA), perfluorooactane sulfamidoacetate (PFOSAA), N-ethyl perfluorooctane sulfonamide (N-EtPFOSA), N-ethyl perfluorooctane sulfamidoacetate(N-EtPFOSAA), and N-ethyl perfluorooctane sulfamido ethyl alcohol(N-EtFOSE).

2.8. RNA preparation

Liver samples from the large lobe from each mouse were collected and stored in RNAlater (Life Technologies Grand Island, NY, cat# AM7021) until extraction. Briefly, liver tissue was homogenized in 1 ml of TRI reagent (Sigma/Aldrich, St. Louis, MO cat# T9424) with 1 mm zirconium silicate beads in a Bullet Blender 24 (Next Advance, Inc., Averill Park, NY). Extraction was performed according to manufacturer's protocol. RNA pellets were resuspended in 100 μl of nuclease-free H_2O, and quantified with Ribogreen Quantitation Kit according to the manufacturers protocol (Life Technologies). The quality of the RNA was verified by 2100 Bioanalyzer RNA 6000 Nano kit (Agilent 5067-1511).

2.9. Gene expression analysis

All of the RNA samples were treated with DNase I and quantified using the Ribogreen Quantitation Kit (Life Technologies). DNase I treated RNA was reverse transcribed (ABI cDNA Archive Kit 4322171) and 25 ng equivalent cDNA was amplified in a 12 μl volume using ABI TaqMan Gene Expression Assays (Supplemental Table 1) and ABI Universal Master Mix 4304437. Amplification was performed on an ABI model 7900HT sequence detection system. All samples were run in technical duplicate. β-actin (22.16 ± 0.072) was used as the endogenous control since it did not show significant change among all samples. Data were analyzed by relative gene quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.10. Identification of differentially expressed genes in NextBio microarray datasets

A commercially available gene expression database (http://www.nextbio.com) facilitated the assembly of expression changes in lipid homeostasis genes after exposure to PPARα activators or between PPARα-null and wild-type mice. The NextBio database contains over 123,000 lists of statistically filtered genes from over 18,800 microarray studies carried out in 16 species (as of June 2015). Raw microarray data came mostly from publicly available submissions in Gene Expression Omnibus (GEO). All differentially regulated genes were identified using the criteria in the NextBio analysis pipeline and are described in details in Kuperschmidt et al. (2010). All lists of differentially regulated genes are annotated for Biodesign, Biosource, Chemical Name, Gene, Gene Mode, Phenotype, Tissue, and Study ID facilitating the identification of biosets used in the present analysis.

2.10.1. Additional computational analyses

Heat maps were generated using Treeview software (http://rana.lbl.gov/EisenSoftware.htm).

2.11. Statistical analysis

All results were statistically analyzed using GraphPad Prism (see Materials and methods section). Differences between control and treatment groups were determined using one- or two-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test to determine significance for each treatment relative to control, or Bonferroni post test to determine strain and treatment effects and interactions. All experimental data were represented as means with standard errors. P values < 0.05 were considered significant.

3. Results

3.1. PFAAs increase steatosis and liver triglycerides

To test the hypothesis that PFAAs cause alterations in lipid homeostasis leading to steatosis, wild-type and PPARα-null mice were exposed to one of four PFAAs for 7 days. Mice were also exposed to the prototypical PPARα activator WY-14,643 (WY), which is known to induce most, if not all, cellular and transcriptional effects through activation of PPARα (Corton et al., 2014). Fig. 1A and B shows that treatment for seven days had no impact on body weights either in wild type (WT) or in PPARα-null mice, in agreement with the previous data from our laboratory for PFOA and PFOS (Rosen et al., 2010; Wolf et al., 2008). Absolute liver weights and liver to body weight ratios were significantly increased in all treatment groups in wild-type mice (Fig. 1C and E). These increases did not occur in PPARα-null mice treated with WY but were observed in PPARα-null mice exposed to PFOA, PFNA, and PFHxS (Fig. 1D and F). Morphometric analysis showed significantly increased cell size and significantly decreased DNA content per mg liver weight in WT mice treated with each chemical (Fig. 2A and C) indicating hepatocyte hypertrophy. In PPARα-null mice, cell size was significantly increased and DNA content was significantly decreased after PFOA, PFNA, or PFHxS treatment (Fig. 2B and D), while exposure to WY in PPARα-null mice had no effects on these parameters.

Livers were examined for changes in lipid content and cellular morphometry by Oil Red O staining (Fig. 3A–J). Little, if any, staining was observed in the control livers from wild-type mice. In contrast, the livers from control PPARα-null mice exhibited extensive steatosis consistent with other studies (Corton et al., 2014). In wild-type mice, all chemicals led to varying degrees of steatosis. PFNA and PFHxS exposures lead to striking Oil Red O staining, while PFOA exposures led to milder effects. Surprisingly, WY also led to mild induction of steatosis. Given the extensive background steatosis in the control PPARα-null mice, it was difficult to observe any further increases in staining between the control and treated PPARα-null mice in the absence of quantitation. Morphometric analysis showed significantly increased staining in WT liver in all treatment groups (Fig. 3K). Staining was dramatically increased in control PPARα-null mice compared to control wild-type mice. In PPARα-null livers, increases in chemical-induced staining over control levels were observed after PFNA and PFHxS but not WY or PFOA exposures. Significantly elevated TG levels were found in all PFAA-exposed wild-type mice (Fig. 4). In control PPARα-null mice, there was an increase in the TG content over that in control wild-type mice ($p < 0.05$). Treatment

