Perfluorinated Carboxylic Acids with Increasing Carbon Chain Lengths Upregulate Amino Acid Transporters and Modulate Compensatory Response of Xenobiotic Transporters in HepaRG Cells

Joe Jongpyo Lim,* Youjun Suh,* Elaine M. Faustman, and Julia Yue Cui

Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, Washington

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ABSTRACT

Perfluorinated carboxylic acids (PFCAs) are widespread environmental pollutants for which human exposure has been documented. PFCAs at high doses are known to regulate xenobiotic transporters partly through peroxisome proliferator-activated receptor alpha (PPARα) and constitutive androstane receptor (CAR) in rodent models. Less is known regarding how various PFCAs at a lower concentration modulate transporters for endogenous substrates, such as amino acids in human hepatocytes. Such studies are of particular importance because amino acids are involved in chemical detoxification, and their transport system may serve as a promising therapeutic target for structurally similar xenobiotics. The focus of this study was to further elucidate how PFCAs modulate transporters involved in intermediary metabolism and xenobiotic biotransformation. We tested the hepatic transcriptomic response of HepaRG cells exposed to 45 μM of perfluorooctanoic acid, perfluorononanoic acid, or perfluorodecanoic acid in triplicates for 24 hours (vehicle: 0.1% DMSO), as well as the prototypical ligands for PPARα (WY-14643, 45 μM) and CAR (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime [CITCO], 2 μM). PFCAs with increasing carbon chain lengths (C8-C10) regulated more liver genes, with amino acid metabolism and transport ranked among the top enriched pathways and PFDA ranked as the most potent PFFA tested. Genes encoding amino acid transporters, which are essential for protein synthesis, were novel inducible targets by all three PFCAs, suggesting a potentially protective mechanism to reduce further toxic insults. None of the transporter regulations appeared to be through PPARα or CAR but potential involvement of nuclear factor erythroid 2-related factor 2 is noted for all 3 PFCAs. In conclusion, PFCAs with increasing carbon chain lengths up-regulate amino acid transporters and modulate xenobiotic transporters to limit further toxic exposures in HepaRG cells.

SIGNIFICANCE STATEMENT

Little is known regarding how various perfluorinated carboxylic acids modulate the transporters for endogenous substrates in human liver cells. Using HepaRG cells, this study is among the first to show that perfluorinated carboxylic acids with increasing carbon chain lengths upregulate amino acid transporters, which are essential for protein synthesis, and modulate xenobiotic transporters to limit further toxic exposures at concentrations lower than what was used in the literature.

Introduction

Perfluorinated carboxylic acids (PFCAs) have been extensively used in many consumer products, such as Scotchgard and the Teflon brand of products due to their chemical and thermal stability. PFCAs, such as perfluoroctanoic acid (PFOA, C8), perfluorononanoic acid (PFNA, C9), and perfluorodecanoic acid (PFDA, C10), have raised increasing public health concerns due to their highly persistent and bioaccumulative nature and have been detected in ecosystems (Falandysz et al., 2006; Sinclair et al., 2006)(Smithwick et al., 2005; Calafat et al., 2006; De Silva and Mabury, 2006; Eggers Pedersen et al., 2015; Pasanisi et al., 2016; Boisvert et al., 2019; Jarvis et al., 2021). Due to both tissue binding and uptake (Fujii et al., 2015; (Van Rafterghem et al.,1987; Vanden Heuvel et al.,1991a; Vanden Heuvel et al., 1991b), the liver is one of the primary target organs of the toxic effects of PFCAs, including oxidative stress, hepatomegaly, and hepatic dysplidemia as described in detail below (Fujii et al., 2015). The solute carrier organic anion (SLCO) transporters, also known as the organic anion transporting polypeptides (OATP), are involved in the cellular uptake of many drugs and other chemicals (Hagenbuch and Meier, 2004). Using transfected Chinese hamster ovary and human embryonic kidney 293 cells, it was shown that the human OATP1B1, 1B3, and 2B1 can transport PFOA and PFNA (Zhao et al., 2017). An active uptake...
mechanism for the anion of PFOA (PFO) was also identified in rat hepatocytes (Han et al., 2008), although the exact transporters involved need to be further investigated.

The nuclear receptors pregnane X receptor (PXR/NR1I2), constitutive androstane receptor (CAR/NR1I3), and peroxisome proliferator-activated receptor z (PPARz), as well as the transcription factors aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2-related factor 2 (NRF2), are widely recognized as xenobiotic-sensing receptors that transcriptionally modulate various drug-processing genes (Aleksunes and Klaassen, 2012; Cui and Klaassen, 2016; Li et al., 2016). Previous studies on PFCAs have shown that they can activate distinct xenobiotic-sensing receptors, such as CAR and PPARz in a dose- and congener-dependent manner; while most of these observations were from laboratory rodent models (Kudo and Kawashima, 2003; Maher et al., 2005; Cheng and Klaassen, 2008a; Cheng and Klaassen, 2008b; Oshida et al., 2015; Wen et al., 2019), a few studies have also tested the effect of PFCAs on human liver cancer-derived HepaRG cells (Buhre et al., 2013; Abe et al., 2017; Behr et al., 2020; Louise et al., 2020). Specifically, PFOA, PFNA, and PFDA have been shown to activate PPARz, with PFOA having the highest potential of PPARz activation, whereas these PFC compounds also activate PPARg and PPARa with a much weaker potential (Buhre et al., 2013; Li et al., 2020). PFOA also activates CAR in both mouse liver and HepaRG cells (Abe et al., 2017).

While most studies on PFCAs have focused on drug metabolizing enzymes and the pathologic outcomes in human hepatocyte and rodent models, a systematic characterization of all transporters by PFCAs in human hepatocytes is lacking, especially the transporters for endogenous metabolites. In addition, only two studies have investigated PFCAs (PFOA and PFNA) and HepaRG cells both of which used higher concentrations (Behr et al., 2020; Louise et al., 2020) and there is no information regarding how PFDA modulates transporters in HepaRG cells. Therefore, the goal of this study was to fill these critical knowledge gaps. To note, it is important to investigate the effect of PFOA on not only the drug transporters but also the transporters involved in physiologic functions, such as nutrition, because it is increasingly recognized that environmental toxicant exposure may impact intermediary metabolism and lead to complex metabolic diseases (Lubrano et al., 2013; Heindel et al., 2017; Le Magueresse-Battistoni et al., 2018; Pannala et al., 2020). We also aimed to determine the effect of various equivvalent PFCA congeners at non-toxic concentrations on the global transcriptomic response in human hepatocytes to unveil upstream regulators of transporters as well as early toxicological biomarkers in an unbiased manner. We also conducted a systematic comparison between our findings on PFCA-mediated transporter regulation in HepaRG cells and the literature in a dose- and congener-specific manner. The transporter genes included within the scope of the present study are shown in Supplemental Table 1.

Materials and Methods (the overall study design is shown in Fig. 1A)

