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Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells

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ABSTRACT

The cytotoxicity of eight perfluorinated chemicals (PFCs), namely, perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS) was assessed in the human placental choriocarcinoma cell line JEG-3. Only the long chain PFCs – PFOS, PFDoA, PFNA, PFOA – showed significant cytotoxicity in JEG-3 cells with EC50 values in the range of 107 to 647 μM. The observed cytotoxicity was to some extent related to a higher uptake of the longer chain PFCs by cells (PFDoA > PFOS ≫ PFNA > PFOA > PFHxA). Moreover, this work evidences a high potential of PFOS, PFOA and PFBS to act as aromatase inhibitors in placental cells with IC50s in the range of 57–80 μM, the inhibitory effect of PFBS being particularly important despite the rather low uptake of the compound by cells. Finally, exposure of JEG-3 cells to a mixture of the eight PFCs (0.6 μM each) led to a relative increase (up to 3.4-fold) of several lipid classes, including phosphatidylcholines (PCs), plasmalogen PC and lyso plasmalogen PC, which suggests an interference of PFCs with membrane lipids. Overall, this work highlights the ability of the PFC mixture to alter cellular lipid pattern at concentrations well below those that generate toxicity, and the potential of the short chain PFBS, often considered a safe substitute of PFOS, to significantly inhibit aromatase activity in placental cells.

Introduction

Perfluorinated chemicals (PFCs) represent a large group of compounds characterized by a hydrophobic fluorinated carbon tail attached to a polar hydrophilic head. PFCs are classified as perfluorinated sulfonic acids (PFSAs), perfluorinated carboxylic acids (PFCAs), fluorotelomer alcohols (PFTOHs), high-molecular weight fluoropolymers and low-molecular weight perfluoroalkanamides. They are used in a variety of consumer products and have emerged as global environmental pollutants (Stahl et al., 2011). They are resistant to degradation and have a high accumulation potential; thus, once released in the environment, PFCs persist in aquatic organisms (Giesy and Kannan, 2002; Kannan et al., 2002). PFSAs and PFCAs are the most ubiquitous compounds and they have been detected in human blood (Ericson et al., 2007). Typical serum levels of PFOS and PFOA are in the range of 1 to 50 ng/mL, although concentrations up to 300 and 2000 ng/mL have been detected in occupationally exposed workers (Olsen et al., 1998).

Concerns about PFC toxicity have risen due not only to its widespread distribution and persistence in humans and the environment, but also to its toxicity and ability to act as endocrine disrupters and obesogens (Du et al., 2013; Hines et al., 2009). However, while the toxicity of PFOS and PFOA has been deeply investigated in the last decades, other PFC homologs have been rarely studied. PFCs of shorter chain length are expected to have similar functions to PFOS and be less bioaccumulative and less toxic. Thus, Buhrke et al. (2013) showed a positive correlation between carbon chain length of PFCAs and its toxicity in the human hepatocarcinoma cell line HepG2: the short chain length PFBA and PFHxA were less toxic than PFOA. Nonetheless, both PFOS and PFBS promoted expression of the estrogen and the androgen receptor at environmentally relevant concentrations and caused adverse hepato-histological effects in the amphibian *Xenopus laevis* at high concentrations (100–1000 μg/L), which opens the question of whether short chain PFCs are safe substitutes of PFOS (Lou et al., 2013).

Moreover, long chain PFCs can modulate the biosynthesis of gender-specific steroid hormones. Olsen et al. (1998) reported a 10% increase in estradiol levels among occupationally exposed workers who had the highest levels of serum PFOA (>30 ng/mL), although this association was confounded by body mass index. Decreased gene expression of key enzymes and transporters involved in steroidogenesis was observed in male rats exposed to PFDoA and male mice exposed to PFOS (Shi et al., 2007, 2009; Wan et al., 2011). Also, Zhao et al. (2010) showed a decrease in testosterone levels in isolated rat Leydig cells exposed to PFOA. All these studies seem to indicate that long chain PFCAs can act as endocrine disruptors, but their mechanisms of action are still unknown. A recent

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work by Rosenmai et al. (2013) revealed that fluorochemicals present in food packaging materials can affect steroidogenesis through decreased Bzrp and increased CYP19 gene expression leading to lower androgen and higher estrogen levels. CYP19 aromatase plays a key role in catalyzing the irreversible conversion of androgens to estrogens and maintaining the androgen/estrogen hormonal balance. Thus, any interaction of chemicals with the expression of this enzyme or its catalytic activity is very likely to disrupt the internal hormonal balance between androgens and estrogens. In humans, aromatase activity has been reported in gonads, brain, ovaries, testis, placenta, adipose tissue, fetal liver, intestine, skin and brain. Numerous assays have been developed to evaluate the potential effects of chemicals on CYP19 aromatase, among them, the human recombinant microsomal aromatase assay that measures the direct effect of chemicals on aromatase catalytic activity in vitro (Vinggaard et al., 2000). Currently, the human placental choriocarcinoma JEG-3 cell line is also frequently used to assess CYP19 aromatase, since it allows the detection of changes in aromatase gene expression (Huang and Leung, 2009).

The structural resemblance of PFCs to fatty acids and the discovery that they bind to peroxisome proliferator-activated receptors (PPARs), nuclear receptors that play a key role in lipid metabolism and adipogenesis, have recently raised the concern that PFCs may disrupt lipid and weight regulation. Indeed, several studies suggest that exposure to PFOS and PFOA may be associated with increased cholesterol levels in humans (Nelson et al., 2010). Also, developmental exposure to low-doses of PFOA lead to increased weight in adult rats, with increased serum insulin and leptin, an effect not seen in animals exposed to high doses of PFOA (Hines et al., 2009). Interestingly, other environmental chemicals, termed obesogens, have been shown to induce obesity in adulthood after low-dose developmental exposure, while inducing weight loss at higher doses (Grün et al., 2006). In addition, PFOS has been shown to affect membrane properties (e.g. membrane fluidity, mitochondrial membrane potential) at concentrations below those associated with other adverse effects (Hu et al., 2003). Despite these evidences, more in-depth studies on the effects of PFCs on cellular lipidome and the physiological consequences for the cell, are still lacking.

