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DEPARTMENT OF BIOMEDICAL ENGINEERING

THE ROLE OF MOUSE AND HUMAN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-α IN MODULATING THE HEPATIC EFFECTS OF PERFLUOROHEXANE SULFONATE (PFHxS) IN MICE

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SPRING 2024

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biomedical Engineering
with honors in Biochemistry and Molecular Biology

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ABSTRACT

Perfluorohexane sulfonate (PFHxS) is a short-chain perfluorinated alkylated substance (PFAS) used in various industrial processes. PFAS chemicals, including PFHxS, have been detected in the general human population. In rodents, PFAS exposure is associated with liver toxicity and liver cancer through mechanisms that may require nuclear receptors like peroxisome proliferator-activated receptor-α (PPARα), constitutive androstane dione receptor (CAR), or pregnane X receptor (PXR). This study tested the hypothesis that PFHxS causes changes in liver by activating PPARα, CAR or PXR. Wild-type, *Ppara*-null, and *PPARA*-humanized mice were fed either a control diet or one containing 3 mg/kg, or 30 mg/kg PFHxS diets for either 7 or 28 days. Relative liver weight was higher in wild-type, *Ppara*-null, and *PPARA*-humanized mice fed PFHxS in a dose-dependent manner compared to controls. The concentration of PFHxS in serum and liver was increased dose-dependently in all three genotypes and reached levels well above typical levels in humans. PFHxS exposure caused an increase in mRNA levels of the PPARα target genes *Cyp4a10* and *Acox1* after twenty-eight days in wild-type mice and *PPARA*-humanized mice compared to controls. This effect was not observed in similarly treated *Ppara*-null mice. PFHxS exposure did not alter expression of the CAR target gene *Cyp2b10* in wild-type mice after either seven or twenty-eight days. By contrast, expression of *Cyp2b10* was increased by PFHxS in *Ppara*-null and *PPARA*-humanized mice compared to controls. PFHxS exposure did not alter expression of the PXR target gene *Cyp3a11* in wild-type or *Ppara*-null mice after either seven or twenty-eight days. By contrast, expression of *Cyp3a11* was increased by PFHxS in *PPARA*-humanized mice compared to controls. Results from these studies demonstrate that exposure to PFHxS causes an increase in liver weight that is due in part to

activation of PPARa, but activation of CAR and PXR also contribute to this phenotype. *Ppara*-null mice had increased relative liver weights despite no PPARa activation, suggesting that CAR and PXR were responsible for increased weights. A dietary concentration of 30 mg/kg but not 3 mg/kg PFHxS was able to achieve liver and serum concentrations in the range required to activate PPARα, CAR, or PXR. While *PPARA*-humanized mice and wild-type mice both had receptor activation, differences between rodents and humans in terms of CAR/PXR activation and PPARα sensitivity suggest that tumors would not form in humans.

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ACKNOWLEDGEMENTS

This work was supported in part by an unrestricted gift from The 3M Company, and the USDA National Institute of Food and Federal Appropriations under Project PEN04607 and Accession number 1009993 (J.M.P, A.D.P.).

1. Introduction

Perfluorohexane sulfonate ($C_6HF_{13}O_3S$, PFHxS) is a compound that belongs to a group of chemicals known as perfluorinated alkylated substances (PFAS) (Zhong et al., 2022). Within this class of compounds, PFHxS is considered a short-chain PFAS which makes it the end-product of breakdown of fluorotelomers (Presentato et al., 2020). PFHxS, like other PFAS chemicals, is thermally stable, has amphipathic properties, and is chemically stable. These factors make PFHxS a favorable chemical to use in a variety of industrial processes including but not limited to production of aviation hydraulic fluid, textiles, and metal plating. Since there have not been any *in vivo* breakdown reactions identified for fluorotelomers, PFHxS has the potential to accumulate within living systems (Presentato et al., 2020). In fact, PFAS are detected within serum samples of nearly all adults in the United States (Costello et al., 2022). This tendency of PFAS substances to bioaccumulate has led to their designation as persistent organic pollutants (POPs) at the Stockholm Convention (Presentato et al., 2020).

Studies with PFAS chemicals have demonstrated that administration of these chemicals in mice over various time frames has led to hepatomegaly, hepatic lesions, upregulated fatty acid metabolism, and an induction of PPARα signaling (Roth et al., 2020). These studies support the idea that PFAS chemicals cause activation of PPARα and the downstream genes associated with PPARα expression (Intrasuksri et al., 1998; Rosen et al., 2008; Su et al., 2022; Takacs & Abbott, 2007).

Existing studies that have been conducted with PFAS in rodents reveal that exposure is associated with liver injury. This was assessed through using biomarkers of hepatotoxicity found within serum (Costello et al., 2022). Hepatic effects that occur as a result of PFAS are believed to be mediated by nuclear receptors such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor-α (PPARα) (Bijland et al., 2011; Chang et al., 2009; Elcombe, Elcombe, Foster, Chang, Ehresman, & Butenhoff, 2012; Elcombe, Elcombe, Foster, Chang, Ehresman, Noker, et al., 2012). Incidences of liver injury and hepatocellular carcinomas in rodents have sparked interest into studying these receptors with regards to human health.

PPARα is a part of a class of ligand-activated transcription factors called peroxisome proliferator-activated receptors (PPARs). It is a nuclear hormone receptor that is present in rodents and humans, regulating critical functions such as energy homeostasis. PPARα is involved in glucose and lipid homeostasis, cell proliferation and differentiation, and inflammation in the liver. It is involved in mediation of liver cancer in rodents resulting from long-term administration of PPARα agonists (Peters et. al. 2012). Additionally, PPARα activation results in a reduction of triglyceride serum concentration. In the liver, PPARα is involved in FA oxidation providing energy for peripheral tissues and they may have a role in antioxidant pathways. It has also been observed that prolonged activation of PPARα can result in liver cancer in rodents when activation is a result of a pharmacological ligand (Corton et al., 2018; Klaunig et al., 2003; Peters et al., 2005). However, due to species differences between rodents and humans in PPARα signaling, liver cancer is not observed with comparable activation of human PPARα (Corton et al., 2018; Klaunig et al., 2003; Peters et al., 2005).

