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Acot1 is a sensitive indicator for PPARα activation after perfluorooctanoic acid exposure in primary hepatocytes of Sprague-Dawley rats

Hui Liu[a,b,1], Jianshe Wang[a,1], Nan Sheng[a], Ruina Cui[a], Yitao Pan[a], Jiayin Dai[a,*]

[a] Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China
[b] Department of Laboratory Medicine, Bengbu Medical College, Bengbu 233030, PR China

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ABSTRACT

Perfluorooctanoic acid (PFOA) is one of the most commonly detected and persistent perfluoroalkyl substances (PFASs) found in the environment. We found that cell viability and intracellular oxidant stress increased in primary rat hepatocytes exposed to PFOA (100 μM PFOA, 24 h), and mitochondrial superoxide increased from 6.25 μM PFOA treatment group. To screen for sensitive indicators in mRNA level, we investigated global transcriptome profile alteration after PFOA exposure using RNA-sequencing (RNA-seq) in primary rat hepatocytes, and identified 177 gene transcripts (158 upregulated, 19 downregulated) as significantly changed after exposure to 100 μM of PFOA for 24 h (fold change ≥ 2, FDR < 0.05). Quantitative PCR (qPCR) and RNA-fluorescence *in situ* hybridization (RNA-FISH) assays were conducted after PFOA treatment at various doses (0, 0.4, 1.56, 6.25, 25, and 100 μM) and times (6, 12, 18, 24, 48, and 96 h). Acot1 transcripts increased significantly in the 100 μM PFOA group (4500-fold) after 24 h of exposure, and increased remarkably for all time points (24, 48, 72 and 96 h) after exposure to 6.25 μM. Acot1 also responded to lower PFOA doses, with a significant increase found after exposure to 0.4 μM for 96 h. These results imply Acot1 could serve as a sensitive indicator for PPARα activation after PFOA exposure in primary rat hepatocytes.

1. Introduction

Perfluoroalkyl substances (PFASs) are a group of chemicals with fluorinated carbon backbones of varying length. They are widely used as surfactants in industrial processes and consumer products (Calafat et al., 2007). Their carbon-fluoride bonds make them highly stable and environmentally persistent. They are also widespread, with studies reporting their existence in occupational workers and general human populations, as well as in wildlife and environmental matrices (Fromme et al., 2009; Giesy and Kannan, 2001; Kannan et al., 2004; Karrman et al., 2006; Olsen and Zobel, 2007). PFASs are both lipo- and hydrophobic, and therefore bind with liver and serum proteins after absorption (Jones et al., 2003; Luebker et al., 2002; Sheng et al., 2016). Among all PFASs, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two of the most widely detected. In human serum, the half-life of PFOA is 3.8 years (Olsen et al., 2007). Since 2000, PFOA manufacturing practices have been phased out, regulated, or restricted (Lau et al., 2007), and there is evidence of decreasing PFOA serum concentrations in the general population. For example, the United States' National Health and Nutrition Examination Survey (NHANES) showed that serum PFOA levels in 2003–2004 decreased by nearly 25% compared with that in 1999–2000 (Calafat et al., 2007). The average serum PFOA level of 600 adult American Red Cross blood donors from six regional donation centers in 2006 was 3.4 ng/mL (Olsen et al., 2008), representing an approximate 24% decline from samples taken in 2000–2001 from the same donation centers (Olsen et al., 2003a). However, PFOA is still an environmentally persistent and dominant PFAS, which has seen continued production in countries lacking legal restrictions, as well as generation by degradation of its precursor chemicals (Prevedouros et al., 2006; Wang et al., 2009).

The ubiquitous presence and persistence of PFASs in the environment and within the human body have led to extensive toxicological and epidemiological studies to understand the biological effects associated with exposure. Studies on PFASs have demonstrated that these

Abbreviations: DCF, 2′,7′-dichlorofluorescein; Acot, acyl-CoA thioesterase; Cyp4a1, cytochrome P450, family 4, subfamily a, polypeptide 1; DEGs, differentially expressed genes; Ech1, enoyl CoA hydratase 1, peroxisomal; GO, gene ontology; Hadha, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit; KEGG, Kyoto Encyclopedia of Genes and Genomes; PFASs, perfluoroalkyl substances; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PPARα, peroxisome proliferator activated receptor alpha; qPCR, quantitative PCR; ROS, reactive oxygen species; RNA-FISH, RNA-fluorescence *in situ* hybridization; RNA-seq, RNA-sequencing

* Corresponding author.
E-mail address: daijy@ioz.ac.cn (J. Dai).
[1] These authors equally contributed to this work.

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compounds are extremely potent peroxisome proliferators in rodents, especially by activating key lipid metabolism regulation factor, peroxisome proliferator activated receptor alpha (PPARα), to induce peroxisomal β-oxidation, peroxisome proliferation, liver hepatomegaly, and hepatocarcinogenesis (Abbott et al., 2007; Ikeda et al., 1985; Kennedy et al., 2004; Sohlenius et al., 1993). Several epidemiological studies, predominantly in occupational workers, have also indicated that exposure to PFASs might be associated with an increase in serum low-density lipoprotein binding cholesterol, total cholesterol, and/or triglycerides (Frisbee et al., 2010; Olsen and Zobel, 2007; Steenland et al., 2009). In rodents, the activation of PPARα under PFOA exposure is followed by changes in the expression of genes involved in lipid metabolism, fatty acid β-oxidation, and peroxisome proliferation (Guruge et al., 2006; Klaunig et al., 2003). These genes might be useful candidates for indication of PFOA exposure. In this study, we screened for global transcriptome profile alteration in primary rat hepatocytes using RNA-sequencing (RNA-seq), and identified the most sensitive gene as an indicator for PPARα activation after PFOA exposure using quantitative PCR (qPCR) and RNA-fluorescence *in situ* hybridization (RNA-FISH) assays.

