Free Radical Biology and Medicine 87 (2015) 300–311

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Perfluorooctanoic acid exposure induces endoplasmic reticulum stress in the liver and its effects are ameliorated by 4-phenylbutyrate

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ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form
8 June 2015
Accepted 30 June 2015
Available online 6 July 2015

Keywords:
Hepatotoxicity
Liver injury
Oxidative stress
Lipid metabolism
Sodium 4-phenylbutyrate

ABSTRACT

Perfluoroalkyl acids (PFAAs) are a group of widely used anthropogenic compounds. As one of the most dominant PFAAs, perfluorooctanoic acid (PFOA) has been suggested to induce hepatotoxicity and several other toxicological effects. However, details on the mechanisms for PFOA-induced hepatotoxicity still need to be elucidated. In this study, we observed the occurrence of endoplasmic reticulum (ER) stress in mouse livers and HepG2 cells after PFOA exposure using several familiar markers for the unfolded protein response (UPR). ER stress in HepG2 cells after PFOA exposure was not significantly influenced by autophagy inhibition or stimulation. The antioxidant defense system was significantly disturbed in mouse livers after PFOA exposure, and reactive oxygen species (ROS) were increased in cells exposed to PFOA for 24 h. However, *N*-acetyl-L-cysteine (NAC) pretreatment did not satisfactorily alleviate the UPR in cells exposed to PFOA even though the increase of ROS was less evident. Furthermore, exposure of HepG2 cells to PFOA in the presence of sodium 4-phenylbutyrate (4-PBA), a chemical chaperone and ER stress inhibitor, suggested that 4-PBA alleviated the UPR and autophagosome accumulation induced by PFOA in cells. In addition, several toxicological effects attributed to PFOA exposure, including cell cycle arrest, proteolytic activity impairment, and neutral lipid accumulation, were also improved by 4-PBA cotreatment in cells. *In vivo* study demonstrated that PFOA-induced lipid metabolism perturbation and liver injury were partially ameliorated by 4-PBA in mice after 28 days of exposure. These findings demonstrated that PFOA-induced ER stress leading to UPR might play an important role in PFOA-induced hepatotoxic effects, and chemical chaperone 4-PBA could ameliorate the effects.

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1. Introduction

Concerns regarding the effects of perfluoroalkyl acids (PFAAs) on human health and the environment have increased because many fit the defining characteristics of persistent organic pollutants [1]. PFAAs are a group of widely used anthropogenic compounds with unique physical and chemical characteristics that have been incorporated into many products over the past six decades [1,2]. They have been detected in environmental media as well as wildlife and human tissue in many locations all over the world [2]. Recently available data have described the toxicological effects of perfluorooctanoic acid (PFOA), one of the most widely known PFAAs, and previous studies demonstrated significant hepatotoxicity in rodents even at low dose exposure [2,3]. Several medical surveillance studies also found that serum PFOA concentration was positively related to serum alanine aminotransferase (ALT) levels and negatively associated with serum high-density lipoprotein (HDL) levels in humans [4,5]. Additionally, serum PFOA levels have also been suggested to correlate with thyroid disease [6], juvenile asthma [7], lower human semen quality [8,9], and fetal growth reduction [10–12]. Several studies on the connection between serum PFOA concentrations and cancers indicated that higher serum PFOA levels may be related to

Abbreviations: PFOA, perfluorooctanoic acid; ER stress, endoplasmic reticulum stress; 4-PBA, sodium 4-phenylbutyrate; ROS, reactive oxygen species; Cat, catalase; Sod1, superoxide dismutase 1; Sod2, superoxide dismutase 2; Sesn1, sestrin 1; LC3, microtubule-associated protein light chain 3; SQSTM1 (p62), sequestosome 1; CQ, chloroquine; NAC, *N*-acetyl-L-cysteine; Rapa, rapamycin; siRNA, small interfering RNA; eIF2α, eukaryotic initiation factor 2α; CHOP, C/EBP homologous protein; XBP1s, spliced X box-binding protein 1; 18S, 18S ribosomal RNA; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element binding protein; Srebf, sterol regulatory element binding transcription factor; Vldlr, very low density lipoprotein receptor; Cyp4a10, cytochrome P450; family 4, subfamily a, polypeptide 10; Fabp1, fatty acid binding protein 1, liver; Fasn, fatty acid synthase; Hmgcr, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Lpl, lipoprotein lipase; Scd1, stearoyl-coenzyme A desaturase 1; Acox1, acyl-coenzyme A oxidase 1, palmitoyl

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.06.043
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testicular, kidney, prostate, and ovarian cancers and non-Hodgkin lymphoma [13,14]. However, as the possible associations of PFOA exposure with cancers were detected in community settings and also were not supported by results in occupational workers, whether PFOA exposure is related to cancers still unclear [15].

Although PFOA-induced hepatotoxicity is generally accepted, especially in rodents, its exact mechanisms remain unclear. Several studies showed that PFOA and perfluorooctane sulfonate (PFOS) were capable of inducing hepatomegaly, and activation of peroxisome proliferator activated receptors (PPARs) was possibly the first key event [2,16]. However, liver weight increase, fat accumulation, and changes in genes related to fatty acid metabolism were observed in PPARα-null mice after PFOA exposure [17–20], indicating that alternate mechanisms could also be involved in the effects induced by PFOA on the liver. Indeed, several nuclear receptors were previously analyzed *in vitro* and appeared to be activated in the cells of rats more so than in the cells of humans after exposure to PFOA or PFOS [21]. Our previous study also suggested that activation of sterol regulatory element-binding proteins (SREBPs) might play an important role in the effects of PFOA on the liver [22]. Nevertheless, current evidence is still insufficient to completely explain the effects of PFOA on the liver, especially the discrepancy between results from *in vivo* and *in vitro* studies.

Most secreted and membrane proteins fold and mature in the lumen of the endoplasmic reticulum (ER) in eukaryotic cells and are then transported to other organelles, displayed on the cell surface, or released extracellularly [23,24]. ER stress is defined as an imbalance between the load of unfolded and misfolded proteins in the ER and the capacity of the cellular machinery to handle this load [23]. Cells adjust the protein-folding capacity in the ER according to their requirements, thereby ensuring fidelity in protein folding [23,24]. The intracellular signaling pathway that mediates this adjustment is cumulatively termed the unfolded protein response (UPR), which monitors ER condition, sensing the threat of protein misfolding and communicating this information to gene expression programs in eukaryotic cells [24]. The UPR is a concerted and complex cellular response to ER stress, and three different classes of ER stress transducers have been identified for its mediation, including inositol-requiring protein 1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR) like ER kinase (PERK) [23,25]. These transducers represent three principal branches of the UPR, which operate in parallel using mechanisms of signal transduction [24]. The ER chaperone glucose regulated protein (GRP) 78 interacts with the ER luminal domains of IRE1, ATF6, and PERK and dissociates them on ER stress, leading to their activation [25]. ATF6 is delivered to the Golgi apparatus and subjected to cleavage on ER stress, and the cytosolic effector portion of ATF6 liberated and imported into the nucleus results in the activation of a subset of UPR target genes [23]. Both PERK and IRE1 are activated by the transautophosphorylation of their activation loop and in turn further activate other factors, including the phosphorylation of eIF2α by PERK and cleavage and splicing of XBP1 by IRE1 [23]. The UPR leads to three main responses, that is, reduction in the protein load of the ER, increase in the capacity of the ER, or cell death [23].

Accumulating evidence from *in vivo* and *in vitro* studies have suggested that ER stress is involved in toxicological effects, especially apoptosis induced by chemicals [26,27]. Earlier studies reviewed elsewhere also suggest that the UPR is activated in several liver diseases, including fatty liver disease, viral hepatitis, and alcohol-induced liver injury [28]. However, little is known about the connection between the ER stress and the toxicological effects of PFOA. In the present study, we assessed the occurrence of ER stress using several familiar protein markers of the UPR after PFOA exposure both *in vivo* and *in vitro*. We demonstrated that PFOA exposure induced ER stress leading to UPR, and that sodium 4-phenylbutyrate (4-PBA), a chemical chaperone and ER stress inhibitor, could ameliorate the toxicological effects of PFOA.

