Molecular Mechanisms of Perfluorooctanoate-Induced Hepatocyte Apoptosis in Mice Using Proteomic Techniques

Kan Li, † Jie Sun, † Jingping Yang, ‡ Stephen M. Roberts, § Xuxiang Zhang, † Xinyi Cui, * †, Si Wei, * †, and Lena Q. Ma †, ‡

† State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, Jiangsu 210046, China
‡ School of the Medicine, Nanjing University, Nanjing, Jiangsu 210046, China
§ Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida 32611, United States
∥ Soil and Water Science Department, University of Florida, Gainesville, Florida 32611, United States

Supporting Information

ABSTRACT: The stability of perfluorooctanoate (PFOA) coupled with its wide use cause serious concerns regarding its potential risk to human health. The molecular mechanisms of PFOA-induced hepatotoxicity relevant to human health was investigated using both in vivo (mouse model) and in vitro (human hepatocyte cells, HL-7702) techniques. Both male and female Balb/c mice were administered PFOA at 0.05, 0.5, or 2.5 mg/kg-d for 28-d, with serum PFOA concentrations after exposure being found at environmentally relevant levels. Liver samples were examined for histology and proteomic change using iTRAQ and Western Blotting, showing dose-dependent hepatocyte apoptosis and peroxisome proliferation. At high doses, genotoxicity resulting from ROS hypergeneration was due to suppression of Complex I subunits in the electron transport chain and activation of PPARα in both genders. However, at 0.05 mg/kg-d, Complex I suppression occurred only in females, making them more sensitive to PFOA-induced apoptosis. In vitro assays using HL-7702 cells confirmed that apoptosis was also induced through a similar mechanism. The dose/gender-dependent toxicity mechanisms help to explain some epidemiological phenomena, i.e., liver cancer is not often associated with PFOA exposure in professional workers. Our results demonstrated that a proteomic approach is a robust tool to explore molecular mechanisms of toxic chemicals at environmentally relevant levels.

INTRODUCTION

Perfluoralkyl substances (PFASs) are synthetic compounds widely used in industrial and consumer products such as Teflon and Gore-Tex.⁠¹ Due to their strong carbon–fluorine bonds, PFASs are stable and accumulate in the environment, so they are classified as persistent organic pollutants (POPs).²,³ Perfluorooctanoate (PFOA), is one of the most used PFASs and is abundant in the environment, resulting in human exposure through food, housedust, and drinking water.⁴ PFOA has been detected in cord blood and breast milk in the general population⁴,¹⁴ partially due to its long half-life (~4 y) in the human body.⁵,⁶ Because of its high usage in China, increasing serum levels of PFOA in humans have been observed. For example, serum levels increased from 0.08 μg/L in 1987 to 4.3 μg/L in 2002 in Shenyang and up to 15.2 μg/L in 2009 was reported for Nanchang.⁷,⁸ The widespread human exposure to PFOA causes increasing concerns regarding its environmental health risks worldwide.

Recent epidemiology studies showed no correlation of PFOA exposure with escalated liver cancer or clinical liver disease occurrence, but a positive correlation with elevated serum alanine transaminase levels, a sign of hepatocyte damage.⁹–¹¹ In vitro assays using human hepatocellular carcinoma HepG2 cells showed that PFOA caused apoptosis,¹²,¹³ which was proven by proteomic assay using isobaric tags for relative and absolute quantitation (iTRAQ) following PFOA exposure in normal human hepatocyte cells (HL-7702). Signs of apoptosis like hepatocyte-nuclear condensation is also observed in PFOA-treated mouse liver.¹⁴ However, the mechanisms of PFOA-induced hepatocyte apoptosis in mammalian hepatocytes are still unknown. Some attributed this to lipid accumulation in the nucleus of mouse liver, while hyperaccumulation of reactive oxygen species (ROS), which triggered a mitochondrial signaling pathway, was the mode of action in HepG2 cells, with ROS origination being unclear.¹⁴ Another critical issue of
Mechanistic studies using animal models of both sexes have been mainly confined to rats, where PFOA caused only minor toxicity in females due to rapid metabolism through an active secretory mechanism, which has not been observed in humans.14–16 In this study, we investigated hepatotoxicity from subchronic PFOA exposure in both male and female Balb/c mice (in vivo assay), and the liver proteome using iTRAQ to better understand the underlying molecular mechanisms. Unlike previous genomic studies using microarrays—a transcriptome method,17–19 iTRAQ can directly identify and quantify changes in protein expression level.20 Since questions often remain when extrapolating data from rodents to humans,21 human hepatocyte cells (HL-7702, in vitro) were also exposed to PFOA to further explore the underlying molecular mechanisms of hepatotoxicity relevant to human exposure.