HepaRG Cell Culture and Chemical Exposure. We obtained the HepaRG cells from Biopredic with permission under the material and transfer agreement. The HepaRG cells were seeded at a density of 2.6 × 10^5/cm^2 in six-well plates (Tissue Culture Treated, Corning 3516) in William’s medium E supplemented with a growth medium supplement (Catalog #ADD711, Triangle Research Laboratories, NC), GlutaMAX-I (1X), as well as penicillin (100 IU/ml) and streptomycin (100 µg/ml). To differentiate the HepaRG cells into a hepatocyte-like morphology, two weeks post-seeding, cells were transferred to new plates containing the same medium with a differentiation medium supplement (Catalog #ADD721, Triangle Research Laboratories, NC), as well as penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were cultured under this differentiation condition for another two weeks, and the medium was renewed every 2 or 3 days. Prior to the exposure to chemicals, the cell culture medium was switched to the Williams’ medium E with induction supplement (HPRG740, Life Technologies, Carlsbad, CA) for 24 hours. The fully differentiated HepaRG cells were then exposed for 24 hours in triplicates to vehicle (0.1% DMSO), the selective CAR agonist 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO), C_14H_14ClN_3O_2S, CAS Number 338404-52-7, Sigma-Aldrich, Catalog No. CS240, 2 µM, the selective PPARz agonist WY-14463 (4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, C_14H_14ClN_3O_2S, CAS Number: 50892-23-4, Sigma-Aldrich, Catalog No. C7081, 45 µM), PFOA (perfluorooctanoic acid; CAS Number: 335-67-1; CF_3(CH_2)_6COOH; Sigma-Aldrich, Catalog No. 171468; 45 µM), PFDA (perfluorodecanoic acid; CAS Number: 335-76-2; CF_3(CH_2)_7COOH; Sigma-Aldrich, Catalog No. 17741; 45 µM), or PFNA (perfluorononanoic acid; CAS Number: 375-95-1; CF_3(CH_2)_9COOH, Sigma-Aldrich, Catalog No. 394459; 45 µM). The concentration of PFOA (45 µM) was selected based on literature evidence of no or minimal cellular toxicity, and upregulation of CAR- and PPARz-targeted P450s (Buhre et al., 2013; Abe et al., 2017; Behr et al., 2020; Louise et al., 2020). Specifically, regarding HepaRG cell viability, PFOA exposure for 24 hours remained non-toxic even at a concentration as high as 750 µM, which was the highest concentration tested (Behr et al., 2020); PFNA exposure for 24 hours remained non-toxic at 100 µM, whereas a decrease in cell viability was observed at 200 µM and above (Louise et al., 2020). The HepaRG cell viability at 24 hours of PFDA exposure was not known; however, it has been demonstrated that in HepaRG cells, which is another liver cancer-derived cell line, the IC50 at 72 hours post PFDA exposure was 15 µM (Buhrke et al., 2013). It is known that prolonged incubation of HepaRG cells with PFCAs results in greater reduction of cell viability (Louise et al., 2020), and HepG2 cells are more sensitive than HepaRG cells to PFCA-induced toxicity at the same concentration and exposure time (Buhrke et al., 2013; Louise et al., 2020). While it has been reported that there is a correlation between the cytotoxicity potential of a PFCA compound and its carbon chain length at high concentrations and prolonged incubation time points (Buhrke et al., 2013), we did not expect cellular toxicity at the low concentration of 45 µM PFDA at 24 hours. Regarding CAR- and PPARz-signaling, it has been shown that 48-hour incubation of PFOA up-regulated cytochrome P450 family 2 subfamily B member 6 (CYP2B6, CAR-target gene) and cytochrome P450 family 4 subfamily A member 11 (CYP4A11, CAR-target gene), whereas the up-regulation was lost at 100 µM (Abe et al., 2017); thus, at 24 hours, we expect that a slightly higher concentration is required, but it needs to be below 100 µM. Therefore, given the literature evidence of the effect of PFCA concentrations and incubation time on cellular toxicity and nuclear receptor activation, we selected 45 µM for all 3 PFCAs. All 3 PFC congeners were set at equal molar concentrations to compare the potency in modulating the transporter gene expression and the general impact on the hepatic transcriptomic response. The selection of the CITCO and WY-14643 concentrations

**ABBBRiVATIONS:** ABC, ATP-binding cassette; AhR, aryl hydrocarbon receptor; ALDO, aldolase; ATF, activating transcription factor; BA, bile acid; BCRP, breast cancer resistance protein; CAR, constitutive androstane receptor; CITO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CYP, cytochrome P450; ENO2, enolase 2; ESR, estrogen receptor; FDR-BH, false discovery rate–Benjamini-Hochberg adjusted p-value; FGF, fibroblast growth factor; FOX, forkhead box; FUT1, fucosyltransferase 1; GNMT, glycine N-methyltransferase; HDAC, histone deacetylase; HIF, hypoxia inducible factor; HNF, hepatocyte nuclear factor; MCCC1, methylcrotonyl-CoA carboxylase subunit 1; MDR, multidrug resistance protein; NFE2L2, nuclear factor erythroid 2 like 2; NRF2, nuclear factor erythroid 2-related factor 2; NTCP, sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptides; PDK, pyruvate dehydrogenase kinase; PFCA, perfluorinated carboxylic acid; PFDA, perfluorodecanoic acid; PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; PFNA, perfluorononanoic acid; PFOA, perfluoroctanoic acid; PGR, progesterone receptor; PPARGC1A, PPARG coactivator 1 alpha; PSAT, phosphoserine aminotransferase; PXR, pregnane X receptor; RELA, RELA proto-oncogene; SLC, solute carrier; SLCO, solute carrier organic anion; SP1, specific protein 1; TF, transcription factor; WY, WY-14643.
Fig. 1. (A) Experimental design: HepaRG cells were exposed to 0.1% DMSO (vehicle control), 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO, constitutive androstane receptor ligand), WY-14643 (WY, peroxisome proliferator-activated receptor a ligand), perfluorooctanoic acid (PFOA), perfluorooctanoic acid (PFNA), or perfluorodecanoic acid (PFDA). RNA was extracted and whole transcriptome RNA sequencing was conducted. The transcriptomic changes following each chemical exposure, as well as the predicted upstream regulators, were quantified. The mRNA levels in genes involved in liver functions, i.e., xenobiotic metabolism, transporters, bile acid metabolism, amino acid metabolism, and carbohydrate metabolism were assessed. A specific focus of the
was based on the observations of the mRNA up-regulation of the prototypical CAR- and PPARα target genes (Supplemental Fig. 1).

RNA Isolation. Cells were washed with PBS and re-suspended in RNA-Be reagent (Tel-Test, Inc., Friendswood, Texas). RNA was isolated using RNA-Be reagent (Tel-Test, Inc., Friendswood, Texas, Catalog No. CS-501B, 1 ml/well), according to the manufacturer’s instructions. RNA concentrations were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) at 260 nm. The RNA integrity was assessed by visualizing the 18S and 28S RNA bands under UV light using formaldehyde-agarose gel electrophoresis. In addition, an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) was used to quantify the concentration of the RNA samples and confirm the RNA integrity. Samples with RNA integrity numbers (RIN) above 8.0 were used for RNA-Seq.

Reverse Transcription and quantitative polymerase chain reaction (RT-qPCR). Total RNA was reversely transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). The resulting cDNA products were amplified by qPCR using the SsoAdvanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The primers for all qPCR reactions were synthesized by Integrated DNA Technologies (Coralville, IA), and primer sequences are shown in Supplemental Table 2. Data are expressed as % of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Differential expression was determined by ANOVA followed by Duncan’s post hoc test (p < 0.05).

RNA Sequencing. In triplicates, the cDNA library was constructed using an Illumina TruSeq Stranded mRNA kit (Illumina, San Diego, CA) using the poly-A tail selection strategy. The RNA fragmentation, first- and second-strand cDNA syntheses, end repair, adaptor ligation, and PCR amplification were performed according to the manufacturer’s protocol. The cDNA libraries were then validated for quantity and integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) before sequencing. Reads were sequenced using a 50 bp paired-end sequencing per the Illumina manufacturer’s protocol. The FASTQ files were de-multiplexed and concatenated for each sample. Quality control of the FASTQ files was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequenced reads from the FASTQ files were then mapped to the human reference genome (National Center for Biotechnology Information [NCBI GRCh38 Ensembl 103]) using HISAT2 version 2.1 (Kim et al., 2019). The sequencing map files were converted to count files/map (SAM) files were converted and sorted to binary alignment/map (BAM) format using SAMTools version 1.8 (Li et al., 2009). Binary alignment/map files were converted to count files by featureCounts using the GRCh38.103lift3.0. The raw and analyzed RNA-Seq data are deposited to the NCBI GEO database.

Data Analysis. Differential expression analysis was performed using DESeq2 (Love et al., 2014). The differentially expressed genes were defined as having a false discovery rate—Benjamini-Hochberg adjusted p-value (FDR-BH) < 0.05 in the chemical-exposed groups as compared with the vehicle-exposed control group. Up- or down-regulated genes were defined as having an absolute fold change (chemical exposure group over vehicle-exposed group) greater than 1.5. Differentially regulated genes were overlapped with a reference Kyoto Encyclopedia of Genes and Genomes database containing genes of specific categories of functions, namely xenobiotic biotransformation, transcription factors, oxidative stress, and inflammation. Venn diagrams were plotted for differentially regulated genes comparing different exposure groups using the R package VennDiagram (Chen and Boutros, 2011). Hierarchical clustering was performed on all differentially expressed genes using the R package ComplexHeatmap (Ou et al., 2016). Lists of up- and down-regulated genes were used as input for gene ontology enrichment using the R package topGO (Alexa and Rahnenfuehrer, 2021), and the list of genes in the unfiltered expression table was used as the background. RNA-seq count matrix was normalized to transcripts per million. Genes were considered expressed if the transcripts per million (TPM) of each gene were greater than the total sample number and if the variance was greater than 1. Up- and downregulated genes, as well as transporters, were subject to upstream regulator prediction with adjusted p-value less than 0.1 using the enrichR function on the ToppFun Transcription factor database (Chen et al., 2013; Han et al., 2018). Bar plots and box and whiskers plots were created using ggplot2 (Wickham, 2016).