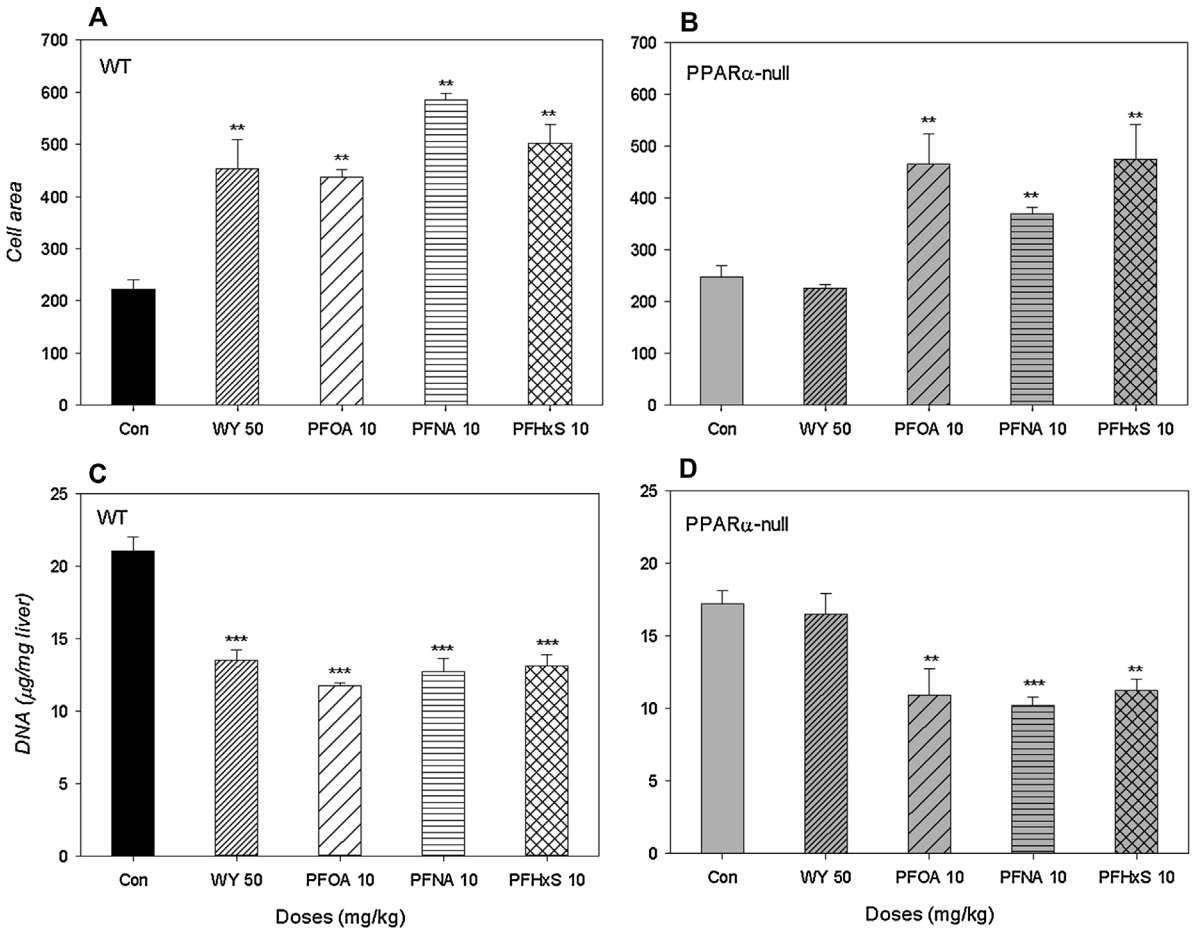

Fig. 2. Determination of cell size and DNA content after exposure.
A and B. Analysis of cell area. The data were analyzed with 2-way ANOVA and Bonferroni post test to determine strain and treatment effects and interactions, and also one-way ANOVA and Dunnett's multiple comparison test to evaluate treatment effects within the strain. C and D. Analysis of DNA content in ug DNA per mg of liver. One-way ANOVA with Dunnett's multiple comparison test was used to determine significance for each treatment relative to control. Data are mean ± SE, where n = 4, ** represents $p < 0.01$ and *** represents $p < 0.001$.

with PFOA or PFNA further increased the TG content in the PPARα-null mice with only PFNA increases becoming significant. Exposure to WY or PFHxS did not further increase TG content in the PPARα-null mice. In summary, all of the PFAAs increased steatosis and TG accumulation in the wild-type mouse livers. Only PFNA also increased steatosis and TG accumulation in PPARα-null mice (Table 2).

3.2. Effect of PFAAs on mitochondrial fatty acid β-oxidation

The efficiency of fatty acid β-oxidation in rat liver mitochondria can be estimated by measuring the rate of oxygen consumption in the presence of palmitoylcarnitine (PC). This substrate is imported into the mitochondrial matrix by the acylcarnitine transporter (CPT1 and CPT2) and subsequently oxidized through the β-oxidation pathway in the matrix of mitochondria. Inside the matrix space, PC is converted into palmitoyl-CoA by carnitine acyltransferase II, which is then oxidized to acetyl-CoA to deliver reducing equivalents to two complexes of the respiratory chain, Complex I and Complex III. Under conditions of uncoupled respiration (following the addition of 2,4,-dinitrophenol (DNP)), the maximal rate of mitochondrial respiration in the presence of PC is a direct measure of the activity of the β-oxidation multienzyme complex and/or the rate of PC penetration into mitochondrial matrix. Hence, if a compound inhibits the transport of PC, the activity of carnitine acyltransferase II in the mitochondrial matrix, and/or the β-oxidation enzymes, the compound would decrease the rate of uncoupled respiration. None of the perfluorochemicals tested decreased the rate of PC-supported respiration in the presence of DNP, indicating that none of the compounds inhibited either the transport, intramitochondrial activation, or β-oxidation of fatty acids by isolated rat liver mitochondria in vitro. A compound may also inhibit Complex I or Complex III directly, which may complicate the interpretation of the data. However, this complication can be resolved by comparing the rates of palmitoylcarnitine oxidation by uncoupled mitochondria to that of alternate substrates of Complex I and Complex III, such as glutamate/malate or succinate.

The above mentioned considerations were taken into account in designing our experimental procedure, as illustrated in Fig. 5: a) control curve with maximum uncoupling using DNP and specific complex I (glutamate + malate) and III (succinate +rotenone) activators, b) mild uncoupling with a surfactant mixture (C-228, c) maximum uncoupling with test compound (PFOSA), and d) mild uncoupling combined with inhibition of Complex I/III with test compound (PFOSAA). First, we estimated the maximal rate of PC oxidation by uncoupled mitochondria by measuring the maximal rate of PC-supported uncoupled respiration (the uncoupling was achieved by adding 2,4-DNP at a concentration that induced the maximal rate of respiration in the presence of glutamate + malate). The additions of glutamate + malate (Complex I-dependent substrates) and succinate (Complex III substrate) yielded the values for

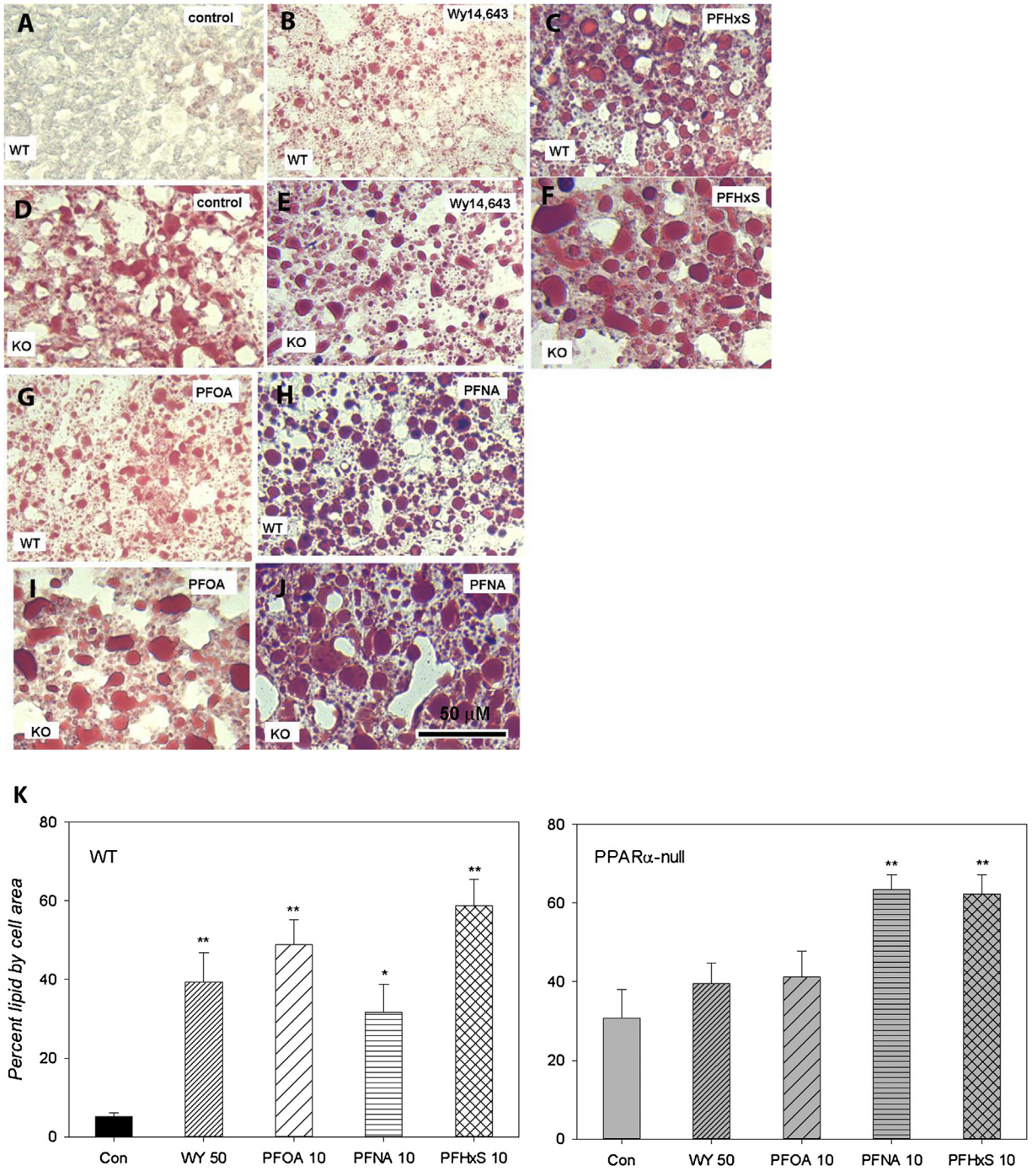

Fig. 3. Lipid accumulation in the livers of control and treated mice.
A–J. Six μM thick frozen sections from each liver were prepared, thaw mounted onto glass slides, stained with Oil Red O and two regions of each section were photographed. Representative images for each treatment (all dosed at 10 mg/kg) and strain are shown. K. Quantitation of lipid accumulation from the images. The data were analyzed with 2-way ANOVA and Bonferroni post test to determine strain and treatment effects and interactions, and also one-way ANOVA and Dunnett's multiple comparison test to evaluate treatment effects within the strain. Data are mean ± SE, where n = 4, * represents $p < 0.05$ and ** represents $p < 0.01$. Significant comparison control vs treated within same strain.

