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Manhai Long, Mandana Ghisari & Eva Cecilie Bonefeld-Jørgensen

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Effects of perfluoroalkyl acids on the function of the thyroid hormone and the aryl hydrocarbon receptor

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Abstract Perfluoroalkyl acids (PFAAs) are perfluorinated compounds that widely exist in the environment and can elicit adverse effects including endocrine disruption in humans and animals. This study investigated the effect of seven PFAAs on the thyroid hormone (TH) system assessing the proliferation of the 3,3',5-triiodo-L-thyronine (T3)-dependent rat pituitary GH3 cells using the T-screen assay and the effect on the aryl hydrocarbon receptor (AhR) transactivation in the AhR-luciferase reporter gene bioassay. A dose-dependent impact on GH3 cells was observed in the range 1×10^{-9} – 1×10^{-4} M: seven PFAAs (perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorooctanoic acid, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and perfluorododecanoic acid (PFDoA)) inhibited the GH3 cell growth, and four PFAAs (PFOS, PFHxS, PFNA, and PFUnA) antagonized the T3-induced GH3 cell proliferation. At the highest test concentration, PFHxS showed a further increase of the T3-induced GH3 growth. Among the seven tested PFAAs, only PFDoA and PFDA elicited an activating effect on the AhR. In conclusion, PFAAs possess in vitro endocrine-disrupting potential by interfering with TH and AhR functions, which need to be taken into consideration when assessing the impact on human health.

Keywords Perfluoroalkyl acids · Thyroid hormone · Aryl hydrocarbon receptor · Transcriptional activity · In vitro

Abbreviations

ADHD	Attention deficit hyperactivity disorders
AhR	Aryl hydrocarbon receptor
AhR-tact	AhR transactivity
AR	Androgen receptor
CV	Coefficient of variation
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
EDCs	Endocrine-disrupting compounds
EC ₅₀	Half maximal effect concentration
EC ₁₀₀	Maximum effect concentration
ER	Estrogen receptor
HAHs	Halogenated aromatic hydrocarbon
LDH	Lactate dehydrogenases
LOEC	Lowest observed effect concentration
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenols
PFAAs	Perfluoroalkyl acids
PFCs	Perfluorinated compounds
PFCAs	Perfluorocarboxylated acids
PFSAs	Perfluorosulfonated acids
POPs	Persistent organic pollutants
PPAR	Proliferator-activated receptor
PE	Proliferative effect
REP	Relative potency
RPE	Relative proliferative effect
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TH	Thyroid hormone
TR	Thyroid hormone receptor
T3	3,3',5-Triiodo-L-thyronine
T4	Throxin
T-screen	Thyroid hormone-dependent GH3 cell growth
TTR	Transthyretin

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M. Long (✉) · M. Ghisari · E. C. Bonefeld-Jørgensen
Centre for Arctic Health and Unit of Cellular and Molecular
Toxicology, Department of Public Health, Aarhus University,
Bartholins Alle 2, Building 1260,
8000 Aarhus C, Denmark
e-mail: ml@mil.au.dk

Introduction

Perfluorinated compounds (PFCs) are a group of synthetic organic chemicals being produced over several decades for different industrial and commercial applications such as food-stuff packaging, nonstick cookware, waxes, paints, cosmetics, and water and oil repellents (Kissa 2001). The most common and well-studied PFCs are the perfluoroalkyl acids (PFAAs). PFAAs consist of a carbon backbone 4–14 atoms in length and a charged functional group composed of either sulfonates (termed as perfluorosulfonated acids, PFSA) or carboxylates (termed as perfluorocarboxylated acids, PFCAs). This molecular arrangement gives PFAAs their desirable lipophilic and hydrophilic properties. Due to resistance against degradation in the environment and bioaccumulation in the food chain, PFAAs widely exist in the environment, and human exposure to PFAAs is ubiquitous (Suja et al. 2009). Human exposure to PFAAs is through dietary intake, drinking water, inhalation, and dermal contact (Haug et al. 2011; Fromme et al. 2009; Trudel et al. 2008; Fromme et al. 2009; Haug et al. 2011). PFAAs have received attention in the recent years as a novel group of persistent organic pollutants (POPs) and perfluorooctane sulfonate (PFOS), and its salts have recently been added to Annex B of the Stockholm Convention on POPs (Bonefeld-Jorgensen et al. 2011; Stockholm Convention 2009). Unlike many other POPs such as dioxins, polychlorinated biphenyls (PCBs), and organochlorine pesticides which accumulate in lipid-rich tissues, PFAAs bind to blood proteins and are primarily detected in human liver and kidney (Maestri et al. 2006; D'Eon J et al. 2010). Some PFAAs have been detected in the lungs, thyroid, gonads, adipose tissue, pancreas, skeletal muscles, and brain as well as in breast milk and umbilical cord blood (Apelberg et al. 2007b; Haug et al. 2011; Maestri et al. 2006; Needham et al. 2011; Tao et al. 2008). The half-lives of human serum elimination have been estimated to be 3.8, 5.4, and 8.5 years for perfluorooctanoic acid (PFOA), PFOS, and perfluorohexane sulfonate (PFHxS), respectively (Olsen et al. 2007).

