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MOLECULAR TOXICOLOGY

High perfluorooctanoic acid exposure induces autophagy blockage and disturbs intracellular vesicle fusion in the liver

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Abstract Perfluorooctanoic acid (PFOA) has been shown to cause hepatotoxicity and other toxicological effects. Though PPARα activation by PFOA in the liver has been well accepted as an important mechanism of PFOA-induced hepatotoxicity, several pieces of evidence have shown that the hepatotoxic effects of PFOA may not be fully explained by PPARα activation. In this study, we observed autophagosome accumulation in mouse livers as well as HepG2 cells after PFOA exposure. Further in vitro study revealed that the accumulation of autophagosomes was not caused by autophagic flux stimulation. In addition, we observed that PFOA exposure affected the proteolytic activity of HepG2 cells while significant dysfunction of lysosomes was not detected. Quantitative proteomic analysis of crude lysosomal fractions from HepG2 cells treated with PFOA revealed that 54 differentially expressed proteins were related to autophagy or vesicular trafficking and fusion. The proteomic results were further validated in the cells in vitro and livers in vivo after PFOA exposure, which implied potential dysfunction at the late stage of autophagy. However, in HepG2 cells, it seemed that further inhibition of autophagy did not significantly alter the effects of PFOA on cell viability. Although these findings demonstrate that PFOA blocked autophagy and disturbed intracellular vesicle fusion in the liver, the changes in autophagy were observed only at high cytotoxic concentrations of PFOA, suggesting that autophagy may not be a primary target or mode of toxicity. Furthermore, since altered liver autophagy was not observed at concentrations of PFOA associated with human exposures, the relevance of these findings must be questioned.

Keywords Perfluorooctanoic acid · Autophagy · Proteome · Vesicle fusion

Introduction

Perfluoroalkyl acids (PFAAs) are widely used anthropogenic compounds, which have been incorporated into many products over the past six decades due to their unique physical and chemical characteristics (Lau et al. 2007; Lindstrom et al. 2011). Because many of these compounds fit the defining characteristics of persistent organic pollutants (POPs), concerns regarding their impact on human health and the environment have increased (Lindstrom et al. 2011). As one of the most widely known PFAAs, the toxicological effects of perfluorooctanoic acid (PFOA) have been extensively studied, with hepatotoxicity found in rodents even at low-dose exposure (Lau et al. 2007; Yan et al. 2014). Medical surveillance studies have also found PFOA exposure to be positively associated with serum alanine aminotransferase (ALT) levels and negatively related to serum high-density lipoprotein (HDL) levels in fluorochemical production workers (Gallo et al. 2012; Wang et al. 2012). Activation of peroxisome proliferator-activated receptor α (PPARα) has been widely accepted as a critical mechanism

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for PFOA-induced hepatomegaly and disturbance of lipid metabolism (Lau et al. 2007; Takacs and Abbott 2007), and our previous study observed significant activation of PPARα in livers of mice exposed to PFOA for 28 days (Yan et al. 2015). However, liver weight increase as well as perturbation of fatty acid metabolism still could be observed in PFOA-exposed PPARα-null mice (Minata et al. 2010; Rosen et al. 2008; Wolf et al. 2008), and this evidence implies that there may be potential mechanisms other than PPARα activation in PFOA-induced hepatotoxicity.

Autophagy is an evolutionarily conserved catabolic cellular process dependent on lysosomes for its execution and includes macroautophagy, microautophagy, and chaperone-mediated autophagy (Kim and Lee 2014; Malhi et al. 2010). Macroautophagy (referred to as autophagy hereafter) is characterized by the formation of a double-membrane-bound structure that sequesters cellular organelles or cytoplasm into a structure called the autophagosome, which then fuses with the lysosome for degradation of the sequestered material (Kim and Lee 2014; Malhi et al. 2010). At least four major protein complexes are required for the formation of autophagosomes: Atg1–Unc-51-like kinase (ULK) complex, which initiates formation; class III phosphatidylinositol 3-kinase (PI3K) or Vps34 complexes, which are required for autophagosome nucleation; autophagy-related (Atg) protein 9 (Atg9) and its trafficking machinery, which may play an important role in delivering vesicles and lipids to expand the autophagosome membranes at all stages; and ubiquitin-like proteins Atg12 and Atg8/LC3 and their conjugation machinery, which are required for autophagosome expansion and closure (Dall'Armi et al. 2013; Lamb et al. 2013).

Autophagy occurs at low basal levels in most cells and contributes to the regular turnover of cytoplasmic components (Rautou et al. 2010; Shintani and Klionsky 2004). It can be rapidly induced by a change in environmental conditions, such as nutrient depletion, growth factor withdrawal, or high bioenergetic demands (Rautou et al. 2010; Shintani and Klionsky 2004). In conditions of nutrient deprivation, several cellular signaling pathways are modulated upon different nutrient deficiencies, which lead to the initiation of autophagosome formation (Kim and Lee 2014). Autophagy has also been suggested to interplay with apoptosis, which has been well reviewed elsewhere (Delgado et al. 2014; Marino et al. 2014). Evidence from mice with systemic or tissue-specific deletion of autophagy-related (Atg) genes suggests that autophagy dysfunction might contribute to certain disease phenotypes, such as cancer, neurodegeneration, liver disease, and metabolic disease (Jiang and Mizushima 2014; Shintani and Klionsky 2004). The possible roles of autophagy in health and disease can serve to protect cells but may also contribute to cell damage (Shintani and Klionsky 2004).

Earlier reviewed studies suggest that autophagy also plays an important physiological role in the liver, particularly in regard to energy and nutrient balance, misfolded proteins removal, and major subcellular organelle turnover under normal and pathophysiological conditions (Rautou et al. 2010; Yin et al. 2008). However, little is known about the connection between autophagy and the toxicological effects of PFOA. In the present study, we assessed the occurrence of autophagy and its potential relevance to hepatotoxic effects after PFOA exposure. We demonstrated that the dysfunction of lysosome–autophagosome fusion led to the blockage of autophagy after PFOA exposure.

Materials and methods

Animal treatment

Male Balb/c mice (aged 6–8 weeks) were purchased from Beijing Vital River Experimental Animals Centre (Beijing, China), with all experimental manipulations described in our previous study (Yan et al. 2014). Briefly, mice were randomly divided into six groups and dosed with either Milli-Q water or PFOA diluted in Milli-Q water at doses of 0.08, 0.31, 1.25, 5, and 20 mg/kg/day via gavage for 28 days. The PFOA doses were chosen based on earlier toxicological studies and our previous experiments (Yan et al. 2014). All animal treatments were approved by the Committee on the Ethics of Animal Experiments from the Institute of Zoology, Chinese Academy of Sciences (Permit Number: EET-015-08-2012) and in accordance with the Guiding Principles in the Use of Animals in Toxicology, which were adopted by the Society of Toxicology in 1989.

Full materials and methods were described in supplementary information.