the maximum uninhibited rates of Complex I and Complex III dependent respiration, respectively (curve a). The other three curves of Fig. 5 illustrate three possible scenarios of effects on the uncoupling efficiencies of the compounds of interest:

- Curve b – If a compound such as C-228, a mixture of surfactant compounds, does not inhibit PC oxidation but is a weak, non-specific uncoupler of oxidative phosphorylation compared to 2,4-DNP, addition would stimulate the rate of coupled respiration (prior to adding DNP). However, the respiration rate in the presence of 2,4-DNP plus C-228 (a mixture of surfactant) is nearly identical to that in the presence of DNP alone (compare with curve a);
- Curve c – If a compound such as perfluorooctane sulfonamide (PFOSA) is more efficient than DNP at uncoupling mitochondrial oxidative phosphorylation, the compound produces the

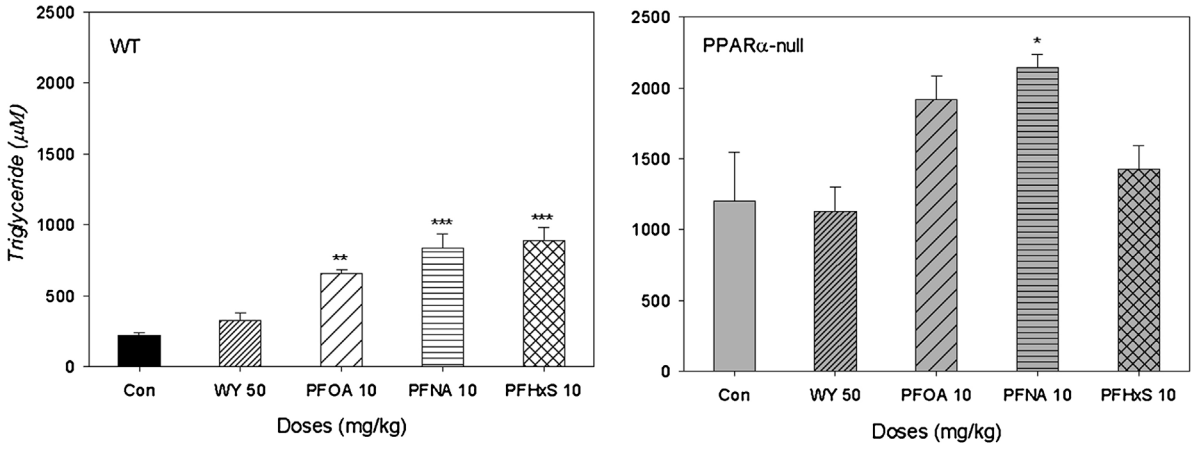

Fig. 4. Alterations of triglyceride levels in the livers of treated mice.
One-way ANOVA with Dunnett's multiple comparison test was used to determine significance for each treatment relative to control. Data are mean ± SE, where n = 4, * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$.

maximal rate of respiration when added to mitochondria by itself. This rate cannot be further stimulated by adding DNP (compare the respiration rates before and after the adding DNP).

- Curve d – The perfluorooctane sulfamidoacetate (PFOSAA)-stimulated rate of respiration is also insensitive to DNP, but the rate of oxygen consumption is only about half of the maximal rate (30 nmol/min/mg after DNP addition compared to 47–51 nmol/min/mg for curves a, b, & c). Furthermore, the respiration rate was also suppressed following the additions of glutamate + malate (Glu) and succinate + rotenone (Suc). All of this indicates that PFOSAA is an inhibitor of Complex I (and possibly Complex III) of the respiratory chain, rather than an inhibitor of fatty acid (PC) transport and/or oxidation.

For PFOS and PFOA the effects were qualitatively similar to those illustrated for C-228 (curve b). The amides and N-EtFOSA at 50 μM were fairly strong uncouplers of oxidative phosphorylation and affected respiration (curve c). Although the results demonstrate that some of the PFAAs, PFOSAA for example, inhibit complex I and/or II of the respiratory chain (curve d) while others uncouple mitochondrial respiration from oxidative phosphorylation (PFOSA and N-EtPFOSA; curve c), none of the tested PFAAs directly inhibited fatty acid

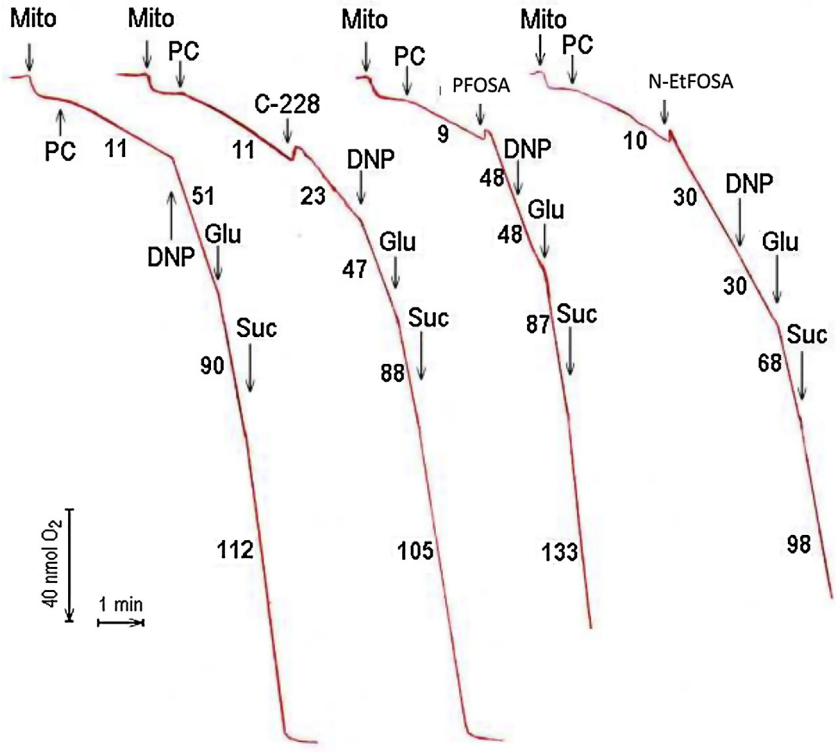

Fig. 5. The effect of representative perfluorooctane compounds on respiration of isolated rat liver mitochondria oxidizing palmitoylcarnitine.
For medium composition and additions see Methods. Mitochondrial protein content was 1 mg/ml. Numbers near the curves indicate the rate of respiration, nmol $O_2 \times min^{-1} \times mg^{-1}$ protein. Each curve from left to right are a, b, c, and d as indicated under results section. Abbreviations: Mito, mitochondria; PC, palmitoylcarnitine; DNP, 2,4-dinitrophenol; C-228, a mix of surfactants as a positive control; Glu, glutamate + malate; Suc, succinate + rotenone; FOSA; N-EtFOSA. For explanations, see the text.

supported respiration by isolated rat liver mitochondria (Supplemental Table 2). Based on the experimental design, this indicates that the PFAAs did not inhibit either mitochondrial fatty acid transport (CPT 1 or 2) or β-oxidation directly.