### MATERIALS AND METHODS

**PFOA Treatment of Mice.** PFOA (98%) was from the Tokyo Chemical Industry (Tokyo, Japan). Six-week old Balb/c mice were from the Aiermaite Company (Suzhou, China). The reasons for choosing Balb/c mice included their gene purity due to the inbred line, which is suitable for toxicity studies on molecular signal pathways, and their tame character making them easy for oral gavage. In addition, abundant toxicity data about Balb/c mice are available,22–25 helpful for mechanism exploration in this study. After 2-w acclimation, mice were divided into four groups, with 60 mice in each group (30 males and 30 females), and raised under standard animal housing conditions (12 h light/dark cycle, 22 ± 2 °C, and 50 ± 5% humidity). The control mice were gavaged with corn oil without PFOA (0.1 mL/d), while the experimental mice were administered PFOA in corn oil at 0.01, 0.1, or 0.5 g/L, equivalent to 0.05, 0.5, and 2.5 mg/kg-d bw/d, for 28 d.

**PFOA Determination and Ultrastructural Changes.** After 28 d exposure, mice were sacrificed to collect liver and blood samples. Liver tissues were isolated, fixed in 4% paraformaldehyde, and sectioned using haematoxylin and eosin staining. For TEM, tissues were quickly dissected and fixed in phosphate-buffered 2.5% glutaraldehyde, followed by 1% osmium tetroxide fixation at 4 °C. After dehydration using an aqueous-acetone series and embedding in Epon 812, tissues were sectioned ultrathin, which were examined using a JEM-2000EX transmission electron microscope (Olympus, Japan).

Liver and serum samples of 10 mice from each treatment (total 30 mice in a treatment) were pooled together and homogenized to generate 3 sample sets for all experiments except for JC-1, which was examined immediately after sacrifice. PFOA in the liver and serum samples was extracted and measured following Li et al.26 13C4-PFOA (>99%, Wellington Laboratory, Canada) was added as an internal standard. The recovery efficiency for liver samples spiked at 10 µg/kg was 79 ± 4.1%.

**JC-1 Flow Cytometer Test, Caspase-9 Activity, and ELISA Assay of 8-OHdG, p53, and PPARα.** The ΔΨm changes in mice livers were evaluated using a JC-1 ΔΨm Detection Kit (Beyotime Institute of Biotechnology, China) on a FACSCalibur flow cytometer (BD Bioscience C-6, San Jose, CA). The ratios of aggregated JC-1 and monomeric JC-1 represent ΔΨm of tissue samples. The details about the JC-1 flow cytometer test can be found in the Supporting Information (SI).
The activity of Caspase-9 was measured by cleaving selective substrates acetyl-Leu-Glu-His-Asp P-nitroanilide (Ac-LEHD-PNA) using a Caspase-9 detection kit (Beyotime Institute of Biotechnology). After being dispersed, the liver tissue was incubated with Ac-LEHD-PNA (10 μL) for 60 min at 37 °C, cleavage of the substrate was detected using a UV−vis spectrophotometer (UV2450, Shimadzu, Japan) at 405 nm. Activities of Caspase-9 were expressed as changes in LEHDase activity.

8-OHdG concentration, and p53 and PPARα activity were tested using ELISA kits (Yifei Xue Biotech, China) on an ELISA reader (Thermo, U.S.A.) following standard operation procedures detailed in the SI.

**In Vitro Cell Culture and Exposure.** Human liver (HL7702) cells were obtained from Chinese Academy of Science Type Culture Collection (Shanghai, China) and incubated in RPMI 1640 medium supplemented with 10% FBS. The cells were detached from the monolayer by incubating in 0.25% trypsin and 0.53 mM EDTA for 5 min at 37 °C, equally...
dividing it into 12 parts, and seeding it on 12 plates, 3 plates for each treatment. PFOA stock solution was prepared in RPMI 1640 medium, and added to plates to achieve 1.0, 2.5, or 7.5 µg/mL for each treatment. The cells were collected after 24 h, and 8-OHdG, Caspase-9 and protein extraction were conducted using the Elisa kit (SI).

