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Hepatic Carboxylesterases are Differentially Regulated in PPAR α -Null Mice Treated with Perfluorooctanoic Acid

Xia Wen^{#*}, Angela A. Baker^{#*}, Curtis D. Klaassen[†], J. Christopher Corton[‡], Jason R. Richardson^{§,2}, and Lauren M. Aleksunes^{*,†,¶,§,2}

^{*}Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, Piscataway, NJ 08854, USA

[†]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98195, USA

[‡]Integrated Systems Toxicology Division, NHEERL/ORD, US-EPA, Research Triangle Park, NC 27711, USA

[§]Robert Stempel School of Public Health and Social Work, Florida International University, Miami, FL, USA (JRR)

[¶]Environmental and Occupational Health Sciences Institute, Rutgers University, Piscataway, NJ 08854, USA

[&]Center for Lipid Research, New Jersey Institute for Food, Nutrition, and Health, Rutgers University, New Brunswick, NJ 08901

[#] These authors contributed equally to this work.

Abstract

Hepatic carboxylesterases (Ces) catalyze the metabolism of drugs, environmental toxicants, and endogenous lipids and are known to be regulated by multiple nuclear receptors. Perfluorooctanoic acid (PFOA) is a synthetic fluorochemical that has been associated with dyslipidemia in exposed populations. In liver, PFOA can activate nuclear receptors such as PPAR α , and alter the metabolism and excretion of chemicals. Here, we sought to test the ability of PFOA to modulate Ces expression and activity in the presence and absence of the PPAR α receptor. For this purpose, male C57BL/6 NCrI mice were administered PFOA (1 or 3 mg/kg, po, 7 days) and livers collected for assessment of Ces expression and activity. PFOA increased Ces1 and 2 protein and activity. Notably, PFOA increased *Ces1d*, *1e*, *1f*, *1g*, *2c*, and *2e* mRNAs between 1.5- and 2.5-fold, while it decreased *Ces1c* and *2b*. Activation of PPAR α by PFOA was confirmed by up-regulation of *Cyp4a14* mRNA. In a separate study of PFOA-treated wild-type (WT) and PPAR α -null mice, induction of *Ces 1e* and *1f* mRNA and in turn, Ces1 protein, was PPAR α -dependent. Interestingly, in PPAR α -null mice, *Ces1c*, *1d*, *1g*, *2a*, *2b*, and *2e* mRNAs and Ces2 protein were up-regulated by

Reprint Requests Lauren M. Aleksunes, Pharm.D., Ph.D., DABT, Department of Pharmacology and Toxicology, Rutgers, the State University of New Jersey, Ernest Mario School of Pharmacy, 170 Frelinghuysen Road, Piscataway, NJ 08854, Phone: +1 (848) 445-5518, Fax: +1 (732) 445-0119, aleksunes@eohsi.rutgers.edu.

²Denotes equal senior contributors

Conflict of Interest

The authors do not have conflicts of interest to declare.

PFOA which contributed to sustained up-regulation of Ces activity, although to a lower extent than observed in WT mice. Activation of the CAR and PXR receptors likely accounted for up-regulation of select Ces1 and 2 subtypes in PPAR α -null mice. In conclusion, the environmental contaminant PFOA modulates the expression and function of hepatic Ces enzymes, in part through PPAR α .

Keywords

PFOA; PPAR α ; Ces; Carboxylesterase

1. Introduction

Hepatic carboxylesterases (rodent Ces/human CES) are Phase-I metabolizing enzymes known for their ability to hydrolyse ester, thioester, carbamate, and amide bonds within chemicals. In human and mouse livers, CES1/Ces1 is the predominant CES with higher mRNA expression compared to CES2/Ces2 (Hosokawa et al. 2008; Jones et al. 2013). Substrates of CES enzymes include antiplatelet drugs, angiotensin converting enzyme inhibitors, HMG-CoA reductase inhibitors, central nervous system stimulants, and insecticides (Potter and Wadkins, 2006). In addition to xenobiotic biotransformation, CES1 (also known as cholesteryl ester hydrolase) has been recognized for its ability to metabolize cholesterol in the liver. Overexpression of CES1 in mice lowers hepatic triglyceride levels (Xu et al., 2014). Likewise, gain of hepatic CES2 expression enhances fatty acid oxidation and represses lipogenesis function in mice (Li et al., 2016). In fact, CES2 can hydrolyse triglycerides and diacylglycerols *in vitro* (Ruby et al., 2017). Collectively, these data point to CES enzymes as important mediators of both xenobiotic and endobiotic metabolism.

For more than a decade, we and others have investigated the transcriptional regulation of Ces enzymes in order to identify novel mechanisms underlying drug-drug and drug-toxicant interactions that can impact xenobiotic disposition and action. One important regulator of chemical disposition in the liver is the peroxisome proliferator-activated receptor alpha (PPAR α). In fact, the PPAR α ligands, di-(2-ethylhexyl)-phthalate and clofibrate, have been shown to induce hepatic Ces activity in mice and rats (Hosokawa et al., 1994; Parker et al., 1996). Subsequent analysis demonstrated that the PPAR α agonist GW7647 can up-regulate the mRNA levels of specific Ces subtypes, namely *Ces 1d*, *1e*, *1f*, *2c* and *2e* (Jones et al. 2013). Two additional hepatic transcription factors, the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), have also been shown to regulate the expression of Ces enzymes (Rosenfeld et al., 2003; Xu et al., 2009; Staudinger et al., 2010). Treatment of mice with either a CAR (1,4-bis-[2-(3,5-dichloro-pyridyloxy)]benzene, TCPOBOP) or PXR activator (pregnenolone-16 α -carbonitrile) enhances the liver mRNA expression of *Ces 1d*, *2a*, and *2c* (CAR targets) and *Ces 1c*, *1d*, *1g*, *2a*, *2c*, and *2e* (PXR targets), respectively (Baker et al., 2015). Likewise, hepatic *Ces* mRNA expression can be also altered by activators of the aryl hydrocarbon receptor (AhR) and the nuclear factor E2-related protein 2 (Nrf2) transcription factor (Zhang et al., 2012). Collectively, these data point to the ability of xenobiotics to modulate Ces expression and activity by influencing the hepatic expression of *Ces* subtypes through multiple transcriptional regulators.