The effect of PFOA and PFOS on mitochondrial fatty acid oxidation was further investigated using the Seahorse Bioscience® technology system for assessing the effect on human liver cell respiration using palmitate-BSA as substrate. Neither PFOA nor PFOS inhibited palmitate-supported respiration in HepG2/C3A human liver cells (Supplemental Figs. 1 and 2). In fact, high concentrations of either PFAA (25 μM and 50 μM) stimulated cell respiration, but this was not reflective of fatty acid oxidation since the same result was observed when palmitate was omitted and etomoxir, which irreversibly inhibits CPT1, added. The significance of this result is that it extends the observations for isolated mitochondria, wherein neither PFOA nor PFOS interfered with either the mitochondrial translocation of palmitoylcarnitine nor its oxidation, to include an absence of an effect on acyl CoA synthase or carnitine acyltransferase (CPT1 and CPT2) activities. Acyl CoA synthase represents the first step in the activation of palmitate to form the CoA-ester, which is then transesterified to the carnitine ester that is translocated across the mitochondrial membranes by CPT1 and CPT2 delivering the activated fatty acid to the intramitochondrial β-oxidation pathway. Therefore, not only do PFOA and PFOS not affect the oxidation or translocation of palmitoylcarnitine by the mitochondrial fraction, they also do not affect the activation of palmitate within the cytosolic compartment of the cell.

3.3. Effects of perfluorinated chemical exposure on expression of lipid metabolism genes

To better understand the molecular basis for the steatosis caused by PFAA exposure, the expression of genes that control major pathways of lipid synthesis and degradation were examined from microarray data. Microarray comparisons generated in our lab came from experiments in wild-type and PPARα-null male mice that were closely matched with the experiments described in the present study: PFOA at 3 mg/kg/day for 7 days (from Gene Expression Omnibus Accession # GSE9786); PFNA at 3 mg/kg/day for 7 days (from GSE55756); PFHxS at 10 mg/kg/day for 7 days (from GSE55756); PFOS at 10 mg/kg/day for 7 days (from GSE22871). An additional study came from mice treated with WY at 0.1% in the diet for 5 days (from GSE8295). In wild-type mice, exposure to all of the compounds led to consistent increases in the expression of genes that are the typical targets of PPARα including peroxisomal, mitochondrial, and microsomal fatty acid oxidation, as expected (Fig. 6A). Treated wild-type mice also exhibited increases in a number of fatty acid transporters that increase uptake of fatty acids into hepatocytes including *Cd36, Slc27a1, Slc27a2, Slc27a4,* and *Vldlr*. It should be noted that although the expression of the *Vldlr* gene is readily detected in the liver, the Vldlr protein is unlikely expressed to any appreciable extent (Reddy et al., 2011). Increases in the genes involved in fatty acid and triglyceride synthesis were also observed for all of the compounds. Almost all of the cholesterol synthesis genes were increased by PFHxS, whereas WY, PFOS, and PFNA increased only subsets of these genes. PFOA appeared to be unique in that none of the cholesterol synthesis genes were affected.

The expression of the lipid metabolism genes was examined in PPARα-null mice. Almost all of the gene expression changes induced by WY were abolished in PPARα-null mice. In contrast, many of the changes observed in wild-type mice were retained in PPARα-null mice after exposure to PFOS, PFOA, PFNA and PFHxS, albeit at generally reduced levels. These included alterations in peroxisomal and mitochondrial fatty β-oxidation and ω-oxidation genes thought to be dependent on PPARα for regulation. A number of these genes have been characterized before as PPARα-independent after PFAA exposure (Rosen et al., 2008b). Mechanisms for how these genes are regulated in PPARα-null mice include activation by PPARβ or PPARγ (Rosen et al., 2010). Increased expression of some of the fatty acid and TG synthesis genes were also retained in the PPARα-null mice after exposure to PFOS, PFNA and PFHxS and to a lesser extent, PFOA. PFOS appeared to be unique in that all of the cholesterol synthesis genes were increased in expression in PPARα-null mice. *Cyp4a* family members (*Cyp4a10, Cyp4a14, Cyp4a21*) were expressed to greater levels in the null mice exposed to the two compounds which caused steatosis (PFNA, PFHxS) compared to the two compounds which did not (PFOA, WY). After exposure to PFNA expression of *Cyp4a10* or *Cyp4a14* 106-fold and 44-fold, respectively. PFHxS induced expression of *Cyp4a10* or *Cyp4a14* 52-fold and 24-fold, respectively. This contrasts with no significant changes in these genes after exposure to PFOA or WY in the null mice.

Efforts were made to determine if any of the changes in fatty acid, TG and cholesterol synthesis genes were associated with changes in the expression of transcription factors that regulate these pathways. Increased expression of *Pparg* was observed after exposure to PFNA and PFHxS (Fig. 6B). There were consistent decreases in expression of *Chrebp (Mlxipl), Hnf4a, and Ppargc1a* for all five chemicals. Expression of *Foxa2, Fxr, Lxra, Lxrb, Rxra, Shp, Srebf1,* and *Srebf2* genes was decreased or not changed after exposure. In PPARα-null mice PFOS, PFNA and PFHxS retained the decreases in the expression of *Hnf4a and Ppargc1a.* The remaining pairwise chemical-gene interactions were not consistent.

Changes in the expression of genes examined in the microarray experiments described above were also observed by RT-PCR (Table 1). These included increases in the expression of the fatty acid transporter *Cd36* and stearoyl-CoA desaturase-1 (*Scd1),* in all of the treatment groups in wild-type mice. The fatty acid synthase gene *Fasn* was increased in expression in all but the PFNA treatment group. Increased expression of these genes was observed in PPARα-null mice treated with PFOA and PFHxS. Increased expression of *Scd1* was also observed in PFNA-treated PPARα-null mice.

Expression of additional genes involved in lipid homeostasis were determined by RT-PCR. CIDEC binds to lipid droplets and restricts lipolysis while favoring storage. Increased expression of *Cidec* was observed in the livers of wild-type mice from all treatment groups. In PPARα-null mice, *Cidec* was increased in all PFAA groups. The expression of two lipases was examined. The endothelial lipase *Lipg* was significantly increased in only the PFOA and PFHxS groups in wild-type mice, and in PPARα-null mice, *Lipg* was decreased in only the PFNA group. Lipoprotein lipase (*Lpl*) was not significantly altered in any group. The expression of *Pparg* was increased only by PFHxS in wild-type mice and by PFOA and PFHxS only in PPARα-null mice.

In summary, the microarray analysis showed that in addition to the typical gene targets of PPARα that were increased (i.e., peroxisomal and mitochondrial fatty acid β-oxidation and microsomal fatty acid ω-oxidation), there were parallel increases in the expression of fatty acid transport genes, as well as fatty acid and triglyceride synthesis genes after exposure to the compounds in wild-type mice. Increased expression of a subset of these genes was retained after exposure to PFOS, PFOA, PFNA and PFHxS in PPARα-null mice.

3.4. Contrasting changes in lipid metabolism genes in untreated PPARα-null mice compared to PFAA treated mice

Decreased expression of PPARα has been associated with basal- and fasting-induced steatosis (summarized in Corton et al., 2014).

