Enhanced cytotoxicity of pentachlorophenol by perfluorooctane sulfonate or perfluorooctanoic acid in HepG2 cells

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HIGHLIGHTS

• PFOS and PFOA could enhance the cytotoxicity of PCP to HepG2 cells.
• PFOS displayed stronger synergistic cytotoxicity with PCP than PFOA.
• Cytotoxic enhancement might be via strengthening the phosphorylation uncoupling of PCP.
• PFOS and PFOA might cause membrane disruption and improve the uptake of PCP.

ABSTRACT

Chlorinated phenols and perfluoroalkyl acids (PFAAs) are two kinds of pollutants which are widely present in the environment. Considering liver is the primary toxic target organ for these two groups of chemicals, it is interesting to evaluate the possible joint effects of them on liver. In this work, the combined toxicity of pentachlorophenol (PCP) and perfluorooctane sulfonate (PFOS) or perfluorooctanoic acid (PFOA) were investigated using HepG2 cells. The results indicated that PFOS and PFOA could strengthen PCP’s hepatotoxicity. Further studies showed that rather than intensify the oxidative stress or promote the biotransformation of PCP, PFOS (or PFOA) might lead to strengthening of the oxidative phosphorylation uncoupling of PCP. By measuring the intracellular PCP concentration and the cell membrane properties, it was suggested that PFOS and PFOA could disrupt the plasma membrane and increase the membrane permeability. Thus, more cellular accessibility of PCP was induced when they were co-exposed to PCP and PFOS (or PFOA), leading to increased cytotoxicity. Further research is warranted to better understand the combined toxicity of PFAAs and other environmental pollutants.

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1. Introduction

Pentachlorophenol (PCP) and other chlorinated phenols are widely present in the environment. PCP has been used throughout the world for purposes as varied as antimicrobial agent and wood preservative (Dong et al., 2009). In China and other developing countries, PCP had also been heavily used to kill oncomelania to prevent schistosomiasis (Zhu and Shan, 2009). In fact, albeit its usage is banned or restricted in many countries, its historically worldwide usage and relative persistence make PCP a ubiquitous environmental pollutant. Zheng et al. recently suggested an increase of PCP contamination in environment might occur due to a growing use of Na-PCP to control the reemergence of schistosomiasis (Zheng et al., 2012). PCP had been detected in human body fluids (plasma or urine) of non-occupationally and occupationally exposed individuals (Zhu and Shan, 2009). Thus, the US Environmental Protection Agency (EPA) listed PCP as a priority pollutant, while the International Association for Research on Cancer (IARC) classified PCP as a group 2B environmental carcinogen.

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated chemicals and the two most widely known PFAAs are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), which have been produced for decades and used for a variety of purposes, including surfactants, refrigerants, water repellents and polymers (Lau et al., 2007; Boltes et al., 2012). PFAAs are released into the environment through the usage of PFAAs-containing products or by degradation from their precursors (Liu et al.,...
Due to their thermal and chemical stability and resistance to biodegradation, PFAs are persistent in the environment and have been found extensively in wildlife and human bodies (Song et al., 2012). They may accumulate in human by pathways of drinking water and food or migration from food packaging and cookware, dust in home, etc. (EFSA, 2008) and pose adverse effects to human health. PFOS was listed as a new persistent organic pollutant by Stockholm Convention in 2009 (Watanabe et al., 2009). As a result of the ubiquitous presence of both PCP and PFOS (PFOA) in the environment, it is possible for human to expose to them simultaneously. In the NOWAC (national representative Norwegian Women And Cancer) study, it was reported that the median concentrations of PFOS and PCP in plasma were 20 and 0.77 μg·L\(^{-1}\) in a general female Norwegian population, respectively (Rylander et al., 2010, 2012).