Results

PFOA exposure induced autophagosome accumulation

Consistent with the serum ALT levels in our previous report (Yan et al. 2014), the histopathology results with H&E staining also showed significant hepatocyte swelling and lipid deposits in mice after 28-day exposure, especially at high doses of PFOA (Fig. S1). Microtubule-associated protein light chain 3 (LC3) conversion (LC3 I to LC3 II) was detected by Western blotting to investigate the occurrence of autophagosome accumulation. Both LC3A II and LC3B II were accumulated in livers of mice after 28-day exposure to PFOA, especially at the middle dose of PFOA (Fig. 1a, b). Several autophagy-related genes, especially Atg12, were stimulated in mouse livers by PFOA (Fig. S2).

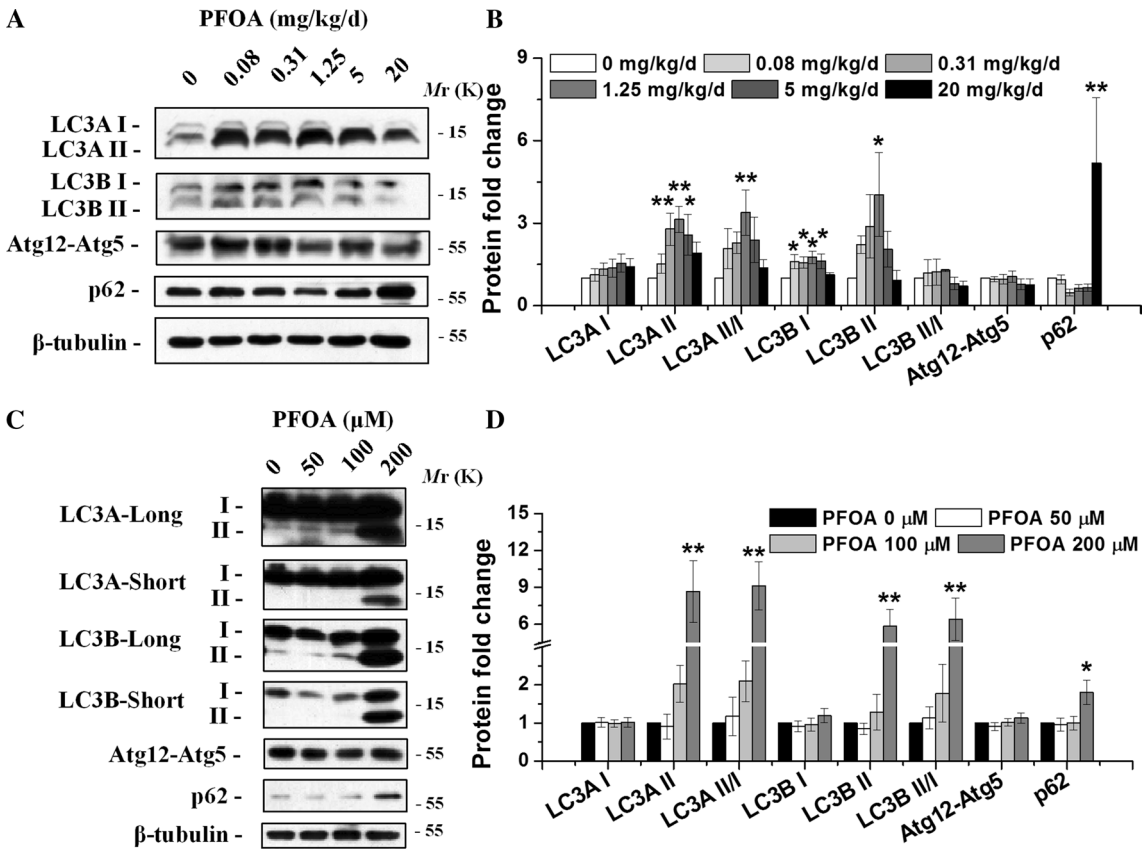


Fig. 1 PFOA exposure induced autophagosome accumulation in mouse livers and HepG2 cells. **a** LC3A, LC3B, Atg12–Atg5, and p62 protein levels were analyzed from whole lysates of livers after 28 days of exposure to PFOA. Band densities of proteins were quantified and shown in (**b**). **c** LC3A, LC3B, Atg12–Atg5, and p62 protein levels were analyzed from whole lysates of HepG2 cells after 72 h of exposure to PFOA. Long indicates the representative images of long exposures, and short indicates short exposures. Band densities of proteins were quantified and shown in (**d**). Data were mean ± SE; $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$

Protein levels of Atg12–Atg5 conjugate and sequestosome 1 (SQSTM1/p62) were further examined. There was no significant change in the protein levels of Atg12–Atg5 conjugate (Fig. 1a, b). However, p62, a selective substrate incorporated into autophagosomes, was accumulated in the livers of mice dosed with 20 mg/kg/day of PFOA (Fig. 1a, b). In the HepG2 cells, according to our MTT results (Fig. S3), we chose PFOA at doses of 50, 100, and 200 μM for further study. Autophagosome accumulation was also observed in HepG2 cells and was significant when dosed with 200 μM PFOA for 72 h (Fig. 1c, d, data for 24- and 48-h exposure not shown). Similar to PFOA-exposed mouse livers, levels of Atg12–Atg5 conjugate were not altered by PFOA in HepG2 cells but p62 showed significant accumulation in cells after being exposed to PFOA for 72 h at 200 μM (Fig. 1c, d).

PFOA exposure did not induce autophagic flux

Immunofluorescence analysis further confirmed LC3B-positive puncta were accumulated in HepG2 cells after PFOA exposure (Fig. 2a). Quantification of the confocal images showed that PFOA exposure significantly amplified the size but not the number of LC3B-positive puncta (Fig. 2b). To assess whether PFOA exposure induced autophagic flux, lysosomal protease inhibitor CQ was used to inhibit autolysosomal degradation. The levels of LC3B II were not significantly changed in HepG2 cells treated with CQ for 72 h in the absence or presence of PFOA (Fig. 2c). To further verify the effect of PFOA on autophagic flux, another autophagy inhibitor bafilomycin A1 (BafA1) was used to block autophagic flux and the results suggested that the levels of LC3B II were not significantly changed in HepG2 cells treated with BafA1 for 72 h in the absence or presence of PFOA (Fig. 2d). These results indicated that PFOA exposure may not induce autophagic flux, and the accumulation of LC3 II may be due to the blockage of the autophagosome degradation step.