2. Materials and methods

2.1. Chemicals, animals, and primary hepatocyte isolation and treatment

We obtained PFOA from Sigma-Aldrich (CAS No. 335-67-1, 96% purity, St. Louis, MO, USA). Hepatocytes were isolated from 200 ± 10 g Sprague-Dawley rats, as described previously (Smedsrod and Pertoft, 1985). The procedures were performed in accordance with the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. Freshly isolated hepatocytes were cultured in William's E medium (Gibco, Life Technology, USA) with 10% fetal bovine serum (FBS), and antibiotics (penicillin-streptomycin). After overnight incubation, the adherent hepatocytes were seeded into tissue culture plates, and then treated with different doses of PFOA (0, 0.4, 1.56, 6.25, 25, and 100 μM, various experiments) dissolved in William's E medium. The cells were harvested at different time intervals of treatment (6, 12, 18, 24, 48, 72, and 96 h, various experiments), and then either fixed in 4% paraformaldehyde for RNA-FISH and immunohistochemical analysis or stored at − 80 °C for RNA and Western blot analysis.

2.2. PFOA content in hepatocytes after exposure

After treatment with different concentrations of PFOA (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h, the PFOA was extracted from hepatocytes using an ion pairing method (Hansen et al., 2001), with a further cleanup process (Taniyasu et al., 2005). The chemical content was quantified using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) according to our previous description (Lu et al., 2016).

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability as the endpoint after PFOA treatment was determined using MTT assay (5 mg/mL). Primary rat hepatocytes were seeded into 96-well tissue culture plates. After the hepatocytes were treated with PFOA at a range of concentrations (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h, MTT was added and the produced formazan was measured with a spectrophotometer (BioTek, Synergy H1 Hybrid Microplate Reader, USA) (Hu et al., 2014). Cell viability (%) was calculated as follows: viability (%) = $(OD_{treated}/OD_{control}) \times 100\%$.

2.4. Oxidative stress injury measurement

The intracellular oxidant intensity and mitochondrial superoxide in the hepatocytes after PFOA treatment (0, 0.4, 1.56, 6.25, 25, and 100 μM, 24 h) was determined by analysis of H_2DCFDA (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and MitoSOX Red reagent (Molecular Probes, Life Technologies, Eugene, OR, USA) according to the manufacturer's protocols. Upon oxidation, H_2DCFDA is metabolized to fluorescent 2′,7′-dichlorofluorescein (DCF). MitoSOX Red reagent is only oxidized by superoxide and exhibits red fluorescence. After PFOA treatment, H_2DCFDA and MitoSOX reagent was added into the culture media. After incubation, hepatocytes were then washed and fluorescence was measured with a Synergy H1 Hybrid Microplate Reader (BioTek, USA).

2.5. RNA isolation, library preparation, and sequencing

Four samples in control and 100 μM PFOA for 24 h (two for each group) were used for RNA-sequencing. Total RNA of the hepatocytes in the control and 100 μM PFOA treatment (24 h) groups was isolated using the RNeasy plus Mini Kit (Qiagen, Culver City, CA, USA) followed by DNase I (Invitrogen, Life Technologies, Carlsbad, CA, USA) treatment according to the manufacturer's protocols. Total RNA was then commercially sequenced in Novogene (Beijing, China) using an Illumina HiSeq 2500 platform. Details are available in the Supplementary methods.

2.6. Read mapping and differential transcript analysis

The raw reads were cleaned by removing the adapter sequences and filtering low quality reads, including reads with > 10% of unknown nucleotides and reads with > 50% of low quality bases (Q ≤ 20). The reads were mapped to whole genome reference sequences with the TopHat program (v2.0.12) (Trapnell et al., 2009). Transcript expression was calculated with RPKM (reads per kb per million mapped reads) (Mortazavi et al., 2008). Differential transcription analysis was tested using the DEGseq program (v1.12.0) (Wang et al., 2010). *P*-values were corrected using the false discovery rate (FDR) in multiple hypothesis testing (Benjamini and Hochberg, 1995). We used the absolute value of $\log_2(RPKM_{treatment}/RPKM_{control}) \geq 1$ (fold change ≥ 2 or ≤ 0.5) and FDR < 0.05 as the thresholds for differentially expressed genes (DEGs) under treatment. The DEGs were subjected to annotation and functional classification against the NCBI database. Enrichment analyses in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology (GO) terms were carried out using David online tools (www.david.ncifcrf.gov), and FDR < 0.001 was considered as significantly enriched after treatment.

2.7. qPCR of mRNA

To screen the most sensitive genes in response to PFOA treatment in primary rat hepatocytes, five dominantly changed PPARα target genes (*acyl-CoA thioesterase 1* (*Acot1*) and *2* (*Acot2*), *cytochrome P450, family 4, subfamily a, polypeptide 1* (*Cyp4a1*), *enoyl CoA hydratase 1, peroxisomal* (*Ech1*), and *hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit* (*Hadha*)), as well as PPARα itself, were selected for real-time qPCR detection in the control and 0.4, 1.56, 6.25, 25, and 100 μM PFOA exposure groups treated for 24 h. Acot1, Cyp4a1, and Acot2 were further selected for qPCR assay in hepatocytes after exposure to 100 μM of PFOA for 6, 12, and 18 h, as well as after exposure to 0.4, 1.56, and 6.25 μM of PFOA for 48, 72, and 96 h. The qPCR was performed with the LightCycler®480 qPCR system (Roche Diagnostics GmbH, Switzerland), and *Gapdh* was used as the internal control for normalization. Primer sequences are listed in Supplementary Table S1. The comparative CT ($2^{-\Delta\Delta CT}$) method was used to obtain the fold change of mRNA levels (Livak and Schmittgen, 2001).

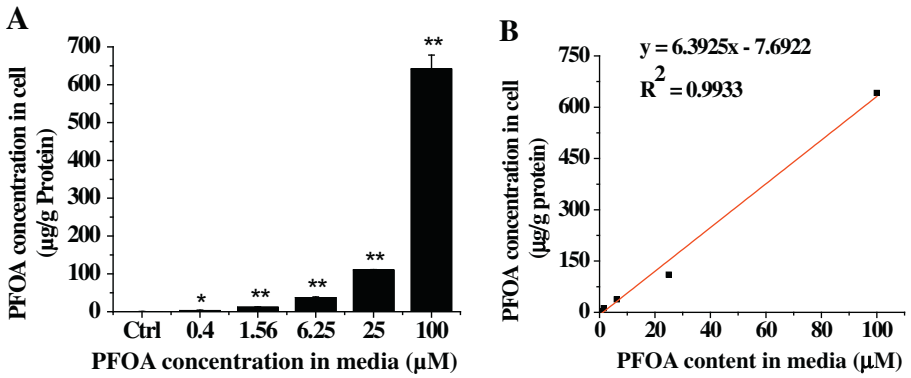

Fig. 1. Levels of PFOA in primary hepatocytes after exposure. (A) Level of PFOA in primary rat hepatocytes after exposure to different doses of PFOA in media. (B) Regression between PFOA levels in cells and media. Rat primary hepatocytes were isolated from rat liver, and cells were exposed to media with different concentrations of PFOA (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h; cells were then harvested, washed with PBS, and the PFOA levels were detected using UPLC-MS/MS. Results were obtained from 3 biological replicates, and presented as Mean ± SEM; *P* value was calculated using analysis of variance (ANOVA), followed by Tukey's HSD test. (* $P < 0.05$; ** $P < 0.01$).