Quantitative Proteomics and Western Blotting. As mentioned above, three pooled sample sets were generated for each treatment. Two of the sample sets were ground to fine powder in liquid nitrogen as bioreplicates of iTRAQ, and the third set was used for Western Blotting. The protein extraction, iTRAQ labeling, detection, and data processing were conducted following Chen et al.27 (SI). The meaningful cutoff values for up-regulated or down-regulated proteins were ≥1.5-fold and ≤0.67-fold, respectively.28–30 Cutoff values were also used to estimate the quantitation confidence between technical replicates.

A duplicate of the cultured cells was processed similarly for Western blotting analysis. Western blotting was performed using standard methods,31,32 with antibodies to AOX, CYPIVA10, NDUV2, NDUA9, and PDCDS. GAPDH was used as house-keeper genes (inner control). Detailed information regarding two antibodies for each protein and their respective reaction conditions is provided in the SI.

Statistical Methods. All proteins showed that significant expression changes were individually searched in the databases of Webolknowledge, NCBI, and Uniprot from 2012 to 2017. The KEGG database (http://www.genome.jp/kegg/), the STRING database (http://string-db.org/), and the Gene Ontology database (http://geneontology.org/) were used to group the identified proteins. Figures were drafted by Origin9.0 or R software, and the statistical analysis was conducted in SPSS 18.0, and R software using Pearson correlation with ANOVA or t test.

# RESULTS AND DISCUSSION

After 28 d exposure to PFOA, male and female mice showed little difference in body weight gain except that the 2.5 mg/kg-d mice showed less body weight gain than the controls after 21 d (Figure 1A). However, all exposed mice excluding the 0.05 mg/kg-d males showed a significant increase in liver weight (Figure 1B), a sign of hepatomegaly, common in rodents after PFOA exposure.33,34 PFOA concentrations in both liver and serum increased with PFOA dose, with 40% in males, and also a higher percentage of hepatocytes for both assays) and dose-dependent manner (p < 0.05 for both assays) and dose-dependent manner (p < 0.01, r = 0.84–0.96, Figure 2H,I), consistent with previous in vitro studies.32 In 2.5 mg/kg-d mice, gender differences were insignificant with both having Caspase-9 activity 3-fold greater than control mice, and cells mtΔΨ dissipation were 25% for male and 39% for females. However, in 0.05 mg/kg-d mice, the Caspase-9 activity was elevated by 72% in females compared with 40% in males, and also a higher percentage of hepatocytes with mtΔΨ dissipation being observed in females than males (12% vs 5.7%; Figure 2H,I). The significant differences in both cases indicated more damage of the mitochondrial membrane in female than males at 0.05 mg/kg-d PFOA (p < 0.05). This is consistent with apoptosis observed microscopically in PFOA-exposed mice (Figure 2G), suggesting that, at 0.05 mg/kg-d PFOA, females were more sensitive to PFOA-induced apoptosis than males.

Liver Proteome Revealed p53 Activation Induced Apoptosis, and Complex I Not PPARα Activation was the Major Source of ROS in Females at 0.05 mg/kg-d PFOA. To gain more insight into the mechanisms of PFOA-induced liver toxicity in mice, iTRAQ was used to compare the expression of liver proteins in PFOA-exposed mice. A total of 1797 proteins were identified in duplicate analyses. After filtering with changed fold threshold ratios of ≥1.5 or ≤0.67,28–30 633 unique proteins were differentially expressed, with 418 and 344 proteins being changed dose-dependently in males and females, respectively (Table S2). The number of changed proteins increased with PFOA dose (Table S1 and Figure S1). At the highest dose of 2.5 mg/kg-d, both males and...
females showed a wide range of changed proteins (>400). However, at lower doses of 0.05 and 0.5 mg/kg-d, ∼300 proteins were changed in females compared to <100 in males, indicative of a gender difference with females being more sensitive at lower PFOA doses.