PFOA exposure impaired proteolytic activity

To investigate how PFOA exposure affected autophagy, we further examined the effects of PFOA on lysosomal

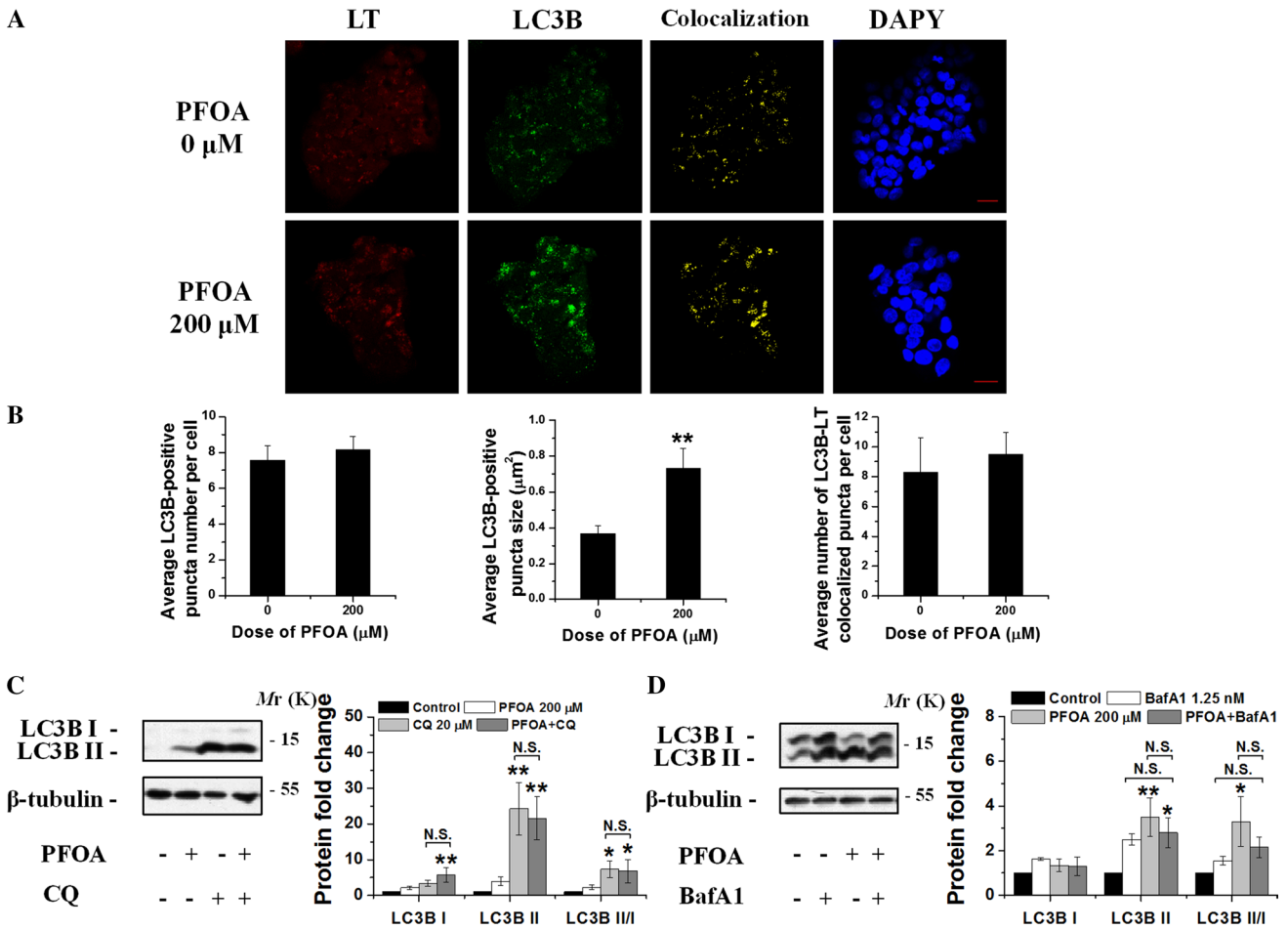


Fig. 2 PFOA exposure did not induce autophagic flux. **a** Endogenous LC3B was assessed under confocal microscopy. Lysosomal content was reflected by LysoTracker red (LT) staining, and DAPI staining was used to reflect the nucleus. *Scale bar* 20 μm. **b** Average LC3B-positive puncta number, average LC3B-positive puncta number, and average LC3B-LT colocalized puncta number from HepG2 cells were analyzed using Image-J software. **c** HepG2 cells were treated with PFOA (200 μM) in the absence or presence of chloroquine (CQ, 20 μM) for 72 h. Total cellular lysates were subjected to Western blotting for LC3B. Band densities of proteins were quantified and shown in the *right panel*. **d** HepG2 cells were treated with PFOA (200 μM) in the absence or presence of bafilomycin A1 (BafA1, 1.25 nM) for 72 h. Total cellular lysates were subjected to Western blotting for LC3B. Band densities of proteins were quantified and shown in the *right panel*. Data were assessed from three independent experiments and shown as mean ± SE; $*p < 0.05$; $**p < 0.01$; *N.S.* no significance

functions. Proteolytic activity was measured by staining HepG2 cells with DQ red BSA after PFOA exposure. Results showed that proteolytic activity was decreased in HepG2 cells treated with 200 μM PFOA for 72 h (Fig. 3a), which was also the dose at which p62 accumulation was observed (Fig. 1c, d). The activity of lysosomal hydrolase cathepsin B was significantly increased in HepG2 cells treated with 200 μM PFOA for 72 h (Fig. 3b). Acidic vesicles in cells exposed to PFOA for 72 h were detected and quantified by AO staining, with results indicating that acidic vesicles modestly increased at high doses of PFOA (Fig. 3c), and from the immunofluorescence analysis results (Fig. 2a), the LysoTracker staining also showed stronger after PFOA exposure especially in the sites where autophagosomes accumulated. These results implied that PFOA impaired proteolytic activity without causing inactivation of lysosomal proteolysis in HepG2 cells, and to some extent the cells may have overcompensated the blockage of autophagy after exposure to PFOA for 72 h. Cathepsin B activity increased at the dose of 1.25 mg/kg/day but decreased at 5 and 20 mg/kg/day in the livers of mice after PFOA exposure (Fig. S4A), implying that PFOA exposure at high doses for 28 days may repress hepatic lysosomal function in mice. Other than autophagy, the ubiquitin-proteasome system (UPS) is also a major intracellular protein degradation system (Nedelsky et al. 2008). We further assessed the activity of proteasome by ubiquitinated protein levels. Levels of ubiquitinated protein were changed

