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Effects of individual polychlorinated naphthalene (PCN) components of Halowax 1051 and two defined, artificial PCN mixtures on AHR and CYP1A1 protein expression, steroid secretion and expression of enzymes involved in steroidogenesis (CYP17, 17β-HSD and CYP19) in porcine ovarian follicles

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ABSTRACT

In this study we tried to answer a question which component of Halowax 1051 is responsible for, observed in previously published study, androgenic effects of the mixture, and whether it is possible to draw conclusions about the action of mixtures by examining the effect of an indicator congener.

Ovarian follicles were incubated with individual congeners of an artificial mixture for 6–24 h. At the end of the incubation period, media were collected for determination of progesterone (P4), androstenedione (A4), testosterone (T) and estradiol (E2) levels by enzyme immunoassay, and follicles were retained for an examination of aryl hydrocarbon receptor (AHR), cytochrome p450 enzymes (CYP1A1, CYP17, CYP19), and 17β-hydroxysteroid dehydrogenase (17β-HSD) protein expression by Western blotting.

CN73 in dose 50 pg/ml after 6 h had no effect and decreased AHR expression after 24 h, while at dose 400 pg/ml increased AHR protein expression after 6 h of exposure which remained elevated after 24 h. CN74 and CN75 at both concentrations tested (25 and 50 pg/ml) stimulated AHR protein expression after 6 h and decreased it after 24 h of exposure. Individual congeners induced a rapid increase in CYP1A1 protein expression, with a rank order of efficacy of CN73 > CN74 = CN75. All congeners increased P4/A4 and T/E2 secretion ratios in association with a decrease in the A4/T ratio, pointing to androgenic and anti-estrogenic properties of PCNs in ovarian follicles. The most potent congener in this context was CN73. The effects of mixtures were comparable to those of CN74 and CN75, and were not as strong as those observed for CN73.

Collectively, these data suggest antagonistic actions of single congeners in a mixture, indicating that the actions of a mixture cannot be predicted based on the actions of individual congeners.

1. Introduction

Polychlorinated naphthalenes (PCNs) are persistent and ubiquitous chemicals in the environment. They have been used in a variety of industries owing to their dielectric, water-repellent and flame-retardant properties, and their high physico-chemical stability (Jakobsson and Asplund, 2000). Although commercial production of PCNs ceased in 1980s (IPCS, 2001), they are formed as a byproduct of the synthesis of other organochlorine compounds (Liu et al., 2014). As a result, humans continue to be exposed to many environmental sources of PCNs, including solid waste (Phan et al., 2013), ambient air (Xu et al., 2014), seafood, meats, and fats (Llobet et al., 2007; Martí-Cid et al., 2007).

We previously reported that Halowax 1051 components: CN73 (1,2,3,4,5,6,7-heptaCN), CN74 (1,2,3,4,5,6,8-heptaCN) and CN75 (1,2,3,4,5,6,7,8-octaCN), exert androgenic properties in ovarian follicles through a direct, stimulatory action on 17β-hydroxysteroid dehydrogenase (17β-HSD) activity and protein expression, and an inhibitory action on CYP19 activity and protein expression (Gregoraszczuk et al., 2011). Moreover, we demonstrated rapid activation of phase I metabolic enzymes with simultaneous inhibition of phase II metabolic enzymes, suggesting local metabolism of Halowax 1051 in the ovary (Barć et al., 2013).

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Hooth et al. (2012) showed that more highly chlorinated mixtures produce biological effects similar to those of 2,3,7,8-tetrachlorodibenzopdioxin (TCDD), including induction of the liver cytochrome P450-associated enzymes, ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH). Genes encoding cytochrome P450s of the 1A family are well-known target of the aryl hydrocarbon receptor (AHR), and the corresponding proteins may therefore serve as biomarkers for the toxicity of dioxins and dioxin-like chemicals. In addition, induction of CYP1A1, linked to increased metabolism of estrogens, may result in action on steroidogenesis.

These studies raise questions about which component of Halowax 1051 is responsible for the observed androgenic effects of this mixture, and whether it is possible to draw conclusions about the action of mixtures by examining the effect of an indicator congener. The main CN constituent of Halowax 1051 is CN75 belonging to the octachloronaphthalene homologue group. The other two congeners, 73CN and 74CN, totaling 10% of mixture, belong to the heptachloronaphthalene homologue group. Congener that most significantly contributes to TEQs (Toxic Equivalent) is CN73 (Noma et al., 2004).

There are a number of reasons why the actions of chemical mixtures are not often studied. In addition to simple additive effects (Ramamoorthy et al., 1997), interactions between different chemicals in a mixture may result in either diminished (Donnelly et al., 1998) or augmented effects compared with those expected on the basis of their individual chemical structures or known toxicity profiles.

In this study, we examined the effect of CN73, CN74 and CN75, alone or in combination, at two different doses (environmentally relevant doses, determined based on the levels of these compounds in blood plasma samples (Park et al., 2010), and at a dose corresponding to proportion of these compounds in the Halowax 1051 mixture (Falandysz et al., 2006)) on CYP1A1 and AHR protein expression and biological response based on steroid secretion by ovarian follicles.

As in our previous studies on the effects of various xenobiotics on ovarian function, we obtained porcine ovarian follicles from sexually mature animals undergoing a natural estrous cycle. We also used this model in a previous study that examined the effect of Halowax 1051 on ovarian steroidogenesis (Gregoraszczuk et al., 2011). The proposed method is more physiologically relevant than the culture of individual cell types or cell lines.