The toxicokinetic studies about PCP and PFOS (or PFOA) have revealed that both kinds of environmental pollutants could be enriched in liver, moreover, PCP could undergo metabolism in liver. Once absorbed, PCP exhibits a small volume of distribution and the highest bioconcentration factor occurs in liver rather than other organs, tissue or body fluids (Geyer et al., 1987). Its metabolism via oxidative dechlorination to more toxic tetrachlorohydroquinone (TCHQ) also occurs primarily in liver (Wang et al., 2001). As for PFOS and PFOA, animal studies indicated that they are also well absorbed orally, but are not metabolized; they are also distributed mainly to liver and serum, with the level in liver being several times higher than in serum (Lau et al., 2007).

In line with their pharmacokinetic properties, studies demonstrated that PCP and PFAs are highly toxic, with liver as the major target organ. PCP is a potential carcinogen, which can induce liver tumors in test animals. Its oxidative dechlorination metabolites might play an important role in PCP’s toxicity through oxidative damage to biomacromolecules (Zhu and Shan, 2009). In addition, PCP, but not its metabolites, can exert cytotoxicity by uncoupling oxidative phosphorylation (EPA U. S., 2010). As for PFOS and PFOA, it has been shown that they are associated with liver enlargement in addition to hepatocellular adenomas in rodents (Lau et al., 2007). Toxic studies on humans are sparse. However, epidemiological studies conducted on 47092 adults showed that there exists potential risk on human liver in the population with elevated PFOA exposure (Gallo et al., 2012). Data on human cell lines in vitro presented evidences that PFOS and PFOA may pose adverse effects in terms of oxidative damage, membrane disruption or interference of endogenous enzyme activity (Kleszczynski and Skladanowski, 2009; Eriksen et al., 2010; Narimatsu et al., 2011). The studies on hepatic activity of PFAs using primary human hepatocytes demonstrated that multiplicity of nuclear receptor activation, not limited to PPAR (peroxisome proliferator-activated receptors)-agonistic mode of action, might be associated to the hepatotoxicity (Bjork et al., 2011; Rosen et al., 2013).

Since both PCP and PFOS (or PFOA) can be enriched in human liver and cause hepatotoxicity, they might present joint adverse effects to human liver. Therefore, our study aimed to evaluate the possible joint effects of PCP and PFOS (or PFOA) using HepG2 cells as a model of human hepatoma cells. Compared to primary human hepatocytes, HepG2 cell is a relatively easy-to-handle tool and has been used for individual PCP or PFOS (or PFOA) toxicity studies. Hence, our results could be compared with the results reported in previous studies. Based on the previous research results of their individual mode of actions, a series of experiments were conducted to disclose the mechanism for the combined effect, helping us to understand the risk of PFOS and PFOA to human health when they are present with other xenobiotics.

2. Materials and methods

2.1. Reagents

Pentachlorophenol (PCP) was purchased from Dongfang Chemical Company (Tianjin, China). Perfluorooctane sulfonate (PFOS) was obtained from Wellington Laboratories (Guelph, Canada). Perfluorooctanoic acid (PFOA) was bought from Alfa Aesar (Ward Hill, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), O-Phthalaldehyde (OPA), 2,7-dichlorofluorescein-diacetate (DCFH-DA), Rhodamine 123 (Rh-123), Fluorescein diacetate (FDA), Tetrachlorohydroquinone (TCHQ) were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), RPMI1640 culture medium and fetal bovine serum were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA).

2.2. Cell culture and chemical treatments

The HepG2 cell line was obtained from the cell bank of Chinese Academy of Science (Shanghai, China). The cells were maintained in RPMI1640 with Earle’s salts and l-glutamine in a humidified incubator under 5% CO\(_2\) at 37 °C. This medium was supplemented with 10% (v/v) fetal bovine serum, 100 IU mL\(^{-1}\) of penicillin and 100 μg mL\(^{-1}\) of streptomycin. The cells were seeded in culture dishes or plates and grown to 80–95% confluence. For single-substance test, the cells were exposed to the solution of PCP or PFOS (or PFOA) at predetermined concentrations for 3 h. For the joint effect studies, increasing concentrations of PCP with PFOS (or PFOA) at a fixed concentration, at which PFOS or PFOA did not present observed toxicity in MTT assay. For the tests with TCHQ alone or plus PFOS (or PFOA), fetal bovine serum was not used in the medium and incubation time was 2 h. The stock solutions of PCP and PFOS or PFOA (200 mM) were prepared in DMSO. A series of working solutions (0, 700, 800, 900 and 1000 μM for PCP and 0, 100, 200, 300 and 400 μM for PFOS or PFOA) were prepared by diluting the stock solutions with culture medium. The control was carefully adjusted to contain the same amount of DMSO as the working solution which had the largest volume of the stock solution.