2. Materials and methods

2.1. Chemicals and antibodies

Perfluorooctanoic acid (PFOA, CAS number 335–67–1, 96% purity), chloroquine diphosphate salt (CQ, CAS number 50–63–5, 98% purity), sodium 4-phenylbutyrate (4-PBA, CAS number 1716–12-7, 98% purity), 4-phenylbutyric acid (CAS number 1821–12–1, 99% purity), and *N*-acetyl-L-cysteine (NAC, CAS number 616–91–1, 99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin solution (Rapa, dissolved in DMSO, 1 mg/mL) was purchased from the Beyotime Institute of Biotechnology (Nantong, China). Antibodies used in this study are listed in Table S1. As the LC3B antibody we used is more specific in HepG2 cell lysates while the LC3A antibody is more specific in mouse liver lysates, we detected LC3B in HepG2 cells and LC3A in mouse livers to assess autophagosome accumulation in this study. Primers for real-time PCR analysis are listed in Table S2. All other chemicals used were of the highest grade commercially available.

2.2. Animal treatment

Male Balb/c mice (aged 6–8 weeks) were obtained from Beijing Vital River Experimental Animals Centre (Beijing, China) and the experiment was performed as described in our previous study [3]. In brief, mice were randomly divided into six groups and treated by oral gavage for 28 days with Milli-Q water or doses of PFOA (0.08, 0.31, 1.25, 5, and 20 mg/kg/day) diluted in Milli-Q water. Another 40 mice were randomly assigned into four groups and treated two times a day with different chemicals by oral gavage for 28 days. In the morning, mice were dosed with Milli-Q water or 5 mg/kg/day PFOA. In the afternoon, water-treated mice were dosed with Milli-Q water and PFOA-treated mice were dosed with Milli-Q water, 125 mg/kg/day 4-PBA, or 250 mg/kg/day 4-PBA. The 4-PBA solution used in the animal experiments was prepared by titrating equimolecular amounts of 4-phenylbutyric acid and sodium hydroxide to pH 7.4 [29]. The doses of 4-PBA selected for animal treatment were as per previous studies [30]. All animal treatments were approved by the Committee on the Ethics of Animal Experiments from the Institute of Zoology, Chinese Academy of Sciences, and in compliance with the Guiding Principles in the Use of Animals in Toxicology, which were adopted by the Society of Toxicology in 1989.

2.3. Serum biochemical assay

Serum lipids and enzymes were detected using a HITAC7170A automatic analyzer (Hitachi, Japan) following standard spectrophotometric methods ($n=6$).

2.4. Cell culture and treatments

The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in 25 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2. Solid chemicals were dissolved as stock solutions in serum-free DMEM, filter-sterilized (0.22 μm Millipore filter, Millipore, USA), and stored at 4 °C. According to the doses used in each experiment, stock solutions were diluted to final concentrations with DMEM containing 10% FBS before use. Cells were plated with an appropriate density in tissue

culture plates. The medium was replaced with media containing PFOA, CQ, Rapa, or 4-PBA overnight after plating. To analyze the role of reactive oxygen species (ROS) in PFOA-induced toxicological effects, HepG2 cells were pretreated with 10 mM NAC for 2 h and then exposed to PFOA.

2.5. Determination of cell viability and cell cycles

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Steinheim, Germany) method. Briefly, cells were seeded in 96-well plates and treated with MTT for 4 h before termination of the experiment. The cell viability percentage compared with the control group was calculated using optical density (OD) at 570 nm. The release of intracellular lactate dehydrogenase (LDH) into culture medium is an indicator of irreversible cell death attributed to cellular membrane damage [31]. Here, cytotoxicity was measured by the LDH activity in the supernatant using an LDH Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instructions. In brief, LDH activity in the cell culture supernatants after treatment was measured. LDH activity from untreated cell lysates was also measured to determine total LDH content. LDH activity in culture medium was measured to determine background LDH content. OD was measured at 490 and 630 nm of the reference. Cytotoxicity was calculated by: Cytotoxicity (%) = [Cell supernatant ODs – Control (untreated) cell supernatant ODs]/[Control (untreated) cell lysate ODs – Control (untreated) cell supernatant ODs] × 100. Cell cycles were measured using a Cycle and Apoptosis Analysis Kit (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instructions. In brief, cells were collected and fixed in cold 70% ethanol at 4 °C overnight after treatments. Fixed cells were stained with propidium iodide (PI) solution from the kit and the fluorescence distribution was measured by flow cytometry. The cell cycle distribution was then analyzed using ModFit software (Verity Software, Topsham, ME, USA).

2.6. Detection of neutral lipids change in cells

Neutral lipid change in HepG2 cells was detected by flow cytometry after staining with the fluorescent neutral lipid dye BODIPY 493/503 (Molecular Probes, Carlsbad, CA, USA) as described in our previous study [22].