Transporter Gene Selection and Visualization. Within the transporter superfamily, there are two major clusters of transporter genes in humans and rodents, namely the transport ATPases (including P, V, F, and ABC families) and the solute carrier (SLC) families (Pedersen, 2005; Doring and Petzinger, 2014). Based on the literature, the transporters investigated in this study are summarized in Supplemental Table 1. Individual differentially regulated transporter genes were plotted using Sigma Plot (SPSS Inc., Chicago, IL).

Results

Effect of PFCA Exposure on the Hepatic Transcriptional Changes in HepaRG Cells. To assess how exposure to various PCFAs modulates the hepatic transcriptome in HepaRG cells, RNA-Seq was performed in DMSO-, PFOA-, PFNA-, and PFDA-exposed HepaRG cells (n = 3 per group) as described in MATERIALS AND METHODS. Because it is known that PCFAs activate the nuclear receptors PPARα and CAR, as a positive control, HepaRG cells exposed to the prototypical PPARα ligand (WY-14643, abbreviated as “WY”) and the prototypical CAR ligand (CITCO) were also included (n = 2 per group). As shown in Supplemental Fig. 1, selected members of the first four CYP families were tested as positive controls for the effects of the prototypical PPARα and CAR ligands as well as PCFAs by RT-qPCR. At the selected concentrations, CITCO was the only chemical that upregulated the mRNA of cytochrome P450 family 1 subfamily A member 2 (CYP1A2), which is a prototypical AhR-target gene but can also be upregulated by CAR activation. The prototypical CAR-target gene CYP2B6 was upregulated by CITCO and all 3 PCFAs but not altered by WY. The mRNA of cytochrome P450 family 3 subfamily A member 4 (CYP3A4) was upregulated by CITCO and tended to be upregulated by the other chemicals but was not statistically significant. To note, CYP3A4 is a prototypical PXR-target gene but can also be upregulated by CAR activation. The mRNA of the prototypical PPARα-target gene CYP4A11 was upregulated by WY and all 3 PCFAs but not by CITCO (Supplemental Fig. 1). In summary, as expected, the prototypical ligands activated the corresponding nuclear receptors, whereas all PCFAs appeared to activate CAR and PPARα but not AhR, whereas a moderate trend of PXR-activation was noted but was not statistically significant.

As shown in Fig. 1B-1F, principal component analysis (PCA) was performed on the normalized, filtered, and Z-transformed gene expression matrix. For all exposures, the first two principal components (PCs) explained at least 85% of the total variation in the filtered gene expression matrix. All PC1 and PC2 explained 78% of the total variation in the combined exposure groups of liver cells (Supplemental Fig. 2A). PCFAs tended to be clustered together, apart from DMSO (Supplemental Fig. 2A). The coordinates of the WY- and CITCO-exposed groups tended to be clustered together, although no clear groups were observed (Fig. 1B and 1C). On the contrary, HepaRG cells exposed to PFOA, PFNA, or PFDA clustered distinctly from the DMSO-exposed control group (Fig. 1D-1F), suggesting that the PFCA exposure resulted in greater transcriptional differences.

present study was to assess the regulation of various xenobiotic and endobiotic transporters by perfluorinated carboxylic acids and their predicted upstream transcription factors. PCA results showing the first two principal components comparing DMSO to WY (B), CITCO (C), PFOA (D), PFNA (E), and PFDA (F). G. Venn diagram comparing CITCO, WY, and PFOA. F. Venn diagram comparing CITCO, WY, and PFNA. H. Venn diagram comparing CITCO, WY, and PFDA. I. Venn diagram comparing PFOA, PFNA, and PFDA. K. Number of differentially regulated genes as defined by false discovery rate—Benjamini-Hochberg adjusted p-value < 0.05 and absolute fold change > 1.5 by each chemical.

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than the exposure to prototypical nuclear receptor ligands, likely through PPARα/CAR-independent mechanisms.

Venn diagrams were used to visualize the commonly and differentially regulated genes following exposure to PFCAs and how the PFCA effects compare with the effects of WY or CITCO (Fig. 1G-1J). For the three PFCAs, most of the PFCA-regulated genes did not overlap with the WY- or CITCO-regulated genes. Within the commonly regulated genes between the nuclear receptor ligands and PFCAs, the transcriptomic signatures of PFCA exposure were more similar to that of WY exposure than CITCO exposure (Fig. 1G-1I), indicating that PPARα activation is a more prevalent mechanism in the transcriptional regulation of these genes than CAR activation. Common targets (31 genes) by all 3 PFCAs were also identified (Fig. 1J). These commonly regulated genes by all 3 PFCAs were involved in amino acid metabolism and carbon utilization (Supplemental Fig. 2B and Supplemental Table 3A). Among all 3 PFCAs, PFNA and PFDA had the greatest number of commonly regulated genes (170), with PFOA having the least number of overlapping differentially regulated genes (Fig. 1G). Commonly regulated genes by PFNA and PFDA were enriched in tRNA aminoacylation and carbohydrate metabolism (Supplemental Fig. 2C and Supplemental Table 3B). To assess the transcriptomic impact of PFCAs with varying carbon chain lengths, the number of differentially regulated genes was compared. Overall, the transcriptomic response was associated with carbon chain length of PFCAs with PFDA (C10) having the most prominent effect on the liver transcriptome (411 upregulated genes and 433 downregulated genes) (Fig. 1K). This was followed by PFNA (119 upregulated and 112 downregulated genes) and PFOA (29 upregulated and 19 downregulated genes) (Fig. 1K). In contrast, CITCO and WY produced minimal effect on the liver gene expression, in general (6 upregulated and 2 downregulated genes for CITCO, and 21 upregulated and 3 downregulated genes for WY) (Fig. 1K). In summary, most PFCA-mediated dysregulated genes were distinct from PPARα- and CAR-mediated pathways, whereas within the commonly regulated genes between PFCAs and the nuclear receptor ligands, the PPARα-signaling appears to be more involved than the CAR-signaling in PFCA-mediated transcriptomic response. All three PFCAs commonly regulated genes involved in amino acid synthesis and amino acid transport, whereas PFNA and PFDA were also commonly regulated in tRNA activity and carbohydrate starvation. These results suggest that transporter-mediated protein synthesis and carbohydrate metabolism genes are critical targets in human hepatocytes following exposure to PFCAs.

Individual Transcriptomic Alterations Following Exposure to PFOA, PFNA, or PFDA. To compare the effect of the prototypical nuclear receptor ligands and different PFCAs on the hepatic transcriptome, differentially regulated genes were analyzed for each exposure group. As expected, CITCO upregulated the CAR-target gene CYP2B6 (Supplemental Fig. 3A and Supplemental Table 4A). In addition, CYP1A2, and CYP3A4 were also upregulated by CITCO. Gene ontology enrichment results of differentially regulated genes included upregulation of the epoxygenase P450 pathway and drug metabolism processes by CITCO (Supplemental Fig. 3B and Supplemental Table 4B). Also as expected, WY upregulated cytochrome P450 family 4 subfamily A member 22 (CYP4A22), a prototypical target of PPARα. Other genes upregulated by WY include fibroblast growth factor 21—another known PPARα-target (Inagaki et al., 2007), as well as pyruvate dehydrogenase 4 (PDK4), fatty acid binding protein 4 (FABP4), and transmembrane protein 50B (Supplemental Fig. 3C and Supplemental Table 4C). At the pathway level, WY exposure

Fig. 2. (A) Volcano plot showing differentially regulated genes by perfluorooctanoic acid relative to DMSO. Top 5 up- (B) and down- (C) regulated gene ontology terms from perfluorooctanoic acid. Color gradient represents false discovery rate-adjusted p-value. Vertical line shows statistical threshold (false discovery rate–Benjamini-Hochberg adjusted p-value < 0.05). Differentially regulated genes (false discovery rate–Benjamini-Hochberg adjusted p-value < 0.05 and absolute fold change > 1.5) were used for all plots.
upregulated genes involved in multiple lipid metabolism-related pathways (Supplemental Fig. 3D and Supplemental Table 4D). No gene ontology terms were downregulated by CITCO or WY.