Within this context, the aim of this study was to comparatively determine the cytotoxicity and ability to disrupt CYP19 aromatase activity of five perfluorinated carboxylic acids and three perfluorosulfonates of different chain lengths in the human placental choriocarcinoma cell line JEG-3. Special emphasis was placed on the analysis of cellular lipids by ultra-high performance liquid chromatography/mass spectrometry (UPLC/MS) in an attempt to roughly characterize major alterations of cellular lipids following exposure to a mixture of PFCs. This was designed as an exploratory analysis to be more fully investigated in future experiments with individual PFCs. The PFCs selected for the study were: perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS).

Materials and methods

Chemicals and reagents. Minimum Essential Medium, fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin G, streptomycin, PBS and trypsin–EDTA were from Gibco BRL Life Technologies (Paisley, Scotland, UK). PFBA, PFHxA, PFOA, PFNA, PFDoA and PFHxS were purchased from Sigma-Aldrich (Steinheim, Germany), except PFBS and PFOS which were obtained from Fluka (Austria). Stock standard solutions and serially diluted test solutions were prepared in ethanol, except for PFOS which was prepared in dimethyl sulfoxide (DMSO). These compounds were added to the complete growth medium so that the final solvent concentration never exceeded 0.4% (v/v), which was not cytotoxic. Perfluoro-n-(1,2,3,4-$^{13}C_4$)octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$)octanesulfonate (m-PFOS) from Wellington Laboratories (Ontario, Canada) were used as surrogate standards. HPLC grade water, ethanol (>99.8%) and acetonitrile (>99.8%) were purchased from Merck (Darmstadt, Germany).

Cell culture. JEG-3 cells derived from human placental carcinoma were obtained from American Type Culture Collection (ATCC HTB-36). They were grown in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate and 50 U/mL penicillin G/50 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. Cells were routinely cultured in 75 cm^2 polystyrene flasks. When 90% confluence was reached, cells were dissociated with 0.25% (w/v) trypsin and 0.9 mM EDTA for subculturing and experiments. Experiments were carried out on confluent cell monolayers.

Cell viability. Two fluorescent dyes were used to monitor cell viability on JEG-3 cells (Schirmer et al., 1997). Metabolic activity was monitored with Alamar Blue (AB™, resazurin) and membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM). Cells were seeded at a rate of 25,000 cells per well (96-well plate) and allowed to attach overnight at 37 °C, 5% CO_2. After 24-hour exposure to PFCs, the medium was replaced by 100 μL of a solution of AB™/CFDA-AM, incubated for 1 h, and cell viability was measured using a fluorescence plate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths of 530/590 nm for AB™ and 485/530 nm for CFDA-AM. Results were recorded as relative fluorescence units.

Three independent sets of experiments were performed for each PFC. PFBA, PFBS, PFHxA and PFHxS were tested at 500 μM whereas PFOA, PFNA, PFDoA and PFOS were also tested at lower concentrations to obtain concentration–response curves. Within each experiment, addition of the test compound was done in septuplicate.

Uptake of PFCs. Cells were seeded at a rate of 100,000 cells per well (24-well plate) and allowed to attach overnight in an incubator at 37 °C, 5% CO_2. Cells were then dosed in triplicate with a mixture of eight PFCs at a concentration of 6.0 μM for each compound, and control cells were exposed to the carrier (0.4% ethanol). Given the intensive analytical work required to test eight PFCs individually at different concentrations, we decided to carry out an exploratory assay with a mixture of PFCs at a single non-toxic concentration, high enough to allow the detection of the fraction of PFCs absorbed by the cells. Right after dosing (time zero), and after 1, 3, 5, 8 and 24 h of incubation, the medium was aspirated, and the cells were rinsed with PBS, trypsinized and centrifuged at 3600 rpm for 10 min. The supernatant was aspirated and the cells were stored at −80 °C until analysis. Two independent sets of experiments were performed.

PFCs were extracted from JEG-3 cells following the method of Fernández-Sanjuan et al. (2010), with some modifications. A surrogate standard solution containing m-PFOA and m-PFOS was added to the cell pellets followed by 1 mL of acetonitrile. Samples were shaken and extracted in an ultrasonic bath for 10 min (×3); the supernatant was transferred to a new vial and purified by adding 25 mg of activated carbon and 50 μL of glacial acetic acid. The obtained supernatant was evaporated to dryness and reconstituted in 15% methanol/acetonitrile (60:40) and 85% water.

PFCs were analyzed using an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, USA) connected to a Triple Quadrupole Detector. An XBridge™ C_{18} column (3.5 μm particle size, 50 mm × 4.6 mm, Waters, Ireland) was used as mobile phase residue trap to remove any PFC traces from the mobile phases and tubing. The analysis was performed on an Acquity UPLC BEH C_{18} column (1.7 μm particle size, 50 mm × 2.1 mm, Waters, Ireland) connected to an Acquity UPLC BEH C_{18} (1.7 μm particle size, 5 mm × 2.1 mm, Waters, Ireland) VanGuard™ pre-column at a flow rate of 0.4 mL/min at a column temperature of 40 °C. The mobile phase was 20 mM NH_4OAc/