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Steroid hormone related effects of marine persistent organic pollutants in human H295R adrenocortical carcinoma cells

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ABSTRACT

Persistent organic pollutants (POPs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorobiphenyl (PCB) 126 and 153, perfluorooctanesulfonic acid (PFOS), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), tributyltin (TBT), and methylmercury (MeHg) can be accumulated in seafood and then form a main source for human exposure. Some POPs have been associated with changes in steroid hormone levels in both humans and animals. This study describes the *in vitro* effects of these POPs and mixtures thereof in H295R adrenocortical carcinoma cells. Relative responses for 13 steroid hormones and 7 genes involved in the steroidogenic pathway, and CYP1A1, were analyzed. PFOS induced the most pronounced effects on steroid hormone levels by significantly affecting 9 out of 13 hormone levels measured, with the largest increases found for 17β-estradiol, corticosterone, and cortisol. Furthermore, TCDD, both PCBs, and TBT significantly altered steroidogenesis. Increased steroid hormone levels were accompanied by related increased gene expression levels. The differently expressed genes were MC2R, CYP11B1, CYP11B2, and CYP19A1 and changes in gene expression levels were more sensitive than changes in hormone levels. The POP mixtures tested showed mostly additive effects, especially for DHEA and 17β-estradiol levels. This study shows that some seafood POPs are capable of altering steroidogenesis in H295R cells at concentrations that mixtures might reach in human blood, suggesting that adverse health effects cannot be excluded.

1. Introduction

Environmental persistent organic pollutants (POPs), either historical or currently in use, accumulate in fatty tissues causing seafood to be a main source for human exposure (Bilau et al., 2008; Liem et al., 2000; Montano et al., 2013). This is of major concern, because many of these marine POPs have been related to negative health effects such as reduced cognitive development, immune toxicity, neurological disorders, cancer, and endocrine disruption (Li et al., 2006; Yu et al., 2010). In addition to the well-known mechanism for dioxin-like compounds via the aryl hydrocarbon receptor (AhR) (Landers and Bunce, 1991; Okey et al., 1994), other mechanisms of toxic action have been suggested to cause adverse health effects, including disruption of thyroid (Brouwer et al., 1998) and steroid hormone systems (WHO, 2012). Such mechanisms can be inappropriate activation or antagonism of the nuclear steroid receptors, modulating nuclear receptor coactivators, or interference with key enzymes involved in steroid hormone synthesis and metabolism (Sanderson, 2006). Several POPs have been shown to affect hormone levels in humans and animals (Steinberg et al., 2008; Turyk et al., 2008; Zimmer et al., 2009). To date, most studies focused on the effects of single POPs on levels of only a few different steroid hormones (Ding et al., 2007; Kraugerud et al., 2010, 2011), or extracted mixtures of only partially identified POPs were tested (Montano et al., 2011; Zimmer et al., 2011).

Abbreviations: AhR, aryl hydrocarbon receptor; BDE-47, 2.2′,4,4′-tetrabromodiphenyl ether; CV, coefficient of variation; DHEA, dehydroepiandrosterone; EIA, enzyme immunoassay; HBCD, hexabromocyclododecane; HPA, hypothalamic-pituitary-adrenal; LOQ, limit of quantification; MC2R, melanocortin 2 receptor; MeHg, methylmercury; OECD, organization for economic co-operation and development; PCB, polychlorobiphenyl; PFOS, perfluorooctanesulfonic acid; POPs, persistent organic pollutants; SC, solvent control; S/N, signal-to-noise ratio; SPE, solid-phase extraction; TBT, tributyl chlorotin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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Investigating single compounds is important to elucidate the mechanisms of action as different compounds may counteract each other's mechanisms. Humans, on the other hand, are exposed to mixtures so it is important to elucidate combined actions as well.

In this study we investigated the effects of single POPs and mixtures thereof on steroidogenesis. Eight different POPs present in the human seafood chain were chosen based on their relative abundance in polluted fish species. These include the dioxin-like compounds TCDD and PCB 126 (Hoogenboom et al., 2007); the non-dioxin-like PCB 153 (de Boer et al., 2010); the perfluorinated compound heptadecafluorooctanesulfonic acid (PFOS) (Kwadijk et al., 2010); the brominated flame retardants hexabromocyclododecane (HBCD) (van Leeuwen and de Boer, 2008) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) (Voorspoels et al., 2003); the biocide tributyl chlorotin (TBT) (Stab et al., 1996), and the organometallic MeHg (Maes et al., 2008). The composition of mixtures to which people are exposed depends on the origin and type of polluted seafood that is consumed. Therefore, there is not one mixture that is most relevant to test, but still it is important to test whether compounds in a mixture can induce interactive effects. The *in vitro* model used was the H295R human adrenocortical carcinoma cell line, chosen because it expresses all hormones found in the adult adrenal cortex and the gonads and all key enzymes involved in steroidogenesis (Gazdar et al., 1990). The functionality of this bioassay is consistent with most results observed *in vivo* (Hecker et al., 2006). The endpoints we studied with the H295R model are corticosteroid synthesis and the production of sex steroid hormones. The assay has been fully validated to evaluate effects on testosterone and estradiol production (OECD, 2011). However, more elaborate approaches provide a screen for endocrine disruption and can identify potential targets of the test compound (Rijk et al., 2012). In this study we measure steroidogenesis disruption as hormone production of 13 steroid hormones plus expression levels of 7 key steroidogenic genes for 8 individual POPs and mixtures thereof.