PFOA upregulated genes, including ChaC glutathione specific gamma-glutamylcyclotransferase 1, PDK4, and CYP4A22, and downregulated genes, such as acireductone dioxygenase 1, neurobeachin pseudogene 2, and small EDRK-rich factor 1B (Fig. 2A and Supplemental Table 5A). Upstream regulators from differentially regulated gene information include predicted activated transcription factors (TFs) involved in xenobiotic biotransformation (nuclear receptor subfamily 1 group I member 2 [NR1I2/PXR]), lipid sensing and metabolism (peroxisome proliferator activated receptor alpha and gamma [PPARα, PPARγ, respectively]), signaling molecule regulation (hepatocyte nuclear factor 4 alpha [HNF4α], activating transcription factor 4 [ATF4], forkhead box a2 [FOXA2]), and cell cycle regulation (REL alpha [RELA], MYC proto-oncogene [MYC]), and predicted inhibited oxygen sensing (hypoxia inducible factor 1a [HIF1α]) (Supplemental Fig. 4A and Supplemental Table 5B). Upregulated genes by PFOA were generally involved in transport processes, such as carboxylic acid transport, organic acid transport, organic anion transport, and L-amino acid transport, as well as lipid metabolic processes, as evidenced by the top enriched gene ontology terms from the pathways analysis using TopGO, whereas no significant gene ontology terms were downregulated by PFOA exposure (Fig. 2B-2C, and Supplemental Table 5C).

Genes upregulated by PFNA include ChaC glutathione specific gamma-glutamylcyclotransferase 1, phosphoserine aminotransferase 1 (PSAT1), and fibroblast growth factor 21, and down-regulated genes included chaperonin containing TCP1 subunit 8 pseudogene 1 (Fig. 3A and Supplemental Table 6A). As shown in Supplemental Table 6B, xenobiotic biotransformation regulators (aryl hydrocarbon receptor [AHR], NR1I2/PXR), lipid sensing and metabolism (PPARα), signaling molecule regulation (ATF4, estrogen receptor 1 [ESR1], progesterone receptor [PGR], forkhead box m1 [FOXM1]), cell cycle (REL, specific protein 1 [SP1], SWI/SNF-related matrix associated actin dependent regulator of chromatin subfamily A member 4, transcription factor 3 [TCF3], CCAAT/enhancer binding protein alpha [CEBPA]), and immune response (nuclear factor kappa B subunit 1 [NFKB1]) were predicted to be activated. Transcription factors involved in lipid sensing and metabolism (PPARγ coactivator 1 alpha [PPARGC1A/PGC1α]) and oxygen sensing (hypoxia inducible factor 1 subunit alpha [HIF1α]) were predicted to be inhibited. Signaling molecule regulators (HNF4α and ATF5) and xenobiotic biotransformation regulator (nuclear receptor subfamily 1 group I member 3 [NR1I3/CAR]) were also predicted to be upstream regulators (Supplemental Fig. 4B). Genes upregulated by PFNA were involved in tRNA aminoacylation, amino acid metabolism, and endoplasmic reticulum stress response (Fig. 3B and Supplemental Table 6C). Downregulated genes by PFNA were related to changes in genes associated with the metabolism of alcohol groups, as well as cellular hypoxic response (Fig. 3C and Supplemental Table 6D).

PFDA-upregulated genes, such as ChaC glutathione specific gamma-glutamylcyclotransferase 1, PSAT1, bile acid-CoA amino acid N-acyl-transferase, and fibroblast growth factor 21, as well as PFDA-downregulated genes, including isopentyl-diphosphate delta isomerase 1, dehydrogenase/reductase 11 (DHRS11), and small EDRK-rich factor 1B are shown in Fig. 4A and Supplemental Table 7A. Activated predicted upstream regulators were involved in signaling molecule

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**Fig. 3.** (A) Volcano plot showing differentially regulated genes by perfluorooctanoic acid relative to DMSO. Top 5 up- (B) and down- (C) regulated gene ontology terms from perfluorooctanoic acid. Color gradient represents false discovery rate-adjusted p-value. Vertical line shows statistical threshold (FDR-BH < 0.05). Differentially regulated genes (FDR-BH < 0.05 and absolute fold change > 1.5) were used for all plots.
regulation (ATF4, SMAD family member 3 and 4 [SMAD3 and SMAD4, respectively], PGR, ESR1, homeobox b7, NKX homeobox 1 [androgen-regulated homeobox], lipid sensing and metabolism (PPARc and PPARa), xenobiotic biotransformation (nuclear factor erythroid 2 like 2 [NFE2L2/NRF2]), oxygen sensing (hypoxia inducible factor 2 subunit alpha [HIF2A/EPAS1]), cell cycle (SMARCA4, histone deacetylase 2 [HDAC2]), tumor protein 53, RNA binding motif protein X linked, Fos proto-oncogene, AP1 transcription factor subunit [FOS], HIX ZBTB transcriptional repressor 1, Krüppel like factor 6, Bon Hipel-Lindau tumor suppressor [VHL], enhancer of zeste 2 polycomb repressive complex 2 subunit), and ER stress (X-box binding protein 1) (Supplemental Fig. 4B and Supplemental Table 7B).

Upstream regulators predicted to be inhibited consist of functions related to lipid sensing and metabolism (sterol regulatory element binding protein 1 and 2, PPARGC1A/PGC1a), bile acid metabolism (nuclear receptor subfamily 1 group h member 4), xenobiotic biotransformation (NR1I2/PXR), and signaling molecule regulation (HNF4a) (Supplemental Fig. 4B and Supplemental Table 7B). Upstream transcription factors related to oxygen sensing (HIF1a), cell cycle (SP1), xenobiotic biotransformation (NR1I2/PXR), as well as lipid-sensing and metabolism (PPARD) were also predicted to be altered (Supplemental Fig. 4C and Supplemental Table 7B).

Following PFDA exposure, the top five up-regulated gene ontology terms included cellular endoplasmic reticulum stress, trNA aminoacylation, and amino acid metabolism (Fig. 4B and Supplemental Table 7B). Downregulated genes were related to alcohol, sterol, and cholesterol metabolism, suggesting deviations from normal hepatic metabolic functions (Fig. 4C and Supplemental Table 7D).

Transcriptomic Changes Related to Hepatic Functions by PFCAs. Gene ontology enrichment of differentially regulated genes by PFCAs was suggestive of changes in endogenous liver functions, in addition to potential dysregulations in transporters (Figs. 2-4). Therefore, to investigate the overall transcriptomic changes involved in endogenous liver functions and linked to the predicted upstream regulators, differentially regulated genes were grouped into categories, namely phase-I and -II metabolism, transporters, bile acid metabolism, amino acid metabolism, and carbohydrate metabolism (Fig. 5 and Supplemental Fig. 4-5). In general, the numbers of differentially regulated genes increased with increasing carbon chain length of the PFCAs, whereas the overlap between the effect of PFCAs and the effect of CAR/PPARα ligands was minimal.

Specifically, as shown in Fig. 5A and Supplemental Fig. 4D-4E, for phase-I and -II drug-metabolizing enzymes, the prototypical CAR ligand upregulated 5 the mRNAs of 5 phase-I enzyme-inducing genes, namely aldehyde dehydrogenase 3 family member A1, cytochrome P450 family 1 subfamily A member 1, CYP1A2, CYP3A4, and CYP2B6. The up-regulation of CYP1A2, CYP3A4, and CYP2B6 observed from the RNA-Seq experiment was consistent with the RT-qPCR results (Supplemental Fig. 1). The prototypical PPARα ligand WY upregulated the phase-I enzymes CYP3A4, alcohol dehydrogenase 1B, arylacatamidine deacetylase, and CYP4A22, and tended to upregulate CYP4A11 but was not statistically significant, and this trend was consistent with the RT-qPCR results (Supplemental Fig. 1). Neither CITCO nor WY altered the mRNAs of phase-II enzymes. Regarding the effects of various PFCAs on the mRNAs of phase-I drug-metabolizing enzymes, most of the PFOA effect was up-regulatory (e.g., CYP3A4,
arylacetamide deacetylase, CYP4A11, CYP4A22, CYP2C8, and CYP2B6). Conversely, most of the PFNA effect was downregulatory (e.g., cytochrome P450 family 2 subfamily C member 19, alcohol dehydrogenase 4, cytochrome P450 family 4 subfamily F member 2, cytochrome P450 family 7 subfamily A member 1 [CYP7A1], alcohol dehydrogenase IB, flavin containing dimethylalanine monooxygenase 5, glutathione S-transferase alpha 2). PFDA had the most effect on the mRNAs of drug-metabolizing enzymes, and most of its effect was down-regulatory (e.g., CYP2E1, CYP2CC9, cytochrome P450 family 4 subfamily C member 19, aldehyde dehydrogenase 7A1, alcohol dehydrogenase 4, cytochrome P450 family 4 subfamily F member 2, CYP7A1, alcohol dehydrogenase IB, aldehyde dehydrogenase 1 family member A1, flavin containing dimethylalanine monooxygenase 5, carboxyl ester lipase), as well as all the differentially regulated phase-II enzymes (Fig. 5A and Supplemental Fig. 4D-4E).