Analysis of functional distribution and category enrichment showed clear dose- and gender-dependent changes in protein expression (Figure 3A). Excluding 0.05 mg/kg-d males, most changed proteins belonged to mitochondria. The fact that the number of affected proteins showed a dose-dependent increase confirmed that PFOA-induced toxicity was mainly related to mitochondrial dysfunction in mouse liver (Table S1; Figure 3A). The gene ontology results showed that after PFOA exposure, 53 molecular functions of proteins were changed (p < 0.05), including oxidoreductase activity, peroxisome proliferator activity, ion binding, and transferase activity, among which 33 functions changed dose-dependently (Table S3 and Figure S2). Unlike the number of changed proteins showing a great difference at a low dose of 0.05 mg/kg-d, females showed a similar number of functional changes (12) compared with males (11). However, only four were the same, most of which were ion binding proteins. For females, the most uniquely changed functions were oxidoreductase activity (4), and the heat map of these protein change levels is shown in Figure 3B. Among them, CYC (cytochrome c, somatic), PDCDS (programmed cell death protein 5), and FIS1 (mitochondrial fission 1 protein) all showed dose-dependent upregulation in PFOA-exposed mice (Figure 3B).

The correlation between exposure dose and protein change levels was conducted with data from both genders, with the correlation coefficients (r) being 0.55, 0.57, and 0.89 (p < 0.05). The first two proteins CYC and PDCDS were up-

Figure 3. Ontology enrichment of most up/down regulated genes, and heatmap of different signal pathways of iTRAQ and ELISA results. (A) Gene ontology enrichment of most up/down regulated genes. The size represents the number of affected proteins in that category, B1, B2, and B3 = 0.05, 0.5, and 2.5 mg/kg-d PFOA exposure. The function of the proteins was created by k-means clustering using Cluster3.0 based on the fold change value of proteins in the listed samples annotated against the KEGG database by using Cluster3.0 software of online tools. The network of the proteins was drawn using protein ontology software DAVID. (B) Heatmap of protein level changes in mouse livers exposed to 0.05, 0.5, or 2.5 mg/kg-d PFOA for 28 d. (C) p53 activity in mouse liver after exposure to PFOA for 28 d, “*” indicates that the p53 activity in the 0.05 mg/kg-d female mice are significantly different from males given the same dose, and from control mice. (D) 8-OHdG concentrations in mouse liver after exposure, “*” has the same meaning with p53 activity. Positive controls were derived by incubating fresh mouse liver with a 5% H2O2 solution for 10 min. (E) Differential expression of the proteins in PPARγ pathway, and their function attribution. (F) PPARγ activity in mouse liver after PFOA exposure. (G) Expression change of subunits of Complex I in the livers of mice exposed to PFOA.
regulated more than 1.5-fold in PFOA-exposed groups excluding 0.05 and 0.5 mg/kg-d male, and for each dose, females changed more than males (Figure 3B). CYC is a protein located in the intermembrane space of mitochondria that induces the cascade when released to the cytoplasm while during p53-induced apoptosis, mtΔΨ dissipates, and CYC is released to cytoplasm, activating the caspase cascades.48 PDCD5 is an early apoptosis indicator in cells undergoing DNA damage, the increased PDCD5 is then translocated to the nucleus and promotes p53 pathway through enhancing p53 stability,49,50 which then activates apoptosis through mtΔΨ dissipation and caspase cascades.51 This is consistent with our data, i.e., PDCD5 was the activator of PFOA-induced apoptosis. The third protein FIS1 showed no change in 0.05 and 0.5 mg/kg-d exposed males, but was up-regulated by 1.4-fold in both doses in females, and by 1.8−2.0 fold in mice at 2.5 mg/kg-d (Figure 3B). The change in FIS1 was associated with p53 activation on mitochondria morphology change,52 consistent with the morphological change based on TEM results (Figure 2A−G).

