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ScienceDirect
Toxicology Letters 166 (2006) 200–211
Toxicology Letters
www.elsevier.com/locate/toxlet

Induction of cytochromes P450, caspase-3 and DNA damage by PCB3 and its hydroxylated metabolites in porcine ovary

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Received 30 May 2006; received in revised form 5 July 2006; accepted 10 July 2006
Available online 14 July 2006

Abstract

Polychlorinated biphenyl (PCBs) levels of tens and hundreds of pg/ml for individual congeners are measured in human follicular fluid. PCB3 (4-chlorobiphenyl), caused a significant increase in estradiol secretion in porcine granulose–theca cell co-cultures and its two metabolites, 4-OH-PCB3 and 3,4-diOH-PCB3, were even more potent than PCB3 itself [Ptak, A., Ludewig, G., Lehmler, H.J., Wojtowicz, A.K., Robertson, L.W., Gregoraszczuk, E.L. 2005. Comparison of the actions of 4-chlorobiphenyl and its hydroxylated metabolites on estradiol secretion by ovarian follicles in primary cells in culture. Reprod. Toxicol. 20, 57–64]. The question is whether these follicle cells are potentially able to metabolize PCB3 to hydroxylated and genotoxic or cytotoxic intermediates. We report here that granulose–theca co-cultures express xenobiotic-metabolizing cytochrome P450 activities, with CYP1A1 > CYP2B ≫ CYP1A2. A significant increase in CYP1A1 and 2B, but not CYP1A2, activity was seen in cells that were exposed to 6 ng/ml PCB3 or 20 nM 17-β-estradiol. An increase in caspase-3 activity, indicative for apoptosis, was only observed in PCB3-exposed cells after 24 h exposure. Genotoxicity, determined with the Comet assay, was initially reduced after 24 h exposure to PCB3 and both metabolites compared to untreated controls, followed by a significant transient increase in Comets at the 4 and 24 h time point with PCB3 and 4-OH-PCB3. 3,4-diOH-PCB3 induced a significant increase only after 72 h of recovery. We hypothesize that these biphasic damage kinetics may be due to cross-links caused by adduct formation. These results show for the first time that granulose–theca cells in co-culture express CYP1A1, 2B and 1A2 activities and that PCBs at concentrations that are reached in the environment induce genotoxicity in granulosa cells.

Keywords: PCB3; Hydroxylated metabolites; Prepubertal pig ovary; CYP; DNA damage; Caspase-3 activity

1. Introduction

Polychlorinated biphenyls (PCBs) are industrial chemicals that were produced as commercial mixtures of chlorinated biphenyls in many countries of the world and sold under various trade names, such as Clophen, Aroclor and Kanechlor, over many decades (Silberhorn

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0378-4274/$ – see front matter © 2006 Published by Elsevier Ireland Ltd.
doi:10.1016/j.toxlet.2006.07.304

et al., 1990). PCBs were incorporated into numerous commercial products and have escaped into the environment. Individuals may be exposed to PCBs via their food (a predominant route of exposure for the average citizen), via the skin (e.g. dermal exposure in capacitor workers), or by inhalation (exposure occurring in contaminated buildings, or near waste sites) (ATSDR, 2000).

A number of toxic signs and symptoms have been identified in individuals suffering occupational and accidental PCB exposures (Cogliano, 1998), and the underlying mechanisms of many of these have been investigated in animal studies. A well-characterized response to PCBs is the induction (increased expression) of a broad spectrum of xenobiotic-metabolizing enzymes (Safe et al., 1985; Safe, 1994), including several cytochrome P450 monooxygenases (CYPs). By virtue of interactions with the aryl hydrocarbon receptor (AhR) (Bandiera et al., 1982), the constitutive and rostane receptor (CAR) (Waxman, 1999), and pregnane-X receptor (PXR) (Hurst and Waxman, 2005), PCBs increase the expression of CYPs 1A, 2B and 3A subfamilies, respectively. The number and position of chlorine atoms on the biphenyl ring determine their interaction with these receptors. In general PCBs that function as inducers of CYPs in rodent livers are *meta*-, *para*-, chloro-substituted (CYP1A), *ortho*-, *para*-substituted (CYP2B) and multi-*ortho* substituted (CYP3A) (Denomme et al., 1983; Parkinson et al., 1983; Schuetz et al., 1998).

CYPs have been characterized in the liver, but have also been detected in the extrahepatic tissues such as lung, prostate gland, uterus, adrenal glands, placenta, kidney, brain and testis (Henderson et al., 1992; Hakkola et al., 1996; Lacroix et al., 1997; de Wildt et al., 1999; Raunio et al., 1999; Zhang et al., 1999; Nishimura et al., 2003). The ovaries also contain CYPs. CYP2E1, CYP2A, CYP2B mRNA and protein are expressed in the mouse ovary (Cannady et al., 2003). Pig and human ovaries contain CYP1A1 and CYP1B1 isoforms (Hammond et al., 1986; Hakkola et al., 1997; Muskhelishvili et al., 2001). Leighton et al. (1995) found CYP1A1 mRNA in porcine ovarian granulosa cells and a porcine ovarian granulosa cells line (MDG2.1). For these reasons the ovaries may play an important role in the metabolism of endogenous and exogenous compounds.