Regarding transporters, as shown in Fig. 5B and Supplemental Fig. 4C, neither CITCO nor WY altered the any transporter mRNAs, whereas PFCAs with increasing carbon chain length differentially regulated more transporter mRNAs, suggesting that PFCAs with longer carbon chains are more potent in regulating transporters, and the regulatory mechanisms are distinct from CAR- and PPARα-mediated pathways. Specifically, PFOA upregulated five and down-regulated one transporters, PFNA upregulated ten and down-regulated three transporters, and PFDA upregulated fourteen and down-regulated eleven transporters (Fig. 5B and S4F). The specific transporter regulatory patterns categorized according to their specific functions are shown in Fig. 6-10.

Bile acid metabolism-related genes were not regulated by CITCO, WY, or PFOA, and most of the PFNA- and PFDA-mediated mRNA changes were downregulatory. Specifically, PFNA decreased the mRNAs of CYP7A1 (rate-limiting enzyme for bile acid [BA]-synthesis), as well as 3b-iodothyronine deiodinase 1, 3b-iodothyronine deiodinase 2, and iso-pentyl-diphosphate delta isomerase 1; PFDA down-regulated most of the differentially regulated genes involved in BA metabolism (Supplemental Fig. 4G and 5A top panel).

Most genes related to amino acid metabolism were not regulated by CITCO or WY, except for a moderate mRNA increase of PSAT1 by WY. The PFCA effect was partitioned into two clusters (Supplemental Fig. 5A bottom panel): genes in cluster 1 were upregulated by PFCAs and genes in cluster 2 were downregulated by PFCAs. For both clusters, the effect was more prominent with increasing carbon chain lengths of the PFCAs. Specifically, PFOA upregulated two genes and downregulated one gene; PFNA upregulated sixteen and downregulated two genes; PFDA upregulated twenty-one genes and downregulated twelve genes (Supplemental Fig. 4H). As shown in Supplemental Fig. 5A, PFOA upregulated genes related to amino acid biosynthesis (e.g., tyrosyl-tRNA synthetase 1 [YARS1], alanyl-tRNA synthetase, PSAT1, glutamic–pyruvic transaminase 2, aicredtate dioxygenase...
1, and methylcrotonyl-CoA carboxylase subunit 1 (MCCC1), and downregulated genes involved in methionine metabolism (e.g., glycine N-methyltransferase [GNMT]) and mitochondrial permeability (e.g., protein phosphatase 1K).

PFNA up-regulated a greater number of genes involved in amino acid metabolism, compared with PFOA, such as asparagine and serine metabolism, and tRNA aminoacylation (e.g., ATP4, tyrosyl-tRNA synthetase 1, phosphoglycerate dehydrogenase, alanine-tRNA synthetase, PSAT1, phosphoserine phosphatase, glutamyl-prolyl-tRNA synthetase 1, isoleucyl-tRNA synthetase 1, threonyl-tRNA synthetase 1, methionyl-tRNA synthetase 1, serine-tRNA synthetase 1, glycyl-tRNA synthetase 1, tryptophanyl-tRNA synthetase 1, and arginine synthetase) and downregulated alpha amino acid metabolism (3-hydroxyisobutyryl-CoA hydrolyase, arginase 1, and adenylosuccinate synthase 1).

PFDA upregulated all genes in the first cluster, which are involved in asparagine, serine, taurine, and glutamine metabolism, as well as carbon-nitrogen and tRNA aminoacyl activity (arginase 2, cysteine-tRNA synthetase 1, ATP4, tyrosyl-tRNA synthetase 1, phosphoglycerate dehydrogenase, alanine-tRNA synthetase, PSAT1, glutamic–pyruvic transaminase 2, phosphoserine phosphatase, glutamyl-prolyl-tRNA synthetase 1, isoleucyl-tRNA synthetase 1, threonyl-tRNA synthetase 1, methionyl-tRNA synthetase 1, serine-tRNA synthetase 1, glycyl-tRNA synthetase 1, solute carrier family 7 member 11 [SLC7A11], tryptophanyl-tRNA synthetase 1, asparagine synthetase, glutamate-cysteine ligase modifier subunit, cystathionine gamma-lyase, 5-phosphohydroxyl-L-lysine phosphohydroxylase, glutamine-fructose-6-phosphate transaminase 1, carbamoyl-phosphate synthetase 2, phosphohydroxypolyglycinamidine synthase, bile acid-CoA amino acid N-acetyltransferase) and most genes in the second cluster, most of which were related to branched chain and alpha amino acid catabolism, and 5-adenosyl methionine binding (3-hydroxyisobutyryl-CoA hydrolyase, procollagen-lysin, 2-oxoglutarate 5-dioxygenase 2, aldehyde dehydrogenase 1 family member A1, arginase 1, cysteine dioxygenase type 1, acetyl-CoA acetyltransferase 1, homogentisate 1,2-dioxygenase, beta-ureidopropionase 1, isoalloxynine dehydrogenase 1, gamma-aminobutyrate and cystathionine betahydroxylase, methyltransferase (GNMT), and mitochondrial permeability (e.g., protein phosphatase 1K, ornithine transcarbamalase, 4-aminobutyrate aminotransferase, glycine-N-acetyltransferase, aldehyde dehydrogenase 7A1, HNF4A, carnabomyl phosphate synthetase 1, cystathionine beta-synthase, adenylosuccinate synthase 1, hydroxycarboxyoxynuclease semialdehyde dehydrogenase, oxoglutarate dehydrogenase, SLC39A8).

Genes involved in carbohydrate metabolism were not changed by CITCO. PKD4 (involved in pyruvate metabolism) was upregulated by WY (Supplemental Fig. 5B). Carbohydrate metabolism-related genes were also grouped into two clusters showing downregulated and upregulated patterns, respectively (Supplemental Fig. 5B). PFOA upregulated two genes and downregulated two genes; PFNA upregulated eight genes and downregulated seven genes; PFDA upregulated twenty-one genes and downregulated 24 genes (Supplemental Fig. 4I). Specifically, as shown in Supplemental Fig. 5B, PFNA upregulated hexose metabolism-related genes (fucosyltransferase 1 [FUT1], PD4, and carnitine palmitoyltransferase 1A), and downregulated 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKBFB4) and enolase 2 (ENO2). The number of dysregulated genes increased, compared with PFOA. PFNA up-regulated genes related to the metabolism of hexose and other carbohydrates, as well as glucagon signaling (e.g., carbohydrate sulfotransferase 3 [CHST3], ATP4, phosphoenolpyruvate carboxykinase 2, FUT1, glycogen phosphorylase B, beta-1,4-galactosyltransferase 5, amyloplastic alpha 2B, PKD4, and carnitine palmitoyltransferase 1A); and down-regulated OMA1 zinc metallopeptidase, aldolase B (ALDOB), ALDOK, lactate dehydrogenase A, chitinase acidic, inositol polyphosphate-5-phosphate 1, PFKBFB4, and ENO2). PFDA dysregulated the greatest number of genes in the carbohydrate metabolism category. PFDA upregulated genes involved in glucose and hexose metabolism and regulation of gluconeogenesis (e.g., hexokinase domain containing 1, O-linked n-acetylgalosamine transferase, insulin receptor substrate 2, FOXK2, ATP4, period circadian regulator 2, phorbol-12-myristate-13-acetate-induced protein 1, nuclear protein 1, DNA damage inducible transcript 4, potassium inwardly rectifying channel subfamily J member 11, sestrin 2, phosphoenolpyruvate carboxykinase 2, FUT1, ATF3, SIK1, FOXK1, and PDK4). PFDA downregulated genes involved in canonical glycosylation and metabolism of NAD, pyruvate, and energy-related metabolites (e.g., ALDOB, glyceral-3-phosphate

Fig. 6. Bar plots showing the up-regulation of ABC transporters by 6-(4-chlorophenylimidazol[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, WY-14643, perfluorooctanoic acid, perfluoroocatonic acid, or perfluorodecanoic acid. Data are expressed as mean ± standard error (SE). Bar plots were made by using Sigma Plot (SPSS, Inc., Chicago, IL). Asterisks represent statistically significant differences as compared with the 0.1% DMSO-exposed vehicle group (DESeq2, false discovery rate–Benjamini-Hochberg adjusted p-value < 0.05).
dehydrogenase 1, glucosylceramidase beta 3, phosphoenolpyruvate carboxykinase 1, myogenesis regulating glycosidase, phosphofructokinase, fructose-bisphosphate A, citrate synthase, oxoglutarate dehydrogenase, pyruvate dehydrogenase E1 subunit alpha 1, malate dehydrogenase 1, glucose-6-phosphate isomerase, triosephosphate isomerase 1, IDH2, pyruvate kinase M1/2, phosphoglycerate mutase 1, PFKFB4, ENO2, aldehyde dehydrogenase 1 family member A1, GNMT, and glycogen synthase 1).