Both CAR and PXR are nuclear receptors highly expressed in the liver and involved in xenobiotic sensing and metabolism. Being members of the NR11 nuclear receptor family, CAR and PXR share many functions related to xenobiotic metabolism and excretion due to having similar target gene profiles. This is due to the receptors sharing similar binding motifs in the promoter region of target genes. Within rodents, CAR is known to cause hepatocarcinogenesis and increased liver cell proliferation (Timsit et. al. 2007). Studies involving treatment of mice with phenobarbital (PB), a CAR agonist, showed hepatocyte proliferation and development of liver tumors. Conversely, PXR activation does not have a direct link to liver cancer. In studies on mice treated with PXR agonist pregnenolone 16α-carbonitrile (PCN) with or without PB, PXR did not promote liver cancer compared to CAR. Instead, PXR attenuated carcinogenesis resulting from CAR activation (Shizu et. al. 2021). Additionally, PB is a drug administered to patients for the management of seizures and alcohol withdrawal (Lindberg et. al. 1992). Despite its long history as a therapeutic, epidemiological investigations of patients suggest PB does not cause significant elevation of liver tumors. Clearly, there is a species difference where hepatocarcinogenesis via CAR/PXR is specific to rodents (Sakamoto et. al. 2013).

Given the existing findings, it is of interest to determine the impact of PFHxS through PPARα-mediated mechanisms and whether there is a species difference between mouse and human PPARα. In the following study, the role of human PPARα, mouse PPARα, CAR, and PXR in changes to the liver will be examined through using wild-type, *Ppara*-null and *PPARA*-humanized mice and treatment groups including control, low PFHxS dose, and high PFHxS dose.

2. Materials and Methods

2.1 Animals and Treatments

Wild-type, *Ppara*-null, and *PPARA*-humanized male mice with a Sv/129 genetic background were used (Akiyama et al., 2001; Cheung et al., 2004; Lee et al., 1995). Mice were age and weight matched and placed in a temperature and light controlled environment (25 ºC, 12-hr light/12-hr dark cycle). PFHxS was provided by the 3M company (St. Paul, MN). Dyets, Inc. (Bethlehem, PA) prepared three diets including a control, 3 mg/kg PFHxS and 30 mg/kg PFHxS diet. An AIN-93G diet formulated for animal growth was used for the control diet in both studies, and both PFHxS diets were formulated in the same manner apart from adding PFHxS at either 3 mg/kg or 30 mg/kg concentrations (Reeves, 1997). Environmental exposure to PFHxS in the general population and occupational exposure in firefighters who work with aqueous film forming foam results in serum PFHxS close to 3.2 ng/mL and 33 ng/mL, respectively (Rotander et. al. 2015). The dietary concentrations of PFHxS were determined with confidential data from the 3M Company in which one strain of mice (CD1) was treated with PFHxS. These data revealed that a dose of 3 mg/kg and 30 mg/kg resulted in PFHxS concentration in the range of 6800 to 110,000 nmol/kg for liver and 19,000 to 96,000 nmol/L for serum. The concentration of PFHxS in the prepared diets was confirmed using LC-MS/MS as previously described (Su et al., 2022; Zhang et. al. 2016). For the 3 mg/kg and 30 mg/kg diets, respectively, actual PFHxS concentration was found to be 2.20 ± 0.13 mg/kg and 25.8 ± 1.47 mg/kg as determined by the 3M Company (St. Paul, MN).

Additionally, there are studies with PFOA (a type of PFAS akin to PFHxS) that demonstrated changes in hepatocellular hypertrophy and PFOA concentration given PFOA diet concentrations ranging from 0.3 mg/kg up to 30 mg/kg (Roth et al., 2020). In reference to previous studies performed by this laboratory along with analysis by other laboratories, the average body weight of Sv/129 adult male mice is 25 grams and the average food consumption on a daily basis is 4 grams (Bachmanov et al., 2002). Therefore, the average daily dosage of PFHxS consumed by mice fed the 0.0003% diet is approximately 0.005 mg/kg body and for 0.003% the average dosage is approximately 0.05 mg/kg body.

To determine whether dietary PFHxS could activate mouse PPARα or human PPARα, 7- and 28-day studies were performed, where 90 total mice were used with 45 mice in each study. Within each 45-mouse cohort, there were 15 male wild-type, 15 *Ppara*-null and 15 *PPARA*-humanized mice. At the end of the study period, mice were euthanized through over-exposure to carbon dioxide, and cervical dislocation was used as a secondary confirmation. Mice were dissected to separate blood and liver tissues. Livers were weighted, snap-frozen in tubes suspended in liquid nitrogen, and stored at – 80 °C until RNA isolation or liver concentration determination. Blood was processed through centrifugation to separate serum that was stored at – 80 °C until serum concentration determination. Five mice from each genotype were used for each treatment group. The same procedure was carried out with mice in the 28-day study.

2.2 Analysis of serum and liver PFHxS concentrations

To quantify PFHxS concentration in liver and serum, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was utilized as previously mentioned (Chang et al., 2012).