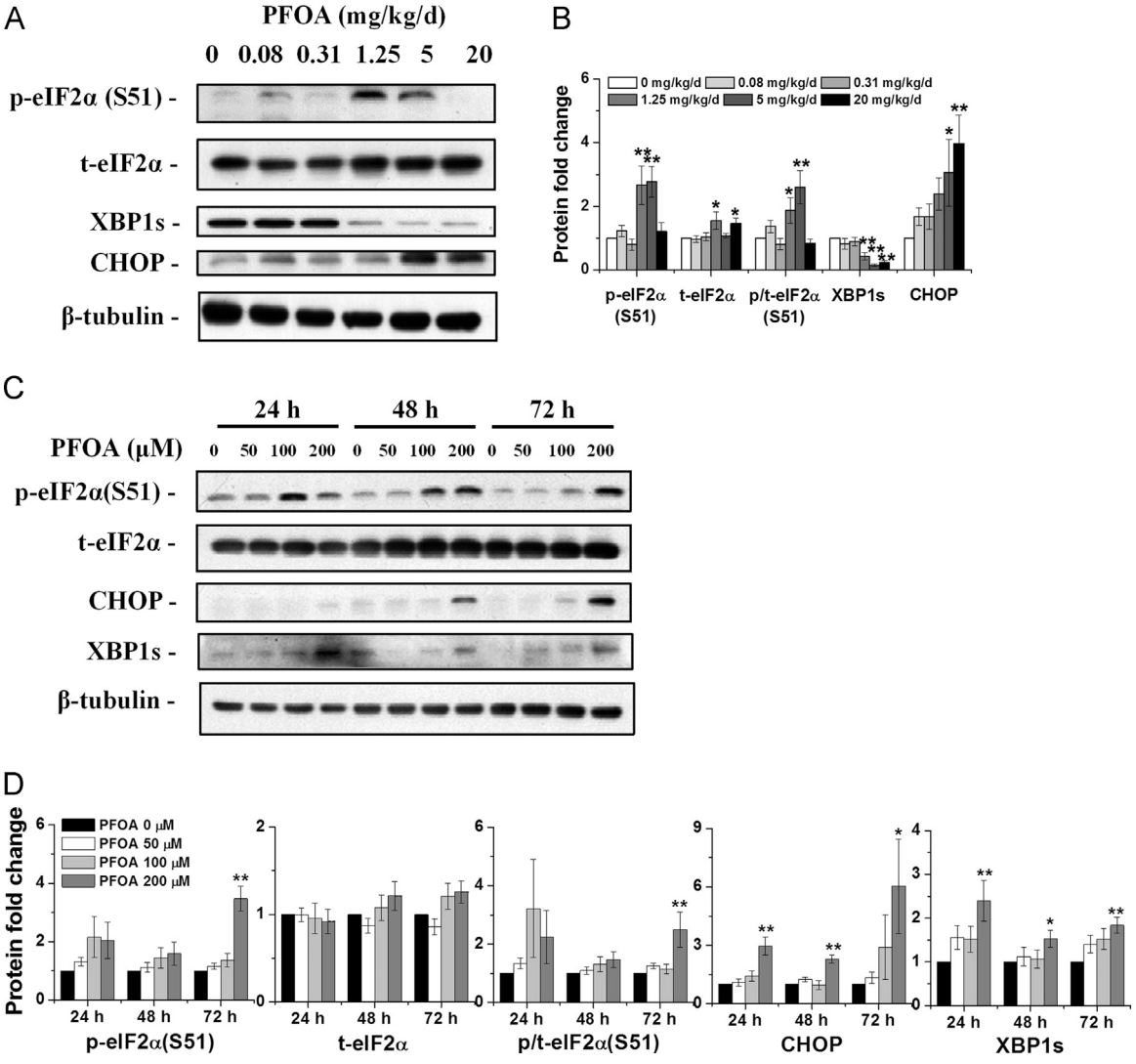

Fig. 1. PFOA exposure induced ER stress. (A) Total tissue lysates from livers of mice exposed to PFOA for 28 days were subjected to Western blotting for proteins involved in ER stress and (B) relative fold change of band densities ($n=3$). (C) HepG2 cells were exposed to PFOA for 24, 48, or 72 h. Total cellular lysates were subjected to Western blotting for proteins involved in ER stress and (D) relative fold change of band densities ($n=3$). Data are shown as means ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$.

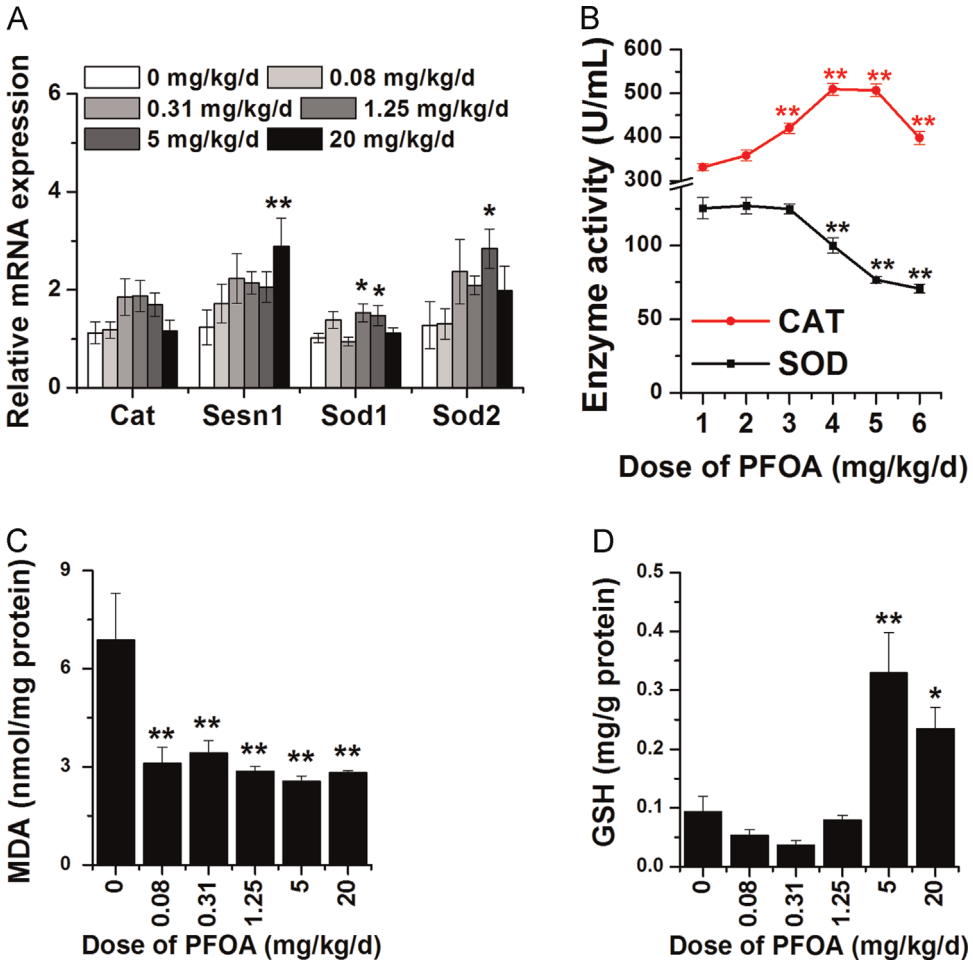

Fig. 2. PFOA exposure disturbed the antioxidant defense system in mouse livers. (A) Expression of oxidative stress-responsive genes including Cat, Sesn1, Sod1, and Sod2 were analyzed in livers of mice after PFOA exposure. (B) Effects of PFOA on SOD and CAT activities in mouse livers. (C) The effect of PFOA on hepatic MDA contents. (D) The effect of PFOA on hepatic GSH contents. Data are shown as means ± SE, $n=6$, * $P < 0.05$, ** $P < 0.01$.

2.7. Detection of ROS change in cells

ROS change in HepG2 cells was detected using a ROS assay kit (Beyotime Institute of Biotechnology, Nantong, China) via the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) method according to the manufacturer's instructions. The superoxide anion change in HepG2 cells was further determined using dihydroethidium (DHE) staining (Beyotime Institute of Biotechnology, Nantong, China). Following treatment, HepG2 cells were cultured with 10 μM DCFH-DA or 5 μM DHE at 37 °C for 30 min. After washing with PBS, cells were lysed in RIPA buffer (Thermo Scientific, Rockford, IL, USA). Fluorescence intensity of the lysates was quantified using a Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA). The excitation/emission of DCFH-DA and DHE staining are 488/525 and 300/610 nm, respectively. Protein concentration of each lysate was determined using a BCA protein assay kit (Tiangen, Beijing, China). The fluorescence intensity was adjusted with protein concentration and results were normalized to the control group.

2.8. Determination of SOD and CAT activities

The total superoxide dismutase (SOD) and catalase (CAT) activities in mouse livers and HepG2 cells after PFOA exposure were analyzed using a SOD assay kit (Beyotime Institute of Biotechnology, Nantong, China) and CAT assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturer's instructions.

2.9. Determination of MDA and GSH contents

The malondialdehyde (MDA) and glutathione (GSH) contents in mouse livers and HepG2 cells after PFOA exposure were analyzed using a MDA assay kit and GSH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturer's instructions.

2.10. DQ–bovine serum albumin staining

DQ red bovine serum albumin (DQ red BSA, Molecular Probes, Carlsbad, CA, USA) was used to measure the intracellular proteolytic activity of HepG2 cells after treatment according to previous studies [32], with some modifications. Briefly, HepG2 cells were cultured with medium containing 10 μg/mL DQ-BSA at 37 °C for 4 h after PFOA exposure and lysed in RIPA buffer (Thermo Scientific, Rockford, IL, USA). Fluorescence intensity of the lysates was quantified with excitation/emission of 590/620 nm. Protein concentration of each lysate was measured by a BCA protein assay kit (Tiangen, Beijing, China) and the fluorescence intensity was adjusted with the protein concentration, respectively. The results were normalized to the control group.