2.3. Viability assay

Cell viability assay was based on a MTT colorimetric assay. MTT was reduced to purple formazan by metabolically active cells. For the assay, a working solution at 5 mg mL\(^{-1}\) of phosphate buffered saline (PBS) was prepared and conserved at 4 °C. After treatment with the chemicals, the cells were incubated in culture medium containing MTT (0.5 mg mL\(^{-1}\)) for 2 h. The medium was then replaced by DMSO to dissolve formazan. The absorbance at 570 nm of the solution was determined using Multimode Microplate Spectrophotometer (Varioskan Flash with dispenser, Thermo Electron Corporation, USA).

2.4. Measurement of intracellular GSH and GSSG contents

Both reduced (GSH) and oxidized (GSSG) glutathione in cells were measured according to Kand’ár R. et al. (2007) with slight modifications. Cold 10% trichloroaetic acid (400 μL) was added into the collected cells resuspended in 200 μL of PBS. After shaking and centrifugation (20000 g, 10 min, 4 °C), the supernatants were transferred into 1.5 mL propylene tubes (50 μL for determination of GSH and 200 μL for determination of GSSG). An Agilent 1200 HPLC apparatus (Agilent Tech. Corp., USA) equipped with a fluorescence detector and a reverse phase Supelcosil LC-18 column (4.6 × 250 mm, 5 μm, Sigma, USA) was used for the separation and detection. The excitation and emission wavelengths of the
detector were set at 340 and 420 nm, respectively. The concentrations of GSH and GSSG in the samples were determined from the calibration curves.

2.5. Detection of reactive oxygen species, mitochondrial membrane potential and permeability of cell membrane

Reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta \Psi_m$) and permeability of cell membrane were measured by flow cytometry using a FACScan flow cytometer (Becton Dickinson Instruments, San Jose, CA, USA). Intracellular ROS production was measured with DCFH-DA. After 3 h treatments, the HepG2 cells were resuspended in 1 mL of PBS and stained for 30 min at 37 °C with 10 µM DCFH-DA and then collected by a pipette and washed twice with PBS, analyzed by flow cytometry according to the method developed by Panaretakis et al. (2001). The $\Delta \Psi_m$ was measured employing the fluorescent dye Rh123. The cells were incubated for 30 min at 37 °C with 5 µM Rh123 and then collected and analyzed by flow cytometry according to Hong and Liu (2004). Permeability of the cell membrane was detected by staining the cells with 20 µg mL$^{-1}$ FDA for 30 min at 37 °C according to Liu et al. (2008).

2.6. HPLC analysis of the intracellular PCP levels

After exposure to 800 µM PCP alone or with addition of 100 µM PFOS or PFOA for 1 h at 37 °C, the cells ($5 \times 10^6$) were collected and washed by PBS for 3 times, then kept frozen at −80 °C. The cell pellet was then thawed and resuspended in 8 mL of PBS, disrupted by sonication at 4 °C and centrifuged at 12000 g for 10 min. The supernatant was collected and PCP was extracted and determined by the method of Levy et al. (2007).