Early studies investigating the regulation of C_{es} enzymes demonstrated that perfluorinated chemicals could induce C_{es} activity. Specifically, perfluorooctanoic acid (PFOA), a synthetic perfluorinated carboxylic acid and fluorosurfactant, was shown in two studies to up-regulate C_{es} activity in rat liver microsomes (Hosokawa and Satoh, 1993; Derbel et al., 1996). The actions of PFOA result, in part, from activation of the transcription factor PPAR α , which is predominantly expressed in liver and regulates fatty acid metabolism (Pyper et al., 2010; Pawlak et al., 2015). PFOA has also been shown to activate CAR and PXR signaling in rodents (Cheng and Klaassen, 2008; Ren et al., 2009; Bjork et al., 2011) as well as estrogen receptor alpha (ER α), PPAR γ , and hepatocyte nuclear factor 4 alpha (HNF4 α) transcription factors in primary human hepatocytes (Zhang et al., 2012; Buhrke et al., 2015). In recent years, there has been increasing interest in the ability of perfluorinated chemicals to not only modulate xenobiotic metabolism but also impart toxicities to humans. PFOA and other related chemicals have been used for decades in commercial applications such as non-stick cookware and carpeting. As a result, PFOA has become an environmental contaminant detectable in drinking water, dust, foods, and also in the serum of the US population (Calafat et al., 2007; Frisbee et al., 2010; Steenland et al., 2010; Gallo et al., 2012). In humans, a growing number of studies have revealed associations between elevated PFOA levels and hypercholesterolemia (Gilliland and Mandel, 1996; Nelson et al., 2010; Steenland et al., 2010; Eriksen et al., 2013; Fitz-Simon et al., 2013; Winquist and Steenland, 2014; Zeng et al., 2015). To date, the exact biochemical and molecular mechanisms underlying the relationship between PFOA and lipid regulation have yet to be definitively established.

The current study was undertaken to elucidate the transcriptional pathways by which environmentally-relevant xenobiotics, such as PFOA, can regulate hepatic C_{es} expression and activity. Specifically, we aimed to determine 1) whether PFOA alters the hepatic expression of C_{es} subtypes and 2) whether C_{es} regulation by PFOA changes in the absence of the PPAR α receptor. Insight into the regulation of C_{es} enzymes is relevant for understanding how environmental chemicals modulate the metabolism of not only drugs and other xenobiotics, but potentially also cholesterol, a lipid mediator implicated in the toxicity of PFOA.

2. Materials and Methods

2.1. Chemicals.

Perfluorooctanoic acid ammonium salt (PFOA) and *p*-nitrophenyl valerate were purchased from Sigma Chemical Co. (St. Louis, MO). Unless specified, all other chemicals and reagents were also obtained from Sigma Chemical Co.

2.2. Animal Treatment.

PFOA was dissolved in deionized water and filter sterilized. *Dose-Response Study*. Adult, male C57BL/6NCR1 mice were purchased from Charles River and administered deionized water or PFOA (1 or 3 mg/kg/d) by po gavage for 7 days. *PPAR α -null Study*. Adult, male wild-type (WT) C57BL/6NTac mice and PPAR α -null mice (n=4-6) were obtained from Taconic Laboratories (Hudson, NY). Groups of WT or PPAR α -null mice (n=4-6) were

administered deionized water or PFOA (3 mg/kg/d) by po gavage for 7 days. Doses of PFOA were selected based on prior mechanistic studies performed by the US Environmental Protection Agency (Rosen et al., 2008; Rosen et al., 2009). Livers were excised, weighed, snap frozen, and stored at -80°C until further analysis. The Rutgers University Institutional Animal Care and Use Committees approved these studies.

2.3. Western Blot Analysis.

Frozen liver samples were homogenized in sucrose-Tris buffer (10 mM Tris-Base, 250 mM sucrose and 1% protease inhibitor cocktail). Tissue homogenates (20 μg protein/well) were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes by iBlot (Life Technologies). After blocking with 5% nonfat dry milk in 0.5% phosphate-buffered saline with 0.5% of Tween 20 (PBS/T), membranes were incubated with a primary antibody against mouse Ces1 (Ab45957, Abcam, Cambridge, MA, 1:2000) or Ces 2 (AF5280, R&D Systems, Inc. Minneapolis, MN, 1:1000) at 4°C overnight followed by incubation with a species-appropriate secondary antibody (Sigma) for 2 h. SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA) was applied to the membranes prior to detection of luminescence using a FluorChem Imager (Alpha Innotech, San Leandro, CA). Target protein band intensities were semi-quantified and normalized to total histone H3 protein expression (4499S, Cell Signaling Technology, Danvers, MA).

2.4. Ces Enzyme Activity Assay.

Ces enzyme activity in mouse hepatic microsomal fractions was determined by the hydrolysis of *p*-nitrophenyl valerate using a continuous spectrophotometric assay as described previously (Baker et al., 2015). Briefly, frozen livers (0.1-0.2 g) were homogenized in buffer (0.05 M Tris-HCl, 1.15% KCl, pH 7.4) and centrifugated at $9000 \times g$ for 20 min at 4°C . The supernatant (*S9* fractions) in homogenizing buffer was further centrifugated at $105,000 \times g$ for 90 min to obtain the microsomal pellet. After washing in buffer (1.15% KCl, 10 mM EDTA, pH 7.4), the pellet was resuspended in 0.25 M sucrose solution. Microsomes (0.5 μg) were then incubated with *p*-nitrophenyl valerate (500 μM). The formation of hydrolysis products was detected via liberation of *p*-nitrophenol by kinetic monitoring at 405 nm for 5 min. Non-Ces mediated hydrolysis was quantified in the presence of paraoxon (1 μM) and subtracted from total activity to calculate specific activity (nmol/min/mg protein). Samples from each mouse ($n = 4-6$ mice) were run in duplicate.

2.5. RNA Isolation and Quantitative PCR (qPCR) Assay.

Livers of WT and PPAR α -null mice were homogenized in RNABee reagent (Tel-Test Inc) and total RNA was isolated using the Qiagen RNeasy Mini Kit (Valencia, CA) according to the manufacturer's protocol. The concentration of total RNA was quantified by UV spectrophotometry at 260/280 nm with a Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific, Wilmington, DE). The mRNA expression of hepatic mouse Ces enzymes (*Ces1c*, *1d*, *1e*, *1f*, *1g*, *2a*, *2b*, *2c*, *2e* and *3a*) as well as transcription factors and their target genes (*Ahr*, *Cyp1a1*; *Car*, *Cyp2b10*; *Pxr*, *Cyp3a11*; *Ppara*, *Cyp4a14*; *Nrf2*, *Nqo1*; *Ppar γ* ; retinoid X receptor alpha (*Rxra*); farnesoid X receptor (*Fxr*); small heterodimer partner (*Shp*); liver X receptor alpha (*Lxra*); Sterol regulatory element-binding protein 1 (*Srebp-1*); *Hnf4*; *Cyp7a1*; *Era*; and glucocorticoid receptor (*Gr*)) were quantified in duplicate by qPCR with

Sybr Green to detect amplified products in a 384-well plate format using a ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Ct values were converted to Ct by comparing to a reference gene β -actin (Livak and Schmittgen, 2001).

2.6. Data Analysis.

Quantitative results were expressed as mean \pm SE (n=4-6) and analyzed by one-way ANOVA with Tukey's multiple comparisons tests using GraphPad Prism software (Version 6; GraphPad Software Inc., San Diego, CA). Significance was set at $p < 0.05$.

