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Congener-specific action of PBDEs on steroid secretion, CYP17, 17β-HSD and CYP19 activity and protein expression in porcine ovarian follicles

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ABSTRACT

The available data on reproductive toxicity of PBDEs are limited. In the present study we evaluated the direct effects of BDE-47, -99 and -100 on porcine ovarian follicular steroid secretions and the activity and expression of enzymes involved in its synthesis. Follicles were exposed to BDE-47 (0.5, 25 and 50 ng/ml), BDE-99 (0.25, 10 and 17.5 ng/ml), or BDE-100 (0.1, 4 and 12.5 ng/ml) for 24 h. Progesterone (P4), androstenedione (A4), testosterone (T) and estradiol (E2) levels in the media were determined by EIA. CYP17, 17β-hydroxysteroid dehydrogenase (17β-HSD) and CYP19 activity was measured by conversion of P4 > A4, A4 > T and T to E2, respectively. Protein expression of CYP17, 17β-HSD and CYP19 was measured by western blot. All of the congeners explored in this study increase testosterone secretion. However, in the case of BDE-47 due to activation of 17 β-HSD and BDE-100 due to activation of CYP17, a corresponding failure to activate CYP19 expression and inhibition of CYP19 activity was seen. The lack of an effect of BDE-99 on the expression and activity of all of the investigated enzymes indicates action on enzymes before progesterone secretion, i.e., STAR or 3β-HSD activity.

1. Introduction

Brominated flame retardants (BFRs) are persistent and ubiquitous chemicals in the environment. They have been found at increasing levels in tissues of wildlife and humans. PBDEs belong to a new class of organohalogenated compounds that show a striking structural resemblance to older classes such as polychlorinated biphenyls (PCBs). Despite placing a ban on the production of Penta- and Octa-BDE in the European Union (EU) market in August 2004, in 10 states in the USA (BSEF, 2007), China (2007) and diminished use of PBDE in Japan by the self-imposed controls of the Japanese Flame Retardants Conference (Akutsu and Hori, 2004), congeners 2,2′,4,4′-tetra BDE (BDE-47), 2,2′,4,4′,5-pentaBDE (BDE-99), and 2,2′,4,4′,6-pentaBDE (BDE-100) are the dominant PBDE congeners generally found in wildlife and humans (Meerts et al., 2001; Sjödin et al., 2003). This phenomenon occurs because lower brominated congeners are more bioaccumulative and persistent (de Wit, 2002). The higher brominated compounds tend to be less mobile in the environment, because of their low volatility, water solubility and strong adsorption on sediments, whereas the lower brominated compounds are predicted to be more water soluble and volatile, and also more bioaccumulative (Watanabe and Sakai, 2003).

These compounds have been shown to share mechanisms of action on the endocrine system (Legler and Brouwer, 2003). Lilienthal et al. (2006) showed that PBDE exposure resulted in pronounced decreases in circulating sex steroids (estradiol and testosterone) in male offspring at weaning as well as in adults. In female offspring, the number of primordial/primary and secondary ovarian follicles was reduced.

To predict the effects of pollutants on animal or human reproductive health, it is necessary to adapt physiological models to include target tissues that not only are responsive to estrogenic compounds but that also produce steroids, including estradiol. In certain cases, the complexity of *in vivo* studies can complicate the interpretation and prediction of substance-induced effects related to reproductive health.

In our laboratory, we collect ovarian follicles from porcine ovaries excised from animals undergoing a natural estrous cycle to study the direct effects of various xenobiotic on ovarian steroid synthesis. This model was used in a previous study (Gregoraszczuk et al., 2003; Grochowalski et al., 2000) for the measurement of tetrachlodibenzo-*p*-dioxin induced accumulation or congener-specific accumulation of PCBs in the ovarian follicular wall. The proposed method offers a system that is more physiologically relevant than the culture of individual cell types or cell lines.