PCBs, especially the less chlorinated congeners, may also be substrates for these CYPs themselves, and the metabolism of PCBs produces oxygen-containing metabolites, i.e. PCB-derived phenols, catechols, quinones, sulfones and bound residues (reviewed in Bergman et al., in press). The cytochrome P450 catalyzed oxidation of lower chlorinated biphenyls, especially mono-, di- and tri-chlorobiphenyls, gives rise to monohydroxy and dihydroxy metabolites (McLean et al., 1996a). These dihydroxy compounds may be further oxidized to reactive metabolites which form adducts with nitrogen and sulfur nucleophiles, including DNA (Amaro et al., 1996; Oakley et al., 1996a; Zhao et al., 2004). CYPs may also be responsible for the biosynthesis and metabolism of endogenous compounds such as steroid hormones, fatty acids and prostaglandins (Waxman, 1988; Nelson et al., 1996; Rendic and Di Carlo, 1997; Gonzalez and Kimura, 1999). CYPs are involved in the metabolism of estradiol to catechol estrogens in the liver (Dannan et al., 1986; Suchar et al., 1996) and in extrahepatic tissues such as uterus (Chakraborty et al., 1990; Paria et al., 1990; Liehr et al., 1995), breast (Telang et al., 1991; Liehr and Ricci, 1996), placenta (Liehr et al., 1995) and ovary (Hammond et al., 1986; Muskhelishvili et al., 2001). Thus, it is possible that PCBs may be metabolized by these enzymes and/or interfere with estrogen metabolism in these extrahepatic tissues by inducing CYP activities.

Furthermore, during PCB metabolism, the formation of reactive oxygen species (ROS) has been detected in cells in culture (Slim et al., 1999, 2000), and in laboratory animals (Pelissier et al., 1990; Saito, 1990). Oxidative stress or DNA damage may act as an initiator of the caspase cascade and apoptosis (Robertson and Orrenius, 2000). Apoptosis is a natural process by which follicles in the ovary degenerate (Hughes and Gorospe, 1991). Caspases are key effector components of apoptosis (Thornberry, 1998). The presence of caspase-3 was shown in granulosa cells of atretic follicles (Boone and Tsang, 1998; Berardinelli et al., 2004). Robles et al. (1999) showed that healthy granulosa cells possess the inactive form of caspase-3, whereas apoptotic granulosa cells possess the active enzyme.

We previously reported that PCB3 and its monohydroxylated and dihydroxylated metabolites 4-OH- and 3,4-diOH-PCB3 at concentrations of 0.06–60 ng/ml significantly increased estradiol levels in the culture medium of granulosa and theca cells derived from follicles of mature animals (Ptak et al., 2005). The rank order of potency in estradiol secretion was 3,4-diOH-PCB3 > 4-OH-PCB3 > PCB3. We also showed that this effect is in part due to increased aromatase activity (Ptak et al., 2006). The current studies were designed to test the hypothesis that porcine ovaries contain CYP1A1, CYP1A2 and CYP2B monooxygenases which could bioactivate PCBs. Moreover, we wished to compare the effects of PCB3 and its metabolites,

as prototypes of lower halogenated PCBs and their metabolites that may be metabolized by such CYPs to DNA-binding and ROS-producing quinone metabolites (McLean et al., 1996a,b, 1998; Oakley et al., 1996a,b; Srinivasan et al., 2001), on caspase-3 activity and DNA damage.

2. Materials and methods

2.1. Test compounds and their characterization

PCB3 (4-chlorobiphenyl) and its hydroxylated metabolites 4′-hydroxy-4-chlorobiphenyl (4-OH-PCB3) and 3′,4′-dihydroxy-4-chlorobiphenyl (3,4-diOH-PCB3) were synthesized using the Suzuki coupling reaction as described previously (Bauer et al., 1995; Lehmler and Robertson, 2001). Stock solutions of these test compounds in DMSO were prepared and added to culture medium immediately before use as described below. The final concentration of DMSO in the medium was always 0.2%. DMSO at this concentration has no effect on steroid secretion and cell viability (data not shown).

Parker Medium 199 without phenol red (M199), fetal bovine serum (FBS, heat inactivated), antibiotic–antimycotic solution (100×), trypsin, Trypan blue, Ac DEVD-pNA and deoxyribonuclease I Type IV (DNAse I) from bovine pancreas were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cell cultures

Porcine prepubertal ovaries from a local abattoir were collected into a bottle filled with sterile saline and transported to the laboratory. Granulosa cells (Gc) and theca interna cells (Tc) were isolated from antral follicles (4–6 mm in diameter) according to the technique described (Stoklosowa et al., 1978). Briefly, Gc were scrubbed from the follicular wall with round-tipped ophthalmologic tweezers and rinsed several times with PBS. After isolation, Gc were exposed to DNAse I (500 units for 1 min) and washed three times in M199, collected, and resuspended in M199 supplemented with 5% fetal bovine serum (M199/FBS). The Tc were prepared from the same follicles by placing the theca layers in a drop of saline under the dissecting microscope. The theca interna was manually separated from the underlying theca externa. Isolated theca interna tissue was then washed with PBS, cleaned, cut with scissors and exposed to 0.25% trypsin in PBS for 10 min at 37 °C. Isolated cells were separated by decantation and the procedure was repeated three times. Finally, the cells were centrifuged and resuspended in M199/FBS. The viability of the cells was determined before seeding by the Trypan blue exclusion test and viability was found to be 60–75% for granulosa cells and 85–90% for theca cells.

For co-culture experiments, granulosa and theca cells were inoculated at concentrations of 4×10^4 and 1×10^4 viable cells/well, respectively, in 96-well tissue culture plates or 12×10^5 and 3×10^5 viable cells/well, respectively, in 48-well tissue culture plates (Nunc). This ratio is similar to that observed in vivo (Gc:Tc = 4:1) according to Stoklosowa et al. (1982). For mono-culture experiments, granulosa cells were inoculated at concentrations of 1×10^5 viable cells/well in 24-well tissue culture plates.