The toxicology of PFAAs has recently been extensively reviewed (Lau 2012). Animal studies have shown that exposure to PFAAs elicited hepatomegaly, hepatocellular adenomas, testicular and pancreatic tumors (Andersen et al. 2008), reproductive (Butenhoff et al. 2004) and developmental deficits (Lau et al. 2004), neurotoxicity (Johansson et al. 2009), immunotoxicity (Fair et al. 2011; Keil et al. 2008; DeWitt et al. 2011, 2009; Peden-Adams et al. 2008), and thyroid hormone alterations (Yu et al. 2009a, b). The developing fetus is especially sensitive to exposure to PFAAs, and PFAAs can transfer from mother to the fetus via the placenta (Needham et al. 2011; Apelberg et al. 2007a). In epidemiological studies, PFOA and PFOS exposure during fetal development inversely affected birth weight, length, and head and abdominal circumference (Fei et al. 2007, 2008; Apelberg et al. 2007b; Washino

et al. 2009) as well as neurodevelopment (Hoffman et al. 2010). Very recently, an association between PFAA exposure at commonly prevalent serum concentrations and a reduced humoral immune response in children was reported (Grandjean et al. 2012). A recent study has shown for the very first time a relation between serum levels of PFAAs and the risk of breast cancer in Greenlandic Inuits (Bonefeld-Jorgensen et al. 2011). Studies have indicated that human fecundity (Fei et al. 2009; Joensen et al. 2009), onset of puberty (Lopez-Espinosa et al. 2011), and thyroid function in adults and children (Ji et al. 2012; Lopez-Espinosa et al. 2012) may be affected by PFAAs. Some PFAAs have been classified as endocrine-disrupting compounds (EDCs) (de Cock et al. 2012).

Little is known about the underlying biochemical mechanisms of action of PFAAs that can account for adverse outcomes observed in toxicological studies. Activation of nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) has been shown to be associated with carcinogenicity, hepatotoxicity, developmental toxicity, immunotoxicity, and endocrine disruption of PFAAs (Lau 2012). As a member of the nuclear receptor family (Jacobs et al. 2003), the thyroid hormone receptor (TR) mediates the thyroid hormones' (THs) actions. Through binding to TR, THs regulate a number of biological processes that are essential for growth, development, and differentiation, especially in the developing brain (Oppenheimer and Schwartz 1997). Disruption of the thyroid system during the fetal and postnatal periods in humans has been shown to cause irreversible mental retardation and neurological deficits (Porterfield 2000; Zoeller 2005). Several studies showed that EDCs can interfere with the binding of THs to TR and may produce complex effects on TH signaling by, e.g., reducing circulating TH levels (Brucker-Davis 1998) and interfering with cell growth and TR function (Ghisari and Bonefeld-Jorgensen 2005; Zoeller 2005). A few experimental studies showed that PFAAs alter TH levels and interfere with brain development in mammals (Johansson et al. 2009; Yu et al. 2009a). PFOS was reported to be able to act as an estrogen receptor (ER) agonist and TR antagonist (Du et al. 2012). However, to our knowledge, the effect of other PFAAs on the TH function in the cellular and molecular levels has not been studied yet.

Many EDCs elicit multiple mechanisms of action, and the cross-talk between nuclear receptors including ER, androgen receptor (AR), TR, and aryl hydrocarbon receptor (AhR) was reported (Porterfield 2000; Safe et al. 1998; Ghisari and Bonefeld-Jorgensen 2005; Fujimoto et al. 2004; Pocar et al. 2005). Therefore, apart from their cell- and tissue-specific ER, AR, or TR agonist or antagonist activities, the involvement of other receptors such as AhR signaling must be taken into account (Safe et al. 2002). Being a ligand-dependent transcription factor, the AhR mediates many toxic and biologic effects of halogenated aromatic

hydrocarbons (HAHs) (Hankinson 1995). Mechanistically, the AhR functions in a manner similar to that of the steroid hormone receptors and stimulates transcription of adjacent genes including CYP1A1 and CYP1B1 (Hankinson 1995; Whitlock 1999). The classical AhR agonists include hydrophobic HAHs such as polychlorinated dibenzo-*p*-dioxins/furans, non- or mono-ortho PCBs, as well as numerous polycyclic aromatic hydrocarbons (PAHs) (Denison and Nagy 2003). However, recent studies have revealed that AhR can be activated by numerous chemicals with little structural similarity and physiochemical characteristics distinct from the classical AhR agonists (Denison and Nagy 2003; Long et al. 2003; Kruger et al. 2008). The studies about the effect of PFAAs on the AhR function are scarce. One study reported that PFOS induced CYP1A4 mRNA expression in chicken embryo hepatocytes (Watanabe et al. 2009). PFOA was reported to upregulate the expression of AhR in rare minnow gills (Liu et al. 2008). PFOS and PFOA produced an increase CYP1A1 expression in salmon kidney and liver (Mortensen et al. 2011). To our knowledge, there is no study for the effect of other PFAAs on the AhR function, especially in mammals.