We previously showed that the ovary is a target tissue for PBDEs (Gregoraszczuk et al., 2008a,b). An environmentally realistic mixture of POPs extracted from the liver oil of the Atlantic cod (*Gadus morhua*) had a stimulatory effect on testosterone and

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17β estradiol secretion in co-cultured porcine ovarian follicular cells (Gregoraszczuk et al., 2008a). To determine which compounds in this natural mixture were responsible for the stimulation of E2 secretion, we exposed cells to BDE-47, -99, -100, -209, DDT, and DDE alone or in different combinations at environmentally relevant concentrations. It has been shown that the overall effect of a real-life mixture on the steroidogenic response in ovarian follicular cells could depend on the concentration of DDTs in the mixture (Gregoraszczuk et al., 2008b). Previous studies have also suggested that the stimulatory action of individual congeners of PBDEs on both estradiol and testosterone secretion in ovarian follicular cells is due to direct action on the expression of enzymes responsible for testosterone secretion. Another study demonstrated that it was not possible to reverse the steroidogenic effects of an artificial mixture of PBDEs composed of BDE-47, -99, -100 and -209 by removing reagents from the cell cultures (Karpeta and Gregoraszczuk, 2010).

To gain further insight into the structure-activity relationships and mechanisms of action, we used PBDE congeners at concentrations 20–2500 times lower, which is similar to amounts found in human blood. Epidemiological studies have revealed that the levels of PBDEs are lower in blood than in fish liver (Guvenius et al., 2003; Mazdai et al., 2003).

To better define the mechanism of action of PBDEs in the ovary, we examined the direct action of BDE-47, -99 and -100 on progesterone (P4), androstenedione (A4), testosterone (T) and estradiol (E2) secretion and the effect on steroidogenic enzymes (protein expression and activity) in the porcine ovarian follicles. Pigs and minipigs are becoming a valid alternative to traditional non-rodent species in pharmacological and toxicological studies, because many of their physiological characteristics resemble those of humans (Swindle and Smith, 1998).

2. Materials and methods

2.1. Reagents

M199, fetal bovine serum (FBS, heat-inactivated), antibiotic/antimycotic solution (100X), Tris, Na-deoxycholate, Nonidet NP-40, sodium dodecyl sulfate (SDS), protease inhibitor (EDTA-free), DTT, Tween 20, and bromophenol blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BDE congeners 47, 99, and 100 were purchased from Chiron AS (Trondheim, Norway). Stock solutions of these test compounds were prepared in isooctane (Maliincrodt Baker B.V. – Deventer – Holland) and added to M199 supplemented with 5% FBS. The final concentration of isooctane in the medium was always 0.1%. Inserts for 24-well plates with 1.0 μm pore size and transparent membranes were purchased from BD Falcon Cell Culture Insert, Cat No. 353104 (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. Tissue cultures

Morphologically normal pig ovaries from sexually mature animals (5–7 months of age) were obtained from a local abattoir. Medium antral follicles (4–6 mm) were mechanically isolated from the ovary and collected at days 10–12 of the estrus cycle as described previously by Gregoraszczuk et al. (2000). Estrus was designated as day 0. In each experiment, six ovaries from 3 animals were selected. Since each ovary yielded four to six follicles, the total number of the follicles for each preparation varied between 24 and 36. This procedure was chosen to minimize potential variations between follicles and animals. After isolation, follicles were cut using small scissors to facilitate penetration of the compounds into the tissue. Follicular walls, including theca and granulosa cells and excluding oocytes and follicular fluids, were individually placed in cell culture inserts in 24-well plates with Parker medium (M199) without phenol red supplemented with antibiotic antimycotic solution and 5% FBS. Treatment conditions included three different doses of BDE-47 (0.5, 25 and 50 ng/ml), BDE-99 (0.25, 10 and 17.5 ng/ml), or BDE-100 (0.1, 4 and 12.5 ng/ml). The doses of PBDEs were chosen based on our previous research (Karpeta and Gregoraszczuk, 2010). The follicles were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h of incubation, the medium was separated from the follicles and stored at −20 °C for steroid determination. Whole follicles were frozen at −70 °C for CYP17, 17β-HSD and CYP19 protein expression analysis. Enzyme activity was studied by adding substrates (10^{-7} M) for CYP17, 17β-HSD and CYP19 (P4, A4, and T, respectively) and measuring the amount of the resulting products in the culture medium after a 24 h incubation. Each treatment was conducted in four wells, and each experiment was repeated three times. This method was used by Ohno et al. (2004) for large-scale screening of compounds that can disrupt endocrine function by influencing aromatase activity.