After 24 h incubation in M199/FBS the test compounds, i.e. 6 ng/ml PCB3, 4-OH-PCB3, or 3,4-diOH-PCB3, were added in fresh medium to the wells and the cells were cultured for additional 6, 24 and 48 h with daily changes of the media without (control) or with the test compounds. This concentration of PCB3 and its metabolites was chosen based on our previous results (Ptak et al., 2005) showing that in culture medium from mature antral follicles an increase in estradiol level was noted under these conditions. In some experiments, 17β-estradiol was added to the medium.

At the end of the exposure times, cells were washed with cold phosphate buffer (PBS) and frozen in situ at −70 °C for later determination of CYP1A1, CYP1A2 and CYP2B activity and caspase-3 activity as described below. Cells from mono-culture experiments were collected by trypsinization and used for the comet assay. Every treatment was conducted in quadruplet (4-wells each), and each experiment was repeated three times.

2.3. EROD, MROD and PROD assay

Granulosa and theca cells were seeded in 48-well plates at 12×10^4 and 3×10^4 viable cells/well density, respectively, and allowed to attach for 24 h. Then medium was replaced with fresh medium containing 6 ng/ml PCB3 or 20 nM 17β-estradiol or solvent alone. After 48 h exposure time the medium was removed and the plates with the cells stored at −70 °C for later determination of cytochrome P450 1A1, 1A2 and 2B activity with ethoxyresorufin, methoxyresorufin and pentoxyresorufin as substrates, respectively.

Briefly, frozen cells were lyzed by removal from the freezer and allowed to thaw for 10 min. For the CYP1A1 determination each reaction well received 50 μl BSA solution (1.33 mg/ml in 50 mM Tris, pH 7.2; final reaction concentration) and 100 μl ethoxyresorufin solution (10 μM in 50 mM Tris, pH 7.2; final reaction concentration). Each blank well received 50 μl BSA solution, 100 μl ethoxyresorufin solution and 50 μl Tris buffer (50 mM, pH 7.2). Plates were incubated for 15 min at 37 °C with gentle shaking. Then, to start the reaction, 50 μl NADPH solution (1.67 mM in 50 mM Tris, pH 7.2; final reaction concentration) was added to each reaction well (not to the blank wells). The samples were incubated at room temperature without shaking, and the increase in fluorescence due to the CYP1A mediated oxidation of the non-fluorescent ethoxyresorufin to the fluorescent resorufin was read at 15 min intervals for up to 2 h with a fluorescence plate reader (FLx 800, Bio-Tek, USA) at 530 nm excitation and 590 nm emission wavelength. After 2 h, 90 μg fluorescamine in 100 μl acetonitrile was added to each well to stop the reaction and determination of the protein concentration in each well (400 nm excitation filter and 460 nm emission filter).

The CYP1A2 activity per well was determined as described above except that methoxyresorufin was used as substrate (5 μM in 50 mM Tris, pH 7.2; final reaction concentration) instead of ethoxyresorufin. CYP2B activities were measured with pentoxyresorufin as substrate (5 μM in 50 mM Tris, pH 7.2; final reaction concentration).

All results were calibrated against a resorufin standard curve (0–100 nM, 200 μl final volume) and a BSA standard curve (0–1000 μg, 200 μl final volume) prepared in a 48-well plates.

2.4. Comet assay

Granulosa cells were seeded in 24-well plates at 1.0×10^5 cells/well density and cultured for 24 h to allow for cell attachment. After 24 h, media were replaced and cells were grown in the presence of PCB3, 4-OH-PCB3, 3,4-diOH-PCB3 (6 ng/ml) or 17β-estradiol (0.08 and 6 ng/ml) for the next 24 h. In order to investigate the capacity to repair the potential DNA damage, media were replaced with fresh culture medium and the cells were cultured for 0, 1, 4, 24, 48 and 72 h after removal of the agents.

The cells were collected at the indicated time points by trypsinization and the cell suspensions (about 4×10^4 cells each) were mixed with 0.5% low melting point agarose (LMPA) and spread on microscope slides that were precoated with 200 μl of 1% normal melting point agarose the day before the experiment. After 4 min of solidification on ice, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 and 1% Triton X-100) for 1 h. After rinsing twice with 0.4 M Tris, pH 7.5, the slides were placed side by side in a horizontal slab electrophoresis chamber (Kucharczyk, Poland), covered with freshly prepared eletrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH > 13), and left for DNA unwinding for 40 min at 4 °C. Electrophoresis was conducted at 0.74 V/cm for 30 min in a cold room. The current was adjusted to 300 mA. Samples were neutralized, stained with the DNA-specific fluorochrome propidinum iodide, and analyzed by microscopy. For visualization of the DNA damage, observations were made using a 10× objective (the final magnification was 200×) on an epifluorescence microscope (Olympus IX-50) equipped with appropriate filters. The microscope was linked to a computer through a CCD camera (i.CAM-hrM; sensor SONY ICX) and an image analysis system (Comet Plus from Theta System GmbH, Germany) was used for the quantification of DNA damage. The percentage of Tail-DNA-Content (TDC) was automatically generated for each nucleus. At least two slides per one sample, with 50 randomly selected cells per slide, were analyzed.