2.11. siRNA transfection

HepG2 cells were seeded in tissue culture plates overnight, and then transfected with siGENOME SMARTpool Human autophagy-related gene 5 (ATG5) siRNA or siGENOME Non-Targeting siRNA

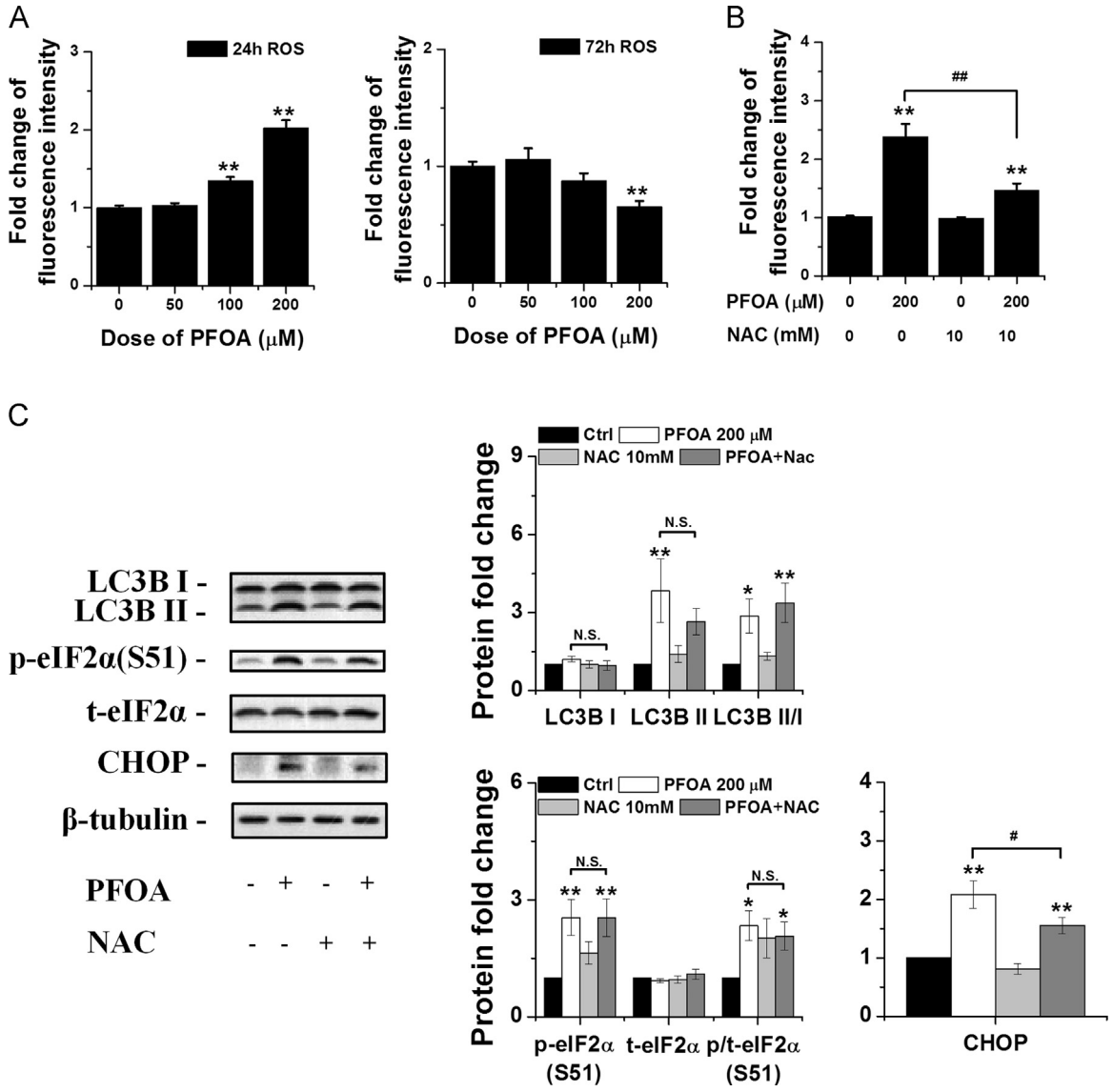

Fig. 3. Effects of ROS elimination on PFOA toxicity in HepG2 cells. (A) Reactive oxygen species (ROS) change in HepG2 cells after exposure to PFOA for 24 or 72 h ($n = 3$). (B) ROS change in HepG2 cells pretreated with *N*-acetyl-L-cysteine (NAC, 10 mM) and then exposed to PFOA (200 μM) for 24 h ($n=3$). (C) LC3B, phospho-eIF2α, total eIF2α, and CHOP expression in HepG2 cells pretreated with NAC (10 mM) and then exposed to PFOA (200 μM) for 24 h. Band densities of proteins were quantified and are shown in the right panel ($n=3$). Data are shown as means ± SE, * (compared with control group) or # $P < 0.05$, ** or ## $P < 0.01$, N.S. indicated no significance.

Pool #2 (25 nM, Dharmacon Research Inc., Lafayette, CO, USA) using Dharma FECT 4 siRNA transfection reagent (Dharmacon Research Inc., Lafayette, CO, USA) according to the manufacturer's instructions. The medium was replaced with medium containing PFOA for 24 or 72 h after 24 h transfection.

2.12. Real-time PCR analysis

RNA isolation and real-time PCR analysis were performed as described in our previous study [22].

2.13. Western blotting

Protein isolation and Western blotting were performed as described in our previous study [22].

2.14. Statistical analysis

All results were statistically analyzed using SPSS for Windows 17.0 Software (SPSS Inc., Chicago, IL, USA). Differences between only two treatment groups were determined via an independent samples *t* test. Differences between more than two treatment groups were determined using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. All experimental data were represented as means with standard errors (mean ± SE). *P* values < 0.05 (* or #) or < 0.01 (** or ##) were considered significant. All represented data from *in vitro* experiments were assessed from at least three independent experiments.

3. Results

3.1. PFOA exposure induced ER stress

Our previous studies suggested significant effects of PFOA on mouse liver after exposure to PFOA for 28 days, including liver enlargement, serum ALT increase, and activation of hepatic SREBPs [3,22]. The content of PFOA was also increased in serum and livers of mice after PFOA exposure [3,22]. To investigate the occurrence