The aim of the present study was to assess in vitro the potential impacts of seven PFAAs on the TH and AhR function. The TH-like activity was assessed using the rat pituitary tumor cell line GH3 expressing intracellular TR and responding to physiological concentration of TH by proliferation (named as T-screen). The AhR transcriptional activity was determined by AhR-luciferase reporter gene bioassay using the stably transfected mouse Hepa1.1 2cR cells. The tested PFAAs were selected based on extent of human exposure and use, detection in human body, potential toxicity, and public concern.

Materials and methods

Compounds and reagents

PFOS, PFHxS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA) were purchased from ABCR (Germany). Perfluorododecanoic acid (PFDoA) was purchased from Sigma-Aldrich (Denmark). The purity of the test compounds

Table 1 Tested perfluoroalkyl acids and their cytotoxicity in the GH3 and Hepa1.12cR cells

Compound	Chemical structure	CAS no. ^a	Purity (%)	Cytotoxicity (M)	
				GH3 cells	Hepa1.12cR cells
PFOS (perfluorooctane sulfonate)		1763-23-1	98	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$
PFHxS (perfluorohexane sulfonate)		355-46-4	98	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$
PFOA (perfluorooctanoic acid)		335-67-1	95	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$
PFNA (perfluorononanoic acid)		375-95-1	97	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$
PFDA (perfluorodecanoic acid)		335-76-2	98	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$
PFUnA (perfluoroundecanoic acid)		2058-94-8	95	$\geq 1 \times 10^{-4}$	$\geq 1 \times 10^{-3}$
PFDoA (perfluorododecanoic acid)		307-55-1	96	$\geq 1 \times 10^{-4}$	$\geq 1 \times 10^{-3}$

^a The CAS no. is for the protonated acid form of the perfluoroalkylates

was above 95 % (Table 1). PFOS, PFHxS, PFOA, PFNA, PFDA, and PFUnA were dissolved in dimethyl sulfoxide (DMSO, BDH Laboratory Supplies, Poole, UK) into stock solutions of 500 mM. PFDoA was dissolved in ethanol (EtOH, 96 % pure) to give a stock solution of 50 mM. All PFAA stock solutions were stored in the dark at room temperature. The same PFAA stock solution was used for both assays and tested in the concentration range from 1×10^{-9} to 1×10^{-4} M by dilution with an appropriate culture medium immediately before assays to give less than 0.1 % (v/v) solvent not affecting the cell viability.

3,3',5-Triiodo-L-thyronine (T3) was obtained from Sigma-Aldrich (Denmark) and used as a dose–response control in the T-screen assay. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, 98 %) was obtained from Cambridge by Bie & Berntsen (Denmark) and used as a dose–response control in the AhR transactivity assay. Some chemicals were harmful, and appropriate personal protective methods and materials were used throughout all experiments; all chemicals were handled avoiding light.

Thyroid hormone-dependent GH3 cell growth (T-screen assay)

The T-screen assay is a useful tool for in vitro detection of agonistic and antagonistic properties of compounds at the level of the TR (Schriks et al. 2006). The assay employs a rat pituitary tumor GH3 cell line, the growth of which is totally dependent on the active thyroid hormone T3. The growth stimulatory effect of T3 is mediated by intracellular expressed TRs, which, upon association with THs and binding to thyroid hormone-responsive elements in the cell nucleus, ultimately lead to gene expression and cell growth. Interaction of compounds with the TRs may result in agonistic effects on cell growth (cell proliferation), whereas interference with the T3–TR association or binding of antagonists to the TRs may result in inhibiting effects on T3-mediated cell growth.

The T-screen assay was performed as described previously (Ghisari and Bonefeld-Jorgensen 2005). GH3 cells were maintained in phenol-red Dulbecco's MEM culture medium (Sigma-Aldrich) supplemented with 10 % (v/v) fetal calf serum (FCS, Gibco, UK), 2 mM Glutamin, 1 mM sodium pyruvate, and 64 mg/l Garamycin (Schering-Plough, Brussels, Belgium) at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air. For the T-screen test, GH3 cells were seeded in 96-well plates (Nucleon Delta, Denmark) at an initial concentration of 3×10^3 cells/well in hormone-free medium (Bio-Rad AG-1×8 resin treated CD-FCS) (Samuels et al. 1979). On the following day, the medium was removed from the cells, and the test medium containing tested PFAAs at the given concentrations was added to the cells. The bioassay was terminated on day 6 by removing the medium from the wells and staining the fixed cells with sulforhodamine-B dye (Ghisari and Bonefeld-Jorgensen 2005).