2.8. RNA-FISH

The probes for *Acot1* RNA-FISH were commercially synthesized by Biosearch Technologies (Petaluma, CA, USA), and hybridization was performed according to the manufacturer's protocols. Briefly, hepatocytes were seeded in 24-well plates with coverslips. After various PFOA treatment doses for different time intervals, the hepatocytes on the coverslips were fixed in freshly prepared paraformaldehyde (3.7% in DPBS). Following ethanol treatment (70%) for 1 h, the coverslips were then hybridized with QUASAR® 570 dye-labeled Acot1 probes overnight at 37 °C in hybridization buffer (90% Stellaris® RNA-FISH hybridization buffer, 10% formamide). After washing, the hepatocytes were then dyed with Hoechst 33342. Finally, the coverslips were mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA, USA) and observed using a confocal laser-scanning microscope (Zeiss LSM 710, Zeiss, Oberko, Germany). The RNA-FISH probe sequences for *Acot1* are listed in Supplementary Table S2.

2.9. Immunohistochemical analysis

The control and PFOA exposure group hepatocytes grown on coverslips in 6-well plates were fixed in 4% paraformaldehyde for 10 min at room temperature. The hepatocytes on the coverslips were incubated with rabbit antibody for Acot1 (gift from Prof. Yamada of Tokyo University, Japan) and then with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probes, Life Technologies, USA). The nuclei were dyed with Hoechst 33342, and finally the cells were observed using a confocal laser-scanning microscope (Zeiss LSM 710, Zeiss, Oberko, Germany).

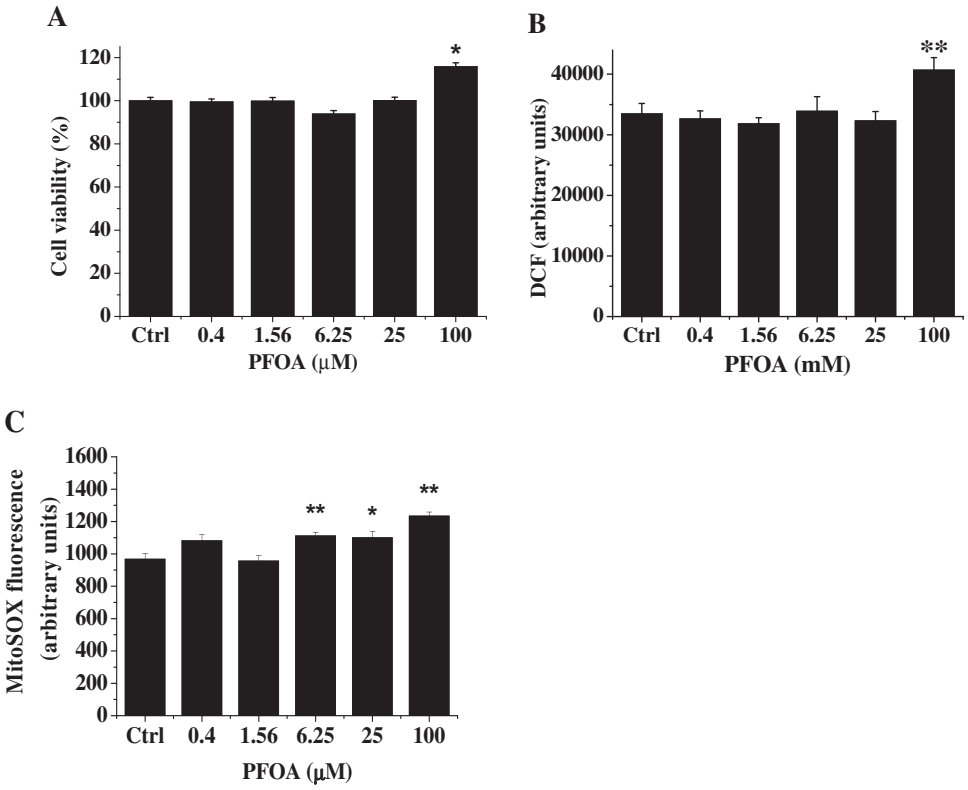

Fig. 2. Cell injury detection after PFOA exposure. Cell viability (A) intracellular oxidant intensity (B) and mitochondrial superoxide (C) in rat hepatocytes after PFOA treatment. After exposure to different doses of PFOA (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h, cell viability was determined using MTT assay, and intracellular oxidant intensity and mitochondrial superoxide were determined by mean fluorescence intensity of fluorescent 2′,7′-dichlorofluorescein (DCF) and MitoSOX fluorescence, respectively. Results were obtained from 3 biological replicates, and presented as Mean ± SEM; *P* value was calculated using analysis of variance (ANOVA), followed by Tukey's HSD test. (* $P < 0.05$; ** $P < 0.01$).

2.10. Western blot analysis

Protein extracts from the control and PFOA exposure group (6.25, 25, and 100 μM for 24 h) hepatocytes were used for Western blot analysis. GAPDH was used as the internal reference protein. The blots were visualized by enhanced chemiluminescence (SuperECL, Tiangen, Beijing, China) on an Image Quant LAS4000 instrument (GE Healthcare, USA) and analyzed with Quantity One software (v4.6.3, Bio-Rad, USA).

2.11. Statistical analysis

Analysis of variance (ANOVA) was used to compare the differences between groups, followed by Tukey's HSD test, with SPSS software (Version 18, SPSS, Inc., Chicago, IL, USA). All data were presented as Mean ± SEM. $P < 0.05$ was considered significant.