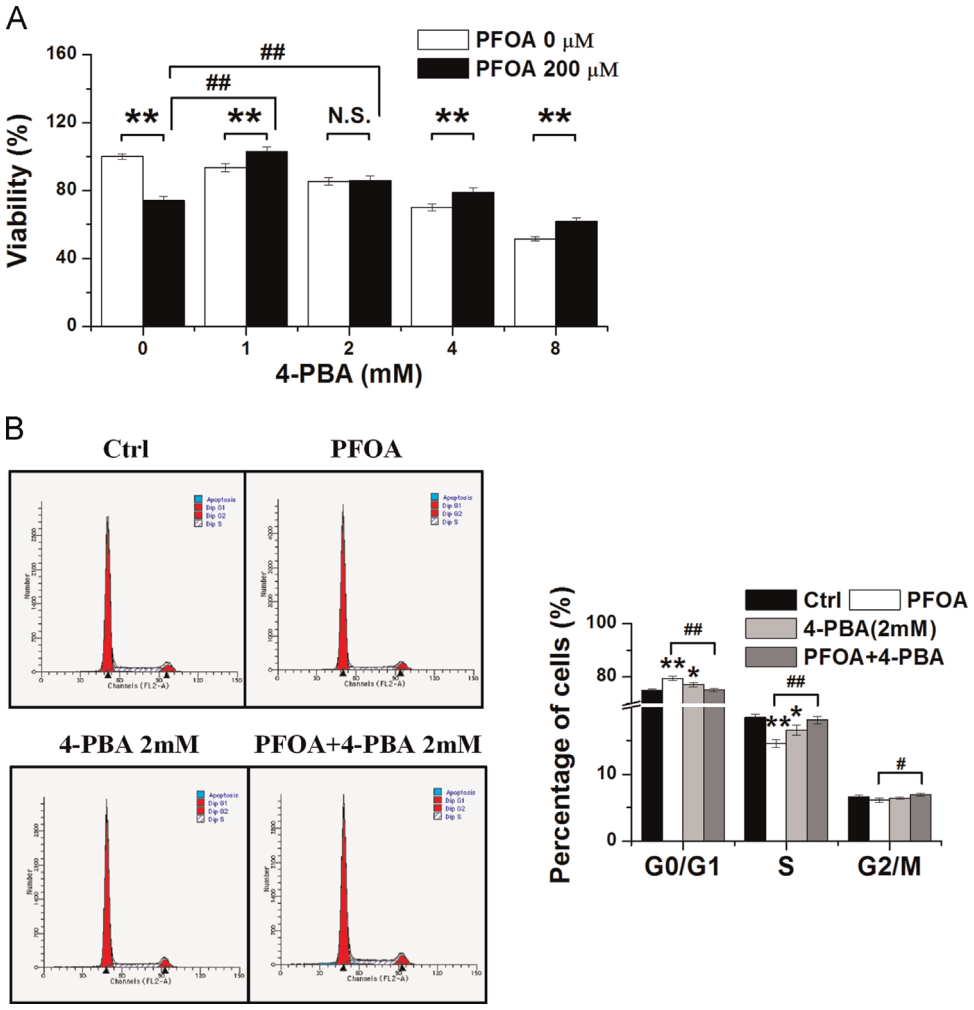

Fig. 4. 4-PBA maintained the viability of HepG2 cells after PFOA exposure. (A) Cell viability of HepG2 cells treated with PFOA (200 μM) in the absence or presence of 4-PBA (1 or 2 mM) for 72 h. (B) Effects of PFOA on HepG2 cell cycles in the absence or presence of 4-PBA (2 mM). Data were assessed from three independent experiments and shown as means ± SE, * (compared with control group) or # $P < 0.05$, ** or ## $P < 0.01$.

of ER stress after PFOA exposure, ER stress markers, including phosphorylated eukaryotic initiation factor 2α (p-eIF2α), C/EBP homologous protein (CHOP), and spliced X box-binding protein 1 (XBP1s) [25], were analyzed in mouse livers and HepG2 cells after PFOA exposure. The p-eIF2α was increased in the livers of mice exposed to PFOA for 28 days at 1.25 and 5 mg/kg/day, CHOP was induced at doses of 5 and 20 mg/kg/day (Fig. 1A and B), but XBP1s was decreased in mouse livers after PFOA exposure, which might be due to liver cell death [28]. CHOP and XBP1s were significantly increased in HepG2 cells exposed to PFOA at 200 μM, especially after long exposure time (Fig. 1C and D). p-eIF2α was also significantly increased after PFOA exposure at 200 μM for 72 h (Fig. 1C and D). These results demonstrated that PFOA exposure induced ER stress.

3.2. Autophagy modulation did not significantly influence ER stress after PFOA exposure in cells

Our previous study showed that PFOA exposure induced autophagy blockage, which will be reported in another paper. To investigate the connection between autophagy and ER stress in PFOA-induced toxicity, autophagy was further inhibited with CQ. Inconsistent with previous studies [33], our results also suggested that CQ itself significantly increased the phosphorylation of eIF2α; however, no significant increase in CHOP was observed (Figs. S1A and B). There were no significant synergistic or antagonistic effects on the phosphorylation of eIF2α when HepG2 cells were treated with both PFOA and CQ (Figs. S1A and B). CHOP was reduced in cells treated with both CQ and PFOA for 24 h but was increased after 72 h compared with the group treated with PFOA only (Figs. S1A and B), which might be due to the cytotoxicity of CQ. We further analyzed the autophagy inhibition on PFOA-induced ER stress using cells transfected with siAtg5 and the results suggested that silencing the expression of Atg5 did not significantly influence ER stress induced by PFOA *in vitro* (Figs. S2A and B). The autophagy stimulator Rapa was also used to investigate the correlation between autophagy and ER stress induced by PFOA exposure. Cell viability was reduced in the presence of Rapa, and no significant change was observed in the viability of cells cotreated with PFOA and Rapa compared with the group treated with Rapa only (Fig. S3A). Autophagosomes were further accumulated in PFOA-exposed cells in the presence of Rapa, the phosphorylation of eIF2α was modestly weakened after PFOA exposure, and the expression of CHOP was not significantly changed (Figs. S3B and C). These results indicated that autophagy stimulation could not satisfactorily relieve the ER stress induced by PFOA exposure.

3.3. PFOA exposure disturbed the antioxidant defense system in mouse livers

Oxidative stress is related to both autophagy and ER stress [34,35]. To investigate whether PFOA exposure induced oxidative

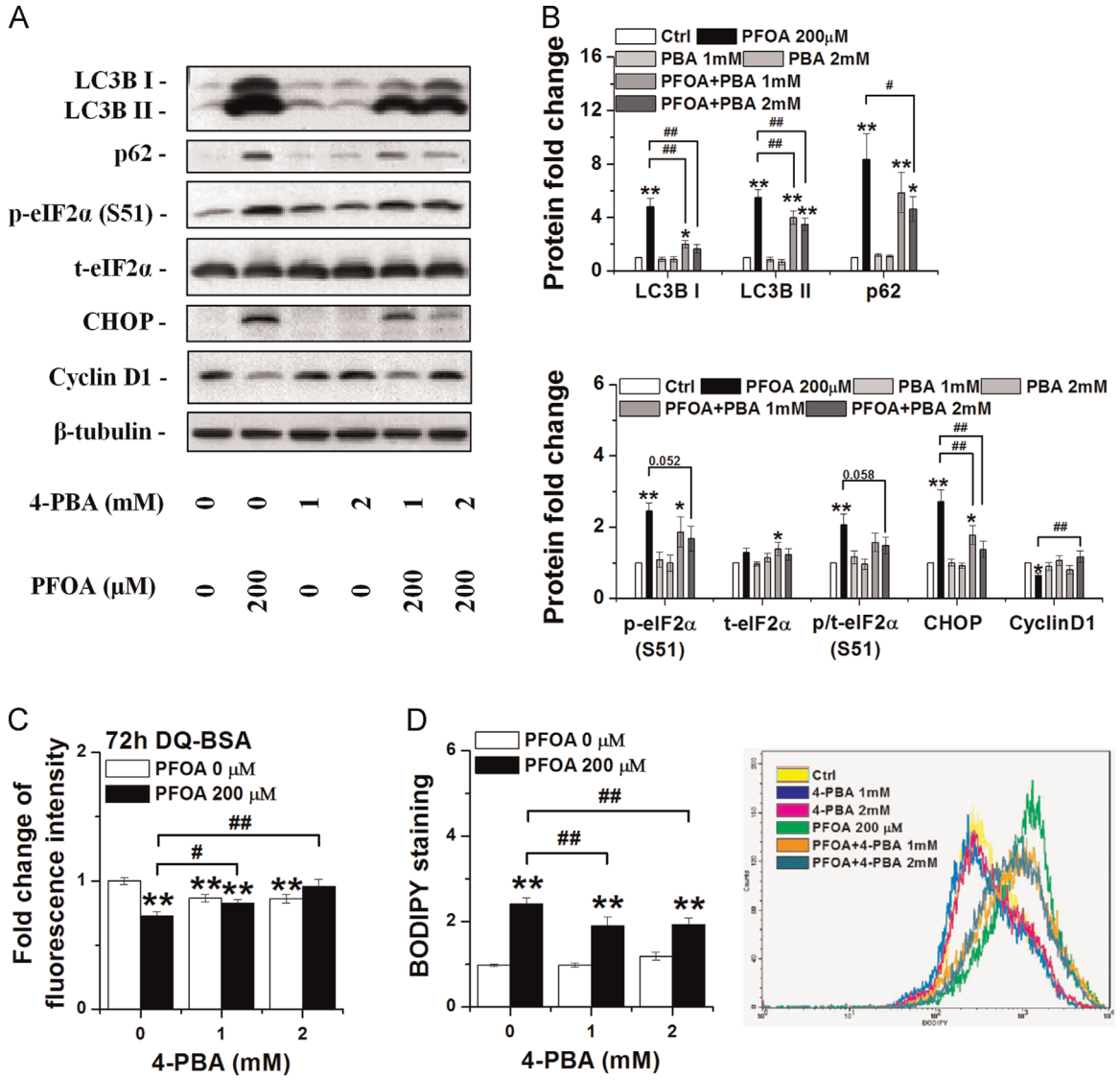

Fig. 5. 4-PBA alleviated the toxicological effects of PFOA on HepG2 cells. (A) HepG2 cells were treated with PFOA (200 μM) in the absence or presence of 4-PBA (1 or 2 mM). Total cellular lysates were subjected to Western blotting for LC3B, p62, phospho-eIF2a (S51), total eIF2a, CHOP, and cyclin D1. (B) Relative fold change of band densities ($n=3$). (C) HepG2 cells were exposed to PFOA (200 μM) in the absence or presence of 4-PBA (1 or 2 mM) and then loaded with DQ-BSA (10 μg/mL) for 4 h. Fluorescence intensity from cleaved DQ-BSA was measured using total cellular lysates. (D) HepG2 cells were exposed to PFOA (200 μM) in the absence or presence of 4-PBA (1 or 2 mM) and then stained with neutral lipid dye BODIPY 493/503. Fluorescence intensity was measured by flow cytometry. Data were assessed from three independent experiments and shown as means ± SE, * (compared with control group) or # $P < 0.05$, ** or ## $P < 0.01$, N.S. indicated no significance.