2.3 Quantitative real-time PCR analysis

Trizol reagent was used to isolate total RNA from mouse liver samples (Invitrogen, Carlsbad, CA). This total RNA was used to generate cDNA using the 2.5 μg total RNA with MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qPCR) analysis was used to measure expression of mRNA encoding acyl CoA oxidase 1 (*Acox1*), cytochrome P450 2b10 (*Cyp2b10*), cytochrome P450 3a11 (*Cyp3a11*), and cytochrome P450 4a10 (*Cyp4a10*). PerfeCTa SYBR green SuperMix (Quantabio, Beverly, MA, USA) was used to perform qPCR reactions in 96-well plates using the Bio-rad CFX Connect Real-Time PCR Detection System (Bio-rad, Hercules, CA). One 96-well plate was used per gene. The following primers were used to quantify mRNAs: Acox1 forward 5′-TGCCTTTGTTGTCCCTATCCGTGA-3′ and reverse 5′-TTACATACGTGCCGTCAGGCTTCA-3′; Cyp2b10 forward 5′-ACCCCACGTTCCTCTTCCA-3′ and reverse 5′-CAGCAGGCGCAAGAACTG-3′; Cyp3a11 forward 5′-GTCAAACGCCTCTCCTTGCTG-3′ and reverse 5′-GGCTTGCCTTTCTTTGCCTTC-3′; and Cyp4a10 forward 5′-TGCCCATGATCACACAGATGGAGT-3′ and reverse 5′-TGAATGTGTCCACCTCAGCACGTA-3′. Given that expression of glyceraldehyde-3-

phosphate dehydrogenase (Gapdh) mRNA is stable between treatments across various PFAS chemicals, expression of target genes was normalized to Gapdh as a housekeeping gene (Bangma et al., 2020). gene using the following primers: forward, 5′-GGTGGAGCCAAAAGGGTCAT-3′ and reverse, 5’-GGTTCACACCCATCACAAACAT-3′.

2.4 Statistical analysis

Statistical significance was determined using the ANOVA and Bonferroni post-hoc test and a $P \leq 0.05$ was used to test for significance (Prism 9.0, GraphPad Software, San Diego, CA, USA). Gene expression was determined through using the delta-delta Ct method and percent of control.

3. Results

Table 1
Average body weight in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$), or humanized Ppara (*PPARA*) mice at over 7 days of treatment with PFHxS (0.0003% and 0.003%).

Genotype	Treatment	Day 0 (g)	Day 7 (g)
Ppara$^{+/+}$	Control	21.3 ± 4.0[a]	24.0 ± 3.8[a]
Ppara$^{+/+}$	3 mg/kg PFHxS	21.1 ± 3.2[a]	22.1 ± 3.2[a]
Ppara$^{+/+}$	30 mg/kg PFHxS	21.9 ± 3.5[a]	24.1 ± 4.4[a]
Ppara$^{-/-}$	Control	21.2 ± 3.4[a]	21.3 ± 3.7[a]
Ppara$^{-/-}$	3 mg/kg PFHxS	20.6 ± 3.6[a]	21.1 ± 3.7[a]
Ppara$^{-/-}$	30 mg/kg PFHxS	21.9 ± 3.8[a]	22.5 ± 4.2[a]
PPARA	Control	22.6 ± 2.4[a]	23.0 ± 3.1[a]
PPARA	3 mg/kg PFHxS	21.9 ± 2.2[a]	22.7 ± 2.7[a]
PPARA	30 mg/kg PFHxS	20.7 ± 2.7[a]	21.8 ± 3.2[a]

Values within each cell represent mean ± SEM. Values within a column with superscripts of a different letter are statistically different, $P \leq 0.05$.

Table 2
Average serum and liver concentrations of PFHxS in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$) or *PPARA*-humanized (*PPARA*) mice over 7 days of dietary PFHxS (0.0003% and 0.003%).

Genotype	Treatment	Liver [PFHxS] (ng/g)	Serum [PFHxS] (ng/mL)
Ppara$^{+/+}$	Control	7.6 ± 11.9[a]	17.9 ± 27.9[a]
Ppara$^{+/+}$	3 mg/kg PFHxS	5600 ± 600[b]	19300 ± 2300[b]
Ppara$^{+/+}$	30 mg/kg PFHxS	135000 ± 20000[c,d]	180000 ± 12000[c]
Ppara$^{-/-}$	Control	5.6 ± 3.3[a]	3.7 ± 8.4[a]
Ppara$^{-/-}$	3 mg/kg PFHxS	5700 ± 1100[b]	19000 ± 2000[b]
Ppara$^{-/-}$	30 mg/kg PFHxS	162000 ± 20000[d,e]	176000 ± 30000[c]
PPARA	Control	2.3 ± 3.2[a]	B L Q
PPARA	3 mg/kg PFHxS	6000 ± 500[b]	20200 ± 2100[b]
PPARA	30 mg/kg PFHxS	188000 ± 20000[e,f]	180000 ± 20000[c]

Values within each cell represent mean ± SEM. Values within a column with superscripts of a different letter are statistically different, $P \leq 0.05$. BLQ means values were below quantifiable limit of 10 ng/mL.

Table 3
Average body weight in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$), or humanized Ppara (*PPARA*) mice at seven day intervals over 28 days of treatment with PFHxS (0.0003% and 0.003%).

Genotype	Treatment	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)	Day 28 (g)
Ppara$^{+/+}$	Control	24.6 ± 3.6[a]	26.0 ± 4.3[a]	27.3 ± 4.6[a]	26.5 ± 4.9[a]	26.7 ± 4.9[a]
Ppara$^{+/+}$	3 mg/kg PFHxS	23.5 ± 1.1[a]	25.9 ± 1.9[a]	27.1 ± 2.2[a]	26.6 ± 2.3[a]	26.5 ± 2.4[a]
Ppara$^{+/+}$	30 mg/kg PFHxS	24.2 ± 2.4[a]	26.9 ± 2.4[a]	27.5 ± 2.2[a]	27.4 ± 2.3[a]	27.8 ± 2.8[a]
Ppara$^{-/-}$	Control	23.0 ± 2.3[a]	24.8 ± 4.3[a]	24.2 ± 3.5[a]	24.7 ± 3.5[a]	24.5 ± 3.4[a]
Ppara$^{-/-}$	3 mg/kg PFHxS	22.8 ± 3.3[a]	24.6 ± 4.0[a]	24.9 ± 4.1[a]	24.9 ± 4.0[a]	24.9 ± 3.9[a]
Ppara$^{-/-}$	30 mg/kg PFHxS	23.4 ± 4.7[a]	24.0 ± 4.0[a]	24.4 ± 4.3[a]	24.9 ± 4.7[a]	25.1 ± 4.8[a]
PPARA	Control	22.1 ± 1.3[a]	23.3 ± 1.6[a]	23.7 ± 2.4[a]	23.6 ±2.2[a]	23.2 ± 2.2[a]
PPARA	3 mg/kg PFHxS	22.5 ± 2.2[a]	23.6 ± 2.2[a]	23.9 ± 2.4[a]	23.6 ± 2.2[a]	23.2 ± 2.5[a]
PPARA	30 mg/kg PFHxS	21.7 ± 1.0[a]	22.5 ± 1.6[a]	23.1 ± 1.9[a]	23.0 ± 1.5[a]	22.3 ± 1.7[a]

Values within each cell represent mean ± SEM. Values within a column with superscripts of a different letter are statistically
different, $P \leq 0.05$.