3. Results

3.1. PFOA contents in hepatocytes after treatment

The PFOA contents in the primary hepatocytes and culture media were quantified by HPLC-MS/MS after exposure (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h. The levels of PFOA in the hepatocytes were 0.26, 3.45, 12.17, 37.72, 110.34, and 641.48 μg/g (normalized to protein) in the control and the 0.4, 1.56, 6.25, 25, and 100 μM treatment groups, respectively (Fig. 1A). The content of PFOA in hepatocytes increased dose-dependently with the level of PFOA in the media (Fig. 1B).

3.2. MTT assay and oxidative stress injury after PFOA treatment

Compared with that of the control, cell viability was not significantly decreased in the 0.4, 1.56, 6.25, and 25 μM PFOA groups after 24 h. However, cell viability was increased in 100 μM PFOA treatment group by 115.9% (Fig. 2A).

The intracellular oxidant stress and mitochondrial superoxide in hepatocytes after PFOA treatment (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h was determined *via* the DCF and MitoSOX assay, respectively. The mean fluorescence intensity of DCF was not significantly altered in hepatocytes after 0.4, 1.56, 6.25, and 25 μM PFOA treatment for 24 h, but was increased in the 100 μM PFOA treatment groups. The mean fluorescence intensity of DCF was > 120% that of the control (Fig. 2B). The MitoSOX fluorescence in the hepatocytes was increased from 6.25 μM PFOA treatment group, and a nearly 130% increase compared with control was observed in 100 μM PFOA treatment group (Fig. 2C).

3.3. RNA-seq data and DEGs

A total of 267,528,966 paired-end reads were generated from the four samples (Supplementary Table S3). After removal of adapter and low-quality sequences, a total of 255,848,506 clean reads were harvested. They were then mapped to the genome sequences, with a mapping rate of 77.9%–79.6% in all four samples. In exploring transcript changes between the treatment and control group, RPKM was adapted to eliminate the influence of variation in gene length and total read numbers. Pearson's correlation test of the transcripts from the four samples showed that PFOA treatment remarkably changed the mRNA expression patterns (Supplementary Fig. S1A). Volcano plot analysis of the transcripts is shown in Supplementary Fig. S1B. Using the threshold of FDR < 0.05 and absolute value of $\log_2(RPKM_{treatment}/RPKM_{control}) \geq 1$, we identified 177 gene transcripts (158 upregulated, 19 downregulated) as significantly altered after exposure to 100 μM of PFOA for 24 h (Supplementary Table S4). Among them, 59 transcripts exhibited a > 4-fold upregulation after treatment, and are separately listed in Table 1.

Table 1
Transcripts (P adj < 0.05) of genes in primary rat hepatocytes showing > 4-fold upregulation after exposure to 100 μM of PFOA for 24 h.

Gene ID	Gene name	log2(Fold change)	P adj
ENSRNOG00000055221	Acot1	10.01	0
ENSRNOG00000015461	Serpine2	6.99	3.71E-33
ENSRNOG00000033680	Cyp2b1	6.33	0
ENSRNOG00000001770	Ehhadh	6.01	2.9E-224
ENSRNOG00000020775	Cyp2b2	5.58	3.2E-229
ENSRNOG00000009565	Pdk4	5.33	0
ENSRNOG00000030154	Cyp4a2	5.15	0
ENSRNOG00000003683	Glp2r	4.83	0.003393
ENSRNOG00000017424	Chrna2	4.80	2.46E-14
ENSRNOG00000009597	Cyp4a1	4.71	0
ENSRNOG00000054181	AC120070.1	4.67	0.00473
ENSRNOG00000009741	Cyp4a2	4.53	0
ENSRNOG00000054603	Pacsin1	4.50	0.012357
ENSRNOG00000019500	Cyp1a1	4.49	1.06E-10
ENSRNOG00000032508	Acot5	4.10	8.37E-41
ENSRNOG00000058340	Krt79	3.93	0.003018
ENSRNOG00000010438	Cpt1b	3.84	0
ENSRNOG00000054955	Adgra1	3.41	8.32E-05
ENSRNOG00000006675	Fabp1	3.32	8.8E-236
ENSRNOG00000017637	Fbp2	3.30	0.000212
ENSRNOG00000033893	Cacna1h	3.25	0.013732
ENSRNOG00000007545	Angptl4	3.05	0
ENSRNOG00000017878	Aldh1a7	2.96	1.4E-296
ENSRNOG00000026848	Tex36	2.88	2.47E-25
ENSRNOG00000017854	Ucp2	2.82	6.3E-231
ENSRNOG00000023557	RGD1563692	2.80	0.009234
ENSRNOG00000015581	Dnah6	2.80	2.1E-14
ENSRNOG00000010134	Acot2	2.75	0
ENSRNOG00000006019	G0s2	2.71	2.25E-56
ENSRNOG00000042352	Dnase1l2	2.71	7.28E-12
ENSRNOG00000019615	Colq	2.71	1.48E-05
ENSRNOG00000023476	Slc16a5	2.66	0.000589
ENSRNOG00000031211	Acsm5	2.65	5.9E-115
ENSRNOG00000006631	Sema3e	2.61	0.005002
ENSRNOG00000016692	Hsdl2	2.60	0
ENSRNOG00000015003	Pex11a	2.59	1E-140
ENSRNOG00000032202	Creb3l3	2.56	0
ENSRNOG00000022572	Pex19	2.50	1.48E-05
ENSRNOG00000008843	Eci1	2.45	0
ENSRNOG00000013589	Cxcl12	2.45	2.22E-34
ENSRNOG00000021526	Slc25a34	2.39	1.08E-06
ENSRNOG00000018461	Pdgfrb	2.37	0.027649
ENSRNOG00000018606	Olr59	2.36	0.00475
ENSRNOG00000020484	Trpm5	2.34	0.004778
ENSRNOG00000002436	Mmd	2.34	0.011801
ENSRNOG00000046864	Acot4	2.32	3.6E-176
ENSRNOG00000055078	Cyp4b1	2.32	0.044693
ENSRNOG00000013766	Acaa2	2.31	0
ENSRNOG00000010580	Acot7	2.31	0
ENSRNOG00000047860	Plin5	2.25	8.62E-18
ENSRNOG00000021201	Txnip	2.22	5E-119
ENSRNOG00000020308	Ech1	2.19	0
ENSRNOG00000008755	Acox1	2.17	6.35E-82
ENSRNOG00000014254	Cpt1a	2.15	0
ENSRNOG00000053460	Acot3	2.13	1.2E-101
ENSRNOG00000034116	Gk	2.12	4.46E-56
ENSRNOG00000020173	Tie1	2.03	0.005002
ENSRNOG00000012307	Mybpc3	2.02	1.91E-24
ENSRNOG00000020546	Lipe	2.02	1.39E-17