At least three independent experiments were carried out, each with four replicates per test concentration. The compounds were tested in the absence and presence of 0.5 nM T3. In each assay, the solvent control (SC, 0.02 % DMSO or 0.02 % EtOH) and 0.5 nM T3 were included as negative solvent and positive controls, respectively. In parallel, a T3 dose–response curve ranging from 10^{-11} to 10^{-8} M was performed.

The average intra-coefficient of variation (CV) and inter-CV of the compounds were approximately 12 and 17 %, respectively.

AhR transcriptional activation bioassay

The cell culture-based AhR transactivation (AhR-tact) reporter gene bioassay is a mechanistically based technique that can detect all the compounds that can activate or inhibit the AhR and thus AhR-dependent gene expression. This assay has proven to be a quick and sensitive assay to detect the AhR-mediated potential of pure chemicals (Garrison et al. 1996; Long and Bonefeld-Jorgensen 2012; Long et al. 2012, 2003; Aarts et al. 1995) environmental matrices, and biological samples (Long and Bonefeld-Jorgensen 2012; Windal et al. 2005).

The Hepa1.12cR cells, stably transfected with the PAH/HAH-inducible luciferase expression vector pGudLuc1.1 (kindly provided by M.S. Denison (University of California, Davis, CA, USA)) that responds in a time-, dose-, and AhR-dependent manner (Garrison et al. 1996), were used for determination of effects on the AhR-tact as described (Long et al. 2006). The Hepa1.12cR cells were maintained with alpha minimal essential medium (α-MEM), supplemented with 10 % FCS (Gibco, UK), 64 mg/l Garamycin (Schering-Plough, Brussels, Belgium), and 400 mg/l geneticin (G418, Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air.

For the AhR-tact bioassay, the PFAAs were tested alone or in co-treatment with 60 pM TCDD. Experiments were carried out with three replicates per tested concentration in two independent experiments. In each experiment, the SC (0.02 % DMSO or 0.02 % EtOH) and 60 pM TCDD were included as negative control and positive control, respectively. The AhR-tact was measured by the reporter luciferase activity expressed in relative light units (RLUs) as described (Long et al. 2006). The average intra-CV and inter-CV of the compounds were approximately 8–9 and 13–14 %, respectively.

Cytotoxicity

Parallel to the T-screen and AhR-tact assays, the PFAAs were tested for cytotoxicity using the protocol of the cytotoxicity detection kit (LDH) from Roche (Hvidovre, DK) as described (Ghisari and Bonefeld-Jorgensen 2005). Only non-cytotoxicity data are described.

Calculation of effect potency for the T-screen and AhR-tact

In T-screen assay, the proliferative effect (PE) was calculated as the percentage ratio between the cell yields obtained with the compounds (PFAAs) versus the cell yields in the compound-free control (SC, 0.02 % DMSO or 0.02 % EtOH). The relative proliferative effect (RPE) which measures the ratio between the maximal cell growth achieved by the test compound and that of T3-EC₁₀₀ were calculated as

$$\text{RPE} = \frac{\text{PE(PFAAs)} - \text{PE(control)}}{\text{PE(T3)} - \text{PE(control)}} \times 100\%$$

Concentration-response curves and EC₅₀ calculations of T3 and PFAAs were performed in Sigma Plot (SPSS, Chicago, IL) using the sigmoid Hill model.

In AhR-tact bioassay, the EC₅₀ of TCDD and PFAAs which elicited dose-dependent agonistic AhR-tact, was calculated by fitting dose-response data to a three-parameter sigmoidal Hill curve using Sigma Plot (SPSS, Chicago, IL) as described (Long et al. 2006, 2003). Since the dose-response curves of PFAAs were not parallel to the TCDD dose-response curve and/or the maximum response were not obtained in the tested concentration range, the tentative AhR-relative potency (AhR-REP) of PFAAs was estimated by comparing TCDD and PFAA concentrations, which induced the same transactivity. The lowest TCDD concentration inducing a significantly higher transactivity compared with that of the solvent control was designated as lowest observed effect concentration (LOEC)-TCDD. The PFAA concentration required to give the same transactivity as LOEC-TCDD was designated as LOEC-PFAA. The tentative AhR-REP was calculated as the following equation:

$$\text{AhR-REP} = \frac{\text{LOEC-TCDD}}{\text{LOEC-PFAA}}$$

Statistical analysis

All experiments were repeated at least two independent times in four (T-screen) or three (AhR-tact) replicates. Mean PE (T-screen) or transactivity (RLU, AhR-tact) of the solvent control (0.02 % DMSO or 0.02 % EtOH) was set to 100 %, and the fold inductions of PFAAs were determined using the PE or RLU. Results are reported as means ± SD. The statistical analysis was performed in SPSS 13.0 (SPSS Inc, Chicago, IL). Due to inequality of variance and relatively few data points per concentration, nonparametric statistics was performed. The Kruskal-Wallis test was used to compare differences between different concentrations, and the Jonckheere-Tepstra test was used to analyze for a linear trend between concentrations and response. If one or both tests showed a significant

difference ($p \leq 0.05$), the Mann-Whitney test was used to compare the difference between each test concentration and the respective control.