3. Results

3.1. Expression and activity of hepatic Ces in PFOA-treated WT mice.

As expected, the body weight of mice treated by PFOA did not change, while the liver weight increased in a dose-dependent manner (Supplementary Fig 1A). Compared to vehicle-treated mice, PFOA (1 or 3 mg/kg) increased Ces1 and 2 protein expression (Fig 1A). PFOA also increased hepatic Ces enzyme activity, as demonstrated by enhanced hydrolysis (100-200%) of *p*-nitrophenol valerate in PFOA-treated WT mice (Fig 1B).

Because multiple Ces subtypes contribute to its hydrolytic activity, the hepatic mRNA expression of *Ces1* (1c, 1d, 1e, 1f, 1g), *Ces2* (2a, 2b, 2c, 2e), and *Ces3a* subtypes were quantified in vehicle- and PFOA-treated mice (Fig 2). PFOA treatment elevated *Ces1d*, *1e*, *1f*, *1g*, *2c*, and *2e* mRNAs between 1.5- and 2.5-fold, while it decreased *Ces1c* and *2b* mRNAs by 50-80%. *Ces2a* and *3a* mRNAs were expressed but not significantly changed by PFOA treatment (Fig 2 and data not shown for *Ces3a*).

3.2. Transcription factor and target gene expression in livers from PFOA-treated WT mice.

Previous studies demonstrated that PFOA activates nuclear receptors including PPAR α and CAR (Poole et al., 2001; Rosen et al., 2008) that may alter hepatic Ces expression/activity (Baker et al., 2015). As shown in Fig. 3, hepatic activation of PPAR α and CAR by PFOA (3 mg/kg) in WT mice was confirmed by up-regulation of *Cyp4a14* and *2b10* mRNAs up to 500- and 6-fold, respectively. Notably, the expression of *CAR* and *PPARa* mRNAs did not change significantly. Interestingly, PFOA induced *Nrf2* and *Nqo1* mRNA levels between 50% and 300%, suggesting PFOA may also activate the Nrf2 signaling pathway. Likewise, *Pxr* mRNA was up-regulated by PFOA; however, no significant changes in its target gene *Cyp3a11* were observed. The mRNA levels of other transcription factors and their target genes including *Ahr*, *Cyp1a1*, *Ppar γ* , and *Rxra* did not change significantly after PFOA treatment.

3.3. Liver and body weight in PFOA-treated PPAR α -null mice.

Compared to vehicle-treated mice, the body weight of WT and PPAR α -null mice treated by PFOA did not change, while the liver weight was significantly increased by 50-100% in WT mice (Supplementary Fig 1B). In PPAR α -null mice, PFOA also increased liver weights albeit to a lesser degree (25%).

3.4. Messenger RNA and protein expression of hepatic Ces in PFOA-treated PPAR α -null mice.

Mice lacking the *PPAR α* gene had significantly lower basal protein expression of hepatic Ces1, but not Ces2, compared to WT mice (Fig 4A). As expected, hepatic Ces1 and 2 protein expression was significantly increased by PFOA in WT mice, and also to some degree in PPAR α -null mice (Fig 4A). Notably, the induction of Ces1 in PFOA-treated PPAR α -null mice was significantly lower than in WT mice. Compared to the vehicle-treated group, WT mice treated with PFOA exhibited a 200% increase in hydrolysis of p-nitrophenyl valerate, a Ces substrate. In PPAR α -null mice, PFOA also increased p-nitrophenyl valerate hydrolysis, but to a lower extent than WT mice (Fig 4B).

To determine whether the absence of PPAR α altered the regulation of Ces subtypes, *Ces* mRNAs were quantified in PFOA-treated WT and PPAR α -null mice livers (Fig. 5). Consistent with Fig. 1, PFOA increased hepatic *Ces1d*, *1e*, *1f*, *1g*, and *2c* mRNAs between 100-350% in WT mice. In PPAR α -null mice, similar increases were also observed for *Ces1d* and *1g*, but not for *Ces1e*, *1f*, and *2c* mRNAs. Interestingly, *Ces1c* mRNA was decreased 60% in PFOA-treated WT mice, but elevated in PFOA-treated PPAR α -null mice. Likewise, PFOA treatment elevated *Ces2a*, *2b*, and *2e* mRNAs only in PPAR α -null mice. Taken together, these data illustrate that WT and PPAR α -null mice exhibit different patterns of Ces mRNA induction in response to PFOA and that the up-regulation of *1e*, *1f*, and *2c* genes occurs in a PPAR α -dependent manner.

3.5. Messenger RNA expression of transcription factors and target genes in livers from PFOA-treated PPAR α -null mice.

To further explore alternate pathways that could regulate Ces subtypes following PFOA treatment, the expression of other transcription factors and their downstream target genes were profiled. As expected, PPAR α -null mice displayed a marked reduction in the basal expression of *Cyp4a14* mRNA (0.25% of WT levels) (Fig 6). PFOA induced hepatic *Cyp4a14* mRNA expression in PPAR α -null mice, but to a lesser extent, compared to WT mice. In contrast, *Cyp2b10* and *Cyp3a11* mRNAs exhibited greater induction by PFOA treatment in PPAR α -null mice (120-fold for *Cyp2b10* and 5.7-fold for *Cyp3a11*) compared to WT mice (11-fold increase for *Cyp2b10* and 1.7-fold increase for *Cyp3a11*), suggesting that the absence of PPAR α leads to a greater activation of CAR and PXR pathways by PFOA. The mRNAs for other nuclear receptors were minimally changed or unchanged, except for *ER α* which was increased 100% after PFOA treatment in PPAR α -null mice, but not in WT mice (Supplementary Fig 2).

4. Discussion

The current study assessed the ability of the environmental contaminant PFOA to alter the expression and activity of Ces enzymes in mouse livers. Mice lacking PPAR α were used to determine whether the differential regulation of Ces subtypes occurred via PPAR α signaling. Oral exposure to PFOA for 7 days stimulated prototypical rodent responses to peroxisome proliferators including hepatomegaly and significant induction of *Cyp4a14* mRNA. Interestingly, PFOA also induced hepatic Ces1 and 2 protein expression and increased the

hydrolysis of the general Ces substrate, *p*-nitrophenol valerate. By comparison, mice lacking PPAR α had impaired induction of Ces1 protein expression and Ces activity following PFOA most likely due to their inability to up-regulate *Ces1e* and *1f* mRNAs. Nonetheless, a number of Ces subtypes were still elevated by PFOA in PPAR α -null mice (*Ces1d*, *1g*) or only induced in the absence of PPAR α , namely *Ces 2a*, *2b*, and *2e*. In fact, up-regulation of some Ces subtypes in PPAR α -null mice likely explains why Ces enzyme activity was still elevated, although to a lesser extent, compared to WT mice treated with PFOA. *P*-nitrophenol valerate is a general substrate of Ces enzymes and has been shown to be a substrate of both Ces1/CES1 and Ces2/CES2 in dogs, monkeys, and humans (Williams et al., 2011). While CES1 and 2 share some common substrates such as *p*-nitrophenol valerate, there are distinct specificities for each enzyme. CES1 substrates often contain a small alcohol group (eg., clopidogrel) and a bulky acyl group whereas CES2 substrates include a large alcohol group and a small acyl group (eg., irinotecan) (reviewed in Wang et al., 2018)). It is conceivable that evaluation of subtype-specific substrates of Ces would reveal greater differences in metabolism, particularly for Ces1, between WT and PPAR α -null mice treated with PFOA. By comparison, we hypothesize that Ces2-mediated metabolism would be similar between PFOA-treated WT and PPAR α -null mice. Likely, alternative transcription factor pathways including CAR and PXR that were strongly induced by PFOA in PPAR α -null mice may be responsible for differences in Ces subtype regulation between the two genotypes. Collectively, the data from this study highlight novel mechanisms of xenobiotic regulation of hepatic Ces in the absence and presence of the PPAR α nuclear receptor.