3.4. Pathway enrichment and GO term analysis of DEGs

Enrichment analysis was further carried out for the DEGs. After PFOA treatment, a high percentage of DEGs were assigned to “monocarboxylic acid metabolic process”, “fatty acid metabolic process”, “lipid metabolic process”, “oxoacid metabolic process”, and “carboxylic acid metabolic process” terms in the Biological Processes (BP). The significantly affected pathways after PFOA treatment were the “PPAR signaling pathway” and “fatty acid metabolism”. For the “PPAR signaling pathway”, at least 21 DEGs were included, which were

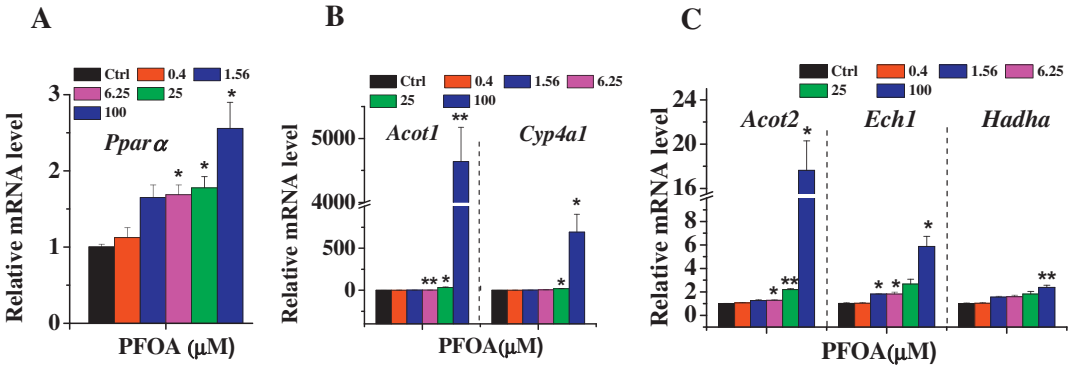

Fig. 3. Verification of changed genes with qPCR. (A) Level of PPARα, (B) Levels of Acot1 and Cyp4a1, (C) Levels of Acot2, Ech1, and Hadha. Some PPARα regulated genes, which were all significantly changed in primary rat hepatocytes after 100 μM PFOA exposure for 24 h, were selected to verify the transcriptome results. Their expressions after exposure to 0.4, 1.56, 6.25, 25, and 100 μM of PFOA are shown. Results were obtained from 3 biological replicates, and presented as Mean ± SEM; *P* value was calculated using analysis of variance (ANOVA), followed by Tukey's HSD test. (* $P < 0.05$; ** $P < 0.01$).

Acot1, *Acot2*, *Cyp4a1*, *enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase (Ehhadh)*, *acyl-CoA oxidase 1, palmitoyl (Acox1)*, *carnitine O-octanoyltransferase (Crot)*, *enoyl-CoA delta isomerase 1 (Eci1)*, *acyl-CoA synthetase long-chain family member 1 (Acsl1)* and *3 (Acsl3)*, *solute carrier family 27 member 2 (Slc27a2)*, *hydroxyacyl-CoA dehydrogenase (Hadh)*, *acetyl-CoA acyltransferase 2 (Acaa2)*, *carnitine palmitoyltransferase 1A (Cpt1a)*, *1B (Cpt1b)* and *2 (Cpt2)*, *hydroxysteroid (17-β) dehydrogenase 4 (Hsd17b4)*, *carnitine O-acetyltransferase (Crat)*, *acyl-CoA synthetase medium-chain family member 5 (Acsm5)*, *Ech1*, *Hadha*, and *acetyl-Coenzyme A acyltransferase 1B (Acaa1a)*. Their mRNA levels were all upregulated after PFOA treatment.

3.5. Transcriptional levels of selected PPAR target genes

To screen sensitive genes in primary rat hepatocytes in response to PFOA treatment, five dominantly changed PPARα target genes, *Acot1*, *Cyp4a1*, *Acot2*, *Ech1*, and *Hadha*, as well as *PPARα* itself, were selected for real-time qPCR analysis after various PFOA treatment (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h. All six genes increased in the 100 μM groups, compared with those in the control (Fig. 3). Among the five PPARα target genes, changes in the *Acot1* transcripts after 24 h treatment were the most remarkable, with > 4500-fold increases in the 100 μM group, compared with that in the control. In addition, *Cyp4a1* and *Acot2* increased > 690-fold and 18-fold after 24 h in the 100 μM PFOA groups, respectively, compared with that in the control. Among the five PPARα target genes, *Acot1*, *Ech1*, and *Acot2*, as well as *PPARα* itself, increased significantly, even in the 6.25 μM PFOA treatment group after 24 h.

To screen for the most sensitive indicator, *Acot1*, *Cyp4a1*, *Acot2*, and PPARα itself were further selected for qPCR assay of the hepatocytes after short-term (6, 12, and 18 h) exposure to 100 μM of PFOA. The expression of all four genes increased, especially that of *Acot1*, which increased > 500-fold compared with that of the control after treatment with 100 μM of PFOA for only 6 h, (Fig. 4A). The qPCR assays of *Acot1*, *Cyp4a1*, *Acot2*, and *PPARα* were further carried out in the primary rat hepatocytes after exposure to 0.4, 1.56, and 6.25 μM of PFOA for 48, 72, and 96 h (Fig. 4B). Although *Acot2* was also sensitive to low dose PFOA treatment, with an increase in the 6.25 μM PFOA exposure group after 48 h, the most sensitive gene (*Acot1*) was upregulated at all time points (48, 72, and 96 h) after exposure to 6.25 μM of PFOA. In