In short, the data from proteomic analysis suggested that PFOA-induced apoptosis in mouse liver was through p53 signal pathway to counteract the DNA damage, so we further tested the p53 activity and 8-OHdG concentration. Accordingly, the p53 activity was also dose-dependent (Figure 3C; p < 0.01, r = 0.91), with female hepatocytes showing more p53 activity than males in 0.05 mg/kg-d mice. The 8-OHdG is an oxidized nucleoside of DNA used as an indicator of oxidative DNA damage.53 In PFOA-exposed mouse liver, the 8-OHdG concentration was significantly increased in all groups excluding 0.05 mg/kg-d males. At 0.05 and 0.5 mg/kg-d, females showed higher 8-OHdG concentrations than males (69−87 vs 51−73 ng/g), suggesting PFOA at lower doses caused stronger genotoxicity in females than males (Figure 3D). The DNA damage also indirectly linked the ROS hypergeneration to apoptosis, via activation of PDCD5-mediated p53 signal pathway.49 In short, PFOA exposure caused ROS hyper-generation in mouse liver, especially for females, leading to 8-OHdG generation with PDCD5 activating p53 to induce apoptosis.

It is suggested that ROS from PPARα activation is an important trigger for apoptosis in PFOA-exposed hepatocytes.13 However, in 0.05 mg/kg-d females, no up-regulation of proteins associated with fatty acid β-oxidation, the major PPARα ROS source, was observed (Figures 3E and S2, and Tables S3 and S4).54 A second ROS source from the PPARα signal pathway, microsomal fatty acid ω-hydroxylation (cytochrome P450 4a family, CYPIVA), was also inactive in 0.05 mg/kg-d females (Figures 3E and S2, Tables S3 and S4). The gene ontology analysis also showed that PPA was significantly changed in all exposed groups, except for 0.05 mg/kg-d females (Table S3 and Figure S2). These data
indicated that ROS generation in 0.05 mg/kg-d females was probably not related to the PPARα signal pathway (Figure 3E). In addition to fatty acid β-oxidation and ω-hydroxylation, other PPARα-regulated genes were also unchanged, or oppositely regulated in 0.05 mg/kg-d females (Figure 3E), confirming that PPARα was not activated in 0.05 mg/kg-d females. In contrast, PFOA induced activation of the PPARα signal pathway at all doses in males, including 0.05 mg/kg-d. For example, fatty acid β-oxidation enzyme THIKA (3-ketoacyl-CoA thiolase A), as well as CYPIVA10 and CYPIVA14 were all up-regulated in 0.05 mg/kg-d males (Figure 3E). Based on our data, we hypothesized that PPARα was involved in the apoptosis of 0.05 mg/kg-d males but not 0.05 mg/kg-d females. At higher PFOA doses of 0.5 and 2.5 mg/kg-d, both males and females showed impact on PPARα-regulated proteins (Figure 3E).

Hence, the PPARα signal pathway may have contributed to the ROS generation in 0.5 and 2.5 mg/kg-d females. These findings are further confirmed by Elisa assay of PPARα (Figure 3F). In 0.05 mg/kg-d females, no significant changes were observed for both PPARα activity and PPARα-associated proteins, thus confirming PPARα was not activated in females. In short, PPARα cannot be an ROS source in 0.05 mg/kg-d females, and other signal pathways may be involved.

In scrutinizing the proteomic profile, one cluster of proteins was closely related with ROS generation. It is the ETC (electron transport chain) (Tables S3 and S4 and Figure S2), which catalyzes electron transfer from oxygen.55 The ETC consists of three macromolecular complexes, i.e., Complexes I, III, and IV, with the high energy gradient involving Complex I and III, which constitute the primary source of intracellular ROS.56,57 In our proteomic results, many ETC proteins were regulated in 0.05 mg/kg-d females (Figure 3E). For other complexes, only two subunits were up-regulated in all exposed-mice (%< 0.01, r = 0.7) and showed significant differences in females and in 2.5 mg/kg-d males, excluding NDUS2 at 0.05 mg/kg-d females (Figure 3G). Other subunits, like NDUA4, NDUB10, NDUS1, and NDUS8, also showed significant down-regulation in females and 2.5 mg/kg-d males. Only two subunits were up-regulated, including ACP (∼1.5 folds) in all mice excluding 2.5 mg/kg-d males, and NDUV2 (∼1.8 folds) in 0.05 and 0.5 mg/kg-d females (Figure 3G). For other complexes, only cytochrome c (CYC) showed dose-dependent up-regulation (Figures 3B and S4, and Tables S2 and S4). As the entry point of oxidative phosphorylation, Complex I catalyzes electron transfer from NADH to ubiquinone or coenzyme Q10. During this process, if electrons leak from the ETC, then superoxide anion (O2−) is generated, making Complex I a major source of ROS.57,58 Our proteome results showed a correlation between ROS accumulation and Complex I subunit suppression, including NDUA9, NDUB6, NDUB10, and NDUS2 (Figure 3G).59−64 Lower expression levels of these subunits leads to ROS generation and causes related diseases in both animals and humans.59−64 For the two up-regulated subunits, ACP overexpression is correlated with ROS accumulation,65 while NDUV2 acts as an antioxidant that accepts excess electrons and reduces ROS generation from Complex I.66