2.7. Biochemical assays

Another index of the membrane permeability is the leakage of lactate dehydrogenase (LDH), which was measured using the CytoTox-ONE Homogenous Membrane Integrity Assay Kit (Promega, Madison, WI, USA). The lipid peroxidation was assessed by measuring the content of malondialdehyde (MDA) using Cellular MDA Determination Kit (Jiancheng Bioengineering Institute, Nanjing, China). Intracellular adenosine triphosphate (ATP) amount was measured using ATP Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The CYP3A4 enzyme activity was measured by the CYP3A4 Activity Fluorescence Quantitative Detection Kit (Genmed Sciences Inc., MA, USA). The protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rochford, IL, USA).

2.8. Statistical analysis

All the results were presented as mean ± SD of the data from at least three independent experiments. Significance between the cells exposed to the combined PCP and PFOS (or PFOA) and exposed to the individual PCP were assessed using one-way analysis of variance (ANOVA) with the post hoc LSD test.

3. Results and discussion

3.1. Combined cytotoxicity assessment

Fig. 1A illustrates the viability of the cells which were exposed to PFOS or PFOA solution alone for 3 h. The cell viability was not affected by PFOA at all the tested concentrations (100, 200, 300 and 400 µM), but was slightly inhibited by PFOS at 300 and 400 µM. In the other words, both PFOS and PFOA with a concentration lower than 200 µM did not affect the cell viability in 3 h. The results agree with the study of Florentin et al., who conducted MTT assay for 1 h and 24 h (Florentin et al., 2011). But Hu and Hu reported that exposure to 150 µM PFOS for 24 h could cause a decrease of cell viability by 8%, while treatment with 100 µM PFOS or PFOA for 24 h did not show significant impacts on the cell viability (Hu and Hu, 2009). Thus, based on the cell viability assay of this study and literature data, the no-observed-effect concentration of both PFOS and PFOA to the cell viability for 3 h treatment was determined to be 100 µM.

As shown in Fig. 1B, the viability of the cells which were exposed to PCP alone for 3 h decreased in a dose-dependent manner and the survival rate of the cells exposed to PCP at 700, 800, 900 and 1000 µM was 90.1%, 85.2%, 52.3%, and 20.5% of the control without PCP, respectively (Fig. 1B). When 100 µM PFOS was added to the PCP solution at the four levels (700, 800, 900, 1000 µM), the cell viability decreased further and the survival rate was 71.9%, 42.3%, 24.3% and 41.0% of the cells treated with PCP alone at the same concentration, respectively. This suggests that PFOS could stimulate the adverse effect of PCP to the cells. PFOA displayed a slightly weaker effect to cell viability than PFOS. Incubation of 100 µM PFOA with PCP, the cell survival rate decreased to 94.4%, 73.4%, 60.5% and 58.9% of the individual PCP treatments, respec-
tively. The above results suggest that PFOS and PFOA could enhance the toxicity of PCP to the liver cells.

It has been shown that the antioxidative mechanisms of small molecular antioxidants such as glutathione (GSH) could be overwhelmed by PCP, PFOS or PFOA, which could induce the production of excessive ROS (Liu et al., 2007; Zhu and Shan, 2009). Therefore, as indicators of cytotoxicity, GSH/GSSG and ROS were also measured. In the single treatment with 100 μM PFOS (or PFOA) for 3 h, no significant difference was observed between the control and test groups, suggesting that 100 μM PFOS (or PFOA) does not affect the antioxidative function of the cells. However, once 100 μM PFOA was added to the PCP solutions, the ratio was reduced by 58.7% and 69.5% compared to the individual PCP (800 and 900 μM) tests, respectively. Greater reduction of 65.3% and 86.6% were observed when the cells were co-exposed to PCP (800 and 900 μM) with 100 μM PFOS (Fig. 2A). The results further indicate that both PFOS and PFOA could enhance the toxicity effects of PCP to human liver cells and PFOA displayed stronger effect than PFOS.