Table 4
Average serum and liver concentrations of PFHxS in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$) or *PPARA*-humanized (*PPARA*) mice over 28 days of dietary PFHxS (0.0003% and 0.003%).

Genotype	Treatment	Liver [PFHxS] (ng/g)	Serum [PFHxS] (ng/mL)
Ppara$^{+/+}$	Control	13.6 ± 6.2[a]	14.1 ± 24.5[a]
Ppara$^{+/+}$	3 mg/kg PFHxS	17000 ± 1200[b]	47000 ± 4000[b]
Ppara$^{+/+}$	30 mg/kg PFHxS	271000 ± 4900[c]	239000 ± 24000[c]
Ppara$^{-/-}$	Control	26.3 ± 22.7[a]	B L Q
Ppara$^{-/-}$	3 mg/kg PFHxS	47000 ± 67000[a,b]	70400 ± 70400[a,b]
Ppara$^{-/-}$	30 mg/kg PFHxS	201000 ± 17000[d]	235000 ± 64000[c,d]
PPARA	Control	19.2 ± 16.1[a]	22.6 ± 8.5[a]
PPARA	3 mg/kg PFHxS	19000 ± 3000[b]	50000 ± 3200[b]
PPARA	30 mg/kg PFHxS	250000 ± 18000[c]	156000 ± 18000[d,e]

Values within each cell represent mean ± SEM. Values within a column with superscripts of a different letter are statistically different, $P \leq 0.05$. BLQ means values were below the quantifiable limit of 10 ng/mL.

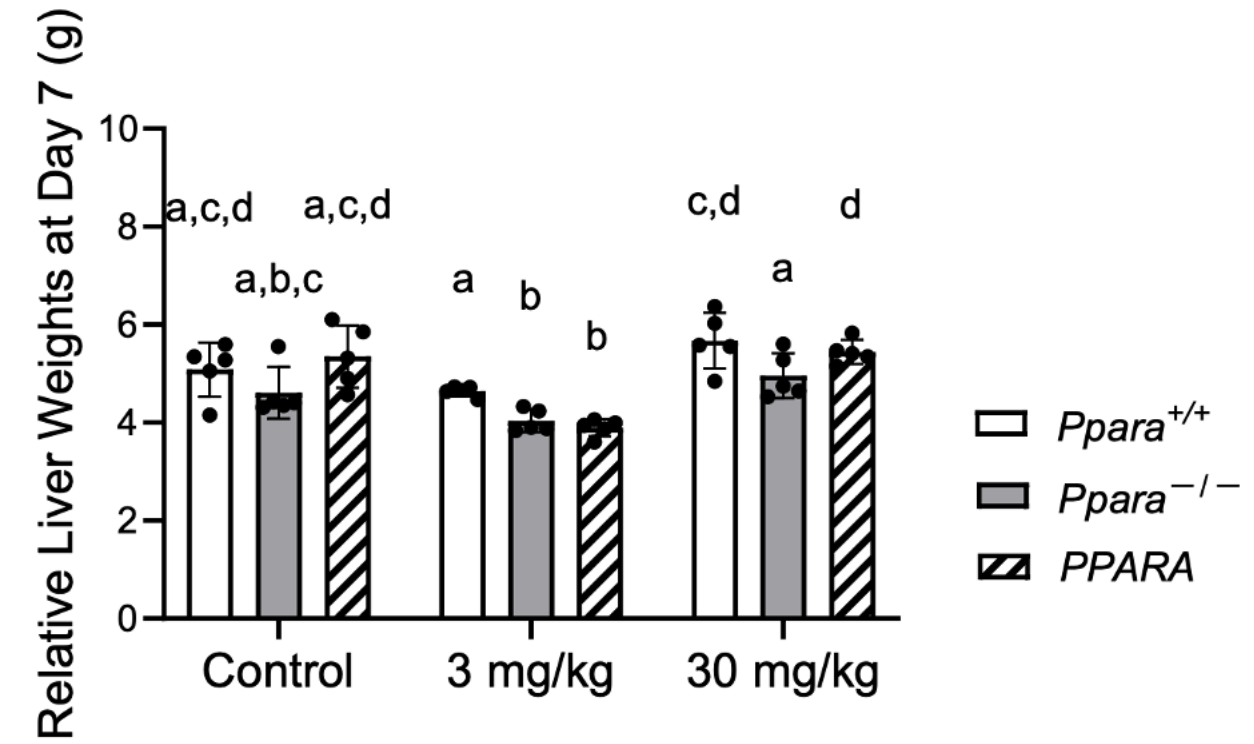

Fig. 1. Average relative liver weight in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$), or *PPARA*-humanized (*PPARA*) mice after seven days of dietary PFHxS (0.0003% or 0.003%). Values represent the mean ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