ROS leakage from Complex I or PPARα causes oxidative DNA damage and increases 8-OHdG concentration. The trends of 8-OHdG generation is consistent with observations of more suppression of Complex I (Figure 3D, p < 0.001, r = 0.89 for all four subunits) and activation of apoptosis (Figure 2HI, p < 0.01, r = 0.9) for both Caspase-9 and JC-1 assays in females than males at lower doses. The dose-dependent down-regulation of Complex I suggested that Complex I was probably a major ROS source in PFOA-exposed mouse liver, which was also supported by Western blotting of selected proteins from three clusters (Figures 4A, and S8). Protein expression changes in Western blot, although with different folds, followed the same trends as the iTRAQ results, corroborating the iTRAQ findings (Figures 3 and 4). We also analyzed other signal pathways associated with ROS hypergeneration, such as PXR, however, no upregulation was observed while counter-effects such as EBr did not stop the ROS generation (Tables S3 and S4 and Figure S2). A further clustering of the biofactors and proteome changes also confirmed our hypothesis that the dissipation of mtΔΨ triggered p53 activation and induced the caspase cascade (Figures S4 and S5). Although PFOA accumulation in mouse liver induced PPARα activation, it ranked second in mtΔΨ dissipation, following p53 and Caspase 9 activation (Figure S5).

Combining all results, we concluded that PFOA-induced ROS hypergeneration in mouse hepatocytes was probably through both suppression of Complex I and PPARα activation in high doses at 0.5 and 2.5 mg/kg-d; while at the lowest dose of 0.05 mg/kg-d, suppression of Complex I pathway was the primary source of ROS for females and PPARα activation for males, with females showing more oxidative stress than males (Figure S6).

**PFOA Caused Similar Effects in Human HL-7702 Liver Cells.** It is known that PFOA-induced hepatic effects are different between humans and rodents based on the PPARα-dependent changes. However, considering the conservation of mitochondrial biochemistry in evolution, especially the key functional components such as ETC, the PFOA-induced apoptosis could be relevant for human hepatocytes. A study showed that the human hepatocyte cell HL-7702 proteomics change was correlated with apoptosis after PFOA exposure; however, the associated mechanism was unclear. To test this hypothesis, i.e., this PPARα-independent mechanism was also effective in human hepatocytes, studies were conducted using HL-7702 cells.67 After PFOA exposure, HL-7702 cells showed significant increase in apoptosis in both early and late stage at doses of 7.5 and 2.5 μg/ml (Figure S7). Western Blot analysis showed that, similar to mice, PFOA caused NDUA9 suppression in HL-7702 cells (Figure 4B). PFOA caused dose-dependent increases in 8-OHdG and Caspase-9 concentrations in HL-7702 cells after 24 h exposure, being significant at the two highest concentrations (Figure 4C,D; p < 0.01). It is known that PFOA-induced dose-dependent 8-OHdG generation is accompanied by DNA strand breaks and micromolecule in human cancer HepG2 cells, which are associated with ROS hypergeneration. This is similar to our observation based on in vivo test. These effects are not limited to hepatic and cancer cells. Zhao et al.68 found that treatment of A549 cells (a hybrid of Chinese hamster ovary cells with human lymphocytes) with PFOA for 16 d significantly increased ROS level, along with increases in Caspase-3, -7, and -9 activities. However, a parallel experiment on mitochondrial-DNA deρ-expression changes in Western blot, although with different folds, showed no significant change, suggesting that ROS probably originated from ETC.68 Furthermore, mutagenic effects are significantly induced in PFOA-exposed A549 cells, while largely decreased in ρ0 A549 cells or A549 cells cotreated with ROS inhibitor, demonstrating ROS generation is sufficient to interaction with DNA.68 Thus, this mechanism may be
applicable to various human cells, which may be due to the conservation of ETC in different organs.