2. Materials and methods

2.1. Experimental procedure

Pig ovaries from sexually mature animals were obtained from a local abattoir. The estrous cycle phase was determined according to established morphological criteria (Gregoraszczuk and Slomczynska, 1998). Antral follicles (5–7 mm) were mechanically isolated from ovaries collected on days 10–12 of the estrous cycle, as described previously by Gregoraszczuk et al. (2000). After isolation, follicles were cut using small scissors to allow better penetration of the compounds into the tissue. Follicular walls, including theca externa, theca interna and granulosa cells, but without oocyte and follicular fluids, were individually placed in 24-well plates containing M199 medium without phenol red, supplemented with antibiotic–antimycotic solution and 5% fetal bovine serum (FBS). The follicles were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 12 h to allow adaptation to culture conditions. Culture media were then changed and follicles were incubated for an additional 6–24 h with environmental doses of the individual PCN congeners (CN73, CN74, CN75) or artificial mixture (400, 25, 75 and 500 pg/ml for CN73, CN74, CN75 and artificial mixture, respectively) (Park et al., 2010) or at doses corresponding to their proportion in Halowax 1051 (50, 50, 900 and 1000 pg/ml for CN73, CN74, CN75 and artificial mixture, respectively) (Falandysz et al., 2006). At the end of the incubation period, media were frozen at −20 °C for steroid hormone determination, and follicles were stored at −70 °C for Western blot analysis.

2.2. Steroid secretion analysis

The concentrations of progesterone (P4), androstendione (A4), testosterone (T) and estradiol (E2) in the media were determined by enzyme immunoassay (EIA) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Germany; EIA-1561 for P4 assay, EIA-3265 for A4 assay, EIA-1559 for T assay, EIA-2693 for E2 assay). All samples were tested in duplicate in the same assay. The sensitivities of individual assays were 0.045–40 ng/ml for progesterone (P4), 0.019–10 ng/ml for androstenedione (A4), 0.083–16 ng/ml for testosterone (T), and 9.7–2000 pg/ml for estradiol (E2). The intra- and inter-experimental coefficients of variation were 4.34% and 6.99% for P4, 5.6% and 12.1% for A4, 3.28% and 6.71% for T, and 2.71% and 6.72% for E2. Cross-reactivity was 0.9% between T and A4 and <0.1% between T and E2. Cross-reactivity amongst P4, E2, A4, and T was 0%, but cross-reactivity with estrone was reported by the manufacturer to be 0.2%.

2.3. Immunoblot analysis

The effects of individual PCN congeners on AHR, CYP1A1, CYP17, 17β-HSD and CYP19 protein expression were examined by first homogenizing follicular walls twice in 50 μl of ice-cold lysis buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.5% sodium dodecyl sulfate (SDS), and EDTA-free protease inhibitors. The lysates were clarified by centrifugation at 15,000 rpm at 4 °C for 30 min, and the protein concentration in lysates was determined with the Bradford reagent (Bio-Rad Protein Assay; Bio Rad Laboratories, München, Germany), using bovine serum albumin (BSA) as a standard.

Samples containing an equal amount of protein were separated by SDS-PAGE and electrotransferred to PVDF (polyvinylidene difluoride) membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Non-specific binding was blocked by incubating blots in 5% dry milk and 0.1% Tween-20 in 0.02 M Tris-buffered saline for 1 h. Blots were incubated overnight with antibodies specific to CYP1A1 (sc-9828), AHR (sc-8088), CYP17 (sc-46084), 17β-HSD (sc-26963), CYP19 (sc-14244), all diluted 1:200 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). An anti-β-actin antibody (A5316; Sigma Chemical Co., MO, USA), diluted 1:2000, was used as a loading control. After incubation with the primary antibody, the membranes were washed three times and incubated for 1 h with the horseradish peroxidase-conjugated secondary antibody, P0447 (DakoCytomation, Denmark), for β-actin (diluted 1:5000), and sc-2020 (Santa Cruz Biotechnology Inc.), for AHR, CYP1A1, CYP17, 17β-HSD and CYP19 (diluted 1:2000). Signals were detected by enhanced chemiluminescence (ECL) using the Western Blotting Luminol Reagent (sc-2048; Santa Cruz Biotechnology) and were visualized using a Chemidoc XRS+ System (BioRad Laboratories). Data visualized by chemiluminescence were quantified using a densitometer and analyzed with Image Lab 2.0 Software (Bio-Rad, Laboratories).

2.4. Statistical analysis

Each experiment was performed three times ($n = 3$) with four replicates in each experiment. Because the variation between experiments was small, the results were averaged and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) multiple-range test, using GraphPad Prism 5. All data are expressed as means ± SD. A p-value < 0.05 was considered statistically significant; specific p-values for differences between control and treated groups are indicated in the text and in figures with asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) (Table 1).

3. Results

3.1. Effects of CN73, CN74 and CN75 on AHR protein expression

CN74 and CN75 at both doses used increased AHR protein expression after 6 h of exposition, while inhibitory effect was noted after 24 h. CN73 at dose 50 pg/ml revealed no effect after 6 h of exposition and decreased AHR protein expression after 24 h. At dose 400 pg/ml stimulatory effect on AHR protein expression was observed after 6 h and remained elevated after 24 h (Fig. 1).

3.2. Effects of CN73, CN74 and CN75 on CYP1A1 protein expression

All congeners at both doses increased CYP1A1 protein expression after 6 h of incubation. After 24 h of incubation CN73, CN74 and CN75 at doses: 400 pg/ml, 25 pg/ml and 50 pg/ml respectively had no effect on CYP1A1 protein expression, and down-regulated