As a primary focus of the present study, we examined the effect of PFCAs on various families of transporters in HepaRG cells. Because an advantage of RNA-Seq over other conventional mRNA quantification methods is that it provides a “true measurement” of the actual transcript counts, allowing the comparison of the absolute abundance among different transporter genes, after we unveiled the relative expression pattern of transporters in a heatmap (Fig. 5), we compared the absolute transcript counts and regulation of these transporters under basal and PFCAs-exposed conditions (Fig. 6-10 and Supplemental Fig. 7-10).

ABC transporters are the largest family of the transport ATPases (Supplemental Table 1), and they are involved in the efflux of various xenobiotics and endogenous metabolites. As shown in Supplemental Fig. 7, under basal conditions, among all the ABC transporters, the cumulative mRNAs of the ABCC sub-family were the most abundant (27%), followed by ABCB (19%), ABCA (17%), ABCD (16%), ABCE (10%), ABCF (9%), and ABCG (2%). Because ABCC/MRP and ABCB/MDR are the most important efflux transporters for various drugs and ABCA is important for eliminating cholesterol and oxysterols, the highest abundance of the mRNAs of these two sub-families highlights the importance of hepatocytes in these two functions. As shown in Fig. 6, following chemical exposure, CITCO and WY had no effect on the mRNAs of the ABC transporters. However, PFCAs with increasing carbon chain lengths tended to increase the mRNAs of ABCA3, ABCB3/MPR3, ABCB10/MPR7, and ABCG2/breast cancer resistance protein (BCRP), with the statistical significance observed for PFDA. The upregulation of these ABC transporters may suggest a compensatory response to eliminate the intracellular toxic metabolites following PFCAs exposure.

In addition to the ABC transporters, the mRNAs of other transport ATPases were also detected in HepaRG cells, with ATP5 being the most abundant transport ATPase family (74.9%), followed by ATP6 (12.9%), ATP1 (8.4%), ATP2 (2.6%), and ATP13 (1.8%). Other transport ATPases had minimal expression in HepaRG cells (<1%) (Supplemental Fig. 8). None of these transport ATPases were readily upregulated by the nuclear receptor ligands or PFCAs.

Another major transporter superfamily in humans is the solute carriers (SLCs), which play important roles in transporting endogenous metabolites, nutrients, and drugs. Within the SLC superfamily, the SLCO/OATP transporters are well-known for their uptake of various drugs and other xenobiotics into hepatocytes for further metabolism. As shown in Supplemental Fig. 9, under basal conditions, among all the OATP transporters, the cumulative mRNAs of the SLCO2/OATP2 sub-family were the most abundant (49%), followed by SLCO4/ OATP4 (28%), SLCO3/OATP3 (13%), SLCO1/OATP1 (9%), SLCO5/ OATP5 (1%), and SLCO6/OATP6 (0.03%). The cumulative mRNA of
all SLCO/OATP transporters comprise approximately 0.2% of the mRNA abundance of all SLC transporters (Supplemental Fig. 10). The SLC transporter genes with highest expressed in HepaRG cells are those involved in amino acid transport, which is essential for protein synthesis (44%), followed by those involved in the transport of zinc (8.0%) and iron (2.0%), as well as other nutrients, such as folate, thiamine, and riboflavin (1.7%). While most of the HepaRG cell-expressed SLC transporter genes are involved in intermediary metabolism, a minor percentage of the SLC transporter genes expressed in HepaRG cells are involved in the transport of xenobiotics (ENTs [0.63%], OCTs/OCTNs [0.5%], MATEs [0.2%], and concentrative nucleoside transporter [0.05%]) and BAs (0.3%) (Supplemental Fig. 10).

The PFCA-mediated gene regulation of amino acid transporting SLC transporters—the most abundant SLC members in HepaRG cells, is shown in Fig. 7. Interestingly, while the CAR and PPARα ligands had no effect, PFCA within increasing carbon chain lengths tended to up-regulate genes for nine SLC amino acid transporters, namely SLC1A4, SLC1A5, SLC6A9, SLC7A1, SLC7A2, SLC7A5, SLC7A11, SLC25A29, and SLC43A1, with statistical significance observed for PFDA. Similarly, other SLC transporters that tended to be upregulated by PFCA include those involved in xenobiotic transport (SLC29A3/ENT3 and SLC47A2/MATE2) with statistical significance observed for PFDA (Fig. 8). PFDA also upregulated genes for several SLC transporters for endogenous substrates, including the monocarboxylic acid transporter SLC16A3, the sodium phosphate transporter SLC17A3, the versatile cellular metabolite transporter SLC22A15, the manganese transporter SLC30A10, SLC35F6 (suggested to be a nucleotide-sugar transporter), and the glycerol-3-phosphate transporter SLC37A2 (GeneCards.org) (Fig. 8).

While most of the up-regulated genes for SLC transporters by PFDA are involved in intermediary metabolism, including protein synthesis, the SLC transporters down-regulated by PFCA include the major hepatic BA uptake transporter SLC10A1/NTCP, two OATP uptake transporters for xenobiotics (SLCO2B1 and SLCO4C1), as well as the organic anion/cation transporter SLC22A10 and the sodium-coupled nucleoside transporter SLC28A1 (Fig. 9A). PFDA also downregulated genes for the creatine efflux transporter SLC6A8, the mitochondrial transporters SLC25A1 and SLC25A4 for citrate and adenine nucleotide (GeneCards.org) (Fig. 9B), the sodium bicarbonate cotransporter SLC4A4, the urea transporter SLC14A1, the ascorbic transporter SLC23A1, the equilibrative nucleoside transporter SLC29A4/ENT4, SLC35F4 (unknown function), the zinc transporter SLC39A8, and the lysosomal drug transporter SLC46A3 (Fig. 10).

Upstream regulators for PFCA-mediated regulation of transporters were predicted (Supplemental Fig. 6). NFE2L2/NRF2, which is the major sensor for oxidative stress and electrophiles, was predicted to be the main upstream regulator for transporters regulated by all PFCA (Supplemental Fig. 6A). In addition, upstream factors predicted to be involved in PFNA-mediated transporter regulation include regulators involved in xenobiotic biotransformation (AHR, retinoic acid receptor α [RARα]), cell cycle (SMARCA4, SP1, signaling molecule regulation [msh homeobox 2, EPAS, splicing factor 1, SRY box transcription factor 2, nuclear receptor subfamily 5 group A member 1, PGR, ESR1, TWIST]), and lipid sensing and metabolism (PPARγ) (Supplemental Fig. 6B). Most of these upstream regulators were also predicted to be
involved in PFDA-mediated transporter regulations (PGR, msh homeo-box 2, SMARCA4, SP1, EPAS1, splicing factor 1, RXRa, AHR, SRY box transcription factor 2, nuclear receptor subfamily 5 group A member 1, TWIST1, RARz). In addition, PFDA-mediated transporter regulation also appeared to involve nuclear receptor subfamily 3 group C member 2/glucocorticoid receptor, the major bile acid receptor nuclear receptor subfamily 2 group h member 4, nuclear receptor subfamily 3 group C member 1/mineralocorticoid receptor, nuclear respiratory factor 1, HNF1a, the estrogen receptor 1 (ESR1), spalt-like transcription factor 1, and NK2 homeobox 1 (Supplemental Fig. 6C).

Observations from CAR-null and Nrf2-null mice showed that Nr1I3/CAR and Nfe2l2/Nrf2 receptors are involved in the PFNA- and PFDA-mediated regulation of certain drug transporters in the liver, respectively (Maher et al., 2008; Zhang et al., 2018). Interestingly, Nr1I3/CAR was also a predicted upstream regulator in PFNA-exposed HepaRG cells (Fig. 3B), and Nfe2l2/Nrf2 was also a predicted upstream regulator in PFDA-exposed HepaRG cells (Fig. 4B). Therefore, our findings from HepaRG cells align with the literature reports on PFNA- and PFDA-exposed mouse models. AHR is another important xenobiotic-sensing receptor, and it was only predicted to be activated by PFNA. The effect of PFNA on hepatic transporters is not characterized in AhR-null mice; the effect on PFOA of hepatic transporters is also not characterized in the receptor gene null mice discussed above. However, it is possible that some of the same xenobiotic-sensing receptors are also involved.