Consistent with our findings, an early study demonstrated that PFOA could induce Ces1 enzyme activity in the livers of male rats (Hosokawa and Satoh, 1993). It was subsequently reported that hepatic Ces1 protein expression and enzyme activity were increased in male mice treated by another PPAR α ligand di-2-ethylhexylphthalate (DEHP) for 7 days (Hosokawa et al., 1994). Repeated administration of a high dose of PFOA (100 mg/kg/day for 3 days) also increased microsomal CES RL4 (Ces2) protein and enzyme activity in male rat livers (Derbel et al., 1996). A more recent study demonstrated that treatment of A129/SvJ male mice with the PPAR α activator GW7647 for less than 24 hrs increased the mRNA expression of specific Ces1 (*Ces1d*, *1e*, *1f*) and Ces2 (*2c*, *2f*) subtypes between 2- to 5-fold (Jones et al., 2013). Notably, the two-to four-fold magnitude of *Ces* mRNA induction by PFOA in this study is similar to the fold changes observed in mice treated with GW7647 (Jones et al., 2013). However, not all reports have observed a link between PPAR α activation and Ces up-regulation. In fact, Zhang et al., found that treatment of male C57BL/6 mice with PPAR α activators, clofibric acid, ciprofibrate, or DEHP for 4 days did not alter the mRNA expression of most Ces1 and 2 subtypes (*1c*, *1d*, *1e*, *1f*, *1g*, *2a*, *2b*, *2c*, *2e* and *3a*) (Zhang et al., 2012). The authors speculated that the short exposure time (4 days) might account for this disparity however, it should be noted that different methods of mRNA quantification between studies were used, namely, qPCR vs. branched DNA analysis. Likewise, the source of C57BL/6 mice (Charles River and Taconic vs. Jackson Laboratories) differed between the two studies. Therefore, the regulation of hepatic Ces enzymes by ligand-activated PPAR α *in vivo* may be dependent on the specificity of the ligand, dosing, animal strain, qPCR methodology, and/or duration of treatment.

PFOA treatment resulted in the activation of multiple transcriptional pathways. As expected, PFOA significantly induced the PPAR α target *Cyp4a14* in mouse livers. In PPAR α -null mice, PFOA activated hepatic *Cyp4a14* mRNA much less than in WT mice. Similarly, a related perfluoroalkyl acid, perfluorooctane sulfonate up-regulated *Cyp4a14* mRNA in the livers of wild-type and PPAR α -null albeit to a lesser degree in the PPAR α -null mice (Rosen et al., 2010). It has been speculated that induction of *Cyp4a14* expression in PPAR α -null mice likely results from activation of PPAR β/δ or PPAR γ (Rosen et al., 2010). PFOA and perfluorooctane sulfonate also activated CAR signaling, as evidenced by the 2- to 6-fold up-regulation of *Cyp2b10* mRNA in the livers of WT mice, presented in the current and previous studies (Cheng and Klaassen, 2008; Rosen et al., 2010). Interestingly, *Cyp2b10* and *Cyp3a11* showed much higher induction in PPAR α -null mice in response to PFOA; with a 120-fold increase for *Cyp2b10* and 5.7-fold increase for *Cyp3a11* in PPAR α -null mice, compared to a 11-fold increase for *Cyp2b10* and 1.7-fold increase for *Cyp3a11* in WT mice. The ability to induce CAR target genes to a greater extent in PPAR α -null mice compared to WT mice has been described previously (reviewed in Corton et al., 2014). These findings suggest a unique relationship underlying the regulation of Phase I enzymes by the PPAR α and CAR pathways. We also demonstrate that CAR and PXR were significantly activated in PPAR α -null mice, which may be important for the up-regulation of Ces, particularly *Ces2* subtypes. For example, the CAR agonist TCPOBOP and the PXR ligand PCN increased hepatic mRNA levels of *Ces2a* by 5- and 16-fold, respectively, in WT mice, but not in CAR- and PXR-null mice (Xu et al., 2009; Jones et al., 2013). Using microarray-based gene profiling in human primary hepatocytes and 129S1/SvImj WT mice as well as PPAR α -null mice, it was confirmed that PFOA regulates numerous genes associated with lipid metabolism, inflammation, and xenobiotic metabolism. This was observed in part through the activation of PPAR α as well as PPAR α -independent pathways such as CAR, PPAR γ , and ER α (Rosen et al., 2010; Rosen et al., 2013; Buhrke et al., 2015). Further studies exploring alternative mechanisms including hepatocyte nuclear factor 4 α could also help to understand the interplay between CAR and PPAR α signaling (Beggs et al., 2016).

The mechanisms underlying the dysregulation of cholesterol homeostasis ability following perfluorinated chemical exposure are not entirely clear. The relationship between PFOA treatment and cholesterol levels in rodents is dynamic with reports of both reduced and increased cholesterol levels in PFOA-treated mice and rats depending upon time point during PFOA treatment and withdrawal, genetic strain, sex, as well as composition of rodent diet (Xie et al., 2003; Rebholz et al., 2016; NTP, 2018). While the primary focus of this study was to investigate the ability of PFOA to modulate xenobiotic metabolism through various transcription factor pathways, it is intriguing to consider how induction of CES/Ces enzymes may be an adaptive mechanism by the liver that contributes to and/or counteracts elevations in cholesterol levels. As aforementioned, CES1 can metabolize cholesterol esters and triacylglycerols. Mouse Ces subtypes including *Ces1d*, *1f*, *1g*, and *2c* also possess triacylglycerol hydrolase activity (reviewed in Lian et al., 2018)). Prior studies have suggested that *Ces1d* promotes hepatic lipogenesis in mice whereas *Ces1g* reverse hyperlipidemia (Wei et al., 2010; Lian et al., 2012; Quiroga et al., 2012; Bahitham et al., 2016). Interestingly, both subtypes were induced by PFOA in a PPAR α -independent manner in the current investigation. Future studies in mice lacking individual Ces subtypes may

reveal novel roles for these enzymes to modulate cholesterol levels following treatment with PFOA.