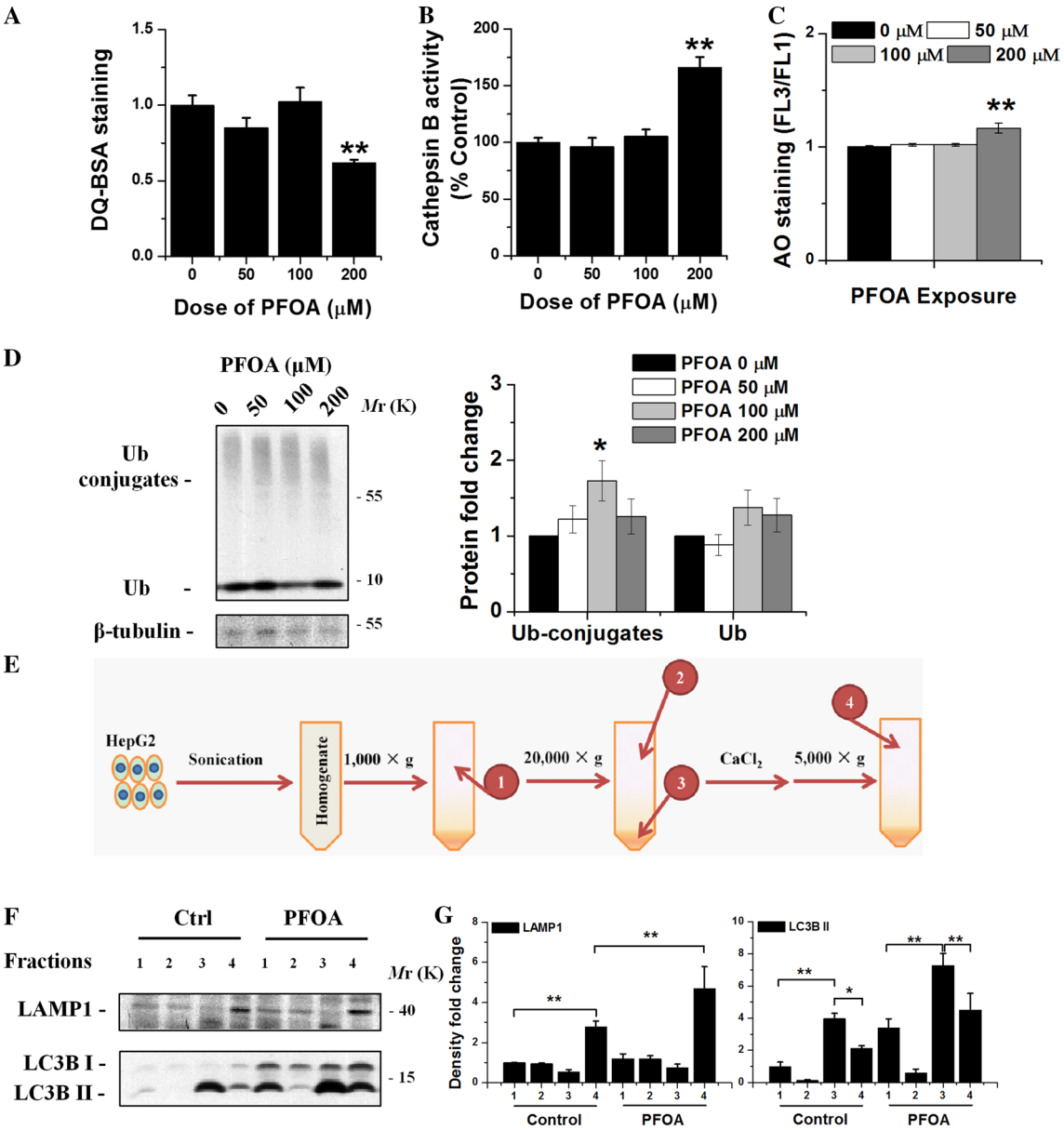


Fig. 3 PFOA exposure for 72 h reduced proteolytic activity in HepG2 cells. **a** HepG2 cells were exposed to PFOA for 72 h and then loaded with DQ-BSA (10 μg/mL) for 4 h. Fluorescence intensity from cleaved DQ-BSA was measured and adjusted with protein concentration, and results were normalized to the control group. **b** HepG2 cells were exposed to PFOA for 72 h, and total cellular lysates were incubated with z-RR-AMC for cathepsin B activity measurement. Fluorescence intensity was measured and adjusted with protein concentration, and results were normalized to the control group. **c** Fold change of acidic vesicles in HepG2 cells exposed to PFOA for 72 h calculated by fluorescence intensity of red and green fluorescences (FL3/FL1) after acridine orange (AO) staining. **d** HepG2 cells were exposed to PFOA for 72 h. Total cellular lysates were subjected to Western blotting for ubiquitinated protein levels. **e** Flow diagram for subcellular fractionation, with numbered fractions collected and stored for further study. **f** HepG2 cells were treated with PFOA for 72 h, and subcellular fractions were prepared. Subcellular fraction lysates were subjected to Western blotting for LAMP1 and LC3B. Band densities of proteins from Western blotting were quantified and shown in (**g**). Band densities of proteins were quantified and shown in the right panel. Data were assessed from three independent experiments and shown as mean ± SE; $*p < 0.05$; $**p < 0.01$

in HepG2 cells after PFOA exposure, but the density fold change increased modestly and was only significant in the 100 μM PFOA group after treatment for 72 h (Fig. 3d). Considering that proteolytic activity was significantly reduced in the 200-μM PFOA group after treatment for 72 h, the modest dysfunction of proteasome may not play a dominant role in the proteolytic activity reduction in HepG2 cells after PFOA exposure. We also analyzed the ubiquitinated protein levels in mouse livers after PFOA exposure, and the results showed no significant change (Fig. S4B).

PFOA exposure altered the expression of cellular vesicle traffic and fusion proteins

Subcellular fractionation of HepG2 cells was carried out following PFOA exposure at 200 μM for 72 h (Fig. 3e). Acid phosphatase activity was analyzed in CLFs and purified lysosomal fractions from HepG2 cells, which showed that lysosomes were enriched after purification (Fig. S5A). Cathepsin B activity from the subcellular fractions of the HepG2 cells showed no cathepsin B release from the lysosomes (Fig. S5B), which suggested that lysosomal membrane permeabilization did not occur in the HepG2 cells after PFOA exposure under the experimental conditions. LAMP1 and LC3 were also examined to assess the distribution of lysosomes and autophagosomes using Western blotting, respectively. Results indicated that autophagosomes were enriched in the CLFs from HepG2 cells (Fig. 3f, g).