Results

Cytotoxicity of the tested PFAAs

The cytotoxicity assay showed that the highest tested concentration (1×10^{-4} M) of PFUnA and PFDoA were toxic to GH3 cells, whereas no cytotoxicity of the GH3 cells was observed for the rest of the tested PFAAs (Table 1). None of the PFAAs show any cytotoxicity to the Hepa1.12cR cells at the tested concentrations during the test duration (Table 1). The given results refer only to effects observed at concentrations not being toxic.

Effects on the thyroid hormone system

T3 dose dependently stimulated the GH3 cell proliferation at 1×10^{-10} – 1×10^{-8} M and induced the maximum proliferation with 12-fold over the solvent control at 1×10^{-8} M (Table 2). The average EC₅₀ of T3 was 0.61 nM (not shown) being similar to our previous study (Ghisari and Bonefeld-Jorgensen 2005).

The effect of seven PFAAs on the GH3 cell proliferation was studied by exposing cells to the single PFAAs in the range of 1×10^{-9} – 1×10^{-4} M in the absence of T3. As shown in Table 2, none of the tested PFAAs induced the GH3 cell proliferation. All PFAAs significantly decreased GH3 cell proliferation compared with the solvent control in the tested range ($p < 0.05$, Fig. 1a). PFOS, PFHxS, PFNA, PFDA, and PFUnA elicited dose dependently decreased GH3 cell proliferation (Fig. 1a).

The competitive effect of PFAAs on the T3-induced GH3 cell proliferation was studied by co-treatment of PFAAs with 0.5 nM T3. It was shown that PFOS, PFNA, and PFUnA significantly decreased the T3-induced GH3 cell proliferation in the range of 1×10^{-8} – 1×10^{-4} M. PFHxS significantly decreased the T3-induced proliferation in the range of 1×10^{-8} – 1×10^{-5} M, but further increased the T3-induced proliferation at 1×10^{-4} M (Table 2, Fig. 1b). PFDA and PFDoA significantly decreased the T3-induced cell proliferation at 1×10^{-4} and 1×10^{-5} M, respectively. No significant effect of PFOA on the T3-induced GH3 proliferation was observed (Table 2, Fig. 1b).

Effects on the AhR function

TCDD significantly induced dose-dependent AhR-tact in the range of 2×10^{-12} – 5×10^{-9} M and the maximum induced AhR-tact was 24-fold over the solvent control (Table 3).

Table 2 Effect of perfluoroalkyl acids in the T-screen assay

Compound	Agonistic response (compound alone)				Competitive response (compound in the presence of 0.5 nM T3)			
	LOEC (M)	MOEC (M)	PE % of SC at MOEC	RPE % at MOEC	LOEC (M)	MOEC (M)	PE % of 0.5 nM T3 at MOEC	RPE % at MOEC
SC			100					
T3	1×10^{-10}	1×10^{-8}	1,165	100	5×10^{-10}	5×10^{-10}	100	100
PFOS	1×10^{-9}	1×10^{-4}	58.76	−3.87	1×10^{-8}	1×10^{-4}	82.04	−1.69
PFHxS	1×10^{-9}	1×10^{-4}	57.06	−4.03	1×10^{-8}	$1 \times 10^{-5}/1 \times 10^{-4}$	84.17/119.17	−1.49/1.80
PFOA	1×10^{-9}	1×10^{-4}	71.17	−2.71	NE	NE	NE	NE
PFNA	1×10^{-9}	1×10^{-4}	69.42	−2.87	1×10^{-8}	1×10^{-4}	79.83	−1.89
PFDA	1×10^{-8}	1×10^{-5}	66.64	−3.13	1×10^{-4}	1×10^{-4}	72.67	−2.57
PFUnA	1×10^{-9}	1×10^{-6}	61.71	−3.59	1×10^{-9}	1×10^{-5}	74.36	−2.41
PFDoA	1×10^{-9}	1×10^{-5}	61.63	−3.60	1×10^{-5}	1×10^{-5}	81.25	−1.76