Extrapolation of findings from the current mechanistic study in mice to humans should be done with caution. The regulatory role for PPAR α in hepatic lipid metabolism has been shown to be well-conserved between mice and humans (Rakhshandehroo et al., 2009). Likewise, the ability of PFOA to activate rodent (Wolf et al., 2014) and human PPAR α and induce Cyp4a/CYP4A isoforms has been demonstrated, although the magnitude of induction of Cyp4a1 in primary rat hepatocytes (59-fold) far exceeds that observed for CYP4A11 in primary human hepatocytes (3-fold) (Bjork et al., 2011). There are additional differences between species. The elimination half-life of PFOA is known to differ significantly between rodents and humans (1 to 9 days in rats, 18 days in mice, and 3.8 years in humans) (Calafat et al., 2007; Lau, 2012). Nonetheless, prior pharmacokinetic analysis in mice has demonstrated that PFOA accumulates in the liver at concentrations higher than the plasma and other tissues such as the kidneys (Lou et al., 2009). Extrapolation of the data generated by Lou et al. (2009) using the same dose (1 mg/kg) and route (p.o.) of PFOA administration as the current study suggests that hepatic concentrations of PFOA between 10 to 30 μ M were achieved and may be a useful starting point for subsequent experiments evaluating the ability of PFOA to up-regulate CES1 and 2 expression in primary human hepatocytes.

In summary, the current study expands our understanding of transcription factors that regulate *Ces* subtype expression in mice following exposure to an environmental contaminant known to activate multiple interrelated signaling pathways. In particular, we have identified specific *Ces* subtypes that are elevated in PPAR α -null mice treated with PFOA and postulate that CAR and PXR contribute to this differential regulation. Future studies are needed to examine whether PFOA similarly induces human CES1 and 2 activity and determine whether up-regulation alters the metabolism and homeostasis of cholesterol esters and triacylglycerol lipids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ahr	aryl hydrocarbon receptor
BHA	butylated hydroxyanisole

CAR	constitutive androstane receptor
Ces	carboxylesterases
DEHP	di-2-ethylhexylphthalate
Era	estrogen receptor alpha
HNF4a	hepatocyte nuclear factor 4 alpha
LXRa	liver X receptor alpha
Nrf2	nuclear factor E2-related factor 2
PCN	pregnenolone-16a-carbonitrile
PFOA	perfluorooctanoic acid
PPARa	peroxisome proliferator activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PXR	pregnane X receptor
Srebp-1	sterol regulatory element-binding protein 1
TCPOBOP	1,4-bis-[2-(3,5-dichloro-pyridyloxy)]benzene
WT	wild-type

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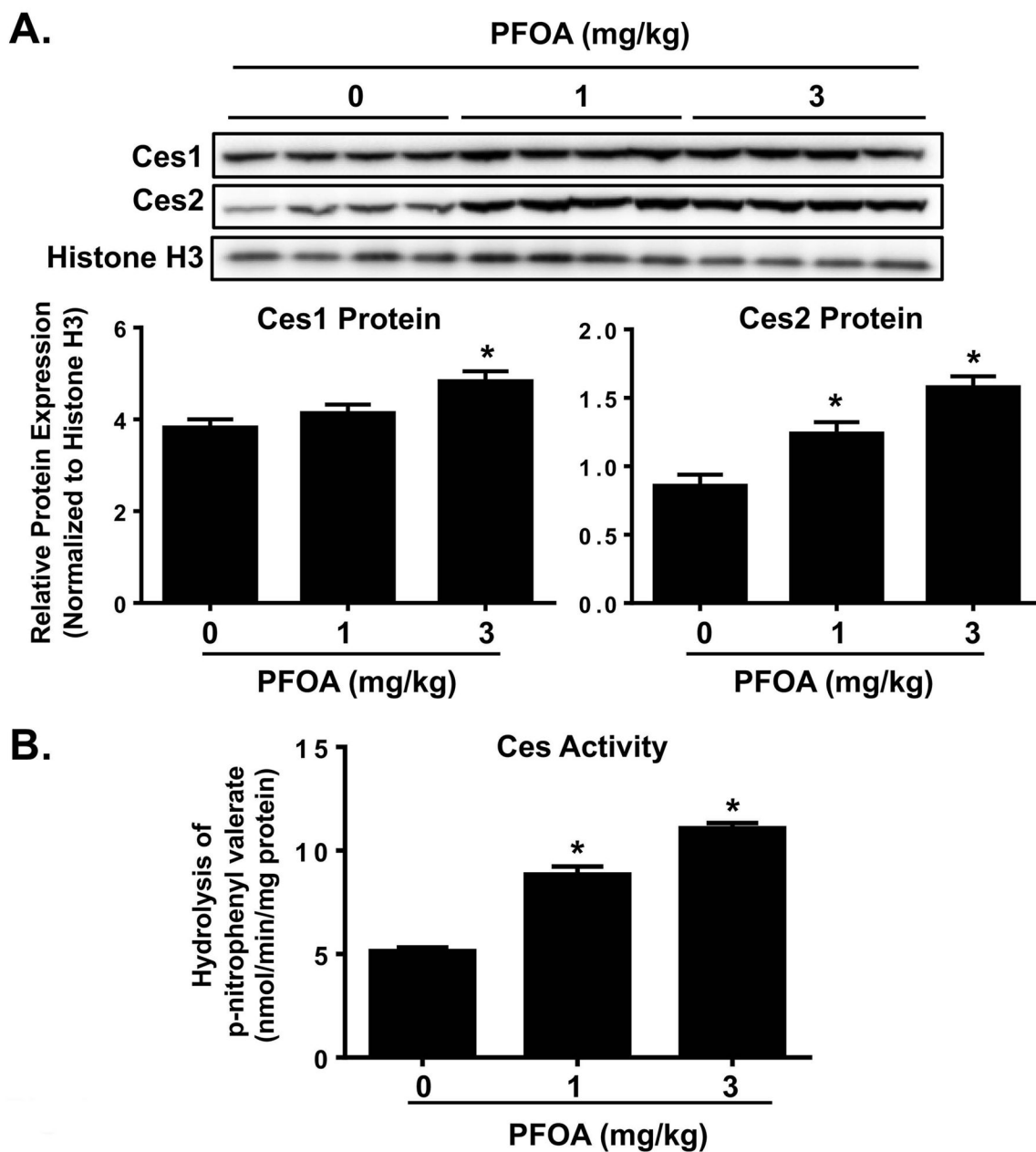


Figure 1. Protein expression and activity of hepatic Ces1/2 in PFOA-treated WT mice. WT mice were treated with PFOA (1 or 3 mg/kg, 7 days, po) and livers were collected. (A) Livers were homogenized and the protein expression of hepatic Ces1/2 was determined by Western blot analysis. Histone H3 was used as a loading control. (B) Ces enzyme activity in mouse liver microsomal fraction was determined by continuous spectrophotometric assay. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated WT mice.