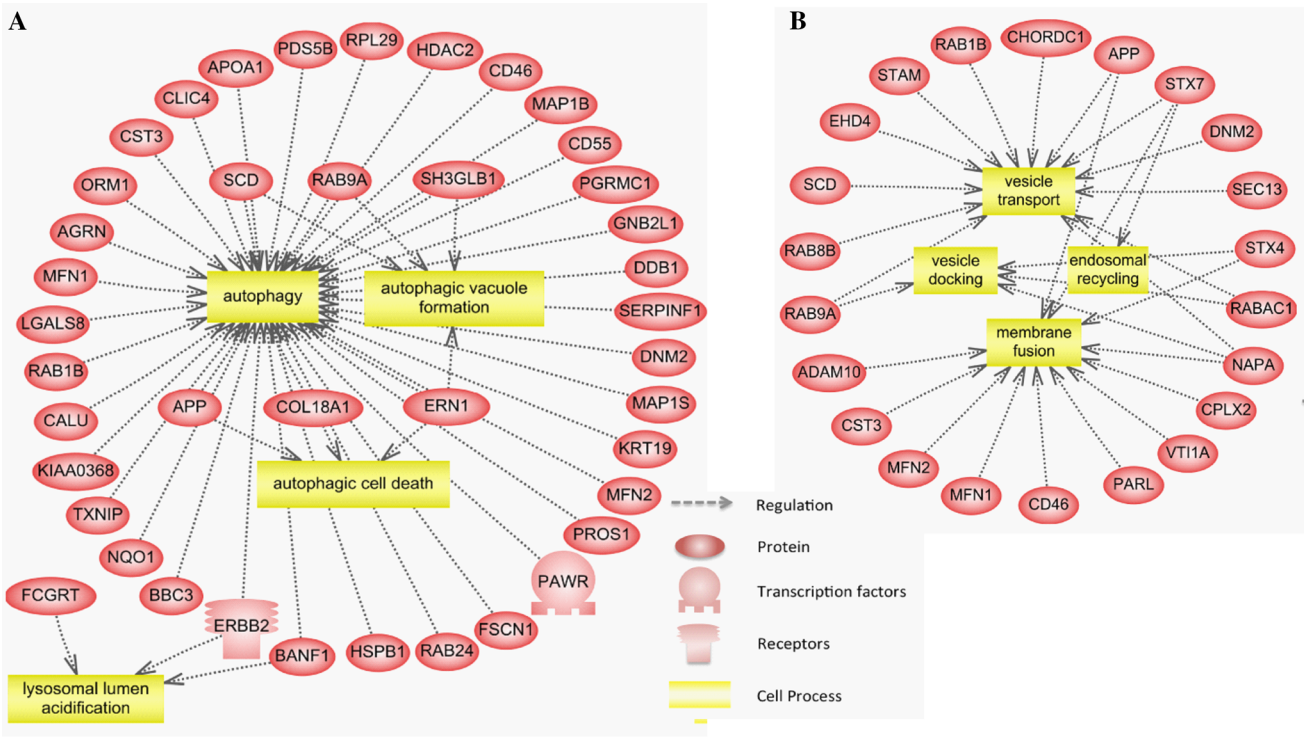
To explore the potential mechanism of autophagosome accumulation induced by PFOA, protein profiles of CLFs from HepG2 cells were analyzed using iTRAQ. From 4890 proteins identified by iTRAQ, there were 698 differentially expressed proteins, including 321 upregulated and 377 downregulated (Table S3). The differentially expressed proteins were further analyzed for their connection to biological processes, with results showing that 41 proteins were correlated with processes involved in autophagy and 22 proteins were related to vesicular trafficking and fusion (Fig. 4a, b). The results also suggested that 9 proteins were related to both autophagy and vesicle fusion, and these 54 differentially expressed proteins as well as their fold changes are listed in Fig. 5c. From the subunits of proton-pumping vacuolar-type ATPase (V-ATPase) identified by iTRAQ in our present study, V-ATPase V_0 *a3* (TCIRG1), which functions in vesicle fusion and autophagy (Belaid et al. 2013; Sun-Wada et al. 2006), was significantly decreased after PFOA exposure, though no significant level change was observed in other subunits (Fig. S6). We then chose *N*-ethylmaleimide-sensitive factor attachment protein α (NAPA) as well as two proteins that increased after PFOA exposure including vesicle transport through interactions with target membrane soluble *N*-ethylmaleimide attachment protein receptors (t-SNAREs), homolog 1A (VTI1A), and syntaxin 4 (STX4) to validate the iTRAQ results using western blot. Results from the subcellular fractions of the HepG2 cells were consistent with the iTRAQ results (Fig. 5a, b). These proteins were further analyzed in total lysates from both HepG2 cells and mouse liver tissues after PFOA exposure. The contents of these proteins were altered in the HepG2 cell lysates after 72 h of PFOA exposure, with changes similar to those observed in CLFs, except VTI1A, and NAPA protein level was reduced even at the PFOA dose of 100 μM (Fig. 5c). STX4 and VTI1A levels were significantly decreased in the livers of mice exposed to PFOA at 1.25 mg/kg/day and higher doses, and liver NAPA protein level was increased even at very low doses of PFOA (Fig. 5d). These results suggested that PFOA might disturb vesicular trafficking and fusion both in vitro and in vivo.

Autophagy inhibition did not change the effects of PFOA on cell viability

Given the significant dysfunction of autophagy after PFOA exposure both in vivo and in vitro, we investigated whether the dysfunction was responsible for cell viability. Viability of HepG2 cells was increased at low doses of PFOA after 24 and 48 h but was reduced at 200 μM PFOA after 48 h and longer compared with the control groups at each time point (Figs. S3 and S7A). However, no significant cell death, as indicated by cytotoxicity, was observed after PFOA exposure for 72 h (Fig. S7B). Further analysis suggested that the decrease in cell viability may be due to cell cycle arrest in G0/G1, indicating that cell growth was inhibited by PFOA (Fig. S7C). Cell viability was significantly decreased in the presence of 40 μM CQ after exposure to 200 μM PFOA for 72 h, and there were no antagonistic or synergistic effects on the viability of HepG2 cells between PFOA and CQ lower than 20 μM (Fig. S7D). CQ induced significant cell death, but the cytotoxicity of CQ and PFOA on HepG2 cells also showed no antagonistic or synergistic effects (Fig. S7E). The Atg12–Atg5 conjugate protein in HepG2 cells was reduced by small interfering RNA (siRNA) of Atg5 after 72-h treatment (Fig. 6a, b). Compared with cells transfected with negative siRNA, LC3B II expression was not significantly altered in cells transfected with siAtg5 and not treated with PFOA, which indicated a potential recover after cultured for 72 h and PFOA may disturb the recover (Fig. 6a, c). Atg5 knockdown induced a modest reduction in cell viability as well as increased cytotoxicity, and no significant change in either viability or cytotoxicity was observed in HepG2 cells transfected with negative siRNA (Neg siRNA) or siAtg5 after PFOA exposure (Fig. 6d, e). These results suggested that further inhibition of autophagy did not influence the effects of PFOA on cell viability, and the reduction in cell viability compared with the control group in HepG2 cells may not be directly attributable to autophagy dysfunction.

Discussion

Autophagy is a crucial cellular pathway for many physiological and pathological processes in the liver (Rautou et al. 2010; Yin et al. 2008). In this study, we found that autophagosomes were accumulated in both mouse liver and HepG2 cells in response to PFOA exposure and that