To determine similarities or differences in the molecular mechanisms of steatosis induced by inactivation of PPARα compared to steatosis induced by the PFAAs, the expression of the lipid metabolism genes discussed above was examined in microarray comparisons (Fig. 7A). Twenty eight PPARα-null vs. wild-type comparisons were examined. Many of the biosets were derived

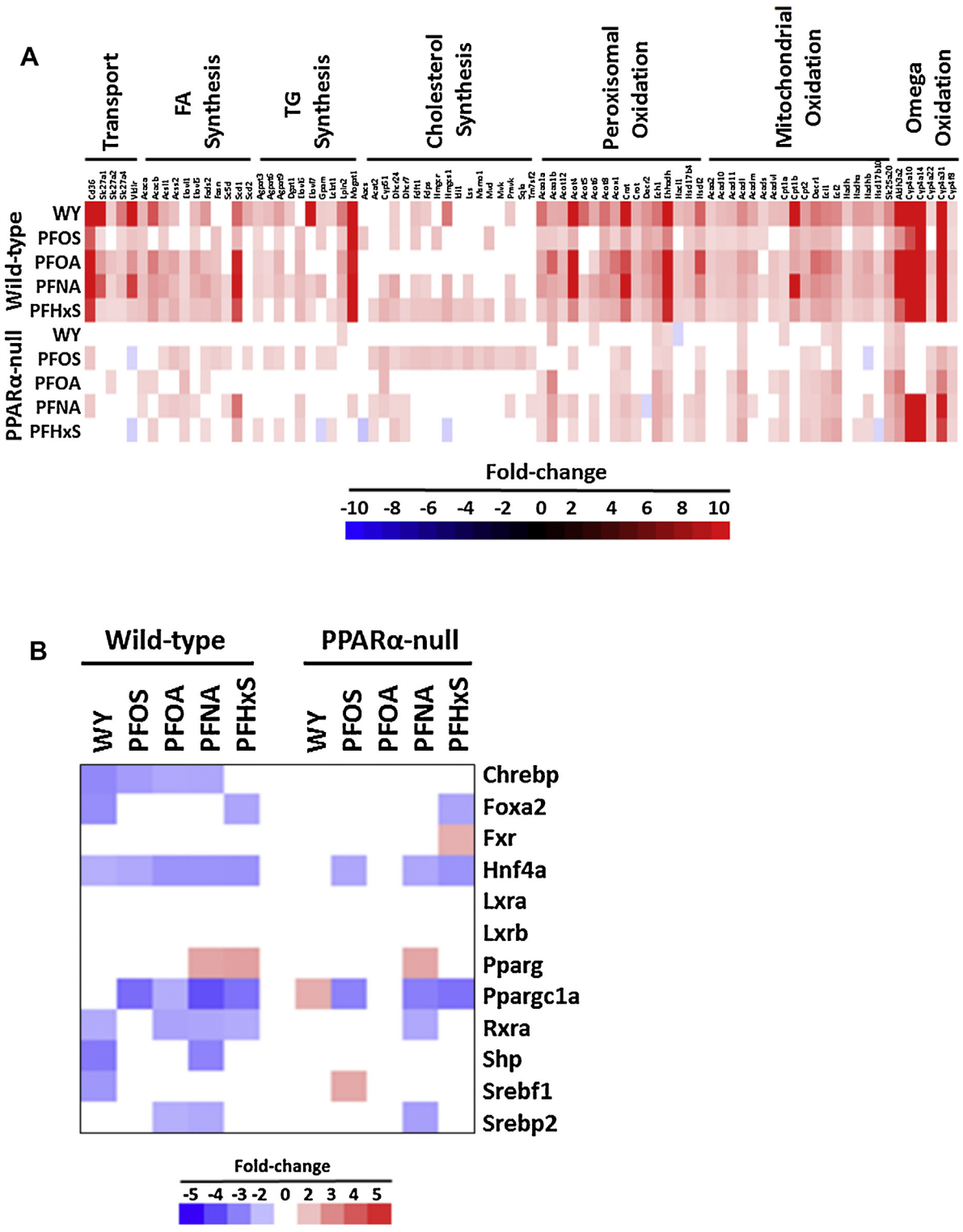

Fig. 6. Coordinated changes in the expression of lipid synthesis and catabolism genes in treated mice.
A. Changes in the expression of the genes in the different functional categories came from microarray experiments that were analyzed using the NextBio Affymetrix array analysis pipeline as described in the Methods. Microarray comparisons came from experiments in wild-type and PPARα male mice that were closely matched with the experiments described in the present study: WY at 0.1% in the diet for 5 days (from GSE8295); PFOS at 10 mg/kg/day for 7 days (from GSE22871); PFOA at 3 mg/kg/day for 7 days (from GSE9786); PFNA at 3 mg/kg/day for 7 days (from GSE55756); PFHxS at 10 mg/kg/day for 7 days (from GSE55756). The microarray analysis shows that although the typical gene targets of PPARα were increased (peroxisomal, mitochondrial and omega fatty acid oxidation), there were also parallel increases in the expression of fatty acid transport and synthesis genes, as well as triglyceride synthesis genes. B. Alterations in the expression of key transcription factors that regulate lipid metabolism. Expression values were derived from the microarray experiments described in A.

Table 1
Expression of lipid homeostasis genes in wild-type and PPARα-null mice after exposure to PFAAs or WY.

Strain	Treatment	Fold Change							
		Apoc3	CD36	Cidec (fsp27)	Fasn	Lipg	Lpl	Pparg	Scd-1
WT	control	1	1	1	1	1	1	1	1
WT	Wy 50 mg/kg	−1.09	18.64^{c}	25.13^{c}	3.02^{b}	1.89	21.82^{c}	1.1	11.49^{c}
WT	PFOA 10 mg/kg	−1.36	47.72^{c}	215.03^{c}	2.64^{a}	3.86^{b}	14.52^{c}	1.56	13.72^{c}
WT	PFNA 10 mg/kg	−2.95^{c}	35.90^{c}	303.14^{c}	1.29	1.33	5.44^{c}	1.19	7.76^{b}
WT	PFHxS 10 mg/kg	−1.46	16.46^{c}	24.14^{c}	3.96^{c}	3.33^{b}	4.54^{c}	2.25^{a}	12.77^{c}
PPARα-null	control	1	1	1	1	1	1	1	1
PPARα-null	Wy 50 mg/kg	−1.01	1.08	−1.18	1.5	1.28	−1.15	−1.19	2.72
PPARα-null	PFOA 10 mg/kg	−1.09	4.32^{c}	8.31^{c}	3.17^{b}	−1.09	2.04	2.19^{a}	32.15^{c}
PPARα-null	PFNA 10 mg/kg	−1.45	2.16	5.62^{c}	1.21	−3.77^{c}	1.25	1.47	20.78^{c}
PPARα-null	PFHxS 10 mg/kg	1.07	3.77^{b}	4.15^{b}	3.32^{b}	1.97	1.57	2.05^{a}	36.79^{c}
PPARα-null	control	1.47	1.07	−1.27	1.11	−3.51^{e}	1.71	2.00^{d}	−6.23^{d}
PPARα-null	Wy 50 mg/kg	1.6	−16.07^{f}	−37.65^{f}	−1.81	−5.19^{f}	−14.77^{f}	1.53	−26.33^{f}
PPARα-null	PFOA 10 mg/kg	1.83	−10.31^{f}	−32.86^{f}	1.33	−14.74^{f}	−4.17^{f}	2.80^{f}	−2.66
PPARα-null	PFNA 10 mg/kg	2.98^{f}	−15.49^{f}	−68.50^{f}	1.04	−17.61^{f}	−2.55^{d}	2.46^{e}	−2.33
PPARα-null	PFHxS 10 mg/kg	2.28^{d}	−4.08^{f}	−7.38^{f}	−1.07	−5.95^{f}	−1.70	1.81	−2.16

Data are mean + SE, where n = 4. Significant comparison control vs treated within same strain: a represents $p < 0.05$, b represents $p < 0.01$ and c represents $p < 0.001$. Significant comparison between strains with the same treatment, where d represents $p < 0.05$, e represents $p < 0.01$ and f represents $p < 0.001$. Significant comparison control vs treated within same Strain: a $p < 0.05$, b $p < 0.01$, c $p < 0.001$. Significant comparison between Strains with the same treatment: d $p < 0.05$, e $p < 0.01$, f $p < 0.001$.

from mice in which both strains were treated with a synthetic triglyceride or a chemical. There were almost universal decreases in the expression of fatty acid catabolism genes across the comparisons, as expected (Corton et al., 2014). The one obvious outlier came from a bioset (GSE6622) in which expression of *Ppara* was decreased using siRNA. Most of the comparisons also exhibited decreases in the expression of fatty acid transport and synthesis genes as well as triglyceride synthesis genes. Approximately half of the comparisons exhibited increases in cholesterol synthesis genes.