In conclusion, the present study using PFOA ([C8]), PFNA ([C9]), and PFDA ([C10]) at a concentration lower than what was used in HepaRG cells from the literature showed that genes associated with amino acid transporters, which are critical for protein synthesis, are novel inducible targets for all three PFCAs. PFCAs with increasing carbon chain lengths had a greater transcriptomic response in HepaRG cells, and the top commonly activated pathways are involved in amino acid metabolism and transport. Commonly activated or predicted to be activated genes for receptors include PPARa, CAR, PXR, and ATF4 by all PFCAs at the global level. At the transporter level, none of the PFCA-mediated transporter gene regulations appeared to be through PPARa or CAR but appeared to be through Nrf2. At equal molar concentrations, PFCA congeners with a longer carbon chain length were shown to be more potent in regulating the expression of transporters for xenobiotics and BAs, and these results suggest a compensatory response to reduce exposures to PFCAs of longer carbon chains by increasing their efflux and reducing their further uptake. In addition, the amino acid transporters were regulated by all PFCAs, and these transporters are critical for protein synthesis and may contribute to chemical detoxification.
Taken together, as shown in Fig. 11, the goal of the present study was to investigate the effect of PFCAs with various carbon chain lengths at an equal molar concentration on transporters involved in intermediary metabolism and xenobiotic biotransformation in human hepatocytes. From a transcriptome-wide scale, we also identified common and unique pathways regulated by various PFCAs, as predicting...
the upstream regulators in an unbiased approach. Because it has been shown in mouse models that certain PFCAs activate CAR and PPAR\textsubscript{z} (Cheng and Klaassen, 2008a and Cheng et al., 2008b), the effects of the prototypical ligands for CAR (CITC) and PPAR\textsubscript{z} were also investigated. To note, WY-14643 has been primarily used as an activator of PPAR\textsubscript{z} (Hsu et al., 1995; Devchand et al., 1996; Staels et al., 1998); however, it also activates PPAR\textsubscript{\textgamma} (Lehmann et al., 1997) and PPAR\textsubscript{\alpha}, although this finding is rare (Schmidt et al., 1992). While all three PFCAs were shown to activate PPAR\textsubscript{z} as evidenced by the upregulation of CYP4A11 (Supplemental Fig. 1) and upstream regulator analysis (Supplemental Fig. 4A-C), PPAR\textsubscript{\textgamma} and PPAR\textsubscript{\alpha} were also predicted upstream regulators for PFOA and PFDA exposures (Supplemental Fig. 4). Therefore, the other PPAR receptors in addition to PPAR\textsubscript{z} may also contribute to the transcriptomic response of HepaRG cells at the global level. However, at the transporter level, neither PPAR\textsubscript{z} nor CAR appeared to be involved in the regulation of transporters by any of the PFCAs at the concentration used in the present study because neither WY nor CITC regulated the transporters that were differentially regulated by PFCAs (Fig. 5B and Fig. 6-10). This is different from previous reports using livers from PFCA-exposed mice showing that PPAR\textsubscript{z} and CAR are necessary for PFCA-mediated regulation of certain uptake and efflux transporters (Cheng and Klaassen, 2008b; Maher et al., 2008) (Table 2). This difference may be due to a lower concentration used in the present study, differences in the nuclear receptors between humans and mice, and/or in vitro versus in vivo settings. However, interestingly, among all three PFCAs, the major oxidative stress sensor Nrf2 was predicted to be a common upstream regulator for transporters (Supplemental Fig. 6). A previous report also showed that Nrf2 is necessary in modulating the PFDA-mediated up-regulation of Mrp3 and Mrp4 in mouse liver. This indicates that oxidative stress may be an important toxicological endpoint in PFCA-exposed hepatocytes. Indeed, it has been reported that oxidative stress and inflammation may contribute to PFOA-induced hepatotoxicity in mice (Yang et al., 2014). In addition, PFNA has been shown to produce immunotoxicity and have persistent effects on the immune system (Rockwell et al., 2013; Rockwell et al., 2017), whereas Nrf2 is known to have an important immunomodulatory effect (Freeborn and Rockwell, 2021). Our study suggests that PFCAs may also activate Nrf2 to regulate the expression of transporters in human hepatocytes as a compensatory response.

**Discussion**

A previous study showed that PFOA downregulated most detected transporters (Table 1) (Behr et al., 2020). The only upregulated transporters reported were the cholesterol efflux transporter ABCG1 and the BA efflux transporter SLC51B/OST\textsubscript{\textgamma} (Table 1) (Behr et al., 2020). Most of the observations from the previous study (Behr et al., 2020) took place at higher PFOA concentrations and/or over a longer incubation time than the present study, with only a few exceptions (PFOA at 10 \(\mu\text{M}\) downregulated ABCG5/6 and MRp3 and upregulated OST\textsubscript{\textgamma}). Correspondingly, at these higher concentrations, PFOA in HepaRG cells increased the major hepatic BAs T-CA and G-CA (250 \(\mu\text{M}\), dilated the bile canaliculi (100 \(\mu\text{M}\), and downregulated the CYP7A1 protein of the rate-limiting enzyme for BA synthesis (100 – 250 \(\mu\text{M}\), suggesting that PFOA has a cholestatic potential at sub-toxic concentrations (Behr et al., 2020). Therefore, although PFOA at the higher concentrations did not reduce HepaRG cell viability (Behr et al., 2020), it may increase cellular stress by disrupting lipid homeostasis. Thus, the discrepancy between this previous study and the present study may be due to dose. Interestingly, in male rats, PFOA increased hepatic triglyceride levels and peroxisomal beta-oxidation between 10 and 20 mg/kg dose ranges (i.e. once daily for 5 days), corresponding to male-specific hepatic accumulation of PFOA (Kudo and Kawashima, 2003). Although the expression of transporters was not determined in the study done in rats (Kudo and Kawashima, 2003), it is possible that the down-regulation of efflux transporters involved in lipid homeostasis may contribute to increased hepatic triglyceride levels. Interestingly, PFOA-exposed mice (40 mg/kg, i.p.) also had decreased expression of several hepatic uptake transporters in the Sko family (Table 2) (Cheng and Klaassen, 2008a), among which Slco1b2/Oatp1b2 has been suggested to contribute to the hepatic uptake of unconjugated BAs (Csanaky et al., 2011). PFOA-exposed mice (40 mg/kg, i.p.) also had increased expression of hepatic efflux transporters Abcc3/Mrp3 and Abcc4/Mrp4 (Maher et al., 2008). These Mrp transporters transport drugs and can also transport BAs during cholestasis (Mennone et al., 2006). Both the down-regulation of uptake transporters and the upregulation of efflux transporters suggest compensatory mechanisms to protect the hepatocytes from further toxic insults from PFOA exposure and indicate that these doses used in mice may be at the higher end.

Although the lipid and drug transporters were not regulated by PFOA at the lower concentration of 45 \(\mu\text{M}\) in HepaRG cells from our study as compared with the literature (Behr et al., 2020), we observed consistent PFOA-mediated up-regulatory patterns for the genes for amino acid transporters SLC1A4, SLC7A1, and the endoplasmic reticulum (ER) inorganic phosphate/glucose-6-phosphate antiporter SLC37A2 (Pan et al., 2011; Lin et al., 2015) at 24 hours at both low (45 \(\mu\text{M}\)) and high (100 \(\mu\text{M}\)) concentrations (Louisse et al., 2020) (Table 1, Fig. 7-8). Thus, PFOA is important in promoting the expression of transporters involved in protein synthesis and ER functions. Several other SLC transporters were not consistently regulated between the present study and the literature (Table 1), and this is likely due to differences in PFOA concentrations, incubation times, and/or culture media components (Behr et al., 2020; Louisse et al., 2020).

