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Activation of the estrogen receptor by human serum extracts containing mixtures of perfluorinated alkyl acids from pregnant women

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ABSTRACT

Humans are exposed to a wide variety of perfluorinated alkyl acids (PFAAs). Several studies have found xenoestrogenic activity of single PFAAs. Studies on mixture effects of the PFAAs are however sparse. In the present study, we aimed to determine the xenoestrogenic activity in human serum extracts containing mixtures of PFAAs.

Recently we developed a method to extract the PFAAs from human serum with simultaneous removal of endogenous hormones and interfering steroid metabolites. We used this method to extract the PFAAs from serum of 397 Danish nulliparous pregnant women followed by analysis of estrogen receptor (ER) transactivation using MVLN cells carrying an estrogen response element luciferase reporter vector. Using 17β-estradiol (E2) concentration-transactivation curves, we calculated the estradiol equivalents (EEQ) for the extracts containing the PFAAs.

Fifty-two percent of the PFAA serum extracts agonized the ER transactivation, and 46% enhanced the E2-induced ER transactivation. We found positive linear concentration-response associations between the ER transactivation and the PFAA serum levels. For the relatively few PFAA extracts that antagonized the ER in the presence of 24 pM E2 (n=38, 10%), we found inverse linear associations between the ER transactivation and the PFAA serum levels. The results indicated that the serum extracts induced the ER in a non-monotonic concentration dependent manner. The median EEQ of the extracts containing the PFAAs corresponds to the effect of 0.5 pg E2 per mL serum.

In conclusion, we observed that most of the extracts containing the PFAA mixtures from pregnant women's serum agonized the ER and enhanced the E2-induced effects in non-monotonic concentration-dependent manners.

1. Introduction

Perfluorinated alkyl acids (PFAAs) are a large group of surfactant chemicals with a wide variety of applications (Prevedouros et al., 2006). The PFAAs are persistent and ubiquitously present in the environment (Buck et al., 2011) as well as in human blood (Karrman et al., 2005) and tissues such as the lungs, liver, kidneys, brain, and bone (Perez et al., 2013). In a recent study, we detected 14 different PFAAs in serum from 1533 Danish pregnant women, and seven of the PFAAs were detected in more than 75% of the samples (Bjerregaard-Olesen et al., 2016a). Strong correlations between PFAA levels in maternal blood during pregnancy and cord

Abbreviations: ANOVA, analysis of variance; BMI, body mass index; CD-FBS, charcoal-dextran stripped fetal bovine serum; CV, co-variance; DHEAS, dehydroepiandrosterone sulfate; DMEM, Dulbecco's modified Eagle's media; E1S, estrone sulfate; E2, 17β-estradiol; EEQ, estradiol equivalents; ER, estrogen receptor; HPLC, high performance liquid chromatography; IQR, interquartile range; KHK, pooled human female serum control; LOEC, lowest observed effect concentration; LOQ, limit of quantification; PFAA, perfluorinated alkyl acid; PFBS, perfluorobutane sulfonate; PFCA, perfluorinated carboxylic acid; PFDA, perfluorodecanoate; PFDoA, perfluorododecanoate; PFDS, perfluorodecane sulfonate; PFHpA, perfluoroheptanoate; PFHpS, perfluoroheptane sulfonate; PFHxA, perfluorohexanoate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; PFOSA, perfluorooctane sulfonamide; PFPeA, perfluoropentanoate; PFSA, perfluorinated sulfonic acid; PFUnA, perfluoroundecanoate; PFTrA, perfluorotridecanoate; PFTeA, perfluorotetradecanoate; POPs, persistent organic pollutants; RLU/prot, relative light units per count of protein; SPE, Solid Phase Extraction; WAX, Weak Anion Exchange; XER, xenoestrogenic receptor transactivation of the serum PFAA extracts alone (i.e. the non-competitive assay); XERcomp, xenoestrogenic receptor transactivation of the serum PFAA extracts upon co-exposure with 24 pM 17β-estradiol (i.e. the competitive assay)

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blood are indicative of fetal exposure (Fei et al., 2007; Kim et al., 2011).

We previously observed that perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), and perfluorooctanoic acid (PFOA) alone as well as in an equimolar mixture with perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and perfluorododecanoic acid (PFDoA) transactivated the estrogen receptor (ER) in MVLN cells (Kjeldsen and Bonefeld-Jorgensen, 2013). PFHxS, PFOS, PFOA, and the equimolar mixture also enhanced the ER transactivation induced by the natural ER-ligand 17β-estradiol (E2) (Kjeldsen and Bonefeld-Jorgensen, 2013). In agreement with our study, others have also reported that the PFAAs induced agonistic (Liu et al., 2007; Henry and Fair, 2013; Benninghoff et al., 2011) and E2-enhancing (Hu et al., 2003; Sonthithai et al., 2015) effects on the ER, but there are also studies finding E2-antagonistic effects (Liu et al., 2007; Henry and Fair, 2013; Kang et al., 2016).

More than 95% of the funding in toxicological research is used for studies on single chemicals (Kortenkamp, 2007) even though it is well known that some compounds may act additive or can modify the actions of other compounds (Kortenkamp and Hass, 2009). For example, using a yeast estrogen screen, Silva et al. (2002) showed that a mixture of eight xenoestrogens at concentrations below their no observed effect concentrations produced a combined estrogenic effect that was much larger than the arithmetic sum of the individual effects. Humans are exposed to very complex mixtures of PFAAs, which complicates the estimation of the actual effects of the PFAAs.

