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

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HIGHLIGHTS

- PFOS and PFOA could enhance the cytotoxity 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.

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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.

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.,

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2011). Due to their thermal and chemical stability and resistance to biodegradation, PFAAs 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 $\mu g\,L^{-1}$ 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, PFAAs 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 PFAAs 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. Lousi. 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 Academic 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 $\mu g\,mL^{-1}$ 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% trichloroacetic acid (400 μL) was added into the collected cells resuspended in 200 μL of PBS. After shaking and centrifugation (20 000 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