2.3. Steroid analysis

The concentrations of progesterone (P4), androstenedione (A4), testosterone (T) and 17β-estradiol (E2) in the media were determined by enzyme immunoassay (EIA) using commercial ELISA kits (DRG Diagnostic, Germany). All samples were run in duplicate in the same assay. The limit of P4, A4, T and E2 assay sensitivity was 0.045 ng/ml, 0.019 ng/ml, 0.083 ng/ml and 9.714 pg/ml, respectively. Inter- and intra-run precisions had coefficients of variation of 4.34% and 6.99% respectively for P4; 12.1% and 5.6% for A4; 6.71% and 3.28% for T and 6.72% and 2.71% for E2. The range of P4 assay was between 0 and 40 ng/ml; for A4 was between 0.019 and 10 ng/ml; for T was between 0 and 16 ng/ml and for E2 was between 0 and 2000 pg/ml. Cross-reactivity of T: to A4 was 0.9%; to E2 < 0.1%. Cross-reactivity of E2: to A4 and T was 0% and to estrone 0.2% (according with DRG EIA-1559 for T assay and EIA-2693 for E2 assay).

2.4. Western blot

For immunoblotting, whole follicles were homogenised twice in 50 μl of ice-cold lysis buffer containing: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% Na-desoxycholate, 0.5% NP-40, 0.5% SDS and protease inhibitory EDTA-free. For each experiment three follicles per treatment group were used. The homogenate were centrifuged at 15,000 × *g* at 4 °C for 30 min, and the protein concentration in the lysates was determined using Bradford reagent (Bio-Rad Protein Assay; Bio Rad Laboratories, Munchen, Germany) with bovine serum albumin (BSA) as a standard. Samples containing 40 μg of total protein were reconstituted in the appropriate amount of sample buffer, which consisted of 125 mM Tris pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM DTT and 0.01% bromophenol blue. The samples were separated by 10% SDS–polyacrylamide gel electrophoresis in a Bio-Rad Mini-Protean II Electrophoresis Cell. After electrophoretic separation, the proteins were electrotransferred to PVDF membranes and washed. Non-specific binding sites were blocked with 5% milk and 0.2% Tween 20 in 0.02 M TBS for 2 h. The membranes were incubated overnight at 4 °C with anti-CYP17, 17β-HSD and CYP19 antibodies diluted 1:200 (sc-46084, sc-26963 and sc-14244, from Santa Cruz Biotechnology). Anti-β-actin antibody (A5316, Sigma Chemical Co.), diluted 1:3000 was used as the loading control. After incubation with the primary antibody, the membranes were washed with TBS and 0.02% Tween 20 and then were incubated for 1 h with donkey anti-goat IgG-HRP (sc-2020 for CYP17, 17β-HSD and CYP19, Santa Cruz Biotechnology) diluted 1:2000 and with a horseradish peroxidase-conjugated secondary antibody (P0447, DakoCytomation, Denmark), diluted 1:5000 for β-actin. Signals were detected by chemiluminescence (ECL) using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology) and visualized using the ChemidocTM XRS+ System (BioRad, Laboratories). All data bands visualized by chemiluminescence were quantified using a densitometer and Image LabTM 2.0 Software (Bio-Rad, Laboratories).

2.5. Statistical analysis

Each treatment was repeated three times ($n = 3$) in quadruplicate. The average of the quadruplet values was used for statistical calculations. Statistical analysis was performed using Graph Pad Prism 5. The data were analyzed by an unpaired t-test. Means marked with asterisks differ significantly ($p < 0.001$; $p < 0.01$; $p < 0.05$).

3. Results

Previous experiments demonstrated that a 48-h exposure to much higher concentrations, such as 1000 ng/ml BDE-47, 500 ng/ml BDE-99, 250 ng/ml BDE-100, and 10 ng/ml BDE-209, did not cause any cytotoxicity as determined by LDH release and caspase-3 activity (Gregoraszczuk et al., 2008b).

3.1. Effect of PBDEs on steroid secretion

After 24 h in culture, the control antral ovarian follicles secreted 22.6 ± 5.6 ng/ml, 0.19 ± 0.02 ng/ml, 1.6 ± 0.2 ng/ml and 1.068 ± 0.251 ng/ml of progesterone, androstenedione, testosterone and estradiol, respectively.

A small dose of BDE-47 (0.5 ng/ml) significantly increased A4 secretion (1.7-fold; $p < 0.01$), and each dose of BDE-47 (0.5, 25 and 50 ng/ml) increased T secretion by 3.2-, 4.4- and 5.5-fold over the control, respectively ($p < 0.001$). All of the investigated doses had no effect on P4 and E2 secretion (Fig. 1a).