Environmental Implication. In this study, apoptosis was triggered by ROS hyperaccumulation originating from PPARα activation and Complex I suppression at high doses of 0.5 and 2.5 mg/kg-d (Figure S6). At the lowest dose of 0.05 mg/kg-d, apoptosis was more serious in female than male mice (Figures 1 and 2). Proteomic profiling revealed that ROS hypergeneration induced by suppression of Complex I was the major pathway to induce apoptosis in female mice at 0.05 mg/kg-d, while PPARα activation was the mechanism for male mice (Figure 3). The different mechanisms between females and males at low exposure dose were possibly due to the fact that the subcellular organelle affected by PFOA was different, with males being main nuclear receptors, especially PPAR, while females were mainly mitochondrial proteins, especially subunits of ETC. However, information about the subcellular distribution of PFOA in different genders of mice has rarely been reported, further studies should be undertaken to test this hypothesis.

In addition, when considering the difference in PFOA toxicokinetics between rodent and human models, the molecular mechanism for PFOA hepatotoxicity involving the p53 pathway has been further confirmed by an in vitro assay using human liver cells HL-7702 (Figure 4). The approach involving in vivo (mouse model) and in vitro test (human cell) to study molecular mechanism under environmental relevant level-exposure makes these findings more relevant to human health.

Our results, i.e., the ROS source in PFOA-exposed females was through suppression of Complex I instead of PPARα activation, may partially answer the epidemiology question as to why no liver cancer was observed in PFOA-exposed workers or residents. In addition, due to the different toxicodynamic and biological responses, PPARα-induced carcinogenesis in rodents does not always occur in humans, which can also explain the lack of liver tumors after exposure to PFOA on some occasions. However, for other organs, the ROS from Complex I may cause variable effects, thus it is unclear if this source of ROS contributes to the elevated occurrence of kidney and testicular cancer in PFOA-exposed populations. Therefore, comprehensive and longitudinal studies to clearly delineate the health effects of PFOA exposure in human populations are desirable.

In this study, the mean serum PFOA concentration in mice exposed to 0.05 mg/kg-d PFOA was 0.97 and 1.2 mg/L for females and males, which was lower than those in professional workers (1.5–6.8 mg/L), and comparable to those in residents near a PFOA factory in China (0.38 and 0.01–2.4 mg/L). When species uncertainty factor (UF) was applied following USEPA, the serum levels of PFOA in this study (0.97–22 mg/L) were lower than the high exposure to humans after using UF = 90. In particular, for the lowest dose at 0.05 mg/kg-d, the serum levels were only 11–13 μg/L, which were slightly higher than the 95% value (9.7 μg/L) of the 2007–2008 American Red Cross blood donors of the general population. Overall, our data indicated that exposure doses used in this study, especially the lowest dose, were environmentally relevant, so the findings should have implications for PFOA-associated health effects in the general population. In addition, the TDI of PFOA recommended by EFSA is 1.5 μg/kg bw for humans based on the dose for mice at 0.3 mg/kg bw and UF = 200. Therefore, since the toxicity effect was observed at 0.05 mg/kg bw (lower than TDI), a question has been raised if the current TDI is safe and whether a lower TDI value may be needed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b02690. Quantitative proteomics, Western-blotting Assay, JC-1, and Elisa Assay of mice liver and cultured hepatocytes, and PFOA exposure suppresses pregx X receptor (PXr) in mice liver; estrogen protective effects and its role in PFOA induced electron transport chain (ETC) damages in mouse liver; and relevance of different factors and proteome changes supports ps3 activation is induced by mtΔΨ dissipation; and protein data (Tables S1–S4 and Figures S1–S9) (PDF) Protein data (XLSX)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: lizyczui@nju.edu.cn (X.C.).
*E-mail: weisi@nju.edu.cn (S.W.).

ORCID

Lena Q. Ma: 0000-0002-8463-9957

Notes

The authors declare no competing financial interest.

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