2. Materials and methods

2.1. Chemicals

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, CAS 1746-01-6), PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl, CAS 35065-27-1), PFOS (heptadecafluorooctanesulfonic acid potassium salt, CAS 2795-39-3), TBT (tributyl chlorotin, CAS 1461-22-9), and MeHg (methylmercury(II) chloride, CAS 115-09-3) were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl, CAS 57465-28-8) was purchased from Promochem (Wesel, Germany). HBCD (hexabromocyclododecane, technical mixture) and BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether, CAS 5436-43-1) were kindly provided by Professor Åke Bergman (Stockholm University, Sweden) within the framework of the EU FIRE project (Schriks et al., 2006). Forskolin (CAS 66575-29-9) and prochloraz (CAS 67747-09-5) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions for all chemicals were prepared in dimethylsulfoxide (DMSO) (Acros Organics, Belgium). Pregnenolone, 17α-OH-pregnenolone, progesterone, 17α-OH-progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol were obtained from Steraloids (Newport, RI, USA). The deuterium labeled internal steroid standards were from CDN isotopes (Point-Claire, Canada). Derivatisation reagent consisted of 1 mg 4-dimethyl-aminopyridine, 5 mg 2-methyl-6-nitrobenzoic anhydride and 3 mg picolinic acid in 1 mL tetrahydrofuran after which 10 μL of triethylamine was added. All were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and exposure

H295R human adrenocortical carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to the standardized protocol approved by the OECD (OECD, 2011). Briefly, cells were routinely grown in 75 cm^2 culture flasks containing DMEM/F12 (without phenol-red, Sigma, Zwijndrecht, The Netherlands) supplemented with 1.2 g/L $NaHCO_3$, 1% Insulin Transferrin Selenium (ITS + premix), and 2.5% NuSerum (BD Biosciences, Bedford, MA) at 37 °C and 5% CO_2 atmosphere. Cells were subcultured when 80% confluency was reached. For subcultivation, cells were washed twice with PBS, detached using trypsin–EDTA (0.25/0.05%, v/v) (Difco, NJ), and seeded in a 1:3 ratio. After thawing frozen stocks (passage 5), cells were cultured for at least four additional passages prior to testing and cells were not used after passage 13. For experiments, 3 mL of 3×10^5 cells/mL was seeded in 6-well plates (Greiner bio-one, Frickenhausen, Germany). After 24 h, medium was replaced by 2 mL exposure medium containing a single POP or a mixture thereof dissolved in DMSO. The final concentration of DMSO in the medium was 0.25%. Three independent experiments were performed and in each experiment three concentrations were tested for both the single POPs and POP mixtures. Concentrations were chosen in two steps, a first selection of concentrations was based on literature study, followed by cytotoxicity testing to ensure experiments were performed at non-toxic concentrations. Two mixtures of different combinations of these compounds were tested, one with only four compounds which all affected steroidogenesis (mixture A) and one with all eight compounds (mixture B). Mixtures A1 and B1 consist of the middle concentration of the concentration range chosen for the individual POP tests based on the absence of cytotoxicity. So mixture A1 consisted of TCDD (30 nM), PCB 126 (3 μM), PCB 153 (3 μM) and PFOS (100 μM) and mixture B1 consisted of these 4 POPs and HBCD (300 nM), BDE-47 (300 nM), TBT (10 nM), and MeHg (30 nM). Mixture A1 or B1 were 3 times diluted to obtain mixture A3 or B3 and 10 times diluted to obtain mixture A10 or B10. Forskolin, a known adenylate cyclase inducer, and prochloraz, a known inhibitor of multiple CYPs involved in steroidogenesis such as CYP17, were run as positive and negative controls. After 48 h of exposure, as suggested by the OECD, the medium was collected and stored at −80 °C until hormone analysis. The cells were immediately lysed in Trizol (Invitrogen, Breda, The Netherlands), transferred to a fresh vial, and stored at −80 °C until RNA extraction.

2.3. Cell viability assays

For cytotoxicity testing, cells were seeded in a 96-well plate using 100 μL of 3×10^5 cells/mL. After 24 h, medium was replaced by 200 μL medium containing the individual POPs or mixtures thereof in triplicate. The highest concentrations tested were 100 nM for TCDD, 10 μM for both PCBs, 600 μM for PFOS, 1 μM for HBCD, 1 μM for BDE-47, 100 nM for TBT, 100 nM for MeHg, and the highest test concentration of both mixtures. Cytotoxic effects from three independent experiments were compared to the solvent control (SC) and Triton X-100 (0.1%) was included as a positive control. After 48 h, cytotoxic effects of the test compounds were evaluated with two cytotoxicity assays that measure different endpoints. The assays applied were the WST-1 cytotoxicity assay (Roche Diagnostics, Mannheim, Germany) based on the activity of mitochondrial dehydrogenase enzymes and the ATPlite assay (PerkinElmer, Groningen, The Netherlands) based on the principle that ATP is present in all metabolically active cells. For