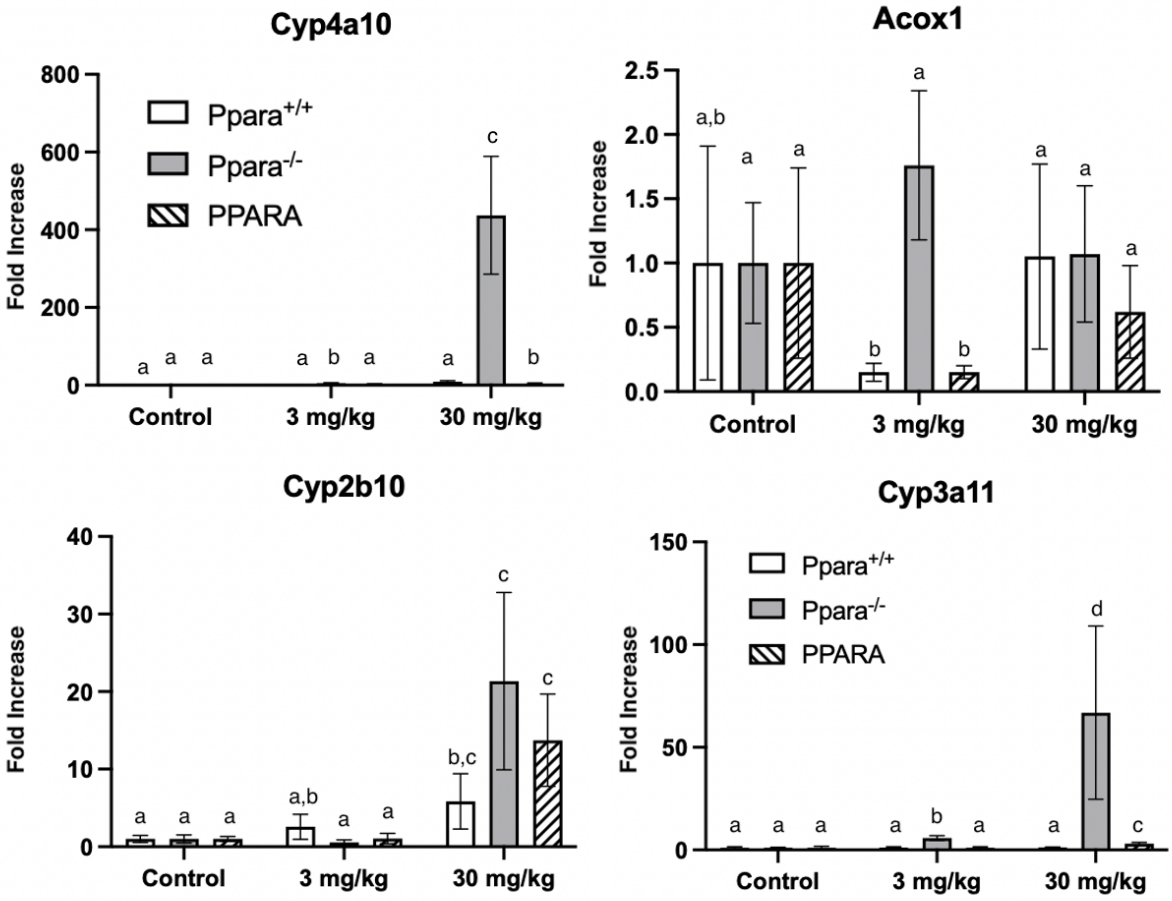

Fig. 2. Relative hepatic expression of the PPARα (*Acox1*, *Cyp4a10*), CAR (*Cyp2b10*), and PXR (*Cyp3a11*) target genes in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$) or *PPARA*-humanized (*PPARA*) mice after seven days of dietary PFHxS (0.0003% or 0.003%). Values represent the mean ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

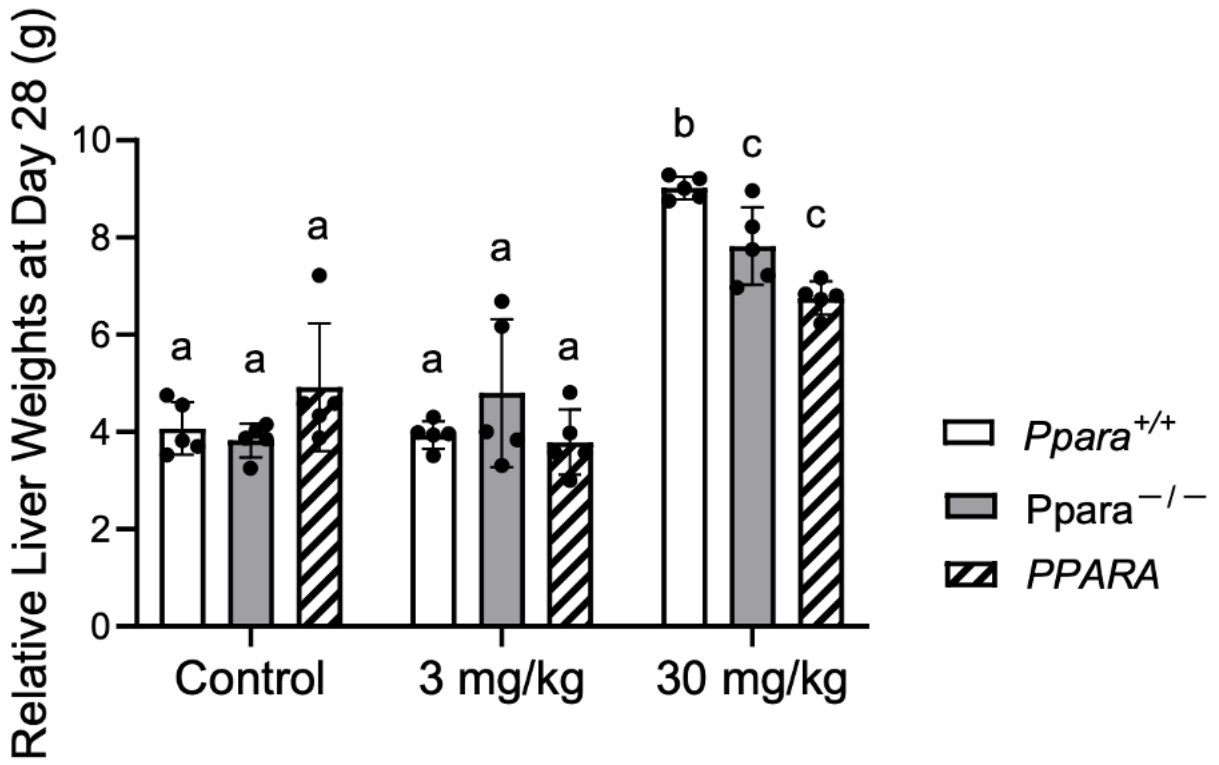

Fig. 3. Average relative liver weight in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$), or *PPARA*-humanized (*PPARA*) mice after twenty-eight days of dietary PFHxS (0.0003% or 0.003%). Values represent the mean ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