SC solvent control (0.02 % DMSO or 0.02 % EtOH); T3 3,3',5-triiodo-L-thyronine; LOEC lowest observed effect concentration, the lowest tested concentration at which a significant effect ($p \leq 0.05$ vs. SC) was detected; MOEC maximum observed effect concentration, the lowest concentration at which a significant ($p \leq 0.05$ vs. SC) maximum effect was detected; PE the proliferative effect as percentage ratio of cell yield with the compound vs. the controls (SC or 0.5 nM T3); RPE relative proliferative effect as described in the “Materials and methods”: $RPE = \frac{PE(PFAAs) - PE(control)}{PE(T3) - PE(control)} \times 100\%$; NE no effect observed

The agonistic effect of PFAAs on AhR-tact was determined by exposure of Hepa1.12cR cells to the PFAAs in the absence of TCDD. At the highest tested concentration, 1×10^{-4} M, PFHxS, and PFUnA significantly decreased the AhR-tact compared to the solvent control ($p < 0.05$, Table 3). Although no obvious cytotoxicity of this concentration was measured in LDH assay, the higher concentration (1×10^{-3} M) of PFHxS and PFUnA elicited cytotoxicity in the cells. Thus, the decreased AhR-tact of PFHxS and PFUnA might be related to the starting of cytotoxicity. PFDoA significantly induced the AhR-tact agonistic response in the range of 1×10^{-9} – 1×10^{-6} M being 114 to 130 % above the solvent control, although no obvious dose–response trend was observed (Table 3). Compared to the potent AhR ligand, TCDD, the potential of PFDoA to induce the AhR-tact was very weak with a tentative AhR-REP of 5×10^{-6} (Table 3). Other PFAAs showed no significant effect on the AhR-tact.

Antagonistic or potentiating effects of the PFCs on the TCDD-induced AhR activity were evaluated by co-treatment of Hepa1.12cR cells with the PFAAs and 60 pM TCDD. PFDA and PFDoA further increased the TCDD-induced AhR-tact in the range of 1×10^{-9} – 1×10^{-7} M and 1×10^{-7} – 1×10^{-6} M, respectively (Table 3). The other PFAAs did not affect the TCDD-induced AhR-tact in the test concentration range.

Discussion

To date, a few studies have shown that PFAAs alter TH levels and interfere with brain development in mammals

(Johansson et al. 2009; Lau et al. 2003; Thibodeaux et al. 2003; Yu et al. 2009a). However, studies on in vitro effects of PFAAs on the TH function are scarce and focused on PFOA and PFOS. To our knowledge, no study has investigated the effect of several other PFAAs on the TH function. The GH3 rat pituitary tumor cells are dependent on the TH for their proliferation in monolayer culture (Samuels et al. 1979; Hohenwarter et al. 1996) and express the endogenous TRs at high amounts and possess all the necessary factors for measuring the biological endpoint of growth. Thus, the effect of a compound on the GH3 proliferation will also include the interactions and cross-talk between different TH actions and other cellular pathways. The GH3 cell line has been widely used as a standard pituitary cell model for TH action (Hohenwarter et al. 1996). In the present study, we employed the T-screen assay to assess the potential effect of two PFSAAs (PFOS and PFHxS) and five PFCAs (PFOA, PFNA, PFDA, PFUnA, and PFDoA) on TH function. The results showed that all seven PFAAs inhibited the basal growth of rat pituitary GH3 cells, and PFOS, PFHxS, PFNA, PFDA, and PFUnA elicited a dose-dependent effect. Four of the PFAAs (PFOS, PFHxS, PFNA, PFUnA) antagonized the T3-induced GH3 cell growth dose dependently. However, for PFHxS, an increase in the T3-induced GH3 cell proliferation was seen at the highest tested concentration. For the observed inhibition of basal GH3 cell growth by PFAAs, the basic mechanisms are not clear. We speculate that PFAAs might compete with the very small background level of T3 in the media to bind to TR and/or other cofactors such as growth hormone and therefore inhibit the basal GH3 cell growth. Nevertheless, more studies are needed to elucidate this hypothesis.

Fig. 1 Effects of perfluoroalkyl acids in the T-screen assay. **a** Effects of perfluoroalkyl acids on the basal cell growth in the absence of T3. GH3 cells were incubated with the given concentration (in molar) of compounds in the absence of T3. Data represent mean $\pm$ SD of at least three independent experiments, each performed in four replicates per concentration. * indicates significantly different from the corresponding control (0.02 % DMSO or 0.02 % EtOH) ($p < 0.05$), # indicates cytotoxic. **b** The effects of perfluoroalkyl acids in the presence of T3. GH3 cells were incubated with indicated concentration (in molar) of compounds in the presence of 0.5 nM T3. Data represent mean $\pm$ SD of at least three independent experiments, each performed in four replicates. * indicates significantly different from the corresponding control (0.02 % DMSO or 0.02 % EtOH plus 0.5 nM T3) ($p < 0.05$), # indicates cytotoxicity