To estimate the effects of PFAA mixtures on the ER at concentrations relevant to humans, we recently developed a method to extract the PFAAs from human serum for subsequent ER transactivation measurements using an MLVN cell assay (Bjerregaard-Olesen et al., 2015). The method consists of solid phase extraction (SPE), high performance liquid chromatography (HPLC), and weak anion exchange (WAX). The SPE-HPLC-WAX method was designed to fractionate the serum compounds such that the PFAAs were collected in a fraction (F3-W2) separated from the lipophilic persistent organic pollutants (POPs) in fraction F1 and from fractions F2.1, F2.2, F2.3, and F3-W1 containing the endogenous steroid hormones. The F3-W2 fraction may contain traces of the sulfated steroid metabolites estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS), but at levels which we recently found did not interfere with the in vitro analysis of the xenoestrogenic effect from PFAAs in the F3-W2 fraction (Bjerregaard-Olesen et al., 2016b). As a precaution against potential interference, the F3-W2 fractions may be combined with the steroid sulfatase inhibitor *STX64* during the in vitro analysis of the xenoestrogenic effects, thus abolishing the conversion of E1S and DHEAS to more potent estrogens such as estrone and androstenediol, respectively (Bjerregaard-Olesen et al., 2016b).

In the present study we aimed to estimate the xenoestrogenic activity of serum extracts containing the actual human serum mixture of PFAAs. This was done by 1) extracting the PFAA fraction (F3-W2) from 397 Danish pregnant women's serum using SPE-HPLC-WAX, 2) analyzing the xenoestrogenic activity in the PFAA fraction (F3-W2) alone, in the presence of E2, and in the presence of STX64 using the MVLN cell assay, and 3) evaluating the dose-response relationship between the serum levels of the PFAAs and the xenoestrogenic activity of the PFAA fractions F3-W2 by a) continuous and categorical linear regression analyses and b) dilution of the PFAA fractions (F3-W2).

2. Materials and methods

2.1. Participants and serum samples

The study included 397 nulliparous women randomly selected from a larger sample set of 801 nulliparous pregnant women, who were enrolled in the Aarhus Birth Cohort from 2011 to 2013 and were included in a recent study (Bjerregaard-Olesen et al., 2016a). The women provided a blood sample between gestation week 11 and 13. Within two hours after blood draw, the blood samples were processed and stored as serum at −80 °C (Mortensen et al., 2013). The women filled out questionnaires with information about height, weight, previous miscarriages, educational level, and country of birth, smoking, and alcohol consumption. All participants gave their consent to the storing of the serum samples in the biobank, and agreed that the serum and information could be used for research.

Serum donated by voluntary women to the blood bank at Aarhus University Hospital, Aarhus, was pooled and used as inter-assay controls which we refer to as KHK.

The study was approved by the Danish Data Protection Agency (ref. 2011-41-6014) and the Danish National Committee on Health Research Ethics (ref. M-20110054).

2.2. Methods

2.2.1. PFAA extraction

The PFAAs were extracted from human serum while simultaneously removing endogenous hormones such as estrone, E2, and testosterone as previously described (Bjerregaard-Olesen et al., 2015). Briefly, 3 mL serum was extracted by SPE on an OASIS HLB cartridge (6 mL, 500cc, Waters, Milford, MA, USA) with elution using 4 mL methanol and 4 mL ethyl acetate. The eluates were concentrated by vacuum centrifugation, extracted two times with hexane: ethyl acetate (9:1) and two times with tetrahydrofuran: hexane (3:2). The supernatants from the latter extraction were evaporated at 30 °C under N_2 until nearly dryness. Upon reconstitution in 315 μL tetrahydrofuran: hexane (4:1), the extracts were fractionated by HPLC. Fraction F3 (containing the PFAAs) was further extracted by WAX on an OASIS WAX cartridge (6 mL, 150 mg, 30 μm; Waters, Milford, MA, USA). Neutral compounds such as estriol and estetrol were eluted in fraction W1 using 4 mL methanol, and the PFAAs were eluted in fraction W2 using 0.1% ammonium hydroxide in methanol. Finally, the fraction W2 was evaporated by vacuum centrifugation, and the dry fractions were stored at −80 °C.

Each extraction batch consisted of 22 randomly selected serum samples, one KHK serum control, and one procedural blank consisting of double distilled water. The KHK was used to secure robustness between the assays. The procedural blank was used to control for potential PFAA-contamination during the extraction as some laboratory materials may contain PFAAs (van Leeuwen and de Boer, 2007).

2.2.2. ER transactivation luciferase reporter gene assay

The ER-transactivation was determined using the stable transfected MVLN cell line (kindly provided by M. Pons, France) carrying the estrogen response element luciferase reporter vector (Demirpence et al., 1993; Pons et al., 1990) as described previously (Hjelmborg et al., 2006; Bonefeld-Jorgensen et al., 2005). Briefly, 8.5×10^4 cells were seeded in each well in a 96-well plate and left at 37 °C in the incubator over night. The next day, the dry PFAA fractions (F3-W2) were reconstituted in 20 μL EtOH:H_2O: DMSO (50:40:10, v/v/v) and 200 μL of Dulbecco's modified Eagle's media without phenol red (DMEM, Lonza) containing 0.5% charcoal-dextran stripped fetal bovine serum (CD-FBS). These solutions