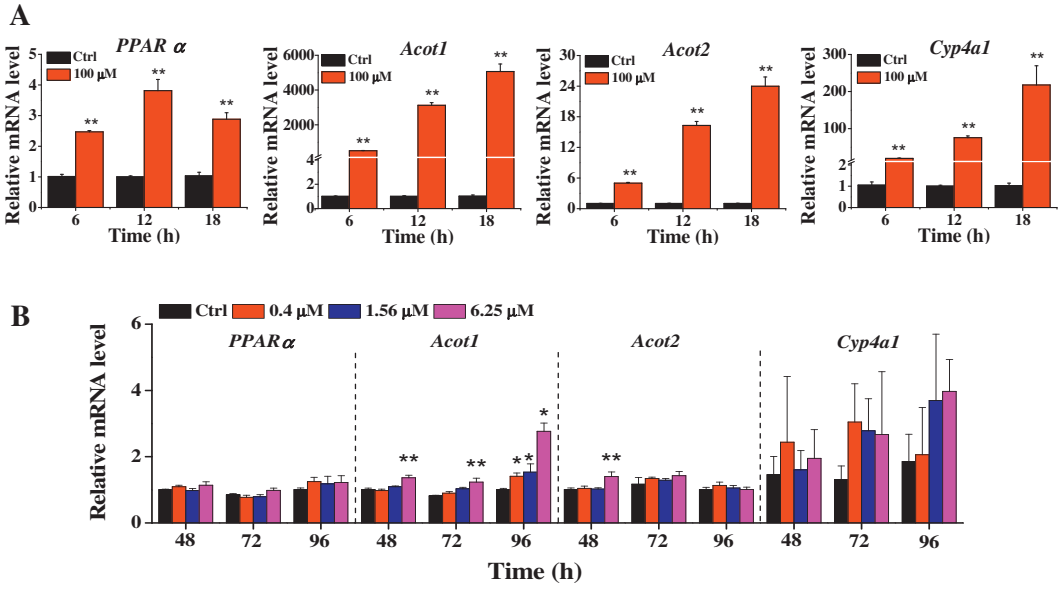

Fig. 4. Time-course mRNA level changes in primary rat hepatocytes after PFOA exposure. (A) mRNA level changes of PPARα, Acot1, Acot2, and Cyp4a1 in primary hepatocytes over different times (6, 12, and 18 h) after exposure to 100 μM of PFOA. (B) mRNA level changes in the above four genes over different times (48, 72, and 96 h) after exposure to 0.4, 1.56, and 6.25 μM of PFOA. The transcriptional factor PPARα and three of its most changed target genes were selected for further time-course expression experiments after PFOA exposure, including their levels after 6, 12, and 18 h of exposure to 100 μM of PFOA, and after 48, 72, and 96 h of exposure to 0.4, 1.56, and 6.25 μM of PFOA. Results were obtained from 3 biological replicates, and presented as Mean ± SEM; *P* value was calculated using analysis of variance (ANOVA), followed by Tukey's HSD test. (* $P < 0.05$; ** $P < 0.01$).

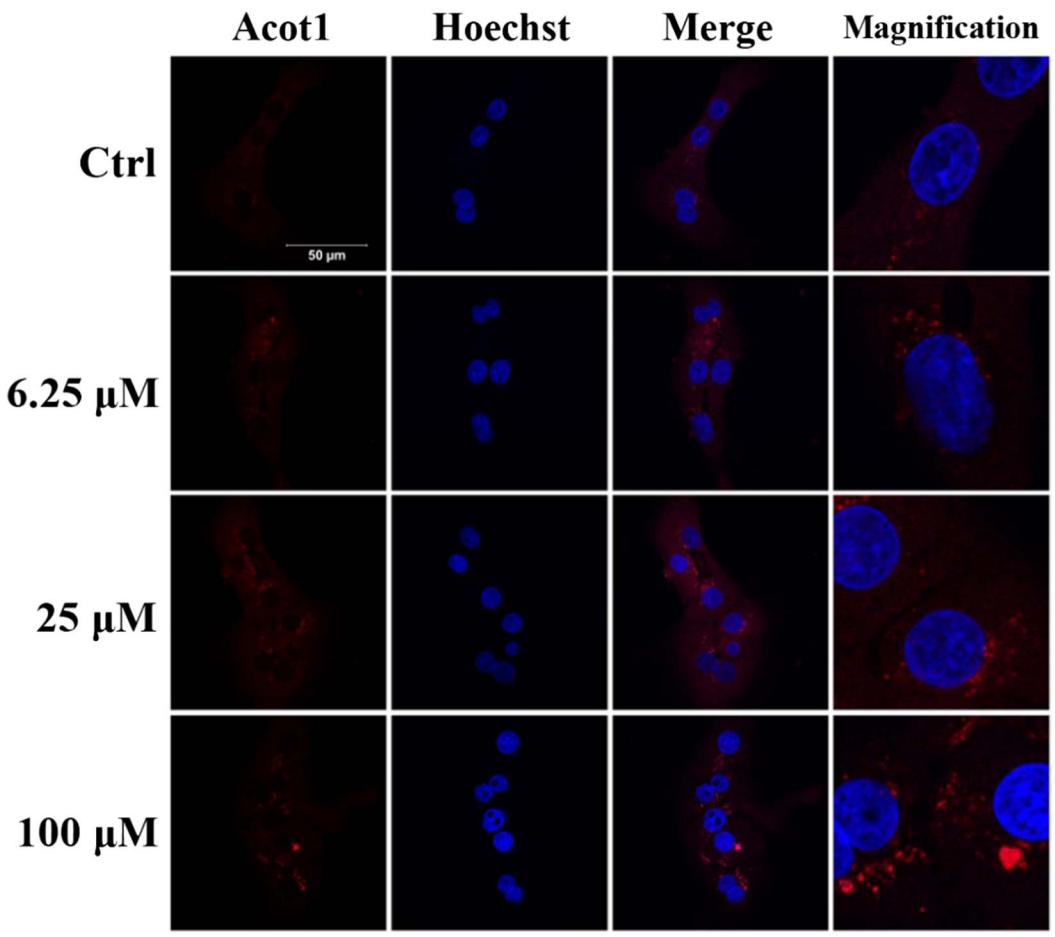

Fig. 5. RNA-FISH of Acot1 in primary rat hepatocytes after exposure to 0, 6.25, 25, and 100 μM of PFOA for 24 h. Rat Acot1 RNA-FISH probes are shown in red, and nuclei are dyed with Hoechst 33,342 (in blue). Rat primary hepatocytes were exposed to 0, 6.25, 25, and 100 μM of PFOA for 24 h. The cells were then fixed and hybridized with RNA-FISH probes of Acot1, and observed using a confocal laser-scanning microscope. The experiment was repeated 3 times with similar results, and a representative image was shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition, *Acot1* responded to low dose PFOA treatment, with its expression level increased after exposure to 0.4 and 1.56 μM of PFOA for 96 h.