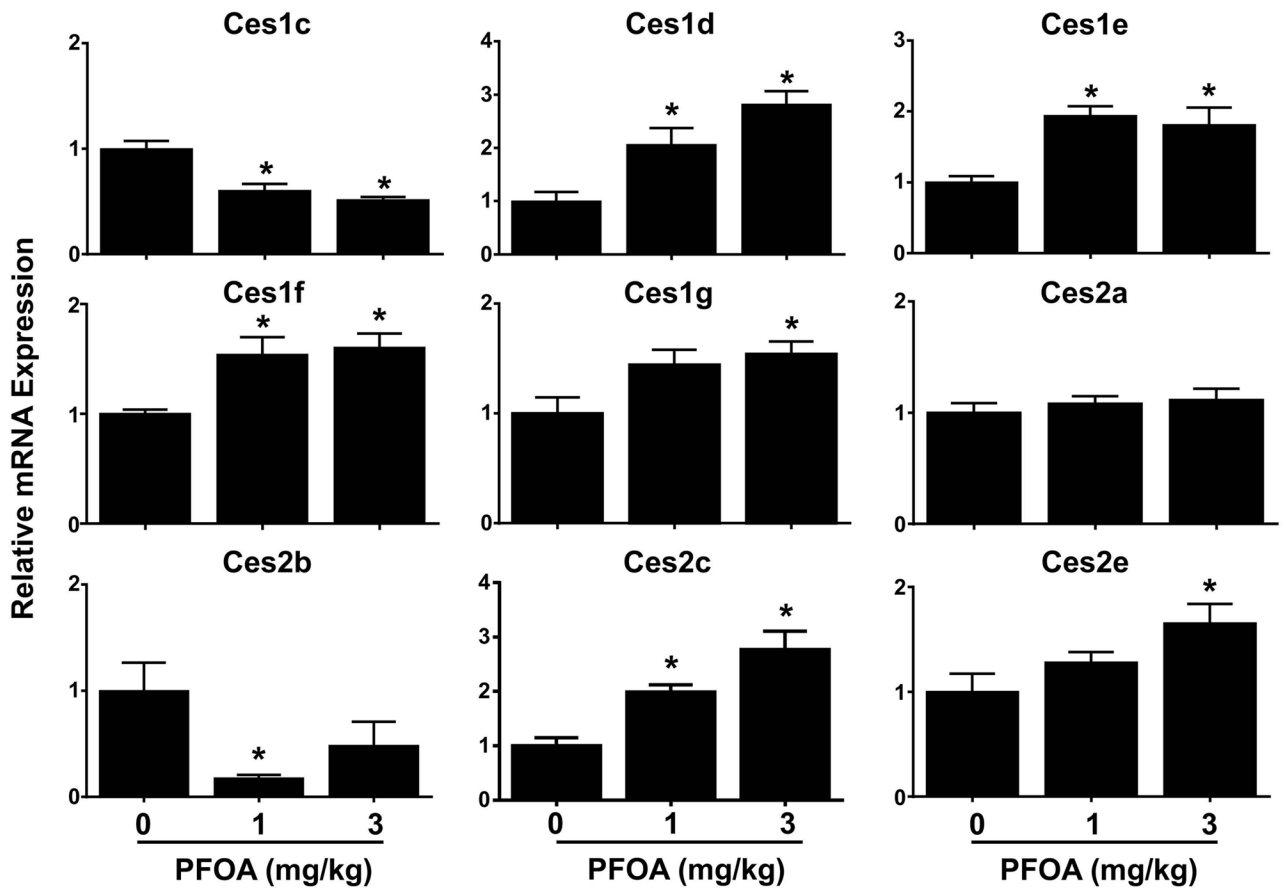


Figure 2. mRNA levels of hepatic Ces in PFOA-treated WT mice.

WT mice were treated with PFOA (1 or 3 mg/kg, 7 days, po) and livers were collected. Messenger RNA expression of *Ces1c*, *1d*, *1e*, *1f*, *1g*, *2a*, *2b*, *2c*, and *2e* was quantified using the qPCR assay and normalized to the housekeeping gene, β -actin. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated WT mice.