The Comet assay, developed by Singh et al. (1988) and modified by others (Tice et al., 2000), is based on the observation that broken DNA strands migrate to the anode during electrophoresis, forming a comet-like picture. The percentage of DNA released into the comet tail is proportional to the frequency of DNA strand breaks (Olive et al., 1990), whereas the head of such a comet contains the unbroken, highly supercoiled, intact DNA. The compactness of the DNA in the comet head seems to depend among others on DNA/highly bound protein interactions and/or on the degree of DNA relaxation caused by the unwinding step during the comet preparation procedure. Thus the induction of DNA–DNA or DNA–protein cross-links inhibits the unwinding and retards the migration of DNA into the gel matrix, thereby reducing DNA migration below the value in untreated control cells. Such physiologically determined higher DNA compactness is observed in cells exposed to a harsher environment, like sperm cells or certain epithelial cells (Singh, 2000), but DNA cross-links were also demonstrated as a result of environmental insults and it was suggested that they can be used as a biomarker of past exposure to harmful agents (Shaham et al., 1996).

2.5. Caspase-3 activity assay

Granulosa and theca cells were seeded in 96-well plates at 4×10^4 and 1×10^4 viable cells/well density. Twenty-four hours later the medium was replaced with fresh medium containing solvent alone or 6 ng/ml PCB3, 4-OH-PCB3, or 3,4-diOH-PCB3. After an exposure time of 6, 24 and 48 h the medium was removed and the plates were stored at −70 °C. As positive control cells were preincubated for 4 h with 2 μM staurosporine, an inducer of apoptosis.

Cells were lyzed in buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT. The assay was carried out by adding 20 μM of Ac DEVD-pNA, a substrate for the colorimetric determination of caspase-3 activity. The reaction mixtures were incubated at 37 °C. After 1 h, absorbance was measured at 405 nm using micro-ELISA plate reader (Bio-Tek Instruments).

2.6. 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 Stastistica 6.0. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) multiple range test. Groups which are significantly different from each other ($p < 0.05$) are indicated in the figures with different letters. Same letters indicate that the data points are not statistically significantly different from each other.

3. Results

Co-cultures of granulosa and theca cells from prepubertal pig ovaries in a ratio of 4:1 were used to assess the basic cytochrome P450 1A1, 1A2 and 2B activity and their inducibility by estradiol and PCB3. This co-culture approach was also used to evaluate apoptosis induction by these treatments using the caspase-3 assay. Genotoxicity as determined with the Comet assay was analyzed in granulosa cell mono-cultures.

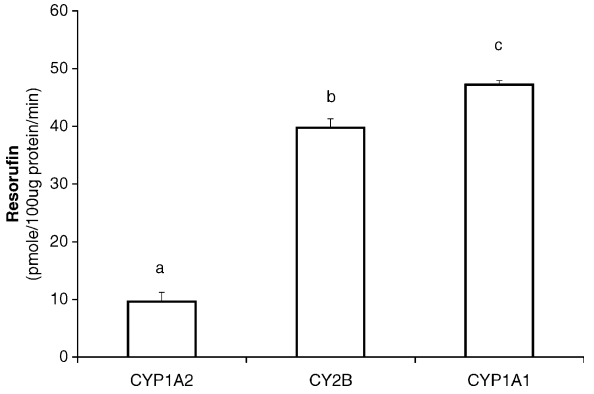

Fig. 1. Basal CYP1A1, 1A2 and 2B activity in porcine ovary, measured by EROD, MROD and PROD assays. Columns representing the means ± S.D. for at least three independent experiments. All means marked with different letters are statistically different ($p < 0.05$) from the respective to blank.

3.1. Basal CYP1A1, 1A2 and 2B activity in prepubertal pig ovary follicle cells

The enzyme activity for CYP1A1, 1A2 and 2B were assayed using the EROD, MROD and PROD assay, respectively. Basal activities were determined on day 3 after granulosa and theca cells had been isolated from pig ovaries and taken into culture.

The basal CYP1A1 activity was 47.4 ± 1.0 pmol/100 μg protein/min (Fig. 1). The CYP2B activity was second highest with 39.7 ± 0.7 pmol/100 μg protein/min and the CYP1A2 activity was comparably low with 8.6 ± 1.6 pmol/100 μg protein/min.

3.2. Effect of PCB3 and E_2 on CYP1A1, 1A2 and 2B activity

Exposure of 1 day old granulosa and theca cells in co-culture to PCB3 (6 ng/ml) or 17β-estradiol (E_2; 20 nM) increased the CYP1A1 activity from 46.8 ± 1.1 pmol/100 μg protein/min in the controls to 53.7 ± 1.8 and 52.3 ± 0.6 pmol/100 μg protein/min, respectively) (Fig. 2a). This increase in activity by 15% and 12%, respectively, was statistically significant ($p < 0.05$).

PCB3 and E_2 also significantly increased the CYP2B activity in these cells by 8% and 7%, respectively, from 40.8 ± 1.1 pmol/100 μg protein/min in the controls to 43.0 ± 1.1 and 43.7 ± 1.6, respectively ($p < 0.05$; Fig. 2b).

No significant increases in CYP1A2 activity was noted under the influence of either PCB3 or E_2 as compared to the control cultures (8.8 ± 0.5 and 9.0 ± 0.1 pmol/100 μg protein/min after exposure to PCB3 and E_2, respectively, versus 8.7 ± 0.3 pmol/100 μg protein/min in control; Fig. 2c).