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Gene Name	Fold Change	Gene Name	Fold Change	Gene Name	Fold Change
ADAM10	0.74 ± 0.03	ERBB2	0.62 ± 0.09	PDS5B	0.84 ± 0.09
AGRN	0.67 ± 0.07	ERN1	1.79 ± 0.04	PGRMC1	0.82 ± 0.04
APOA1	0.49 ± 0.07	FCGRT	0.66 ± 0.01	PROS1	0.26 ± 0.15
APP	0.79 ± 0.13	FSCN1	0.72 ± 0.05	RAB1B	0.81 ± 0.08
BANF1	0.58 ± 0.17	GNB2L1	0.68 ± 0.22	RAB24	1.36 ± 0.04
BBC3	1.69 ± 0.15	HDAC2	0.81 ± 0.14	RAB8B	0.72 ± 0.06
CALU	0.80 ± 0.09	HSPB1	0.79 ± 0.08	RAB9A	1.42 ± 0.08
CD46	0.79 ± 0.02	KRT19	0.52 ± 0.22	RABAC1	0.71 ± 0.17
CD55	2.60 ± 0.45	LGALS8	0.71 ± 0.03	RPL29	1.70 ± 0.48
CHORDC1	1.21 ± 0.17	MAP1B	1.46 ± 0.25	SCD	0.43 ± 0.13
CLIC4	0.75 ± 0.06	MAP1S	0.75 ± 0.12	SEC13	0.82 ± 0.10
COL18A1	0.59 ± 0.11	MFN1	1.65 ± 0.24	SERPINF1	0.54 ± 0.07
CPLX2	2.88 ± 0.17	MFN2	1.64 ± 0.06	SH3GLB1	1.43 ± 0.11
CST3	0.30 ± 0.05	NAPA	0.74 ± 0.02	STAM	1.28 ± 0.13
DDB1	0.78 ± 0.02	NQO1	1.28 ± 0.11	STX4	1.33 ± 0.23
DNM2	0.74 ± 0.02	ORM1	0.61 ± 0.06	STX7	1.25 ± 0.13
KIAA0368	1.41 ± 0.18	PARL	1.61 ± 0.03	TXNIP	0.74 ± 0.10
EHD4	1.30 ± 0.09	PAWR	1.41 ± 0.04	VTI1A	1.29 ± 0.09

Fig. 4 PFOA exposure changed the protein profile of crude lysosomal fractions. Crude lysosomal fractions were prepared from HepG2 cells after 72-h exposure to PFOA, and proteomes were analyzed using iTRAQ. **a** Network of proteins related to autophagy differentially expressed in crude lysosomal fractions of HepG2 cells exposed to PFOA. **b** Network of proteins related to cellular vesicle traffic and fusion differentially expressed in crude lysosomal fractions of HepG2 cells exposed to PFOA. **c** Protein fold change from iTRAQ analysis, shadowed characters are proteins related to autophagy, and red characters are proteins related to cellular vesicle traffic and fusion. Data were assessed from three independent samples from each treatment group and shown as mean ± SD

the blockage of autophagy at the degradation stage may contribute to this. Our previous study suggested the PFOA level in mouse serum in the 0.08 mg/kg/d group was similar to the median serum level of PFOA observed in occupational participants (2.24 vs. 1.64 μg/mL; Wang et al. 2012; Yan et al. 2014). From the LC3 expression results in the mouse liver, autophagosome accumulation occurred after 28-day PFOA exposure, even at the dose of 0.08 mg/kg/day. However, no other results were significantly affected by PFOA in mouse livers at the dose of 0.08 mg/kg/day,

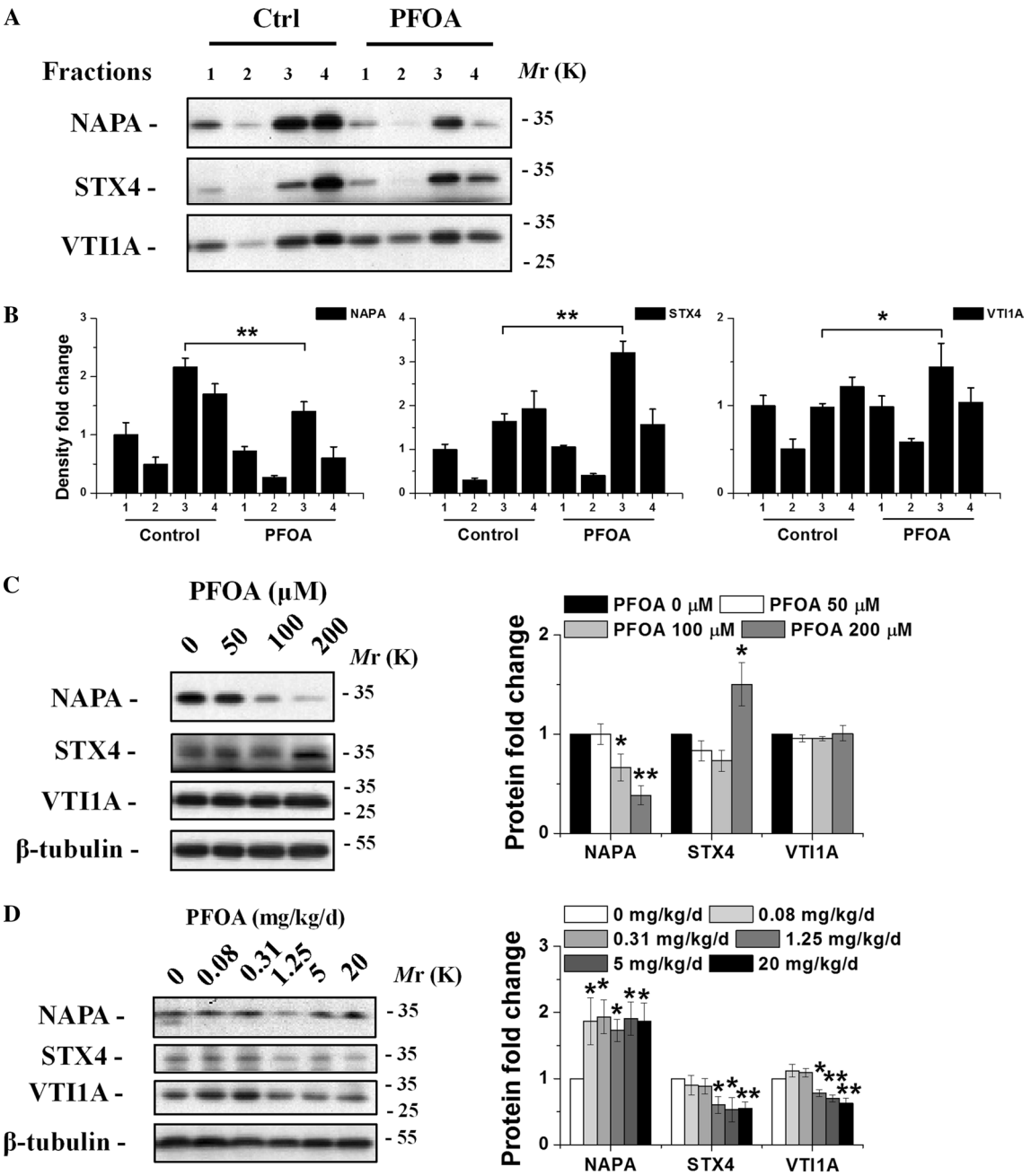

Fig. 5 PFOA exposure altered expression levels of proteins related to cellular vesicle traffic and fusion in crude lysosomal fractions. **a** Subcellular fraction lysates from HepG2 cells (exposed to PFOA at 200 μM for 72 h) were subjected to Western blotting for NAPA, syntaxin 4 (STX4), and VTI1A. Band densities of proteins were quantified and shown in (**b**). **c** NAPA, STX4, and VTI1A expression in HepG2 cell lysates after exposed to PFOA for 72 h ($n = 3$). Band densities of proteins were quantified and shown in the *right panel*. **d** NAPA, STX4, and VTI1A expression in liver lysates of mice after exposed to PFOA for 28 days ($n = 3$). Data were mean ± SE; $^{*}p < 0.05$; $^{**}p < 0.01$

and in vitro study using HepG2 cells also suggested that autophagy disturbance by PFOA occurred with significant reduction in cell viability only at a very high dose, such as one far higher 10 nM, the reported human serum level (Fromme et al. 2009). Thus, one has to admit, the observed effects of PFOA on autophagy are helpful in understanding the potential biomolecular mechanisms of hepatotoxicity induced by high doses of PFOA, but do not seem to be of practical relevance when put in context of human exposure, including occupational exposure.