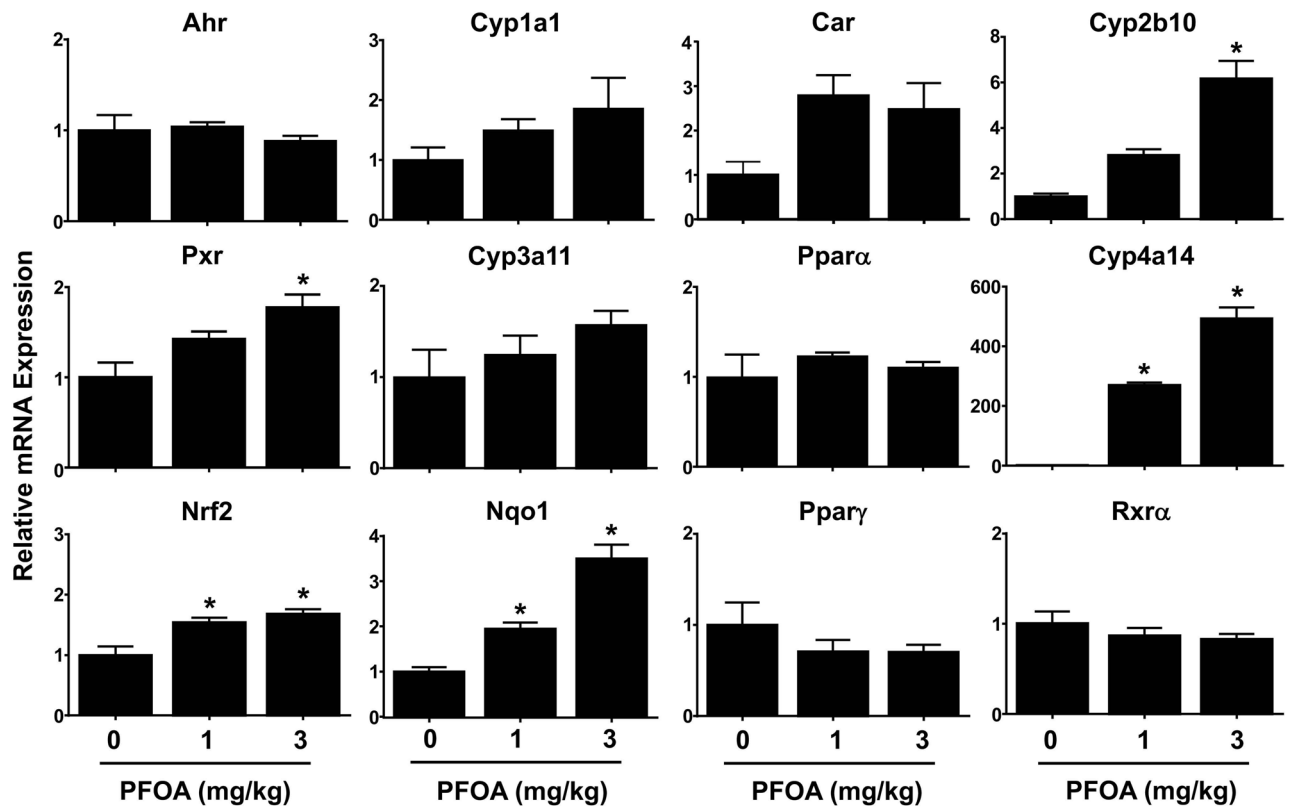


Figure 3. mRNA levels of transcription factors and their target genes in PFOA-treated WT mice. WT mice were treated with PFOA (1 or 3 mg/kg, 7 days, po) and livers were collected. Messenger RNA expression of *Ahr*, *Cyp1a1*, *Car*, *Cyp2b10*, *PXR*, *Cyp3a11*, *PPAR α* , *Cyp4a14*, *Nrf2*, *Nqo1*, *PPAR γ* , and *Rxra* was quantified using the qPCR assay and normalized to the housekeeping gene, *β -actin*. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences (p < 0.05) compared to vehicle-treated WT mice.

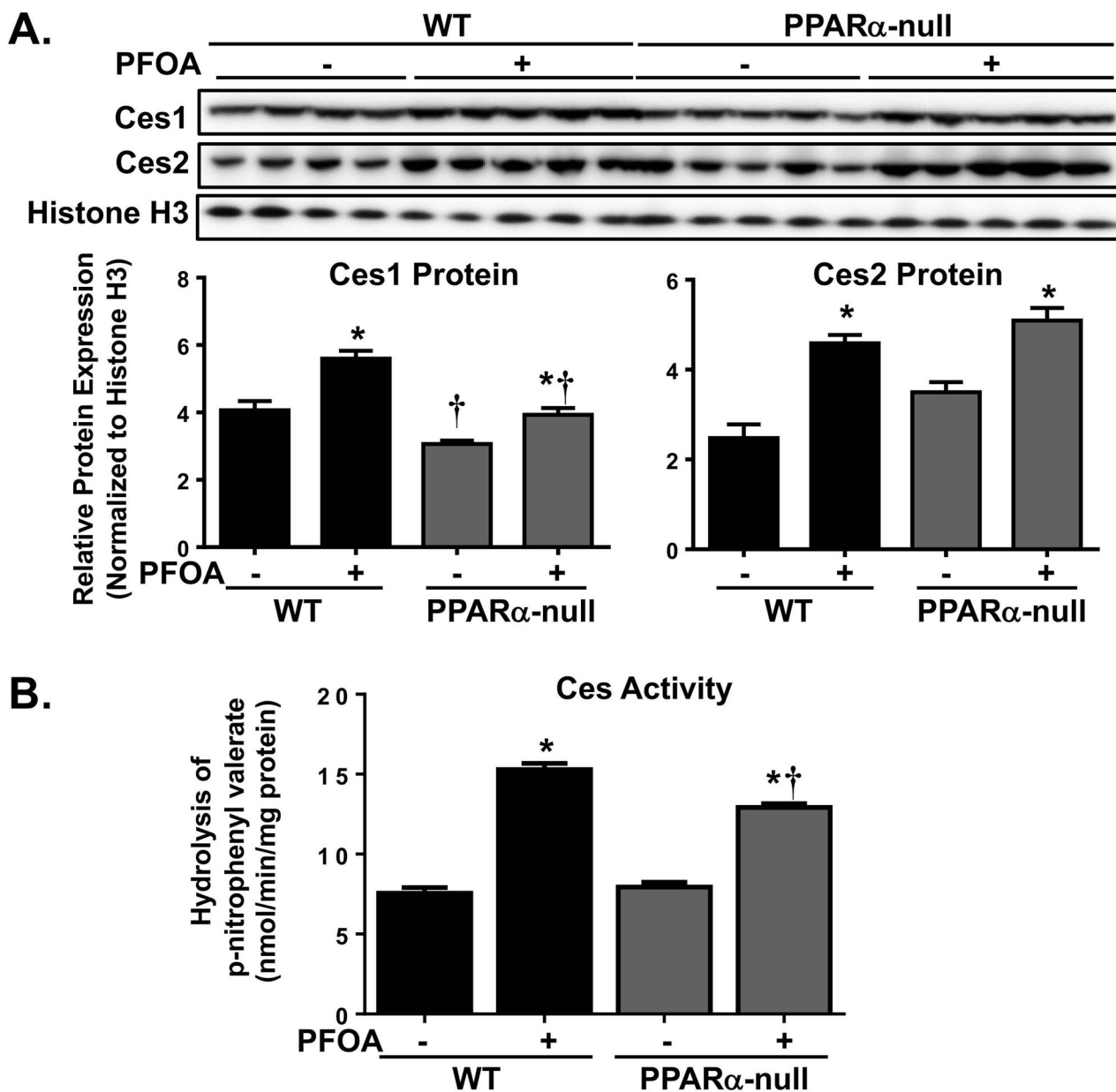


Figure 4. Protein expression and activity of hepatic Ces1/2 in PFOA-treated WT and PPAR α -null mice.

WT and PPAR α -null mice were treated with PFOA (3 mg/kg, 7 days, po) and livers were collected. (A) Livers were homogenized and the expression of hepatic Ces1/2 was determined by Western blot analysis. Histone H3 was used as a loading control. (B) Ces enzyme activity in mouse liver microsomal fraction was determined by continuous spectrophotometric assay. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences (p < 0.05) compared to vehicle-treated WT mice or vehicle-treated PPAR α -null mice. Daggers (†) represent statistically significant differences (p < 0.05) compared to vehicle-treated or PFOA-treated WT mice.