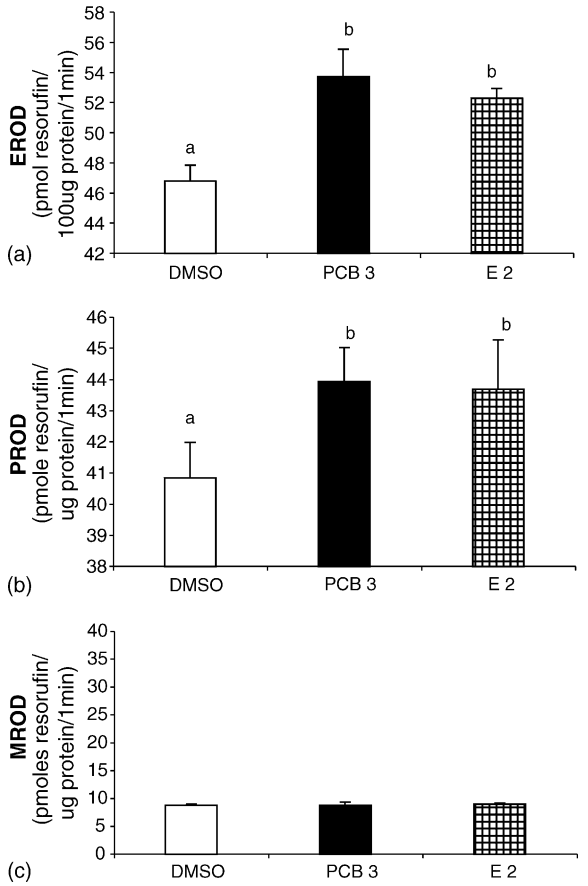

Fig. 2. Effect of PCB3 and 17β-estradiol (E_2) on the CYP1A1 (EROD) (a), CYP2B (PROD) (b) and CYP1A2 (MROD) (c) activity in granulose–theca cell co-culture after 48 h of exposure. Control cultures were treated with medium containing 0.2% DMSO. Columns representing the means ± S.D. for at least three independent experiments. Different letters indicate statistically ($p < 0.05$) differently activities.

3.3. Effect of PCB3 and its hydroxylated metabolites on caspase-3 activity

Granulosa–theca cell co-cultures expressed a caspase-3 activity of 0.041 ± 0.004, 0.038 ± 0.003 and 0.030 ± 0.004. Relative absorbance units (RAU) at the 6, 24 and 48 h datapoint, respectively, which is equivalent to 30, 48 and 72 h in culture after isolation from pig ovary follicles (Fig. 3). We observed that treatment with PCB3 significantly increased the caspase-3 activity by 29% to 0.049 ± 0.003 RAU after 24 h of exposure ($p < 0.05$), but no increase was seen

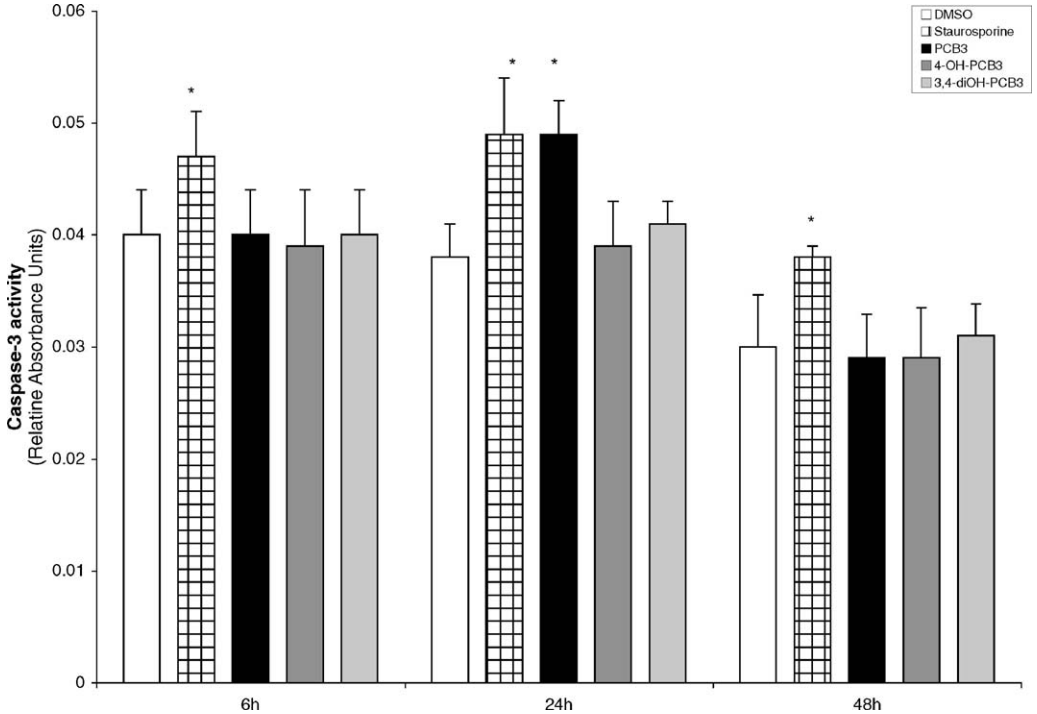

Fig. 3. Effect of PCB3 and its metabolites on caspase-3 activity after 6, 24 and 48 h of exposure. Control cultures were treated with medium containing 0.2% DMSO. Staurosporine treated cells (0.2 μM for 4 h) were used as positive control. Columns representing the means ± S.D. for at least three independent experiments. Asterisk indicates value statistically different from control ($p < 0.05$).

after 6 h or 48 h of exposure. The two hydroxylated metabolites 4OH-PCB3 and 3,4-diOH-PCB3 had no effect on caspase-3 activity at any of the time-points tested.

Staurosporine (0.2 μM) was used as positive control for apoptosis induction. Four hours of exposure to staurosporine effectively increased the caspase-3 activity by 15%, 29% and 27% at the 6, 24 and 48 h time points of the experiment (Fig. 3).