However, accompanying with more decrease of GSH in the co-exposure tests, the intracellular ROS level did not show significant increase (Fig. 2B). In the single test with PCP at 700 and 800 μM, ROS generation was 2.5 and 2.7 times of the control, respectively. Although ROS generation in the single tests with 100 μM PFOS or PFOA increased 1.3–1.7-fold compared to the control, ROS production did not show significant increase compared to individual PCP treatments when the cells were exposed to the mixture of PCP and PFOS (or PFOA) (Fig. 2B). Measurement of MDA, the lipid peroxidation product by ROS (Klaunig et al., 2011), also showed that addition of 100 μM PFOS or PFOA did not significantly increase intracellular MDA levels in the co-exposure tests. It seems that addition of 100 μM PFOS (or PFOA) only caused more GSH depletion, but not significantly increased intracellular ROS levels or intensified the oxidative stress in the co-exposure tests. The results suggest that GSH and other antioxidants might play an active role in the protection of the cells against ROS in the co-exposure tests.

The toxicity of PCP has been attributed to its ability to uncouple mitochondrial oxidative phosphorylation and PCP, not its metabolites, exerts a strong inhibition of electron transport between a flavin coenzyme and cytochrome P450 enzymes (CYPs) (Levy et al., 2007). To assess whether the enhanced cell toxicity of the co-exposure group is attributed to the uncoupling role, two endpoints: mitochondrial membrane potential (ΔΨm) and intracellular ATP contents (Valmas et al., 2008), were measured. Treatment with 100 μM PFOS (or PFOA) did not cause observed ΔΨm alteration, which was in accordance with the data reported by Hu and Hu (2009). However, a dramatic reduction in ΔΨm was observed in the treatment with 800 μM PCP alone and more ΔΨm reduction was induced when 100 μM PFOS or PFOA was added (Fig. 3A). Compared to the cells treated with 800 μM PCP alone, 30.7% and 15.6% decrease in fluorescence intensity were observed in 800 μM PCP plus 100 μM PFOS (or PFOA), respectively. In consistent with the change of ΔΨm, intracellular ATP content in the combination groups also decreased significantly more than the individual PCP group (Fig. 3B). The variations of these two uncoupling indicators were consistent with the enhanced cytotoxicity caused by addition of PFOS (or PFOA). Thus, it is presumed that PFOS and PFOA may strengthen the oxidative phosphorylation uncoupling effect of PCP and thus cause the enhanced cytotoxicity.

3.2. The mechanism of enhanced cytotoxicity caused by addition of PFOS (or PFOA)

Enzyme CYP3A4 plays a crucial role in biotransformation of PCP to more destructive tetrachlorohydroquinone (TCHQ) (Westerink and Schoonen, 2007). Previous studies showed that the expression of CYPs could be up-regulated by PFOS or PFOA in tested animals (Andersen et al., 2008; Cheng and Klaassen, 2008; Liu et al., 2009a; Kim et al., 2011; Nordén et al., 2012). This induction of CYPs in mice liver by PFOA was demonstrated through activation of PPAR and other transcription factors (Cheng and Klaassen, 2008). To examine whether PFOS (or PFOA) increases CYP3A4 protein levels by this induction, the enzyme activity of CYP3A4 treated with PFOS (or PFOA) was measured. The results are shown in Fig. S1 in Supporting information, indicating that PFOS (or PFOA) could not significantly change the CYP3A4 enzyme activity. Therefore, it is expected that 100 μM PFOS (or PFOA) might not promote the biotransformation of PCP via the induction of CYP3A4 under our experimental conditions. Our results suggest that the induction through activation of transcription factors might not play a role in the enhanced toxicity of PCP in the current study. To evaluate the role of cellular PCP metabolite, TCHQ, in the enhanced toxicity of PCP, we made an effort to measure cellular TCHQ amount, but failed due to its instability and low concentration. Furthermore, the combined effects of PFOS (or PFOA) and TCHQ was further accessed with the cells co-exposed to TCHQ (0, 25, 50, 100, 150, 200 μM) and PFOS (or PFOA) at 50 μM. The results (see Fig. S2 in Supporting information) illustrate that adding PFOS (or PFOA) in
the solution of TCHQ could not result in significantly more decrease of cell viability than the individual TCHQ exposures. Thus, the enhanced cytotoxicity of PCP by PFOS (or PFOA) might not be related to the biotransformation of PCP to TCHQ.