In the same comparisons, the expression of a number of transcription factors that control the expression of these genes was examined (Fig. 7B). All but one of the comparisons exhibited decreases in the expression of *Ppara,* as would be expected because the gene was disrupted by homologous recombination in most biosets. There were consistent increases in the expression of *Pparg* in almost all of the comparisons (21 out of 28 comparisons). As determined by RT-PCR, expression of *Pparg* was increased in the control PPARα-null group vs. the control wild-type group in our study (2.00 fold-change; *p*-value < 0.05). Increases in the expression of the *Pparg* gene in PPARα-null mice has been reported previously (Patel et al., 2001; Patsouris et al., 2006). The expression of other transcription factors involved in lipid homeostasis was also examined by microarray analysis. There were no changes or only inconsistent changes (less than half of the comparisons) for *Chrebp, Mlxipl, Foxa2, Fxr, Hnf4a, Lxra, Lxrb, Rxra, Shp, Srebf1,* and *Srebp2* (data not shown).

4. Discussion

Steatosis is the first step in a continuum of chemical-induced adverse effects that, under chronic exposure conditions, include steatohepatitis, fibrosis, impaired liver function, and cancer (Al-Eryani et al., 2015). The steatosis endpoint has been used as the critical effect in EPA's IRIS assessments to determine exposure limits for a number of chemicals (Kaiser et al., 2012). In the present study, we evaluated the ability of a number of structurally-diverse PFAAs to perturb lipid metabolism and induce liver steatosis. Our studies confirm and reinforce previous observations that PFOA causes extensive micro- and macro-vesicular steatosis in hepatocytes (Haughom and Spydevold 1992; Martin et al., 2007; Tan et al., 2013; Wang et al., 2013) and that the steatosis is associated with increases in the accumulation of TG in the liver (Haughom and Spydevold 1992; Kudo and Kawashima 1997; Tan et al., 2013). PFOS has previously been shown to cause steatosis (Martin et al., 2007; Wan et al., 2012; Wang et al., 2014) and increases in TG levels in the liver (Bijland et al., 2011; Wan et al., 2012; Wang et al., 2014). Furthermore, our studies show that PFNA and PFHxS also cause steatosis and increases in TG. PFNA has been shown to cause increases in TG in the livers of mice (Wang et al., 2014) and rats

Table 2
Summary of both PFAA and WY exposure effects on apical endpoints.

Strain	Treatment	Fold Change						
		Body wt	Liver wt	%BW/Lv Wt	Cell area	DNA/mg LV	Lipid accumulation	Liver TG level
WT	control	1	1	1	1	1	1	1
WT	Wy 50 mg/kg	–	↑	↑	↑	↓	↑	–
WT	PFOA 10 mg/kg	–	↑	↑	↑	↓	↑	↑
WT	PFNA 10 mg/kg	–	↑	↑	↑	↓	↑	↑
WT	PFHxS 10 mg/kg	–	↑	↑	↑	↓	↑	↑
PPARα-null	control	1	1	1	1	1	1	1
PPARα-null	Wy 50 mg/kg	–	–	–	–	–	–	–
PPARα-null	PFOA 10 mg/kg	–	↑	↑	↑	↓	–	–
PPARα-null	PFNA 10 mg/kg	–	↑	↑	↑	↓	↑	↑
PPARα-null	PFHxS 10 mg/kg	–	↑	↑	↑	↓	↑	–

Data are fold change of each end point, where n = 4. A-Wild Type and B-PPARα-null. Significant comparison control vs treated within same strain: Up arrow represents significant increase, Down arrow represents significant decrease, and Dash represents no change.

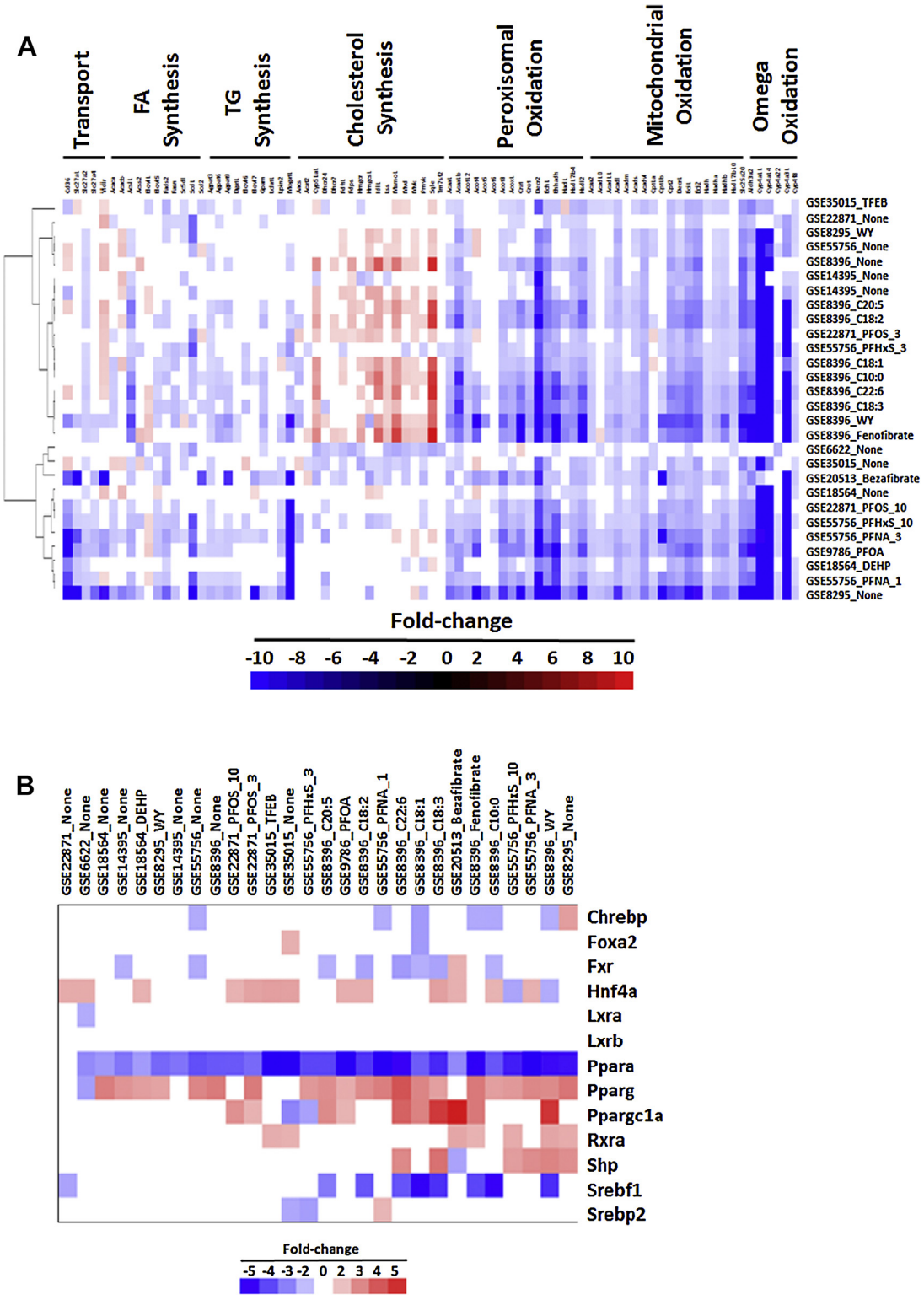

Fig. 7. Effects of *Pparα* inactivation on lipid synthesis and catabolism genes.
A. Biosets derived from comparisons between PPARα-null and wild-type mice were examined for expression of the genes in the indicated functional categories. Many of the biosets were derived from mice in which both strains were treated with the indicated compound at the indicated dose (in mg/kg/day) or synthetic triglyceride, as indicated in the name of the bioset. Biosets were clustered by one-dimensional clustering. B. Expression of the *Ppara* and *Pparg* genes in the PPARα-null and wild-type comparisons described in A.