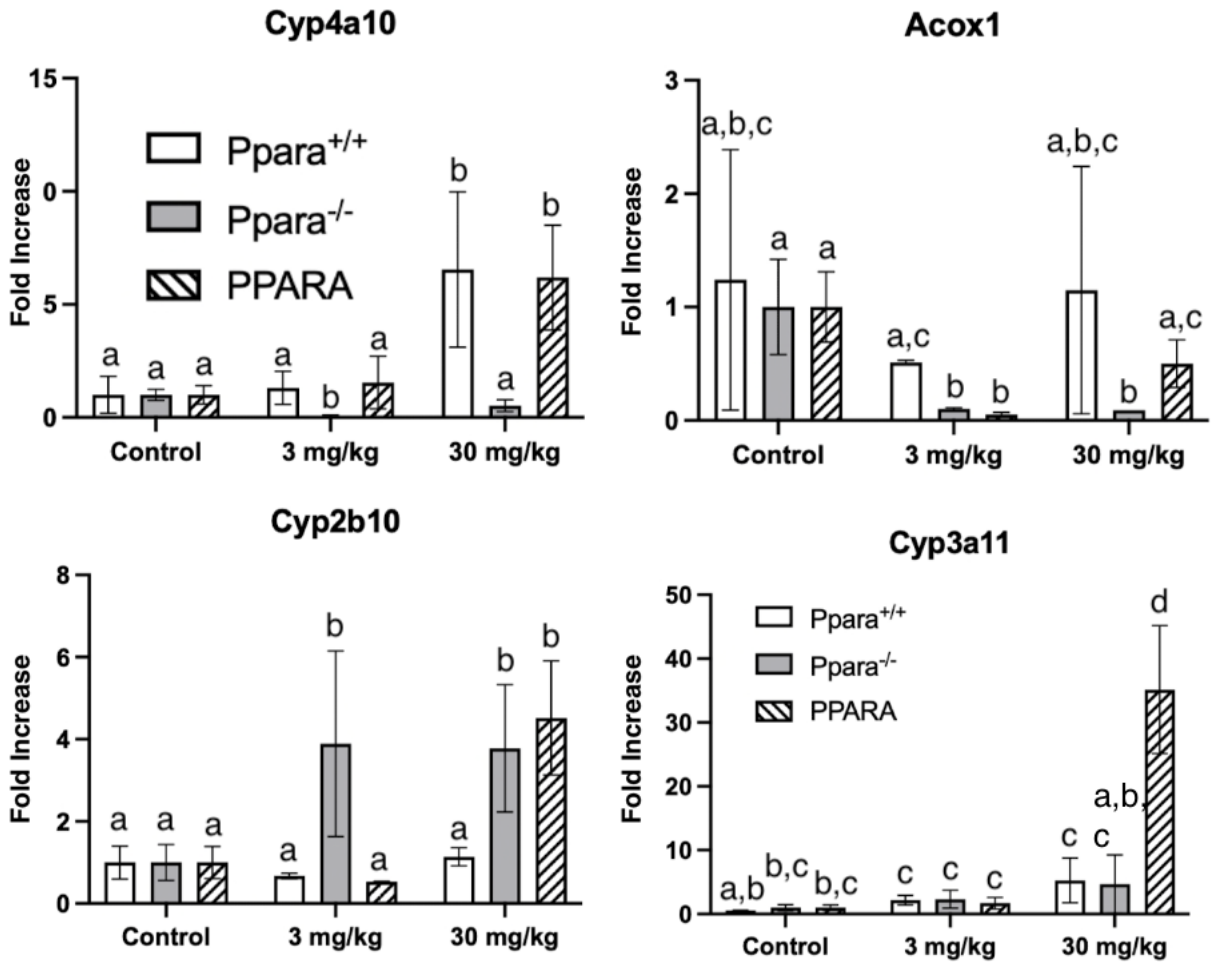

Fig. 4. Relative hepatic expression of the PPARα (*Acox1*, *Cyp4a10*), CAR (*Cyp2b10*), and PXR (*Cyp3a11*) target genes in wild-type (*Ppara*$^{+/+}$), *Ppara*-null (*Ppara*$^{-/-}$) or *PPARA*-humanized (*PPARA*) mice after twenty-eight days of dietary PFHxS (0.0003% or 0.003%). Values represent the mean ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

The beginning and ending average mouse body weight with 7- and 28-day exposure were similar across all genotypes and treatment groups (Table 1, 3). There was no effect of PFHxS on liver weight in response to the 3 mg/kg exposure in wild-type and *Ppara*-null mice, but liver weight was higher in wild-type mice fed 30 mg/kg PFHxS compared to controls after 28 days of exposure (Figure 1, 3). Interestingly, there was no effect of PFHxS on liver weight in response to the 3 mg/kg exposure in *PPARA*-humanized mice, and liver weight was lower in *Ppara*-null and *PPARA*-humanized mice fed 30 mg/kg PFHxS after 28 days of exposure compared to wild-type (Figure 1, 3). Average liver concentration of PFHxS in 3 mg/kg mice with 7- and 28-day exposure were approximately 2000 nmol/kg and 12,000 nmol/kg, respectively. When treated with 30 mg/kg PFHxS over 7 and 28 days, average liver concentration was approximately 60,000 nmol/kg and 100,000 nmol/kg, respectively, across all three genotypes. There was a statistically significant difference between PFHxS concentrations in the liver of wild-type and *PPARA*-humanized mice when treated with 30 mg/kg PFHxS over 7 days (Table 2, 4).