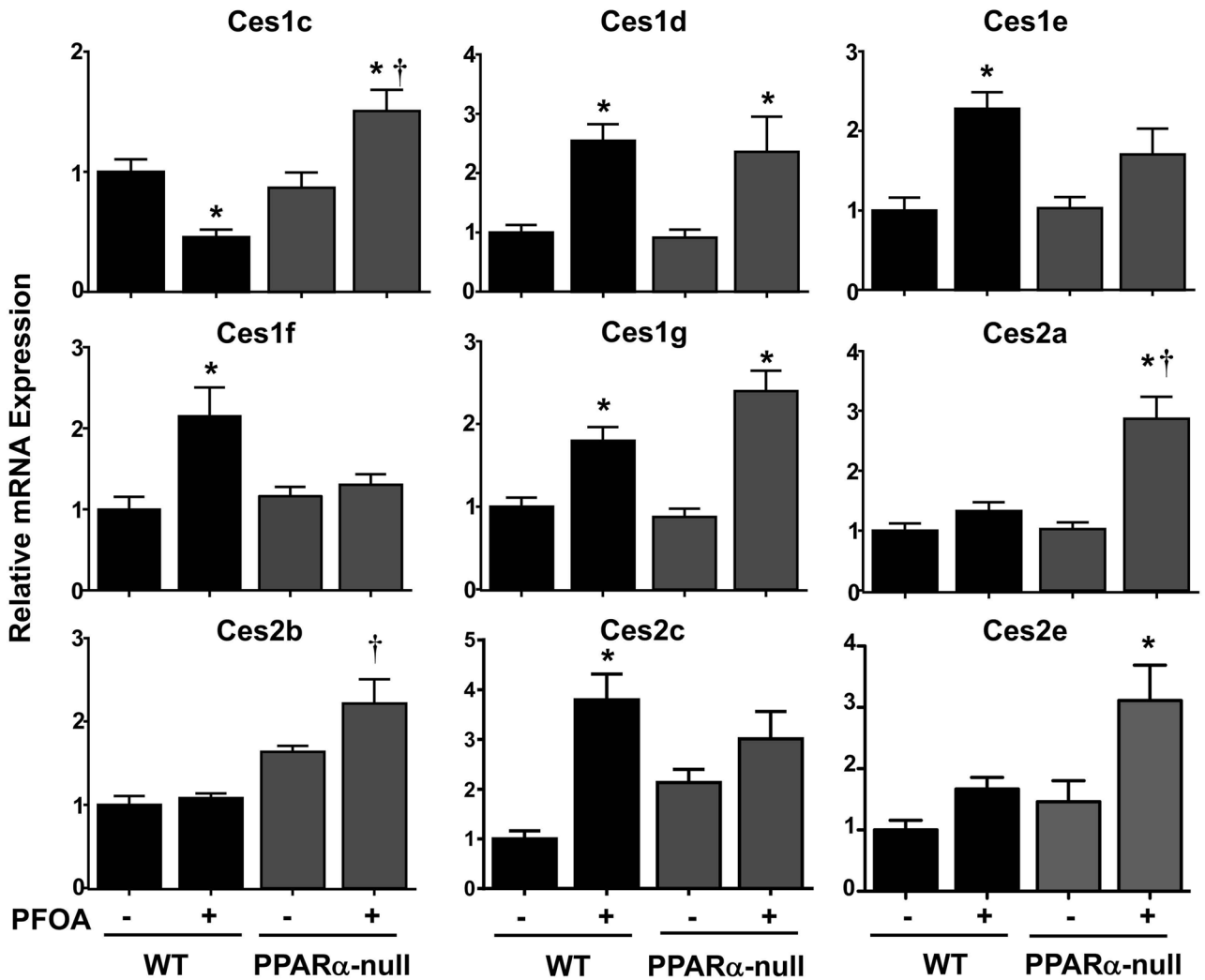


Figure 5. mRNA levels of hepatic Ces in PFOA-treated WT and PPAR α -null mice.

WT and PPAR α -null mice were treated with PFOA (3 mg/kg, 7 days, po) and livers were collected. (A) Messenger RNA expression of *Ces1c*, *1d*, *1f*, *1e*, *1g*, *2a*, *2b*, *2c*, and *2e* was quantified by qPCR assay and normalized to the housekeeping gene, β -actin. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences (p < 0.05) compared to vehicle-treated WT or PPAR α -null mice. Daggers (†) represent statistically significant differences (p < 0.05) compared to PFOA-treated WT mice.

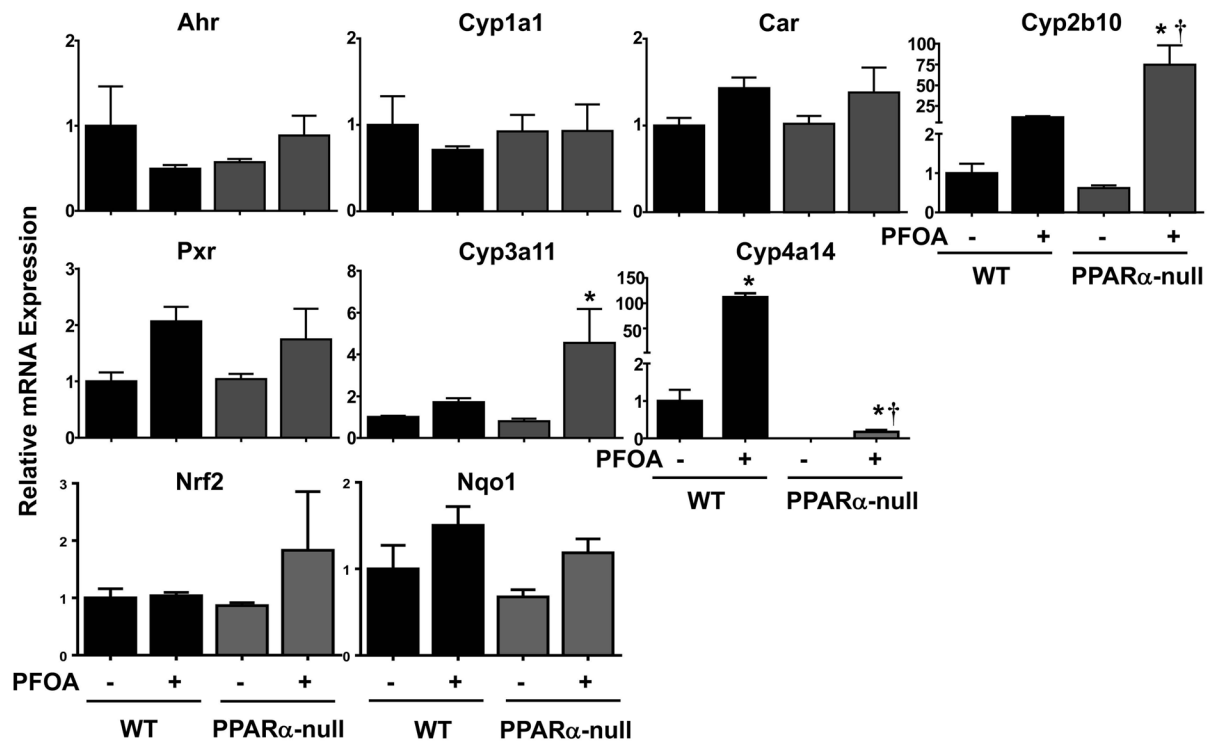


Figure 6. mRNA levels of transcription factors and their target genes in PFOA-treated WT and PPAR α -null mice.

WT and PPAR α -null mice were treated with PFOA (3 mg/kg, 7 days, po) and livers were collected. Messenger RNA expression of *Ahr*, *Cyp1a1*, *Car*, *Cyp2b10*, *PXR*, *Cyp3a11*, *Cyp4a14*, *Nrf2*, and *Nqo1* and was quantified by qPCR assay and normalized to the housekeeping gene, β -actin. Data are presented as mean \pm SE (n = 4-6 mice). Asterisks (*) represent statistically significant differences (p < 0.05) compared to vehicle-treated WT or PPAR α -null mice. Daggers (†) represent statistically significant differences (p < 0.05) compared to PFOA treatment WT mice.