stress in mouse livers, we analyzed the mRNA expression of oxidative stress-responsive genes [36,37] including Cat, Sod1, Sod2, and sestrin 1 (PA25/Sesn1) in the livers of mice after exposure to PFOA for 28 days. Cat mRNA expression was not significantly changed after PFOA exposure (Fig. 2A). Sesn1 mRNA expression increased at the dose 20 mg/kg/day, Sod 1 increased at doses of 1.25 and 5 mg/kg/day, and Sod 2 increased at the dose of 5 mg/kg/day (Fig. 2A). However, mRNA expression changes of these three genes were humble. We further determined activities of antioxidant enzymes, including SOD and CAT, and the results demonstrated that PFOA exposure induced the increase of CAT activity but reduced the total SOD activity (Fig. 2B). A lipid peroxidation marker [38], MDA, was analyzed using the reaction with 2-thiobarbituric acid, and the results indicated that the MDA contents were decreased after PFOA exposure even at the lowest dose in mouse livers (Fig. 2C). GSH is a major endogenous antioxidant in the liver [39], and we also found that GSH contents were increased in livers of mice exposed to PFOA at doses of 5 and 20 mg/kg/day (Fig. 2D). These results suggested that PFOA disturbed the antioxidant defense system in mouse livers after exposure for 28 days. However, no significant oxidative damage was observed.

3.4. No significant change in cells pretreated with antioxidant after PFOA exposure

To explore whether oxidative stress occurred in HepG2 cells after PFOA exposure, we measured the levels of ROS in HepG2 cells exposed to PFOA. Results showed that ROS levels were significantly increased in HepG2 cells after 24 h exposure to PFOA but decreased after 72 h exposure (Fig. 3A). However, the levels of superoxide anion were not significantly altered after PFOA exposure for 24 h (Fig. S4A), and the activities of SOD and CAT as well as contents of MDA and GSH also did not show significant change (Fig. S4B). To investigate whether ROS increase contributed to ER stress, cells were pretreated with an antioxidant, NAC, and then exposed to PFOA for 24 h. The levels of ROS were significantly reduced after 24 h in cells pretreated with NAC compared with

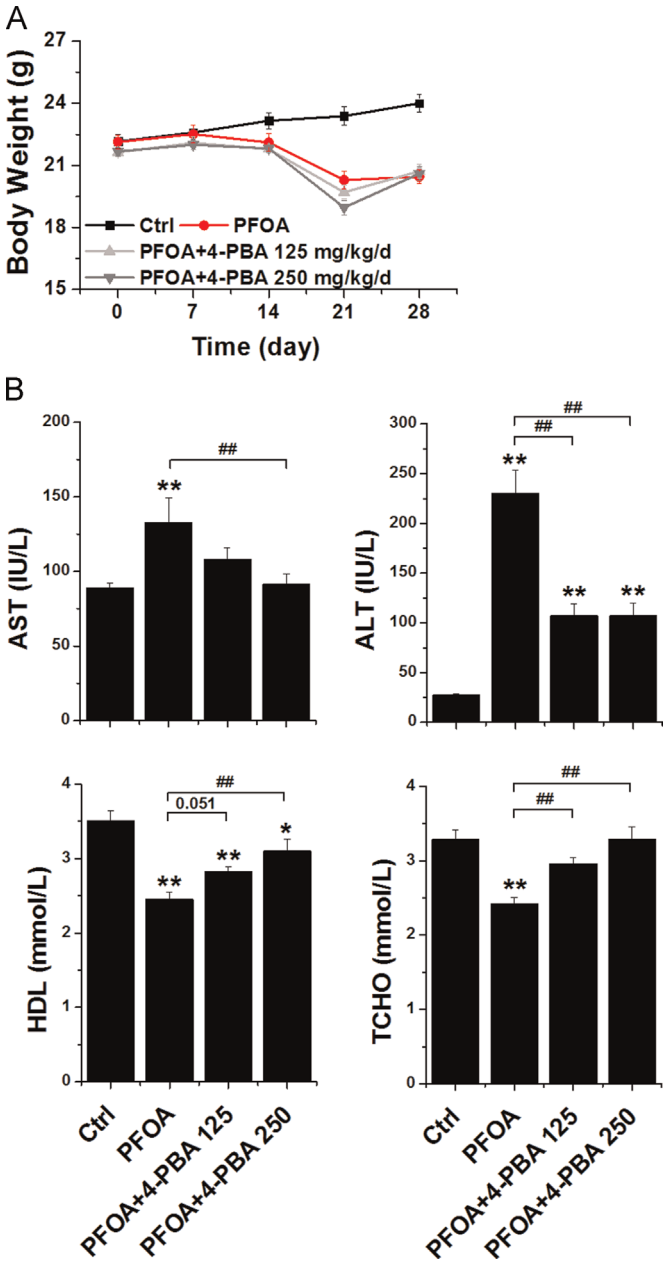

Fig. 6. 4-PBA ameliorated the hepatotoxicity of PFOA in mice. (A) Body weights of mice exposed to PFOA (5 mg/kg/day) in the absence or presence of 4-PBA (125 or 250 mg/kg/day) for 28 days ($n=10$). (B) Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-density lipoprotein (HDL) and total cholesterol (TCHO) content in mice exposed to PFOA (5 mg/kg/day) in the absence or presence of 4-PBA (125 or 250 mg/kg/day) for 28 days ($n=6$). Data are means ± SE, * (compared with control group) or # $P < 0.05$, ** or ## $P < 0.01$.

cells exposed to PFOA directly (Fig. 3B). We further analyzed the expression of LC3B and ER stress markers using Western blotting, which demonstrated that autophagosome accumulation and phosphorylation of eIF2α were not significantly changed in cells pretreated with NAC compared with cells exposed to PFOA directly for 24 h, although the expression of CHOP seemed modestly reduced (Fig. 3C). These results indicated that oxidative stress might not play an important role in ER stress induced by PFOA exposure.

3.5. Sodium 4-phenylbutyrate ameliorated the toxicological effects of PFOA in vitro

Because manipulations on either autophagy or ROS were not satisfactory for PFOA toxicity amelioration, we considered whether ER stress played a more important role in PFOA toxicity. Several studies suggest that chemical chaperones like 4-PBA can reduce ER stress, while alleviating the consequences of its activation [30,40]. Hence, we treated HepG2 cells with PFOA in the absence or presence of 4-PBA. Interestingly, cellular viability was maintained with 4-PBA cotreatment (Fig. 4A). We selected 4-PBA at doses of 1 and 2 mM for further study, and found no significant cytotoxicity of 4-PBA at the two doses (Fig. S5A). Resting cells attributed to PFOA exposure were maintained in the cell cycle in the presence of 4-PBA (Fig. 4B and S5B). The accumulation of autophagosomes and the p62 protein, as well as ER stress, was ameliorated in the presence of 4-PBA (Fig. 5A and B). Earlier research showed that the UPR inhibited cell cycle progression through interference with cyclin D1 translation [41]. Our results also showed that cyclin D1 was reduced after PFOA exposure, and the effect was weakened in the presence of 4-PBA (Fig. 5A and B). The impairment of proteolytic activity after PFOA exposure was improved by 4-PBA (Fig. 5C) and neutral lipid accumulation in HepG2 cells after PFOA exposure was also ameliorated (Fig. 5D).