3.4. Effect of PCB3 and its hydroxylated metabolites on DNA damage

The alkaline comet assay was used to analyze DNA damage induced by PCB3 and/or its hydroxylated metabolites in granulosa cells. Cells were exposed to PCB3, 4-OH-PCB3 and 3,4-OH-PCB3 for 24 h and the percent of DNA in the comet tail (Tail-DNA-Content, TDC) was determined immediately after exposure and after 1–72 h of recovery in normal medium. Fig. 4 shows that all three compounds significantly reduced the TDC immediately after 24 h of exposure and 1 h after removal of the compounds compared to solvent treated controls. In PCB3 and 4-OH-PCB3 treated cultures the TDC value increased significantly during the next hours of recovery time, reaching a maximum of about 140% and 180% of controls for PCB3 and 4-OH-PCB3, respectively, after 24 h of recovery. Two and 3 days after exposure the TDC was no longer significantly elevated compared to the control levels.

The kinetic of DNA damage induction and removal was different in 3,4-OH-PCB3-treated cultures. Here the TDC was increased from 4 to 48 h recovery time, but reached a significant level (∼156% of control) only at the 72 h time point of recovery (Fig. 4).

This biphasic TDC kinetic during the recovery time, below control immediately after exposure and above control after four or more hours without compound was not observed when granulosa cells were treated with 17β-estradiol (Fig. 5). Treatment of granulosa cells with 0.08 ng/ml or 6 ng/ml 17β-estradiol for 24 h did not cause any change in mean TDC value compared to control levels as measured immediately after hormone removal. However, 24 h later, the amount of DNA breaks increased significantly above the level for untreated cells (Fig. 5). These breaks diminished to the control level 48 h after the hormone was removed from the culture medium.

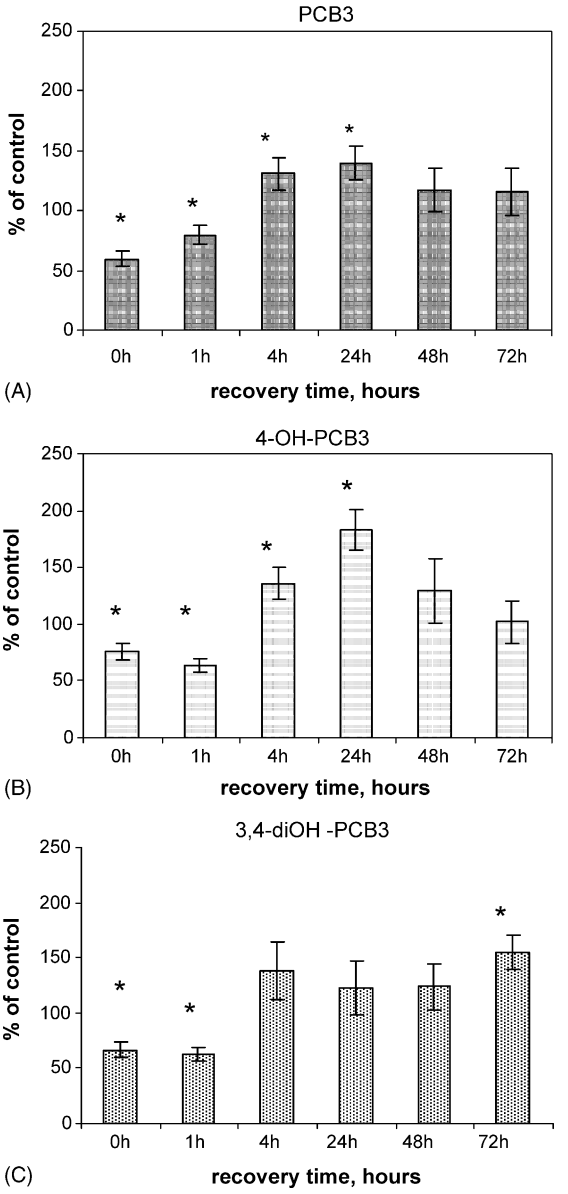

Fig. 4. Change in Tail-DNA-Content (TDC) in percent of control in granulosa cells exposed for 24 h to 6 ng/ml of PC3 (panel A), 4-OH-PCB3 (panel B) and 3,4-OH-PCB3 (panel C) after 0–72 h recovery time in normal medium. Each value presents the mean ± S.D. for at least two independent experiments. The asterisk indicates statistically difference to control ($p < 0.05$).

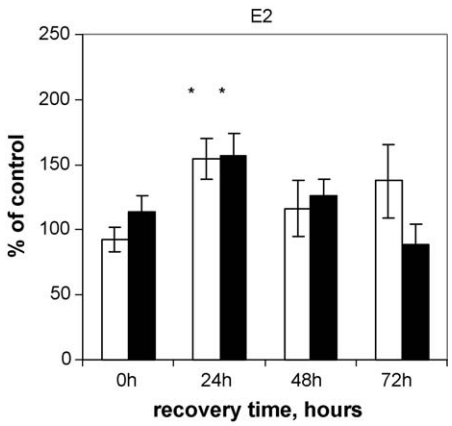

Fig. 5. Change in Tail-DNA-Content (TDC) compared to untreated controls in granulosa cells exposed for 24 h to 0.08 ng/ml (open bars) and 6 ng/ml (black bars) E_2 during the recovery time of 0–72 h. Each value presents the mean ± S.D. for at least two independent experiments. The asterisk indicates statistically different values compared to controls ($p < 0.05$).