For the effect of PFASs, using a reporter gene assay, Du and coworkers reported that PFOS had no agonistic TR activity but inhibited the T3–TR activity in the African green monkey kidney cell line (Du et al. 2012). This is consistent with our result in the rat pituitary GH3 cells which showed that PFOS had no agonistic effect on TR-mediated cell growth but significantly antagonize the T3-induced GH3 cell proliferation. PFOS was shown to compete with T4 for binding sites on human transthyretin (TTR) (Weiss et al. 2009), and a previous study showed that 10^{-5} M PFHxS down-regulated TTR mRNA expression in avian neuronal cells (Vongphachan et al. 2011). TTR is a TH-binding protein in the serum and cerebrospinal fluid carrying the thyroid hormone T4 into the central nervous system where T4 is converted to its more active form T3

(Schreiber 2002). A reduction of TTR expression could result in decreased TH availability to target tissues and thus affect TH-dependent processes. This might partly explain the decreased T3-induced GH3 cell proliferation of PFOS and PFHxS observed in the present study. Vongphachan et al. (2011) also found that PFHxS and T3 had a potential shared mechanism of action, which might explain the potential of 1×10^{-4} M PFHxS to further increase the T3-induced GH3 cell proliferation. Further studies are needed to confirm this interpretation.

Higher serum PFOS and PFHxS was reported to be significantly associated with attention deficit hyperactivity disorders (ADHD) (Hoffman et al. 2010; Stein and Savitz 2011). In addition, PFOS decreased serum T3 and T4 levels in animals (Thibodeaux et al. 2003; Lau et al. 2003), and in humans, PFOS concentrations were negatively associated with total T3 (Dallaire et al. 2009) and fetal T3 (Kim et al. 2011). As disruption of TH functions are related to the development of ADHD (de Cock et al. 2012; Negishi et al. 2005; Simic et al. 2009), previous studies and the present study suggest negative effects of PFOS and PFHxS on the thyroid system, and thus, these compounds might increase the risk of ADHD via TH system interference.

For PFCAs, we observed that PFUnA dose dependently decreased the T3-induced GH3 cell proliferation, and PFDoA antagonized the effect of T3 at high concentration (1×10^{-5} M), suggesting the potential TH interference of PFUnA and PFDoA. PFUnA and PFDoA were found to activate the PPAR α in mouse and rat, respectively (Wolf et al. 2012; Zhang et al. 2008). A previous study showed that activation of PPAR decreased the expression of TR in rat liver (Bonilla et al. 2001). Therefore, whether the observed antagonizing effect of PFUnA and PFDoA in our T-screen test and the TH system in general is related to PPAR α activation needs further study.

Among the seven tested PFAAs, only PFDoA elicited agonistic AhR-tact in the Hepa1.12cR cells, whereas both PFDA and PFDoA further increased the TCDD-induced AhR-tact. AhR regulates an adaptive response pathway to environmental contaminants (Long and Bonefeld-Jorgensen 2012; Schmidt and Bradfield 1996). Specially, AhR up-regulates phase I metabolizing enzymes such as CYP1 that participate in the degradation and elimination of xenobiotics. The AhR also mediates the induction of phase II enzymes which serve as a detoxification mechanism for many mutagens, carcinogens, and other toxic compounds. Several AhR ligands are environmental contaminants that not only serve as agonist of the AhR, but also substrates for the induced phase I enzymes (Denison and Heath-Pagliuso 1998; Stejskalova et al. 2011). AhR has cross-talk with other nuclear receptors such as ER, AR, and TR (Porterfield 2000; Ghisari and Bonefeld-Jorgensen 2005; Fujimoto et al. 2004; Pocar et al. 2005). It has been reported that PFAAs interfere

Table 3 Effect of perfluoroalkyl acids on AhR transactivity

Compound	Agonistic response (compound alone)				Competitive response (compound in the presence of 60 pM TCDD)		
	LOEC (M)	MOEC (M)	% of SC at MOEC	AhR-REP	LOEC (M)	MOEC (M)	% of 60pM TCDD at MOEC
SC			100				
TCDD	5×10^{-12}	5×10^{-9}	2384	1	6×10^{-11}	6×10^{-11}	100
PFOS	NE	NE	NE	NE	NE	NE	NE
PFHxS	1×10^{-4}	1×10^{-4}	86	NE	NE	NE	NE
PFOA	NE	NE	NE	NE	NE	NE	NE
PFNA	NE	NE	NE	NE	NE	NE	NE
PFDA	NE	NE	NE	NE	1×10^{-9}	1×10^{-7}	124
PFUnA	1×10^{-4}	1×10^{-4}	89	NE	NE	NE	NE
PFDoA	1×10^{-9}	1×10^{-6}	130	5×10^{-6}	1×10^{-7}	1×10^{-6}	109