3.6. Sodium 4-phenylbutyrate partially ameliorated the toxicological effects of PFOA in vivo

Based on the effects of 4-PBA observed *in vitro*, we further investigated whether 4-PBA could weaken the effects of PFOA *in vivo*. Body weight decrease and liver weight increase were not influenced by 4-PBA in mice after PFOA exposure for 28 days (Fig. 6A and S6A). Serum biochemical analysis showed that 4-PBA significantly improved liver injury and lipid metabolism (Fig. 6B and S6B). The p-eIF2α ER stress marker was significantly reduced in mouse livers when cotreated with PFOA and 4-PBA, but other proteins related to autophagy or ER stress did not show such obvious improvement (Fig. 7A and B). Our previous study demonstrated that PFOA exposure activated PPARα and SREBPs [22], and thus we further examined the mRNA levels of PPARs and SREBPs as well as their downstream genes. In agreement with serum biochemical analysis, several genes stimulated by PFOA, especially SREBP downstream genes, were alleviated by 4-PBA (Fig. 8A and B). We also analyzed the activities of total SOD and CAT, but no significant change was observed in mouse livers after PFOA exposure in the presence or absence of 4-PBA (Fig. S7A). 4-PBA also did not improve PFOA-induced hepatic MDA reduction, but hepatic GSH seemed to increase after 4-PBA treatment (Figs. S7B and C).

4. Discussion

In this study, we chose factors that play critical roles in the UPR but not ER stress sensors directly, including eIF2α, XBP1, and CHOP, to investigate the effect of PFOA on ER stress. The results demonstrated that PFOA exposure induced UPR in both HepG2 cells and mouse livers after PFOA exposure. Our previous study suggested that the serum PFOA content in mice exposed to PFOA at 0.08 mg/kg/day was similar to the median serum content of PFOA observed in occupational participants (2.24 μg/mL versus 1.64 μg/mL) [3,5]. In this study, we did not observe the UPR in livers of mice in the 0.08 mg/kg/day group, which implied that the ER stress may only occur after exposure to high levels of PFOA. However, whether exposure to PFOA at low doses could induce ER stress after a longer exposure still needs further study. As a transcription factor downstream of all three ER stress transducers [28], CHOP has been reported to mediate cell death by inducing proapoptotic genes [42] and repressing antiapoptotic genes [43]. We observed the expression of CHOP increased in both HepG2 cells and mouse livers after PFOA exposure, but our results in this study also suggested that cell cycle arrest might contribute to the decrease in

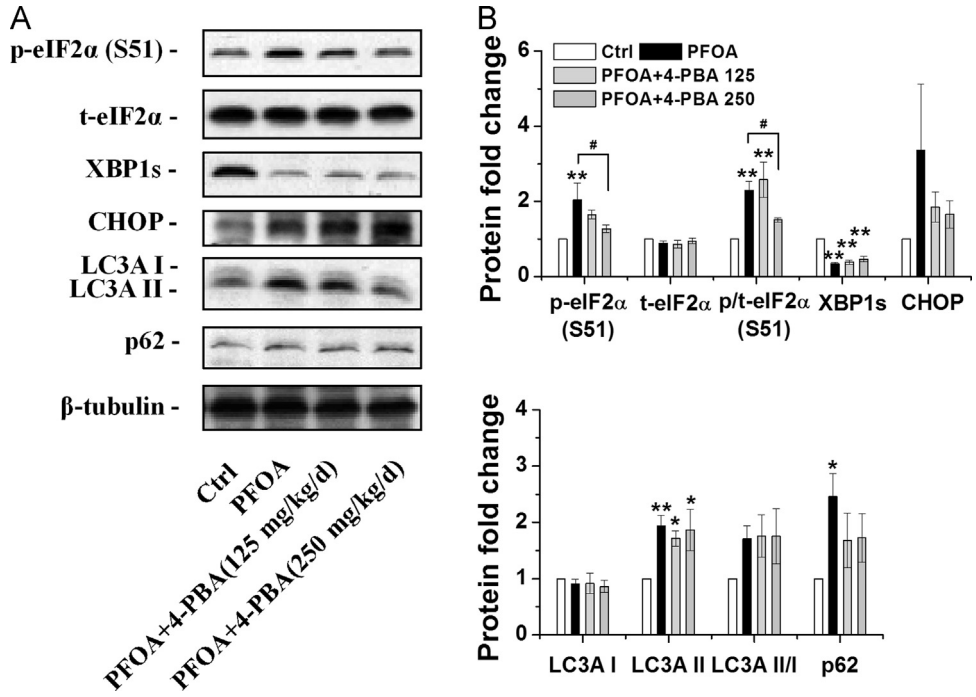

Fig. 7. 4-PBA partially ameliorated ER stress in mouse livers exposed to PFOA. (A) Total tissue lysates from livers of mice exposed to PFOA (5 mg/kg/day) in the absence or presence of 4-PBA (125 or 250 mg/kg/day) for 28 days were subjected to Western blotting for proteins involved in ER stress or autophagy and (B) relative fold change of band densities, ($n=3$). Data are means ± SE, * (compared with control group) or # $P < 0.05$, ** $P < 0.01$.