Autophagosome formation and maturation is highly regulated by a series of distinct steps controlled by

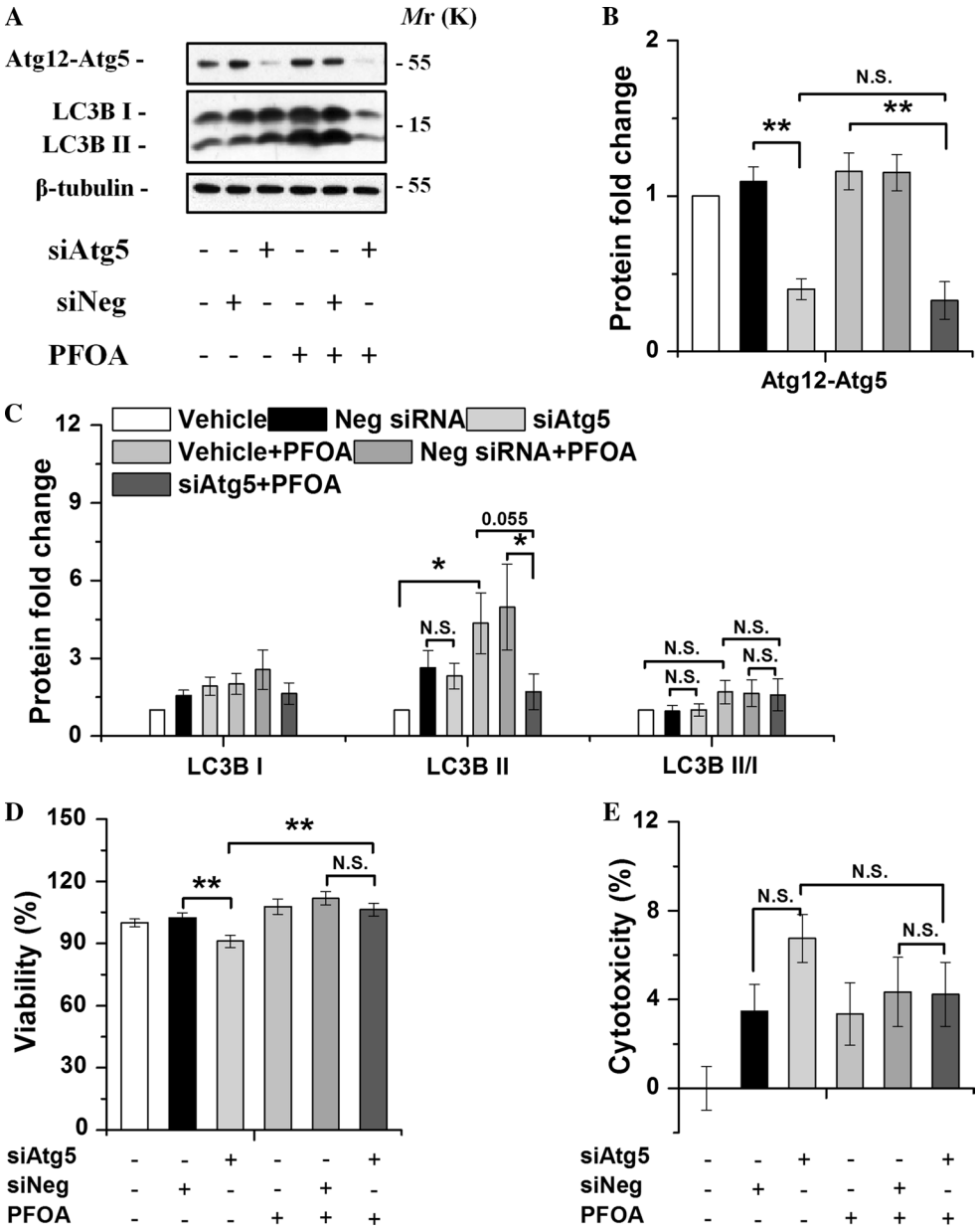

Fig. 6 Atg5 knockdown did not influence the reduction in cell viability induced by PFOA. **a** HepG2 cells were transfected with Atg5 siRNA (siAtg5) or nontargeting siRNA (Neg siRNA) for 24 h and then treated with PFOA (200 μM) for 72 h. Total cellular lysates were subjected to Western blotting for Atg12–Atg5 and LC3B. Band densities of proteins were quantified and shown (**b**, **c**). **d** Cell viability of HepG2 cells transfected with siAtg5 or Neg siRNA for 24 h and then treated with PFOA (200 μM) for 72 h. **e** Cytotoxicity of HepG2 cells transfected with siAtg5 or Neg siRNA for 24 h and then treated with PFOA (200 μM) for 72 h. Data were assessed from three independent experiments and shown as mean ± SE; $^{*}p < 0.05$; $^{**}p < 0.01$; *N.S.* no significance

autophagy-related proteins, and ultimately, the autophagosome is degraded after fuses with endocytic and lysosomal compartments (Kenific and Debnath 2015). The lysosome is the major digestive organelle in most eukaryotic cells, which is involved in a series of biological processes including cell death, signal transduction, and autophagy (Shen and Mizushima 2014). The acidic lumen (pH 4.5–5.0) of the lysosome is important to the activity of acid hydrolases in it, and its basification always leads to lysosome dysfunction and also a reason for autophagy dysfunction (Klionsky et al. 2012; Mindell 2012). In this study, we found PFOA exposure induced autophagosome accumulation in both mouse livers in vivo and HepG2 cells in vitro. However, there was no more accumulation of autophagosomes in cells cotreated with PFOA and neither lysosomotropic agent chloroquine nor V-ATPase inhibitor bafilomycin A1 (BafA1), which reduces vesicle acidification and also blocks the fusion between autophagosomes and lysosomes in cultured mammalian cells, was found to accumulate (Mauvezin et al. 2015). These results indicated that PFOA exposure may result in autophagy blockage, and from the activity of cathepsin B and the change of acid vesicles in cells after PFOA exposure, lysosome inactivation may not the reason for autophagy blockage.