Average serum concentration of PFHxS in 3 mg/kg PFHxS mice over 7- and 28-day exposures was approximately 8,000 nmol/L and 24,000 nmol/L across all three genotypes. When treated with 30 mg/kg PFHxS with 7- and 28-day exposure, average serum concentration was approximately 72,000 nmol/L and 84,000 nmol/L, respectively. Dosimetry showed a general trend of increasing PFHxS serum concentrations as the dosage of PFHxS treatment increased (Table 2, 4).

No effect was seen in wild-type mice treated with 3 mg/kg PFHxS over 7 and 28 days for *Cyp4a10, Acox1, Cyp2b10* and *Cyp3a11* mRNA (Fig. 2, 4). For *Cyp3a11*, wild-type mice over 28 days had higher mRNA levels when treated with 3 mg/kg PFHxS as compared to control (Fig.

4). *Ppara*-null mice with 7-day exposure treated with 3 mg/kg PFHxS had elevated *Cyp4a10* and *Cyp3a11* mRNA (Fig. 2). *Ppara*-null mice treated with 3 mg/kg PFHxS over 28 days had lower levels of *Cyp4a10* and *Acox1* mRNAs as compared to controls. In *Cyp2b10*, *Ppara*-null mice treated with 3 mg/kg PFHxS had higher mRNA levels compared to control (Fig. 4). For *Acox1*, *PPARA*-humanized mice treated with 3 mg/kg PFHxS had lower mRNA levels as compared to control (Fig. 2). In *Acox1*, *PPARA*-humanized mice treated with 3 mg/kg PFHxS had lower mRNA levels as compared to control (Fig. 4).

When treated with 30 mg/kg PFHxS, wild-type mice had elevated mRNA levels only for *Cyp2b10* as compared to controls. *Cyp4a10*, *Acox1*, and *Cyp3a11* were not significantly different compared to controls for 30 mg/kg PFHxS wild-type mice (Fig. 2). When treated with 30 mg/kg PFHxS over 28 days, wild-type mice had elevated *Cyp4a10* mRNA as compared to control. However, for *Acox1*, *Cyp2b10*, and *Cyp3a11*, mRNA levels were not significantly different from controls (Fig. 4). *Ppara*-null mice treated with 30 mg/kg PFHxS displayed elevated mRNA levels in *Cyp4a10*, *Cyp2b10*, and *Cyp3a11* compared to controls. *Acox1* mRNA was not significantly different compared to controls for 30 mg/kg PFHxS *Ppara*-null mice (Fig. 2). *Ppara*-null mice treated with 30 mg/kg PFHxS over 28 days did not show elevated mRNA levels in any gene except *Cyp2b10* (Fig. 4). Finally, *PPARA*-humanized with 30 mg/kg PFHxS mice presented elevated *Cyp4a10*, *Cyp2b10*, and *Cyp3a11* mRNA compared to respective controls. Finally, *PPARA*-humanized with 30 mg/kg PFHxS over 28 days had elevated *Cyp4a10*, *Cyp2b10*, and *Cyp3a11* mRNA compared to respective controls (Fig. 4).

4. Discussion

Average body weight was consistent in mice within both 7-day and 28-day exposures showing no dose-dependent difference in body weight and suggesting no toxicity (Tables 1, 3). Dosimetry did show a clear increase in liver and serum concentration of PFHxS as the treatment of PFHxS increased, suggesting the experiment was performed correctly (Table 2). Given dosages of 3 mg/kg and 30 mg/kg PFHxS, PFHxS concentration was in the range of 6800 to 110,000 nmol/kg in liver and 19,000 to 96,000 nmol/L in serum. Concentrations resulting from 30 mg/kg PFHxS administration were able to achieve PPARα, CAR, and PXR activation which was not seen in mice fed 3 mg/kg PFHxS. Dosimetry from administration of dietary PFHxS to wild-type, *Ppara*-null, and *PPARA*-humanized revealed an important finding when compared to similar studies. In this study, hepatic PFHxS concentrations for mice treated with 3 mg/kg or 30 mg/kg with 28-day exposure was of similar order of magnitude to hepatic concentrations in a study with a different strain of mice over 28 days (Change et. al. 2018). Similarly, serum concentration in mice treated 3 mg/kg or 30 mg/kg at either 7 days or 28 days was of similar order of magnitude to serum concentration in two studies utilizing different strains of mice over 28 days (Change et. al. 2018; Narizzano et. al. 2023). Therefore, these results suggest that there may be no significant differences in PFHxS toxicokinetics between strains of mice used in the respective studies (Sv/129, CD1, and deer mice).

Further, 3 mg/kg and 30 mg/kg PFHxS treated mice with 28-day exposure had higher serum and liver PFHxS concentration compared to mice with 7-day exposure. Thus, there was a time-dependent increase in serum and liver concentrations in both 3 mg/kg and 30 mg/kg PFHxS treated mice. This is distinct from a previous study performed with PFOS, where hepatic and

serum concentrations of PFOS were similar in 7-day and 28-day exposures for both low and high dose groups, suggesting that a steady-state concentration of PFOS had been achieved within the study timeframe (Su et al. 2022). Since a steady-state concentration of PFHxS was not able to be achieved in a similar time frame, these results suggest that PFHxS has a longer half-life than PFOS. Indeed, research conducted over the past two decades suggests that PFOS has a half-life of 5.4 years compared to 8.5 years for PFHxS (Sonnenberg et. al. 2023), but this will require further studies.