(Fang et al., 2012). PFHxS was shown to increased TG in the livers of APOE*3-Leiden CETP mice, which exhibit attenuated clearance of ApoB-containing lipoproteins and exhibit a human-like lipoprotein metabolism on a Western diet (Bijland et al., 2011). Our study is the first to compare the induction of steatosis by PFAAs between wild-type and PPARα-null mice. PFNA and PFHxS were found to increase steatosis beyond the background levels of steatosis in the PPARα-null mice, indicating that at least for these two compounds,

the mechanism of the steatosis is at least partially PPARα-independent. On the other hand, WY did not result in changes in TG levels, consistent with WY either having no effect or acting to decrease steatosis in mice induced by genetic predisposition (Larter et al., 2012) or by various diets (Ip et al., 2003). Although not tested in the present study, perfluorooctane sulphonic acid (PFOSA) increased liver TG (Haughom and Spydevold, 1992). Only perfluorobutane sulfonate (PFBS) had little if any effect on increasing TG levels but with the caveat that the mice were fed a high fat diet and thus already carried a burden of high TG levels (Bijland et al., 2011). Except for PFBS then, increases in TG leading to steatosis is a common adverse event in the livers of mice and rats exposed to structurally-diverse PFAAs.

The underlying mechanism for the accumulation of TG in the livers of PFAA-treated mice and rats has been the focus of several studies. There is prior evidence that a number of nonexclusive mechanisms are involved which include four major mechanisms for chemical-induced steatosis induction (Angrish et al., 2016). These include 1) increases in TG synthesis, 2) increased lipid uptake, 3) decreased TG secretion, and 4) inhibition of mitochondrial fatty acid β-oxidation. Insights into the mechanisms of PFAA-induced effects were facilitated by comparing global transcriptional effects of PFAAs that activate PPARα and cause steatosis to those hypolipidemic agents that activate PPARα but do not cause steatosis.

Increased *de novo* TG synthesis by PFAAs could contribute to steatosis. Analysis of gene expression changes in the liver caused by 4 PFAAs in wild-type and PPARα-null mice showed the coordinated regulation of peroxisomal, mitochondrial, and microsomal fatty acid oxidation genes, which have been shown to be universally induced by all PPARα activators (Fig. 6) (Kersten, 2014). What has not been well documented in previous studies is the simultaneous activation of fatty acid and TG synthesis genes by PPARα activators, which presents a paradox in why conflicting pathways of synthesis and catabolism would be simultaneously activated. PFAAs universally induced increases in the expression of fatty acid and TG synthesis genes in wild-type mice. For PFOS, PFNA, and PFHxS, induction of a subset of the genes, although muted, was retained in the PPARα-null mice. PFOA was unique in that most of the fatty acid and TG synthesis gene expression was abolished in PPARα-null mice. Expression of genes involved in fatty acid synthesis (*Fasn, Scd1*) was confirmed by qPCR (Table 1). These results are consistent with earlier studies, which showed that some of the same genes exhibited increased expression in the livers of rats (Bjork et al., 2008; Martin et al., 2007) and wild-type mice (Yan et al., 2015) given PFOA. PFOA, but not the PPARα activators clofibrate and tetradecylthioacetic acid, was found to increase fatty acid formation from acetate (Haughom and Spydevold, 1992). Our results are in conflict with an earlier study which showed decreases in the expression of some of the fatty acid and TG synthesis genes by PFOS in mice that were fed a high fat diet (Bijland et al., 2011). It has not been previously appreciated that PPARα activators, including WY, increased the expression of the fatty acid and TG synthesis genes as well, similar to the results of earlier microarray studies in which wild-type and PPARα-null mice were treated with WY (Anderson et al., 2004; Knight et al., 2005). In contrast to PFOS, PFNA, and PFHxS, most of the fatty acid and TG genes were not induced by WY in the PPARα-null mice. Based on these profiles, we hypothesize that the fatty acid and TG synthesis genes altered by WY are regulated by PPARα whereas the PFAAs regulate these genes through both PPARα-dependent and -independent pathways. Additionally, the pattern of expression of the synthesis and catabolism genes could not differentiate those compounds that do or do not cause steatosis in wild-type mice.

The mechanistic basis for the induction of the fatty acid and TG synthesis genes by PFAAs and other PPARα activators points to the involvement of the sterol regulatory element-binding proteins (SREBPs) transcription factors. SREBPs are encoded by two genes *Srebf1* and *Srebf2*. The *Srebf1* gene encodes the protein isoform SREBP-1c which regulates genes involved in *de novo* lipogenesis, whereas the *Srebf2* gene encodes the protein SREBP-2, which regulates cholesterol synthesis genes (Xu et al., 2013). There is evidence that PFAAs can modulate the activity of the SREBPs either transcriptionally or through post-transcriptional processing. PFOA was shown to increase the post-translational processing (also called maturation) of SREBP-1c and SREBP-2 to mature active forms that were associated with increases in the expression of SREBP target genes (Yan et al., 2015). PFNA increased the expression of the *Srebf1* gene in rat livers (Fang et al., 2012), PFOA and PFOS increased the expression of *Srebf1* in rat primary hepatocytes (Bjork et al., 2011), and PFOA increased the expression of the *Srebf1* and *Srebf2* genes in the livers of wild-type mice (Yan et al., 2015). In contrast, microarray analysis did not reveal increased expression of either subtype by any of the PFAAs (Fig. 6B). WY increased fatty acid synthesis, decreased cholesterol synthesis and increased proteolytic cleavage of SREBP-1 in the livers of mice that were PPARα-dependent (Knight et al., 2005). While human *SREBF1* expression in liver is directly regulated by PPARα, the mechanism for how the PPARα agonist GW7647 increased the expression of *Srebf1* in rat primary hepatocytes has not been fully delineated (Fernandez-Alvarez et al., 2011). Activation of SREBP through proteolytic cleavage is induced by low levels of cholesterol (Xu et al., 2013). PFOA and other PFAAs may induce activation because of evidence that PFOA (Yan et al., 2015) and fenofibrate (Ducheix et al., 2013) can lower total cholesterol levels in the liver. However, PFOS or PFNA in mice increased (Bijland et al., 2011; Wang et al., 2014) and PFNA in rats did not change (Fang et al., 2012) total cholesterol in the livers of treated animals. An alternative hypothesis includes PPARα-dependent activation of *Scd1*, increases in the ratio of unsaturated to saturated fatty acids, alteration of membrane composition affecting the conformation, and increased activity of the transport protein SREBP cleavage-activating protein (SCAP) allowing the transfer of SREBP-1c from the endoplasmic reticulum to the golgi for cleavage (Knight et al., 2005). Further studies are needed to determine how PPARα activators (in general) and PFAAs (in particular) regulate the expression and activity of SREBPs.

PFAAs may also increase steatosis by perturbing the flux of TG between hepatocytes and the blood either by increasing lipid uptake or by decreasing lipid secretion. PPARα activators, including PFAAs, decrease circulating levels of TG in the blood, which is the basis for the therapeutic effects of a number of drugs marketed to control hyperlipidemia (Staels et al., 1998). Lipid uptake is controlled in part by lipoprotein lipase (LPL), which controls the hydrolysis of core triglycerides (TGs) in chylomicrons and very low-density lipoproteins (VLDLs), producing chylomicron remnants and intermediate-density lipoproteins (IDLs), respectively (Eckel, 1989; Goldberg and Merkel, 2001). LPL requires specific cofactors ApoCII and ApoAV to be fully active (Catapano 1982; Kinnunen et al., 1977). LPL is inhibited by other lipoproteins (ApoCI and ApoCIII). A number of PPARα activators increase LPL activation and TG import by increasing the expression of ApoAV and decreasing the expression of ApoCIII (Staels et al., 1998). Examination of the *Apo* genes from the microarray analysis did not show this type of expression pattern, but rather there were minor decreases in the expression of both *Apoa1* and *Apoa5* after most of the PFAA exposures in wild-type mice (data not shown). PFOS, PFHxS, and PFBS were shown to increase the clearance of triglyceride from the blood, and at least for PFOS and PFHxS, clearance occurs through increases in the activity of LPL (Bijland et al., 2011). Thus, like other PPARα activators, PFAAs increase the uptake of TG from the blood.