PFOS could alter the cell membrane and potentially lead to an increase of the cellular accessibility of other chemicals (Hu et al., 2003). PFOS could significantly increase membrane fluidity of fish leukocytes and reinforce the toxic effects of tetrachlorodibenzo-p-dioxin and estradiol (Hu et al., 2003). PFOS might have considerable impact on the availability of cyclophosphamide to V79 cells, leading to the enhanced genotoxic action of cyclophosphamide (Jembro et al., 2007). PFOS also increased the uptake of PCP in green alga, resulting in synergistic effect with PCP (Liu et al., 2009b). The synergistic effect of co-exposure to PFOS and another chlorinated phenol 2,4,6-trichlorophenol was also observed in green alga (Boltes et al., 2012). Most of these joint studies were limited with respect to PFOS in wildlife. To our knowledge, no previous studies about combined effects of PFOS or PFOA with other xenobiotics on human health have been reported. To examine whether PFOS and PFOA could promote the cellular uptake of PCP, the intracellular concentration of PCP was measured with and without the presence of PFOS (or PFOA). The results are shown in Fig. 4. Compared with the cells without PFOS, those exposed to PCP with PFOS tended to take up significantly more PCP; moreover, PFOA, albeit its impact was weaker than PFOS, could also enhance the cellular concentration of PCP. Correlation between the influence of PFOS (or PFOA) on cell concentration of PCP and the effect on the oxidative phosphorylation uncoupling of PCP in co-exposure tests suggests that PFOS (or PFOA) might increase the cell membrane permeability, leading to relatively higher intracellular concentration of PCP and higher cytotoxicity.
The amphiphilic nature of PFOS or PFOA suggests that their effects could primarily be associated with the membrane functions. To examine whether more cellular accessibility of PCP is attributed to the membrane effects induced by PFOS (or PFOA), the membrane properties of the cells were evaluated by exposing them to 100 μM PFOS (or PFOA) by FDA and LDH tests, which have been used for evaluating membrane permeability or integrity (Liu et al., 2008; Orpesa et al., 2011). The fluorescence intensity in FDA assay decreased about 18% (or 16%) when the cells were treated with PFOS (or PFOA) compared to the control (Fig. 5A). The LDH study also showed that 100 μM PFOS (or PFOA) could cause the damage of cell membrane, resulting in LDH leakage to some extent (Fig. 5B). Like anionic detergents such as sodium dodecyl sulfate, they might preferentially bind plasma membrane fractions (Kudo et al., 2007), and interfere with the membrane/protein interfacial interactions by the membrane solubilization even at concentrations below their critical micelle concentrations (Kragh-Hansen et al., 1998; le Maire et al., 2000; Hu et al., 2003; Kadiyala et al., 2010; Otzen, 2011). For comprehensive understanding the disruptive effects of PFOS (or PFOA) on plasma membrane, further investigations are needed.

4. Conclusions

Even though PFOS (or PFOA) at 100 μM did not show adverse effect to cell viability of HepG2 cells in MTT assay for 3 h, they displayed synergistic effect with PCP to the cytotoxicity of human liver cells. PFOS (or PFOA) could disrupt the membrane and then promote cell membrane permeability, resulting in more cellular accessibility of PCP. Rather than by intensifying oxidative stress or promoting biotransformation, more intracellular PCP might enhance cytotoxicity by exacerbating the uncoupling of oxidative phosphorylation. This study gave some insights on the combination effects of perfluorinated compounds with other xenobiotics to human health. The authors gratefully acknowledge the financial support of Chinese National Natural Science Foundation Grants (21077058, 21077060), Tianjin Municipal Science and Technology Commission (12JCYBJC16000), and the Fundamental Research Funds for the Central Universities.

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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.chemosphere.2013.07.054.

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