A previous study showed that PFNA at a higher concentration (100 \(\mu\text{M}\), 24 h) upregulated several amino acid transporters (SLC1A4, SLC1A5, SLC7A1, SLC7A5, and SLC7A11, and SLC43A1), the monocarboxylic acid transporter (SLC16A13, which transports lactate, pyruvate, branched-chain oxo acids derived from leucine, valine, and isoleucine, as well as ketone bodies acetoacetate, beta-hydroxybutyrate, and acetate), and the ER inorganic phosphate/glucose-6-phosphate antiporter SLC37A2 (Louisse et al., 2020). Interestingly, the present study showed that PFNA at the lower concentration of 45 \(\mu\text{M}\) (24 hours) also up-regulated these transporters (Table 1). It is important to note that the amino acid transporters SLC1A4, SLC7A11, and SLC37A2 were consistently upregulated by both PFOA and PFNA at both low and high concentrations (Louisse et al., 2020) (Table 1, Fig. 7). The consistency between the present study and the literature further confirmed the upregulatory effect of PFNA and PFOA at multiple non-toxic concentrations on transporters that are important for protein synthesis.

To the best of our knowledge, there are no reports on the effect of PFDCA on the hepatic transcriptomic response in HepaRG cells. The present study has provided the first evidence showing that, PFDCA regulated more genes in HepaRG cells than the other PFCA congeners, and most of these genes were uniquely regulated by PFDCA but not the other 2 PFCA congeners (Fig. 1G and 1H). At the transcriptome-wide level, PFDCA was also predicted to activate the most genes that are upstream regulators, including transcription factors involved in xenobiotic metabolism (NFE2L2/NRF2, NR1I3/CAR, NR1I2/PPX3) and lipid metabolism (PPARA, PPARG, PPARD, PPARC1A/PGC1\textalpha, nuclear receptor subfamily 1 group h member 4, and HNF4A). epigenetic modulators (HDAC1, HDAC2, enhancer of zeste 2 polycystic repressive complex 2 subunit), and steroid hormone nuclear receptors (PGR and ESR1), and the tumor suppressor tumor protein 53/P53 (Fig. 4B). At the transporter
level, PFDA regulated more genes for xenobiotic and BA transporters than the other PFCAs, and the upregulation of xenobiotic efflux transporters and downregulation of uptake transporters may suggest a protective mechanism to reduce further toxic insults. Indeed, it has been shown that PFDA exposure increased serum BA concentrations in mice (Cheng and Klaassen, 2008a), whereas the PFOA-mediated down-regulation of the major BA hepatic uptake transporter SLCO10A1/NTCP in both HepaRG cells (Fig. 5B and Fig. 9) and mouse liver (Cheng and Klaassen, 2008a) indicate a compensatory mechanism to prevent excessive BA accumulation in the liver. In summary, among the 3 PFCA congeners tested, PFDA is the most potent in modulating the hepatic transcriptomic response in general and transporter gene expression in particular.

Similar to the inducible effect of amino acid transporters by PFOA and PFNA, PFDA exposure also upregulated many genes associated with amino acid transporters, including SLC1A4, SLC1A5, SLC6A9, SLC7A1, SLC7A2, SLC7A5, SLC7A11, and SLC43A1, as well as the ER inorganic phosphate/glucose-6-phosphate antiporter SLC37A2 (Table 1) (Louisse et al., 2020). This finding is further supported by the transcriptome-wide pathway analysis showing that all 3 PFCAs commonly upregulated amino acid-related pathways, including L-amino acid transport, tRNA for protein synthesis, amino acid activation, cellular amino acid metabolic processes, and response to ER stress (Fig. 2-4).

It has been increasingly recognized that understanding the role of amino acid intake in the pathogenesis of liver diseases is a promising therapeutic strategy because amino acids are involved in a wide spectrum of biological processes. For example, amino acids are essential for protein synthesis, and alterations in amino acid metabolism have been linked to liver disease progression. The present study further supports this notion by demonstrating that PFCAs can modulate amino acid transport gene expression, which may have implications for understanding the role of amino acid metabolism in liver disease.

**Table 1**

<table>
<thead>
<tr>
<th>PFCAs</th>
<th>Exposure Time &amp; Concentration</th>
<th>Transporters</th>
<th>Effect</th>
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<tr>
<td>PFOA</td>
<td>48 h: 250 μM</td>
<td>ABCA1</td>
<td>(Behr et al., 2020)</td>
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<tr>
<td></td>
<td>24 h: 250 &amp; 500 μM</td>
<td>ABCB11/BSEP</td>
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<td></td>
<td>48 h: 50, 100, &amp; 250 μM</td>
<td>ABCCC2/MPR2</td>
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<td>24 h: 500 μM</td>
<td>ABCCC3/MPR3</td>
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<td></td>
<td>48 h: 10 &amp; 50 μM</td>
<td>ABCG1</td>
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<td>45 h: 250 μM</td>
<td>ABCG5</td>
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<td>24 h: 10, 50, 250, &amp; 500 μM</td>
<td>ABCG8</td>
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<td></td>
<td>48 h: 10, 50, &amp; 250 μM</td>
<td>SLC10A1/NTCP</td>
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<td>24 h: 250 &amp; 500 μM</td>
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<td>48 h: 100 &amp; 250 μM</td>
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<td>48 h: 10 &amp; 50 μM</td>
<td>SLC10A1/NTCP</td>
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PFNA 24 h: 250 μM

PFDA 24 h: 250 μM
cellular metabolisms, including the synthesis of lipids and nucleotides as well as the chemical detoxification process (Lee and Kim, 2019). For example, amino acids have been suggested to protect against acetaminophen-induced hepatotoxicity by serving as mitochondrial energy substrates independent from glutathione synthesis in mice (Saito et al., 2010). Branched-chain amino acids have been shown to suppress hepatocellular carcinoma in vitro and are required for immunosurveillance. In addition, in cirrhotic patients, serum branched-chain amino acids are decreased, and administration of branched chain amino acid-rich medicine has shown positive results (Tajiri and Shimizu, 2018). PFNA and PFOA have been shown to increase inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver metabolism – hypoxia, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012). Therefore, at the equal molar concentration of 45 μM, which is a relatively low concentration, the majority of the PFCAs compounds are expected to be taken up into the hepatocytes rather than lingering in the media. Another limitation of the present study was the focus on transcriptomic regulation without validation at the levels of protein expression and functional consequences. Because changes at the RNA level may not always translate into functional changes, additional studies in the figure are needed to address this limitation. Within the context of transporters, future studies are needed to quantify PFCA-mediated changes in both the protein abundance and the cellular localizations; in addition, intracellular and extracellular intermediary metabolites (such as amino acids) should be determined by metabolomics approach using in vitro and in vivo models. It is important to note that smaller changes in gene expression may or may not lead to physiologic consequences. Therefore, caution is needed while interpreting RNA-Seq data from a limited sample size for functional output. In addition, RT-qPCR may be inherently biased because it is based on short primer sequences. For example, the discrepancy in OATP2B1 mRNA levels from RT-qPCR and RNA-Seq results may be due to amplifying only portions of the mRNA regions (RT-qPCR) versus estimating the mRNA abundance using reads from all exons (RNA-Seq).

Despite the difference in the potency of the 3 PFCAs in modulating the hepatic transcriptome, all 3 PFCAs were predicted to activate the genes associated with lipid-sensing nuclear receptor PPARα and the xenobiotic-sensing nuclear receptor Nrf1I2/PXR in HepaRG cells (Fig. 2-4). Consistent with this prediction in HepaRG cells, in vivo studies using nuclear receptor gene null mice showed that PPARα is necessary for the PFNA-mediated regulation of multiple xenobiotic transporters (Zhang et al., 2018), and both PPARα and PXR are necessary for the PFDA-mediated regulation of multiple transporters in the liver (Cheng and Klaassen, 2008a) (Table 2). In addition to PPARα and Nrf1I2/PXR, all three PFCAs were also predicted to activate the master regulator for lipid and drug metabolism–HNF4α, the hypoxia-inducible factor-1α (HIF1α), which is involved in liver fibrosis, inflammation, and cancer (Nath and Szabo, 2012), as well as the activating transcription factor 4 (ATF4), which directs stress-induced gene expression in the unfolded protein response and cholesterol metabolism in the liver (Fusakio et al., 2016)
(Fig. 2–4). Together, these regulatory signatures may serve as early biomarkers for liver injury even at low doses of PFCA exposures.

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Authorship Contributions

Participated in research design: Cui. Conducted experiments: Lim, Suh, Cui. Performed data analysis: Lim, Suh, Cui. Wrote or contributed to the writing of the manuscript: Lim, Suh, Faustman.

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Faustman, Cui.


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Address correspondence to: Julia Yue Cui, 4225 Roosevelt Way NE, Suite 100, Seattle, WA. E-mail: juliacui@uw.edu

PCFAs and Transporters in HepaRG Cells