Suppression of lipid secretion may also contribute to steatosis by PFAAs. TGs are secreted from the liver in the form of very-low-density lipoproteins, into the blood where they mature and function to deliver endogenously derived lipids to peripheral tissues. ApoB, within the VLDL, is the primary organizing protein of the particles required for formation. A number of PFAAs have effects on VLDL and LDL secretion. PFOS and PFHxS, but not PFBS, decreased the secretion of VLDL and VLDL particles containing ApoB (Bijland et al., 2011). Secretion of LDL was decreased by exposure to PFOA (Quist et al., 2015) and PFOS (Wang et al., 2014). Microarray analysis showed that PFNA and PFHxS decreased the expression of the *Apob* gene in both wild-type and PPARα-null mice (data not shown). There is also evidence that PFOA interferes with the association between ApoB and the TG particle (Okochi et al., 1999).

A number of environmentally-relevant chemicals, fatty acids, and drugs inhibit mitochondrial fatty acid β-oxidation (Massart et al., 2013). Inhibition of mitochondrial fatty acid β-oxidation results in increased levels of fatty acids that are used for TG synthesis and, in the absence of compensatory mechanisms of oxidation or transport, results in increased number and size of lipid vesicles. Early studies showed that fatty acids structurally similar to PFAAs cause inhibition of mitochondrial fatty acid β-oxidation, including 2-bromopalmitate, which inhibits carnitine palmitoyltransferase (Chase and Tubbs, 1972) and 4-pentenoic acid which may inhibit acyl-CoA dehydrogenase (Thayer, 1984). PFOS was shown to increase the recovery of radioisotopically labeled palmate in crude mouse liver homogenates, suggesting a possible inhibitory effect on mitochondrial fatty acid β-oxidation (Wan et al., 2012). Circumstantial evidence indicates that PFOA and PFDA caused inhibition of mitochondrial fatty acid β-oxidation in mouse liver, as there was an accumulation of shorter chain fatty acids that are the substrates of mitochondrial fatty acid β-oxidation, but little, if any increases in levels of fatty acids that are substrates of peroxisomal fatty acid β-oxidation (Kudo and Kawashima, 1997). In the present study, we show that 7 PFAAs of varying structure had no direct effect on fatty acid transport, intramitochondrial activation, or β-oxidation in isolated rat liver mitochondria (Fig. 5). Despite the fact that the PFAAs tested in our study do not cause inhibition of mitochondrial fatty acylcarnitine β-oxidation, it is possible that they may interfere with activation of fatty acids by inhibiting either the fatty acyl thiokinase, ATP-dependent Acyl-CoA synthase, or carnitine-palmitoyltransferase (CPT1) of the fatty acid oxidation cascade. Palmitoylcarnitine, used as substrate in this assay, bypasses all three of these steps. Regardless, the mitochondrion is a toxicologically-relevant target of PFAAs. However, the observation that neither PFOA nor PFOS alter palmitate-supported mitochondrial respiration in isolated mitochondria or human hepatocyte cell culture suggests that none of these intermediary steps in fatty acid activation, transport, or oxidation is affected. Indeed, many PFAAs have effects on mitochondrial biogenesis that may be secondary and compensatory to mitochondrial toxicity, in part through uncoupling of oxidative phosphorylation (Berthiaume and Wallace 2002; Starkov and Wallace 2002; Walters et al., 2009).

Peroxisomes and mitochondria share a critical interplay in fatty acid (Schrader et al., 2015; Wanders et al., 2016). Peroxisomes catalyze the oxidation of branched and very long chain fatty acids (VLCFA; >C20) to 6–8 carbon carboxylic acid products. Mitochondria, on-the-other-hand, oxidize medium chain fatty acids (MCFA; C8–C14), including the products of peroxisomal fatty acid oxidation. Thus, peroxisomes and mitochondrial share many of the same enzymatic machinery. Furthermore, the activity in both compartments is increased by activation of PPARα (Cook et al., 2000; Mandard et al., 2004). However, compared to peroxisomes where PPARα-mediated stimulation of fatty acid oxidation reflects primarily a proliferation of peroxisomal bodies, in mitochondria PPARα stimulated fatty acid oxidation is manifest primarily at the transcriptional level to increase the complement of the enzymes (Eggens et al., 1980; Paget 1963). In an acute model (single i.p. injection, 100 mg/kg PFOA, PFOS or N-ethylFOSE) PPARα activation itself has only marginal effects on mitochondrial biogenesis in rat liver (Berthiaume and Wallace, 2002), whereas subchronic oral dosing with PFOA (30 mg/kg, 28 days) caused a doubling of hepatic mitochondrial copy number (Walters et al., 2009).

Although peroxisomes and mitochondria act in concert in the oxidation of fatty acids, the interplay is complementary, but not compensatory; inhibition of peroxisomal fatty acid oxidation is not associated with the stimulation of mitochondrial β-oxidation pathway, and vice versa. Up regulation of one system does not compensate for the inhibition of the other (Djouadi, 2008; Hashimoto et al., 1999). This is evidenced by the current observation that PFAAs cause robust stimulation of peroxisome proliferation in rodents, but have no detectable effect on mitochondrial fatty acid oxidation *in vitro*.

Our microarray analysis showed that *Cyp4a* genes (*Cyp4a10, Cyp4a14, Cyp4a21*) were expressed to greater levels in the null mice exposed to the two compounds which caused steatosis (PFNA, PFHxS) compared to the two compounds which did not (PFOA, WY). Levels of the CYP4A subtypes were highly up-regulated in livers of db/db diabetic mice which exhibit steatosis compared with C57BL/6J mice that do not. Inhibition of Cyp4a enzymatic function using HET0016 ((N-Hydroxy-N0-(4-butyl-2-methylphenyl)-formamidine)), a potent pan-CYP4 inhibitor in db/db diabetic mice on a standard diet and wild-type mice on a high-fat diet reduced features of diabetes including hepatic steatosis, oxidative stress, and endoplasmic reticulum stress (Park et al., 2014). Further work is needed to determine if increases in the Cyp4a family members are involved in PFAA-induced steatosis.

To gain additional insights into the PFAA-induced steatosis, we compared the transcriptional changes in lipid pathways by PFAAs to those that occur upon inactivation of the *Ppara* gene. We capitalized on a large number of comparisons between PPARα-null and wild-type mice in a microarray database that allowed identification of consistent effects across a number of different exposure scenarios. In contrast to the almost universal increased expression of fatty acid oxidation and synthesis genes by PFAAs, the lack of a functional PPARα caused striking decreases in the expression of oxidation genes and modest, but consistent, decreases in expression of the fatty acid and TG synthesis genes (Fig. 7A). While the decreases in the expression of *Ppara* gene could be observed in almost all of the comparisons, there were parallel increases in the expression of *Pparg* (Fig. 7B). Increases in expression of *Pparg* were also observed after exposure to the PFAA (Fig. 6B and Table 1). Thus, the steatosis induced by PFAAs and PPARα inactivation may have increased in *Pparg* in common.

Approximately half of the PPARα-null vs wild-type comparisons exhibited increases in cholesterol synthesis genes that have not been previously associated with defective PPARα. Most of these comparisons came from one study (GSE8396), in which the two mouse strains were administered synthetic triglycerides or WY. There were comparisons from three other studies (GSE14395, GSE22871, GSE55756) that also exhibited coordinated increases in the cholesterol synthesis genes. Cholesterol synthesis in the mouse liver exhibits significant circadian variation with greatest rates of synthesis during the dark phase in wild-type mice; in PPARα-null mice the diurnal variation in synthesis was abolished (Patel et al., 2001). We speculate that these studies that exhibited increases in the cholesterol synthesis genes in the PPARα-null mice had varying sacrifice times that capture these strain differences in cholesterol synthesis. In fact, the mice in the GSE8296 study were sacrificed in the late afternoon, close to the dark phase and the mice in the

GSE14395 study were sacrificed at ZT14, 2 h after the initiation of the dark phase.

5. Conclusions

In summary, our studies highlight the almost universal effects of PFAA exposure on the increase of TG levels and induction of steatosis in the livers of exposed rodents. The precise mechanistic basis for these effects remain elusive. We hypothesize that PFAAs increase steatosis because the balance of fatty acid accumulation/synthesis and oxidation is disrupted to favor accumulation. We speculate that strong PPARα activators like WY do not induce steatosis even though they activate fatty acid and TG synthesis, because fatty acid oxidation dominates over any increases in the synthesis or accumulation of fatty acids and TG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.12.007.

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