SC solvent control (0.02 % DMSO or 0.02 % EtOH); TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; LOEC lowest observed effect concentration, the lowest tested concentration at which a significant effect ($p \leq 0.05$ vs. SC) was detected; MOEC maximum observed effect concentration, the lowest concentration at which a significant ($p \leq 0.05$ vs. SC) maximum effect was detected; NE no effect observed; AhR-REP AhR-mediated relative potency (see “Materials and methods”): $\text{AhR-REP} = \frac{\text{LOEC-TCDD}}{\text{LOEC-PFAA}}$

with the TH system, AR and ER (Du et al. 2012). To date, few studies on the effect of PFOS and PFOA on the AhR function have been reported (Liu et al. 2008; Watanabe et al. 2009; Hu et al. 2003). In the present study, PFOS and PFOA did not affect TCDD-induced AhR-tact in mammal cells, similar to the report of CYP1A induction in chicken embryo hepatocyte (Watanabe et al. 2009). Hu et al. reported that PFOS alone did not induce AhR-mediated luciferase activity in the rat hepatoma H4IIE cell line (Hu et al. 2003), supporting the observation of the present study. However, they found PFOS significantly increased TCDD-induced luciferase activity (Hu et al. 2003), while we did not observe that in the present study. This difference might be related to species (rat vs. mouse), dose (0.6–3 nM TCDD vs. 60 pM TCDD) and exposure time (24 vs. 4 h).

As far as we know, there is no study about the effect of other PFAAs on the AhR function. Of the PFCAs with a chain length of six to nine carbon atoms, longer chain length induced higher level of compound accumulation in the liver and higher toxicity compared with shorter chain length species (Kudo et al. 2006). Moreover, the rate of elimination decreased with increasing carbon chain length and exhibited tremendous species differences (Martin et al. 2003; Olsen et al. 2007). Thus, PFDA and PFDoA, compounds with 10 and 12 carbon atoms, are likely to have a different toxic effect than PFOS and PFOA. Only limited studies of the toxicity of PFDA and PFDoA have been performed (Shi et al. 2007; Zhang et al. 2008; Harris et al. 1989; Kawashima et al. 1995). To our knowledge, we observed for the first time that PFDoA elicited agonistic AhR-tact in the range of 1×10^{-9} – 1×10^{-4} M, and a further increase of the AhR-TCDD-induced AhR-tact. Also PFDA further increased the TCDD-induced AhR-tact. In human cell lines, two peroxisome

proliferator response element sites are located in the CYP1A1 promoter region (Seree et al. 2004; Villard et al. 2011), suggesting that there is a possibility that PPAR α agonists could induce CYP1A1. Previous studies suggested there is cross-talk between AhR and PPAR α in some species (Fallone et al. 2005; Shaban et al. 2004a, 2004b, 2005). It was reported that the expression of PPAR α was induced by PFDoA in rats (Zhang et al. 2008). PFDA is a known PPAR α agonist in mouse cells (Wolf et al. 2008, 2012). Thus, the observed PFDoA and PFDA-induced AhR-tact in the Hepa1.12cR mouse cells in the present study is supported by a previous study (Zhang et al. 2008) and further suggests the existence of AhR and PPAR α cross-talk. However, further studies are needed to explore whether PPAR α is involved in the induction of PFDA and PFDoA on AhR-tact.

Dioxins and THs share common molecular reactivity properties and possibly similar molecular recognition in biochemical systems (McKinney and Waller 1998). Recent data showed that dioxins may inhibit post-receptor events induced by THs (Bogazzi et al. 2003), suggesting an antagonist TR activity of the tested dioxin-like compounds. We observed that PFDoA activated AhR but inhibited the TR function, which might be related to the cross-talk of AhR and TR. Since the cells used in T-screen and AhR-tact bioassay were derived from different tissues, additional cell lines that are thyroid hormone dependent should be concomitantly tested for TR and AhR signal transduction to further explore the cross-talk of AhR and TR.

In summary, we observed that the seven tested PFAAs have the potential to inhibit the function of the TH system, and two of the tested PFAAs affected AhR. Thus, PFAAs might affect the endocrine homeostasis via interfering nuclear receptor pathways including PPAR, ER, AR, TR, and AhR. It should

be noted that the biological endpoint in the T-screen assay determines the proliferative effect of the GH3 cells, which could be influenced by other factors than the more specific TR function (Ghisari and Bonefeld-Jorgensen 2005). It is evident that the GH3 cell growth is dependent on T3, but the ER is involved in basal and T3-induced growth of GH3 cells, possibly as a secondary effect mediated by a T3-dependent ER up-regulation (Ghisari and Bonefeld-Jorgensen 2005). More research is needed in order to fully evaluate the activities of PFAAs and the biological relevance of experimental findings. Future studies may focus on elucidating the mechanisms underlying PFAA action and characterizing the actions of PFAAs in different hormonal environments, and the combined effect of PFAA mixtures. Knowledge gained from these studies may be helpful for the risk assessment of possible adverse effects of the different PFAAs.

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