4. Discussion

PCBs are ubiquitous environmental contaminants and it is well known that PCBs accumulate in human follicular fluid (Jarrell et al., 1993; Foster et al., 1996; Pauwels et al., 1999; Younglai et al., 2002) and in the ovarian follicular wall (Gregoraszczuk et al., 2003a). The levels of five PCB congeners (PCB52, 101, 138, 153, 180) found in the follicular fluid in pig, goats, sheep and cattle was 0.45, 0.55, 2.97 and 3.05 ng/ml, respectively (Kamarianos et al., 2003). Our previously published data using theca and granulosa cells in co-culture showed that PCB3 significantly stimulated estradiol secretion at all concentrations tested (0.06, 0.6, 6.0 and 60 ng/ml) with the highest stimulatory effect of 6 ng/ml (Ptak et al., 2005). We also showed that progesterone secretion was decreased, especially by PCB3 itself, and that the increase in estradiol secretion is caused by an increase in aromatase activity (Ptak et al., 2006). However, still little is known about the capability of ovaries for xenobiotic metabolism in general or about the metabolic activation of PCBs to genotoxic and cytotoxic species in that organ. We therefore analyzed ovarian follicle cells in culture for their CYP activities and their responses to challenges from PCB3 and its metabolites.

4.1. Cytochrome P450 1A1, 1A2 and 2B activity in porcine theca–granulosa co-cultures

Very few studies describe xenobiotic-metabolizing enzyme activities in ovaries. Swine express cytochrome P450 forms similar to those identified in humans and rodents in the liver (Myers et al., 2001), and Leighton et al. (1995) identified CYP1A1 mRNA in porcine ovarian granulosa cells. To our knowledge we show here for the first time the activity of three P450 isoforms, 1A1, 1A2 and 2B in porcine prepubertal ovary cells. The rank order of activity level was CYP1A1 > CYP2B > CYP1A2,

determined with the EROD, PROD and MROD assays, respectively. The activity level of CYP1A1 and CYP2B was five times higher than the level CYP1A2 activity and ranged around 40–50 pmol/100 μg protein/min. This activity is similar to the basal CYP2B activity reported by Cannady and coworkers in mouse ovary (Cannady et al., 2003). These results support the hypothesis that ovarian cells are capable of metabolic activation of xenobiotics.

4.2. Effect of PCB3 and E_2 on CYP1A1, 1A2 and 2B activity

Cytochrome P450 induction is a major characteristic of PCBs and depends strongly on the molecular structures of individual PCB congeners. For example, in mouse liver EROD (CYP1A) activity was significantly induced by TCDD and PCB126, which also induced histologically visible ovarian toxicity, but PCB153 did not show these effects (Johnson et al., 1997). Connor et al. (1995) described the structure-dependent induction of hepatic CYP2B1/2 (PROD) by PCB congeners in female rats, and Dragnev et al. (1994) found a striking dose- and time-dependent increase in hepatic CYP1A1 and CYP1A2 levels in rats after exposure to subchronic amounts of Aroclor 1254. CYP activity can also be induced in cells in culture. For example, Pang et al. (1999) reported that exposure of MCF-7 cells to PCBs 81, 126 and 39 caused highly elevated CYP1A1 and CYP1B1 mRNA levels and caused marked stimulation of E_2 metabolism in this cell line. Exposure to PCB169 resulted in elevated levels of the CYP1A1 and CYP1B1 mRNAs, but did not cause elevated rates of E_2 metabolism.

We previously reported that PCB3 and its monohydroxylated and dihydroxylated metabolites 4OH- and 3,4-diOH-PCB3 significantly increased estradiol levels in the porcine ovarian cells, and that this effect is in part due to increased aromatase activity (Ptak et al., 2006). Moreover, the presented data clearly show that both, PCB3 and E_2, can acts as inducers of CYP1A1 and CYP2B. Although PCB3 is not known to be an efficient CYP inducer, exposure to only 6 ng/ml PCB3 produced a significant increase in both activities, slightly above the increase produced by 20 nM to17β-estradiol. Thus CYP-induction may be another mechanism by which PCB3 changes steroid secretion by these follicle cells. In addition, both estradiol and PCB3 are metabolized by these CYP isoforms and it therefore cannot be excluded that the competition of PCB and the steroid hormone for interaction with CYPs may be a mechanism by which PCBs could inhibit the inactivation of estradiol.

4.3. Effect of PCB3 and its hydroxylated metabolites on caspase-3 activity

Widespread observation of apoptosis in cells from different tissues (Jeon et al., 2002; Tharappel et al., 2002; Howard et al., 2003; Sanchez-Alonso et al., 2003) raises the question of the vulnerability of ovarian follicle cells. Carefully timed and limited apoptosis is a necessary event in the normal function of ovaries (Tilly, 1996), but induction of apoptosis in the ovary at the wrong time and in the wrong cell population could reduce its steroidogenic capacity and cause a loss of the oocytes and thus infertility. Indeed, exposure of bovine luteal cells to PCB77 was reported to stimulate the expression of the pro-apoptotic Bax (Liszewska et al., 2005). Exposure of cumulus–oocyte complexes to 100 pg/ml of a mixture of the coplanar PCBs 77, 126 and 169 induced apoptosis, whereas 84 ng/ml of a mixture of the non-coplanar PCBs 52, 101 and 153 were negative (Pocar et al., 2005). Gregoraszczuk and coworkers, however, did not observe an adverse effect of 0.02–2 ng/ml of a low chlorinated PCB mixture (Delor 103) or a highly chlorinated PCBs mixture (Delor 106) on cell viability and apoptotic cell death in prepubertal porcine ovary cells in vitro (Gregoraszczuk et al., 2005). Moreover, in cells from large porcine follicles, exposure to PCB126 (100 pg/ml) or PCB153 (100 ng/ml) was paralleled by a suppression of caspase-3 activity and a decreased incidence of apoptotic bodies (Gregoraszczuk et al., 2003b).