During the processes of autophagy, autophagosome–lysosome fusion is important for the degradation of autophagosome content, and emerging evidences suggest several groups of proteins involved in membrane fusion

also play a role in late-stage autophagy (Shen and Mizushima 2014). From the results of quantitative proteomic analysis in CLFs from HepG2 cells, we found PFOA exposure altered the contents of factors involved in vesicular trafficking and fusion, including components of SNAREs. SNAREs are suggested as key components of protein complexes that drive membrane fusion, and their effects on lysosome fusion and autophagy have also been identified (Jahn and Scheller 2006; Luzio et al. 2007; Shen and Mizushima 2014). NAPA is a key regulator of SNARE-mediated vesicle fusion and functions in SNARE complex disassembly, with previous research showing that loss of NAPA induces autophagosome accumulation (Jahn and Scheller 2006; Naydenov et al. 2012). In the present study, NAPA was reduced in the CLFs as well as total lysates of HepG2 cells after PFOA exposure. In mouse livers, NAPA expression was also altered after PFOA exposure, although it was increased. These results suggested that PFOA might interfere with SNARE-mediated vesicle fusion, but the effect seems contradictory in vitro and in vivo. Indeed, vesicular trafficking and fusion is an important biological process for membrane-enclosed organelles in eukaryotic cells to communicate with each other (Bonifacino and Glick 2004; Jahn and Scheller 2006). Our results also demonstrated that PFOA exposure reduced proteolytic activity without significant lysosome and proteasome inactivation in HepG2 cells, which implies that interference of factors driving vesicular trafficking and fusion may result in the reduction in proteolytic activity as well as autophagy blockage after PFOA exposure.

Other than SNAREs and their cofactors, which drive most fusion in eukaryotes, several studies suggest that V-ATPase functions in trafficking or fusion (Jahn and Scheller 2006; Luzio et al. 2007). V-ATPase is composed of 14 different subunits and plays a critical role in pH regulation of intracellular organelles (Forgac 2007). In addition to functions in vacuolar acidification, V-ATPase, especially the V_0 domain, has a dramatic effect on vacuolar fusion. The lack of a V_0 domain subunit in *Drosophila melanogaster* has been shown to block vesicle fusion at a step downstream of *trans*-SNARE pairing without significant pH alteration (Forgac 2007; Hiesinger et al. 2005). Furthermore, a lack of TCIRG1 in mice results in defective insulin secretion and autophagosome accumulation (Belaid et al. 2013; Forgac 2007; Marshansky and Futai 2008). In the present study, we found autophagosome accumulation in cells after PFOA exposure, though no significant change was observed in the colocalized puncta number of autophagosomes and lysosomes. The results seemed like PFOA exposure did not affect the tethering of autophagosomes and lysosomes but influenced their fusion, which were unexpectedly consistent with previous studies on V-ATPase (Belaid et al. 2013; Forgac 2007; Marshansky and Futai 2008) and indicated a potential role of TCIRG1 in PFOA toxicity. However, as the mechanisms of V-ATPase on lysosomal fusion and autophagy still remain unclear (Mauvezin et al. 2015) and the downregulation of TCIRG1 in our iTRAQ results seem very modest, whether loss of TCIRG1 after PFOA exposure is involved in autophagy blockage in HepG2 cells in vitro and in mouse livers in vivo still needs further study.

PFOA reduced the cell viability of HepG2, which seemed to result from cell growth inhibition but not cell death induced by PFOA. Autophagy has been suggested to play dual roles in cell life and death (Baehrecke 2005). Apoptosis is a well-known type of programmed cell death, and several reports have suggested a complex relationship between autophagy and apoptosis (Delgado et al. 2014; Marino et al. 2014). In the majority of previous evidence, apoptosis and autophagy are mutually inhibitory and autophagy tended to be anti-apoptotic rather than pro-apoptotic (Marino et al. 2014). Chloroquine has been suggested to inhibit autophagy and trigger apoptosis as well as certain genetic or pharmacologic inhibition of autophagy (Boya et al. 2005). In consistent with previous study, we also found that CQ significantly reduced cell viability and increased cell death. Knockdown of Atg5 also demonstrated significant reduction in cell viability, although cytotoxicity exhibited no significant change. However, PFOA exposure did not induce cell death even though significant autophagy blockage was observed in vitro. Lysosomes play critical roles in autophagy and have also been suggested to participate in cell death control with autophagy (Kroemer and Jaattela 2005). In our results in vitro, we found no significant dysfunction in cellular lysosomes after PFOA exposure, which may partially contribute to autophagy dysfunction without cell death after PFOA exposure. Cell cycle arrest resulting in cell growth inhibition appeared to contribute to the reduction in cell viability compared with the control group after PFOA exposure in HepG2 cells, but further inhibition of autophagy could not further reduce cell viability. Earlier research suggested that cell cycle arrest in G0 and autophagy often occurred in the same cell, but the relationship between these two processes is still not clear (Valentin and Yang 2008). Hence, more evidence is needed to clarify the correlation between autophagy and cell cycle arrest.

Hepatomegaly and dysregulation of hepatic genes related to lipid metabolism were evident in rodents after PFOA exposure (Lau et al. 2007; Yan et al. 2015). We also found neutral lipids accumulation in HepG2 cells after PFOA exposure for 72 h, especially at the dose in which autophagosomes were also accumulated (data not shown). Furthermore, PFOA-induced liver hepatotoxicity appears to result from acting as a PPAR ligand; however, results from PFOA-exposed PPARα-null mice argue against the simple toxicological effects of PFOA through PPARα

activation (Rosen et al. 2008; Wolf et al. 2008). In addition to significant lipid accumulation in PFOA-exposed cells, earlier research has indicated that autophagy can regulate lipid metabolism (Singh et al. 2009), suggesting a probable connection between autophagy and lipid accumulation attributable to PFOA exposure. Previous studies have also shown an elegant regulation of autophagy by nutrient-sensing nuclear receptors, including PPARα and farnesoid X receptor (Lee et al. 2014; Seok et al. 2014). In this study, we observed that several autophagy-related genes were induced by PFOA exposure in livers, but other results demonstrated that the blockage of the autophagosome degradation step played a dominant role in autophagy dysfunction from PFOA exposure. Additionally, further blockage of autophagy seemed to have not significantly changed the effects of PFOA on cell viability, which implied autophagy may not play a dominant role in PFOA-induced hepatotoxicity. Thus, the relationship between PPARα activation and autophagy blockage in mouse livers and HepG2 cells after PFOA exposure still needs further exploration.

Our observations indicated that PFOA exposure induced autophagosome accumulation. Additional in vitro evidence suggested that autophagosome accumulation may be not the result of autophagy activation, but may be caused by the blockage of autophagy at the degradation step. Proteomic analysis showed that PFOA disturbed protein profiles of CLFs from HepG2 cells, and 63 differentially expressed proteins were related to autophagy or vesicular trafficking and fusion, which revealed that a disturbance of vesicular trafficking and fusion after PFOA exposure may contribute to autophagy blockage. Although these findings demonstrate that PFOA blocked autophagy and disturbed intracellular vesicle fusion in the liver, the changes in autophagy were observed only at high cytotoxic concentrations of PFOA, suggesting that autophagy may not be a primary target or mode of toxicity. Furthermore, since altered liver autophagy was not observed at concentrations of PFOA associated with human exposures, the relevance of these findings must be questioned.

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Compliance with ethical standards

Conflict of interest The authors declare there are no conflicts of interest.

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