A dose-dependent increase was observed in relative liver weight in 3 mg/kg treated mice and 30 mg/kg treated mice. This effect was only seen with 28-day exposure where all three genotypes had greater relative liver weights in the 30 mg/kg treatment group as compared to controls and 3 mg/kg treatments. This increase was especially pronounced in the 30 mg/kg wild-type genotype which was statistically higher than 30 mg/kg *Ppara*-null and *PPARA*-humanized treated mice (Fig. 3). This data alone suggests that wild-type mice possibly had a greater degree of PPARα activation. In similar studies that looked at PPARα activation over long-term administration of agonists, there were high incidences of tumor generation seen in wild-type mice that were not seen in *PPARA*-humanized and *Ppara*-null mice (Foreman et al., 2021). In one of these studies, despite both mouse and human versions of the PPARα being activated by a high-affinity human PPARα agonist (GW7647), the wild-type mice experienced a greater degree of hepatomegaly, hepatotoxicity, and hepatocarcinogenesis (Foreman et al., 2021). These differences can potentially help explain why a greater relative liver weight was observed in wild-type mice as compared to *Ppara*-null and *PPARA*-humanized mice.

Wild-type and *PPARA*-humanized mice both had elevated expression compared to respective controls for PPARα target gene *Cyp4a10.* In a prior study involving PFOS, under identical treatment conditions, wild-type mice had greater mRNA levels than *PPARA*-humanized mice (Su et al., 2022). Human PPARα may be more sensitive to activation by PFHxS as compared to PFOS in terms of *Cyp4a10*. However, wild-type and *PPARA*-humanized mice were not significantly different from one another in expression for PPARα target gene *Acox1.* While both *Cyp4a10* and *Acox1* are PPARα target genes, their sensitivities to PPARα activation differ. Through analyzing similar studies that looked at expression of both target genes, *Cyp4a10* generally had a greater magnitude of expression as compared to *Acox1* (Foreman et al., 2021; Su et al., 2022; Yoo et al., 2015). Overall, the data suggests that PPARα activation did occur for wild-type and *PPARA*-humanized mice when treated with dietary PFHxS. However, this does not necessarily mean that tumors would form in human livers when exposed to PFHxS.

Further, CAR target gene *Cyp2b10* had elevated levels of mRNA in *PPARA*-humanized mice, but there was no significant change in expression in wild-type mice. Unexpectedly, there is elevated *Cyp2b10* expression for *Ppara*-null mice compared to control. *Ppara*-null mice lack PPARα which is known to induce expression of CAR, bringing into question the validity of the data (Shizu et. al. 2023). However, it is possible that *PPARA*-humanized mice had CAR activation with administration of dietary PFHxS. For PXR target gene *Cyp3a11*, 30 mg/kg treated wild-type and *PPARA*-humanized mice had elevated levels of mRNA as compared to controls. 30 mg/kg *PPARA*-humanized mice had a fold increase nearly four times that of 30 mg/kg wild-type mice. PXR activation likely did occur with *PPARA*-humanized and wild-type mice with the administration of dietary PFHxS.

While *PPARA*-humanized and wild-type mice did show expression of PPARα target genes, the expression of PPARα is not singularly responsible for hepatic effects. Despite *Ppara*-null mice demonstrating no activation of *Cyp4a10* and *Acox1* as compared to controls, *Ppara*-null mice did undergo a statistically significant increase in relative liver weight in the 30 mg/kg PFHxS treatment group. In fact, they had a greater relative liver weight than *PPARA*-humanized mice (Figure 3). This suggests that mechanisms beyond PPARα activation mediated the hepatic effects observed in the *Ppara*-null group. These mechanisms may include CAR and PXR. Within mice, CAR activation is known to cause development of liver cancer (Timsit et. al. 2017). In studies where mice were treated with CAR agonist phenobarbital (PB), CAR expression led to the development of liver tumors (Shizu et. al. 2021). On the other hand, mice treated with PXR agonist pregnenolone 16α-carbonitrile (PCN), with or without PB, PXR did not promote liver cancer compared to CAR (Shizu et. al. 2021). Rather, PXR served to attenuate liver cancer that resulted from CAR activation (Shizu et. al. 2021). There are also species differences that have implications for CAR and PXR in terms of hepatocarcinogenesis in rodents compared to humans. PB has a long history as a therapeutic for seizures, and epidemiological investigations of patients suggest PB does not cause significant elevation of liver tumors. Clearly, hepatic tumorigenesis via CAR/PXR is specific to rodents and is not seen in humans (Sakamoto et. al. 2013). Further, there are differences in terms of PPARα signaling between rodents and humans. Even though *PPARA*-humanized mice displayed increased expression of PPARα target genes compared to controls, tumor formation that occurs in mice is not believed to be present in humans (Corton et al., 2018; Klaunig et al., 2003; Peters et al., 2005). Studies suggest that mouse PPARα is more sensitive to activation than human PPARα across a wide array of agonists (Corton et al., 2018; Klaunig et al., 2003; Peters et al., 2005). For instance, a study investigating GW7647's activation

of PPARα showed modification in tumor formation in *Ppara*-null and *PPARA*-humanized mice as compared to wild-type mice (Foreman et al., 2021). Histopathology data is currently pending completion and is required to delineate the exact changes that PFHxS caused in the liver, however, the increase in liver weights is likely from hypertrophy and hyperplasia. There is a need for further research to delineate the mechanisms by which PFHxS results in PFAS-induced hepatotoxicity while keeping in mind species differences between rodents and humans.

Quality qPCR data, particularly for the 7-day exposure mice, could not be retrieved during the time frame of this study. RNA isolation was performed twice along with qPCR, yielding highly variable data that did not line up with expected results based on dosimetry and relative liver weight data. The exact step during which mistakes were made is still unknown, but it may potentially involve too many freeze thaw cycles from the – 80 °C freezer, primers being taken out too many times from multiple people performing qPCR, taking on too many samples at one time (leading to initial tubes sitting out while later tubes are being worked on), and lack of attention to detail that led to imprecision and missteps. Overall, the results from this study do support the hypothesis that PFHxS causes changes in liver by activating PPARα, CAR or PXR.

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