3.6. RNA-FISH, immunohistochemistry and Western blot determination of Acot1 level after PFOA treatment

A RNA *in situ* hybridization assay was carried out to show mRNA levels after exposure to 0, 6.25, 25, and 100 μM of PFOA. After 24 h of treatment, the probes signaling *Acot1* were enhanced (Fig. 5). To detect if the Acot1 protein increased after PFOA treatment, immunohistochemical and Western blot analyses were carried out in the primary hepatocytes after exposure to 6.25, 25, and 100 μM of PFOA for 24 h. Similar to the response in transcriptional level, the Acot1 protein increased in a dose-dependent manner after 6.25, 25, and 100 μM PFOA treatment (Fig. 6).

4. Discussion

4.1. MTT assay and oxidative stress injury were not sensitive parameters of PFOA exposure

Cell viability, defined by compound potential for inducing cell death, is a useful endpoint of cytotoxicity. In this *in vitro* study, cell viability evaluation by MTT was carried out in isolated primary rat hepatocytes after exposure to PFOA at a range of concentrations (0, 0.4, 1.56, 6.25, 25, and 100 μM) for 24 h. No significant decrease was observed at the PFOA concentration range of 0.4 to 25 μM after 24 h, indicating low sensitivity of the cell viability assay as an endpoint in evaluating PFOA toxicity toward freshly isolated hepatocytes. However, under 100 μM PFOA treatment for 24 h, rat hepatocytes exhibited increased viability, with values of 115.9%, compared with that in the control. The pro-proliferative effect of PFOA agreed with our previous *in vitro* study on cell line HL-7702, in which exposure to 50, 100, and 200 μM of PFOA for 48 h and 96 h significantly increased cell viability (Zhang et al., 2016).

With H_2O_2-generating flavin oxidases and H_2O_2-degrading catalase, the peroxisome is an organelle essential for maintaining H_2O_2 intracellular homeostasis (Singh, 1996). As a peroxisome proliferator in rodents, PFOA exposure likely results in an increase in reactive oxygen species (ROS) synthesis, leading to oxidative DNA damage (Badr and Birnbaum, 2004; Dzhekova-Stojkova et al., 2001). Several *in vitro* studies with HepG2 and Vero cells have shown that PFOA causes oxidative stress, and the dramatic increase in the cellular content of ROS, such as H_2O_2 and superoxide anions, might be involved in DNA strand breaks, cell cycle arrest, and apoptosis (Fernandez Freire et al., 2008; Panaretakis et al., 2001; Yao and Zhong, 2005). The fluorescence intensity of DCF, which serves as an intracellular oxidant stress indicator, was not significantly changed in the primary rat hepatocytes after treatment with 0.4, 1.56, 6.25, and 25 μM of PFOA for 24 h, though increases were observed in 100 μM treatment group. These results indicate that intracellular ROS are not sensitive parameters in the evaluation of PFOA exposure. However, the intensity of MitoSOX Red, a fluorogenic indicator for mitochondrial superoxide, increased

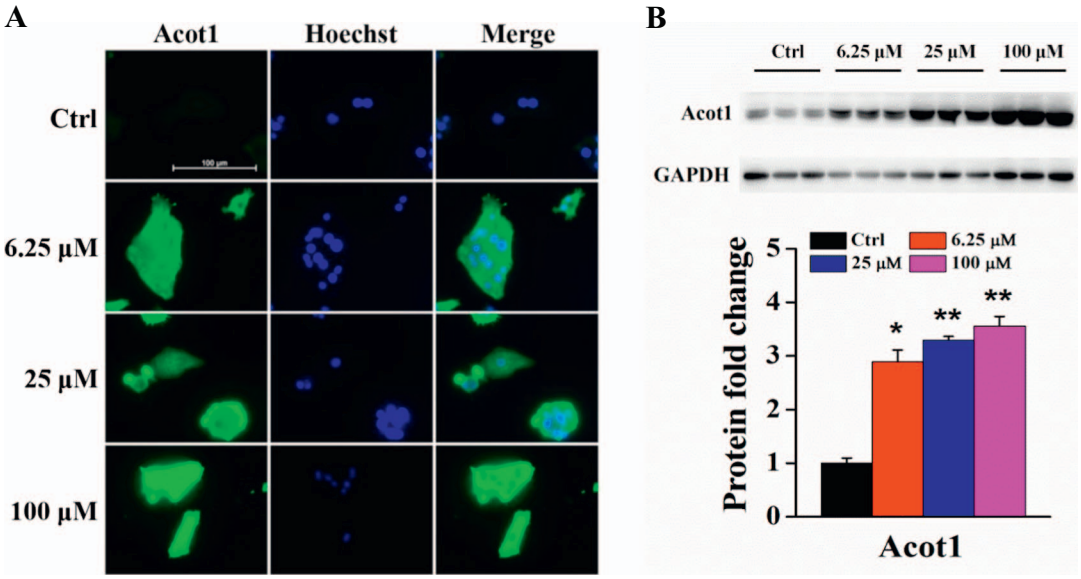

Fig. 6. Acot1 protein levels in primary rat hepatocytes after exposure to 0, 6.25, 25, and 100 μM of PFOA for 24 h. (A) Immunohistochemical analysis. (B) Western blot analysis. (C) Band density of the result. The primary rat hepatocytes were exposed to 100 μM of PFOA for 6, 12, and 18 h, and the cells were then fixed for immunohistochemical analysis or lysed for Western blot analysis. The experiment was repeated 3 times, and a representative image was shown. Results were presented as Mean ± SEM; *P* value was is not shared calculated using analysis of variance (ANOVA), followed by Tukey's HSD test. (* $P < 0.05$; ** $P < 0.01$).

after treatment with 6.25 and 25 μM of PFOA for 24 h, implied that mitochondrial superoxide is more sensitive than intracellular ROS as a whole.