In this study we observed an increase in caspase-3 only in co-cultures of theca and granulosa cells that had been exposed to PCB3 for 24 h. Shorter or longer exposure times were negative. It should be pointed out, that a concentration of 6 ng/ml PCB3 was used here, which is in the range observed in human follicular fluid of non-exposed women and about 1000× lower than in the concentrations used in studies with cells from non-ovarian tissues. This ability of PCBs to induce apoptosis, especially in the prepubertal ovary, may affect the fertility of the organism. It needs to be analyzed whether different PCB congeners act in an additive or even synergistic manner in apoptosis induction.

Metabolism of PCB3 gives monohydroxy and dihydroxy metabolites. As shown here, neither of the 2 PCB-metabolites tested had an effect on caspase-3 activity, which indicates that metabolism may reduce the risk of caspase-mediated apoptotic cell death. Thus the apoptosis-inducing activity may be limited to the parent compound and not shared with the metabolites. However, the lack of apoptosis could also be due to an indirect mechanism. PCB3 metabolites are more potent inducers

of estradiol secretion (Ptak et al., 2005, 2006) which is known as a anti-apoptotic factor in ovarian cells. Thus metabolism of lower chlorinated PCBs may have an anti-apoptotic effect. This may be an advantage with respect to immediate cell survival in the ovary, but may have negative consequences, if cells with damaged DNA are allowed to survive and multiply.

4.4. Effect of PCB3 and its hydroxylated metabolites on DNA damage

An important question for all environmental contaminants is their potential genotoxicity. One very sensitive method for genotoxicity testing that can be used in virtually all cell types and tissues is the Comet assay.

The lowered percentage of DNA in comet tails which was observed in the granulosa cells immediately or shortly (1 h) after treatment with PCB3 or its hydroxylated metabolites reveals an induction of increased compactness by these compounds, indicating the possibility of DNA cross-link formation. It was shown that the production of interstrand cross-links can occur after induction of DNA adducts (Sanchez et al., 2005; Noll et al., 2006). PCBs and their metabolites form adducts with proteins and DNA in vitro and in vivo (McLean et al., 1996b; Oakley et al., 1996b; Schilderman et al., 2000; Pereg et al., 2002; Borlak et al., 2003; Zhao et al., 2004).

Four to 24 h after removal of the compounds an increase in DNA in the comet tails was observed. This increase in DNA breaks 24 h after the cross-link formation may represent transient abasic and/or alkali-labile sites created by cleaved of the damaged bases by a DNA glycosylase in the base-excision repair process of removing of DNA adducts (Hang, 2004). The breaks disappearance after 48 h seems to confirm this suggestion. A second mechanism of the delayed increase in DNA breaks may be by secondary damage induced by reactive oxygen species (ROS), generated during the metabolic oxidation of the PCBs to the corresponding quinones as suggested by Srinivasan et al. (2001). Only the most hydroxylated metabolite, 3,4-OH-PCB3, caused, after initial period of enhancement in DNA compactness, an increase in DNA breaks in granulosa cells up to 72 h after its removal. Alternatively or in addition, the difference in the kinetics of strand break formation and recovery in granulosa cells after exposure to this metabolite may be a result of the higher concentration of 17β-estradiol induced by 3,4-OH-PCB3 as compared to the other two compounds (Ptak et al., 2005), since estradiol itself produces DNA strand breaks. Thus redox cycling of hormone metabolites and/or hydroxylated biphenyl may be responsible for the additional DNA breaks.

The repair process of DNA adducts is not yet fully understood and takes longer time than the repair of other DNA lesions (Gantt, 1987). However, any unrepaired DNA damage may direct cells toward apoptosis. Only PCB3-treatment increased the caspase-3 activity 24 h after its removal, the same time when significant DNA breaks occurred. PCB3 may be metabolized to a DNA-reactive epoxide. The two hydroxylated compounds did not show such a relationship. These metabolites can form a secondary epoxide and/or quinone and they are more likely to form ROS and induce an increase in estradiol. We observed that PCB3 significantly increased point mutations in the livers of transgenic male BigBlue rats, whereas 4-OH-PCB3 was not significant (unpublished results), indicating that the DNA-reactive derivative of PCB3 is more genotoxic than at least one of the monohydroxy-metabolites. Literature reports concerning the genotoxicity of PCBs are controversial. However, differences in metabolism and changes in hormone levels in different tissues may explain some of the different observations after PCB-exposure on the DNA reported in the literature (Silberhorn et al., 1990; Belpaeme et al., 1996; Schilderman et al., 2000; Ludewig, 2001; Hauser et al., 2003).

These results show that ovarian follicle cells express various forms of cytochrome P450 and exposure of follicle cells to PCBs or increased levels of estradiol may increase CYP activity. These CYPs may metabolize environmental contaminants like PCBs to reactive intermediates that could damage the DNA, possibly by adduct and cross-link formation, by generation of ROS, or by increasing the levels of estradiol. Finally, unrepaired adducts of a PCB3-metabolite, possibly an epoxide, may be the trigger of the transient increase in caspase-3 that we observed in these follicle cells. These results, while not fully clarifying the mechanisms of toxicity of PCB and its hydroxylated compounds, suggest that DNA damage, most probably due to the DNA cross-link formation, and the resulting the DNA breaks, are involved, and that ovarian follicle cells are one target of these adverse PCB effects.

Acknowledgements

This work was supported by KBN Grant State Committee number 0431/PO4/04/27. Anna Ptak is the scholar of the Foundation For Polish Science. GL and LWR thank NIH (ES13661, ES05605), EPA (R-82902102-0) and DOD (DAMD 17-02-1-0241) for financial support.

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