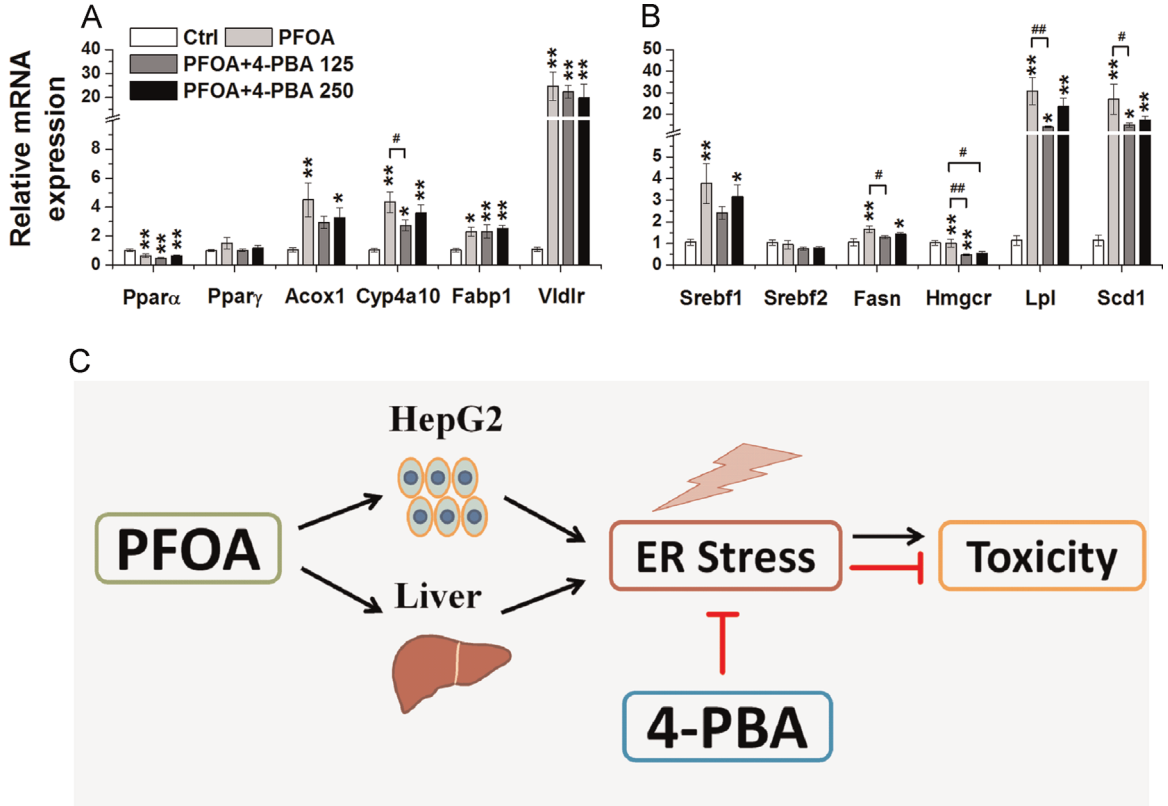

Fig. 8. 4-PBA partially reduced lipid metabolic gene overexpression in mouse livers exposed to PFOA. (A) Relative mRNA expression of PPARs and PPAR-α representative target genes ($n=6$). (B) Relative mRNA expression of SREBPs and SREBPs representative target genes ($n=6$). (C) Possible schematic model of how 4-PBA affects PFOA-induced hepatotoxic effects. Data are means ± SE, * (compared with control group) or # $P < 0.05$, ** or ## $P < 0.01$.

HepG2 cell viability after PFOA exposure, and no significant cell death was observed. In addition, CHOP was not significantly reduced in mouse livers exposed to PFOA in the presence of 4-PBA even though serum concentrations of ALT and AST were significantly reduced. Indeed, a recent study has suggested that genetic depletion of CHOP does not protect mice against CCl_4-induced

liver injury [44]. These findings implied that other potential, unidentified signals were involved in cell death resulting from ER stress.

Our earlier study suggested that PFDoA decreased both total SOD and CAT activities, and increased MDA contents in livers of rats after exposure for 14 days [45]. These results implied that PFDoA impaired the antioxidant defenses and induced lipid peroxidation in rat livers. In this study, we found that PFOA exposure disturbed the hepatic antioxidant defense system with decreased total SOD and increased CAT activities, and we also found increased hepatic GSH and decreased hepatic MDA which suggested that no significant oxidative damage occurred in mouse livers. The different phenomena between the two studies may have resulted from the different lengths of the carbon chain and the exposure duration. Additionally, PFAAs have been considered as PPARα agonists [2], and earlier studies have suggested that PPARα might affect the expression of antioxidant enzymes and protect hepatocytes from potential oxidative damage during fasting [46]. Considering the results from our studies and the potential functions of PPARα in oxidative stress, it seems like PFOA may stimulate oxidative stress after short exposure in mouse livers, but some of the effects are counteracted by the activation of PPARα after longer exposure.

The mechanisms for perturbations in hepatic ER homeostasis include ROS generation, altered membrane lipid composition, hyperhomocysteinemia (HHC) with subsequent protein N-homocysteinylation, and protein aggregation [28]. ROS, including the superoxide anion, hydrogen peroxide and hydroxyl radicals, are normally produced by the metabolism of normal cells [46,47]. An overproduction of ROS is often related to oxidative stress and results in the damage of lipids, proteins, and DNA, and ROS overproduction may induce liver injury when it occurs in the liver [46,47]. Earlier studies suggested that PFOA exposure induced ROS generation which lead to apoptosis and contributed to the reduction of cell viability [48,49]. In this study, we found that PFOA exposure for 24 h increased ROS but not superoxide anions, and lipid peroxidation and apoptosis were not detected based on the unchanged MDA contents and the inconspicuous sub-G1 peak from the results of cell cycle analysis, respectively. We also found that the UPR did not appear improved when pretreated with NAC, which implied that ROS generation might not be critical in PFOA-induced ER stress. The discrepancy between our results and previous studies may be due to different analysis methods and exposure time, and the DMSO used in previous studies as a solvent may also be a potential reason. Additionally, the alteration of antioxidant enzyme activities and MDA contents in livers of mice after PFOA exposure were not improved by 4-PBA, though liver injury seemed to be significantly ameliorated by 4-PBA. These results also implied that disturbance of the antioxidant defense system in livers of mice exposed to PFOA for 28 days may not play a dominant role in PFOA-induced hepatotoxicity.

Accumulating evidence suggests an intriguing connection between the UPR and the hepatic lipid homeostasis [50]. Previous study has also reported on a coordinated, adaptive transcriptional response to hepatic ER stress in progressive human nonalcoholic fatty liver disease [51]. Interestingly, we found that 4-PBA weakened lipid accumulation in HepG2 cells after PFOA exposure and also alleviated lipid metabolism dysfunction in PFOA-exposed mice. Earlier research demonstrated that ER stress could induce hepatic steatosis by stimulating the expression of very low-density lipoprotein receptor (VLDLR) [52]. Our previous study also observed that VLDLR mRNA was significantly increased after PFOA exposure [22]; however, VLDLR expression was not improved by 4-PBA, which might result from the activation of PPARα by PFOA. SREBP activation plays an important role in ER stress-induced lipid metabolism dysfunction [50]. We also found that PFOA stimulated SREBP maturation resulting in SREBP activation, which could be attributed to the reduction in serum total cholesterol [22]. Surprisingly, mRNA levels of SREBP1 and familiar target genes of SREBPs, which were increased after PFOA exposure, were improved by 4-PBA, though the changes were modest. These results suggested an important role of ER stress in PFOA-induced hepatic lipid metabolism perturbation. Lipids, particularly saturated fatty acids, have been suggested to activate several intracellular responses contributing to lipotoxic stress in the ER of the liver [53]. Whether the lipid-like characteristics of PFOA also result in the occurrence of ER stress still needs further investigation.

A complicated connection between autophagy and ER stress has been suggested in previous research [54] and an incomplete autophagy response to hepatitis C is reportedly related to ER stress [55]. In the present study, cells or mice treated with chemical chaperone 4-PBA showed a significant reduction in the toxicological effects of PFOA, implying a critical role of ER stress in PFOA toxicity. However, autophagy manipulation did not significantly influence ER stress in HepG2 cells exposed to PFOA, which implied that autophagy disturbance might not be dominantly responsible for ER stress. Proteolytic activity as well as LC3 II and p62 accumulation were improved in HepG2 cells after PFOA exposure in the presence of 4-PBA, which indicated the potential effects of ER stress on autophagy disturbance after PFOA exposure. These results demonstrated a complicated connection between autophagy and ER stress, and more evidence is needed to elucidate whether ER stress leads to the disturbance of autophagy or the latter initiates the accumulation of proteins in cells, which ignites ER stress. In addition to complex pharmacological actions [56], whether 4-PBA ameliorated the toxicological effects of PFOA primarily as an ER stress inhibitor or through other possible modes of action needs further exploration. A possible schematic model of how 4-PBA affects PFOA-induced hepatotoxic effects is shown in Fig. 8C.

Our observations indicated that PFOA exposure induced ER stress and stimulated the UPR in mouse livers and HepG2 cells. However, neither autophagy manipulation nor antioxidant pretreatment influenced the UPR in PFOA-exposed HepG2 cells. It was intriguing to find that 4-PBA, a chemical chaperone, alleviated both ER stress and autophagosome accumulation *in vitro*. Cell cycle arrest, proteolytic activity impairment, and neutral lipid accumulation were also improved by 4-PBA in HepG2 cells after PFOA exposure. Indeed, several toxicological effects in the livers of mice attributed to PFOA exposure, including lipid metabolism dysfunction and liver injury, were also significantly improved in the presence of 4-PBA. Our results demonstrated that ER stress played an important role in PFOA-induced hepatotoxic effects and that chemical chaperone 4-PBA was able to ameliorate these effects.

Competing financial interests

The authors declare there are no conflicts of interest.

Acknowledgments

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB14040202) and the National Natural Science Foundation of China (Grants 31320103915, 21277143 and 21377128)

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.06.043.

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