4.2. PPARα pathway was influenced by PFOA treatment in rat hepatocytes

Accumulating evidence, including transcriptome studies using microchip and RNA-seq methods, has indicated that the PPARα agonistic effect of PFASs in rodents is involved in hepatomegaly induction and peroxisomal β-oxidation enhancement (Guruge et al., 2006; Martin et al., 2007; Rosen et al., 2008; Shipley et al., 2004; Wang et al., 2016). PPARα influences lipid catabolism by directly controlling the transcriptional level of genes involved in β-oxidation pathways, fatty acid uptake, lipoprotein assembly and transport (Cheung et al., 2004; Lee et al., 1995). Despite extensive hepatotoxicity screening of PFASs, sensitive molecules that can serve as indicators of PFAS exposure remain limited. Using freshly isolated hepatocytes, we identified 177 DEGs after treatment with 100 μM of PFOA for 24 h. Based on the largest category of these DEGs (the "PPAR signaling pathway"), the PPARα agonistic activity of PFOA was further confirmed in isolated primary rat hepatocytes.

In mammals, fatty acids are mainly oxidized *via* β-oxidation in the mitochondria and peroxisomes (Reddy and Mannaerts, 1994; Wanders et al., 2001). PFOA can induce the transcription of fatty acid degradation enzymes (Haughom and Spydevold, 1992; Kudo et al., 2000; Kudo et al., 1999). In the present study, many genes associated with the fatty acid β-oxidation pathway were altered by PFOA, including *Cpt1a*, *Cpt1b*, and *Cpt2*, which are essential for fatty acid translocation across the mitochondrial membrane and conversion from acylcarnitine to acyl-CoA, and *Acox1*, *Eci1*, and *Ech1*, which are involved in straight-chain fatty acid degradation and the peroxisomal oxidation of long-chain fatty acids (Bonnefont et al., 2004; Kersten et al., 2000). In addition, as an alternative pathway to β-oxidation, ω-hydroxylation involves the oxidation of the ω carbon of medium-chain fatty acids and is governed by a subset of Cyp4a enzymes (Johnson et al., 1996). In the present study, Cyp4a1, a member of the Cyp4a enzymes, was remarkably induced by PFOA in the rat hepatocytes.

4.3. Acot1 was a sensitive indicator for PPARα activation after PFOA exposure in rat hepatocytes

Five upregulated genes, as well as *PPARα*, were selected for real-time qPCR analysis under different PFOA doses and exposure times. Acot1 was found to be the most dominantly changed DEG transcript by RNA-seq, and of the most concern. Acot1 belongs to the acyl-CoA thioesterases, which hydrolyze acyl-CoAs to CoA and fatty acids, with free fatty acids and acyl-CoA important metabolic energy sources that serve as bioactive molecules affecting numerous cellular systems and functions (Faergeman and Knudsen, 1997; Hunt et al., 2000; Waku, 1992). Studies have shown that hepatic acyl-CoA thioesterases are strongly induced in rodents by peroxisome proliferators, including PFOA (Kawashima and Kozuka, 1992; Lindquist et al., 1998).

The five selected genes showed a dose-dependent increase, and remarkable upregulations of these genes were observed after 24 h of treatment in the 100 μM PFOA groups. The upregulation of *Acot1* was the most remarkable, with > 4500-fold increase in the 100 μM group, compared with that in the control. Although *Acot1* was significantly induced in isolated rat hepatocytes after 100 μM PFOA treatment for 24 h, we wondered if it responded to PFOA exposure earlier. Our results demonstrated that its mRNA level increased > 500-fold after 6 h of exposure to 100 μM of PFOA, suggesting that Acot1 promptly responds to PFOA exposure. In addition, *Acot1* was upregulated in the primary rat hepatocytes at all time points (48, 72, and 96 h) after 6.25 μM of PFOA exposure, and the upregulation effect was more evident with the extension of incubation time. Furthermore, *Acot1* was upregulated even after exposure to 0.4 μM of PFOA for 96 h.

Serum concentrations of PFOA in the general population are reasonably consistent, ranging from 4 to 5 μg/L (geometric mean) in the USA to 2–8 μg/L in other major industrialized countries (Vestergren and Cousins, 2009). The concentrations in some exposed workers (median concentrations 1100–1300 μg/L) are extremely high (Olsen et al., 2000; Olsen et al., 2003b), with median concentrations of 1636 μg/L reported in exposed workers in Changshu, China (Wang et al., 2012). In the present study, the PFOA doses applied to the rat hepatocytes were 0.4, 1.56, 6.25, 25, and 100 μM, respectively. The concentrations were too high to compare with that in serum samples from the general population, with the lowest dose of PFOA (0.4 μM,

24 h) being three orders of magnitude higher than that in the general population, though it was the same magnitude as that from exposed workers. Therefore, the effects observed in our *in vitro* studies were only obtained with very high PFOA concentrations. Even under 0.4 μM PFOA, only moderate induction of Acot1 was observed in rat primary hepatocytes after 96 h exposure (Fig. 4B). In addition, the comparison between the intracellular PFOA concentrations (in μg/g; normalized to protein) in rodent primary hepatocytes to human blood serum concentrations (in μg/L) may not be appropriate, and if the PFOA concentration in human hepatocytes in the population is available, a comparison between the intracellular PFOA concentrations with the intracellular concentrations in human hepatocytes will be more reasonable. Furthermore, although PPARα target genes, especially *Acot1*, is sensitive to PFOA exposure in rodent primary hepatocytes, PPARα-mediated gene transcription is not shared by human primary hepatocytes and HepG2 cells, thus PPARα-dependent hepatotoxicity in rodents cannot be extrapolated directly to humans (Bjork and Wallace, 2009). The prompt and remarkable response of *Acot1* to short-term PFOA exposure implies that it could serve as a sensitive indicator for PPARα activation after PFOA exposure in primary rat hepatocytes.

5. Conclusions

Cell viability and intracellular oxidant intensity were not sensitive parameters to PFOA exposure. By screening the global transcriptome profile alteration in primary rat hepatocytes after PFOA exposure using RNA-seq, we identified 177 DEGs after treatment with 100 μM of PFOA for 24 h. Following a series of qPCR and RNA-FISH assays, we found that Acot1 was the most sensitive DEG, and increased remarkably not only at all time points (24, 48, 72, and 96 h) after 6.25 μM PFOA exposure, but also after low dose exposure (0.4 μM) for 96 h. We suggest that Acot1 can serve as a sensitive indicator for PPARα activation after PFOA exposure in primary rat hepatocytes.

Transparency document

The http://dx.doi.org/10.1016/j.tiv.2017.05.012 associated with this article can be found in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2017.05.012.

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