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A LOW HALOGENATED BIPHENYL (PCB3) INCREASES CYP1A1 EXPRESSION AND ACTIVITY VIA THE ESTROGEN RECEPTOR BETA IN THE PORCINE OVARY

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Polychlorinated biphenyls (PCBs) have been detected at high levels, up to hundreds of pg/ml, in human ovarian follicle fluid. The effect of PCBs on the ovary and the consequences of exposure are largely unknown. We have previously shown that PCB3 (4-chlorobiphenyl) increases the secretion of estradiol and the activity of cytochrome P450s (CYPs) in ovarian follicle cells. Our goal here is to elucidate the mechanism of CYP induction by this congener. Exposure of porcine follicle cells, a co-culture of theca and granulosa cells, to 6 ng/ml of PCB3 caused an increase in CYP1A1 protein and enzymatic activity, in the same manner as exposure to exogenous 17β-estradiol. No changes were seen in the protein level of the aryl hydrocarbon receptor (AhR), which mediates the first step in the signaling pathway of CYP1A1 induction. However, a strong reduction was seen in the protein level of estrogen receptor beta (ERβ), while no effect was seen on ERα protein levels. These result suggest that: 1) PCB3 acts as an agonist of ERβ but not the Ah receptor in the ovarian follicles, 2) PCB3 is not only an efficacious inducer of CYP1A1 expression and activity, but also a substrate for this enzyme. Changes in the expression level of CYP1A1 not only alter the intensity of the activity of PCB3, but also the activity of estrogen in the ovary.

Key words: *PCB3, E2, prepubertal ovarian follicle, CYP1A1, AhR, ERα, ERβ*

INTRODUCTION

Polychlorinated biphenyls (PCBs) are one of the various classes of environmental contaminants that have been observed to affect ovarian function. Although PCBs can destroy ovarian follicles, the mechanisms by which this

occurs are still unclear. Measurable concentrations of PCBs have been found in follicular fluid and serum from women (1) and farm animals (e.g., cattle, sheep, goats and pigs) (2).

Lower chlorinated PCBs, especially those with one or two free para-positions, are rapidly metabolized and called “episodic congeners”(3). As a many PCBs selectively induce cytochrome P-450 (4), which may catalyze the oxidation of a broad range of endogenous and exogenous substances. One member of the CYP1 family, CYP1A1, has been shown to play an important role in the metabolism of PCBs and endogenous compounds, such as 17β-estradiol (5, 6). CYP1A1 is expressed or induced in numerous extrahepatic tissues, including breast (7), placenta (8), uterus (9) and ovary (10, 11). Thus, it is possible that PCBs might be inducers of or substrates for this enzyme, and/or interfere with estrogen metabolism in these extrahepatic tissues.

Expression of CYP1A1 may be increased through the interaction of compounds with the aryl hydrocarbon receptor (AhR). Many PCBs are known to induce CYP1A1, and thus their own metabolism, through binding to and activation of AhR (12,13). Expression of AhR has been described in the ovary of different species: rat (14), rabbit (15), human (16), mouse (17) and pig (18). However, some evidence suggests that lower chlorinated PCBs are not AhR receptor agonists and may exert their toxic effect via AhR independent mechanisms (19).

CYP1A1 is a major extrahepatic CYP enzyme. Its basal expression is very low in extrahepatic tissues, but it is induced by AhR ligands in almost every tissue studied, including lung, lymphocytes, mammary gland and placenta (20). Very few studies describe xenobiotic metabolizing enzyme activities in ovaries (17, 21 - 23). Swine express CYP forms similar to those identified in humans and rodents (24). To our knowledge there is only one data of Leighton and coworkers (10) showing CYP1A1 mRNA in porcine ovarian granulosa cells and our previously published data showing that three CYP isoforms, 1A1, 1A2 and 2B, in porcine prepubertal ovary cells (25). The rank order of activity was CYP1A1>CYP2B>CYP1A2, determined with the resorufin assays (EROD, PROD and MROD). Moreover, our results clearly showed that PCB3 could act as an inducer of CYP1A1 and CYP2B expression in ovarian follicle cells (25). Therefore, the ovaries may play an important role in the metabolism of endogenous and exogenous compounds. Furthermore, we also showed that PCB3 and its metabolites significantly increased estradiol secretion in porcine prepubertal and mature ovary cells (26, 27).

We performed this study to understand the mechanism by which PCB3 induces expression of CYPs in the ovary and the type of receptor (AhR, ERα or ERβ) that mediates this induction. We analyzed the effect of PCB3 on CYPA1 activity and on the expression levels of CYP1A1, AhR and the two forms of the estrogen receptor. We compared these results to the effect of 17β-estradiol on prepubertal ovarian follicle cells in culture.

MATERIALS AND METHODS

Test compound and other materials

PCB3 (4-chlorobiphenyl) was synthesized using the Suzuki coupling reaction and characterized as described previously (28,29). A stock solution of this compound in DMSO was prepared and added to culture medium immediately before use, as described below. The final concentration of DMSO in the medium was always 0.2%. Parker Medium 199 without phenol red (M199), Fetal Bovine Serum (FBS, heat inactivated), Trypan blue, 4-hydroxytamoxifen and α-naphthoflavone were obtained from Sigma Chemical Co., MO, USA.

Granulosa and theca cell isolation and co-culture

Porcine prepubertal ovaries were obtained from a local abattoir. Granulosa cells (Gc) and theca internal cells (Tc) were isolated from antral follicles (4 - 6 mm in diameter) according to the technique described earlier (30). 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 12 x 10^4 and 3.0 x 10^4 viable cells/well, respectively, in 48-well tissue culture plates or 20 x 10^5 and 5 x 10^5 viable cells/well, respectively, in 24-well tissue culture plates. This ratio is similar to that observed *in vivo* (Gc: Tc = 4:1), according to Stokłosowa (31). Cells were cultured in Parker Medium 199 without phenol red (M199) supplemented with 5% FBS for 24hrs, at 37°C in a humidified atmosphere of 5% CO_2, to allow for attachment.

Experimental design

Cells were cultured without (control) or with the test compounds PCB3 (6ng/ml) and 17β-estradiol (6ng/ml). This concentration of PCB3 was chosen based on our previous findings (26) that in culture medium from mature antral follicles an increase in estradiol levels was noted under the influence of 6 ng/ml of this compound. 4-Hydroxytamoxifen (OH-TMX, blocker of ER), at 10μM, or α-naphthoflavone (ANF, blocker of AhR), at a concentration of 10μM, was added to the medium to confirm activation of ER or AhR.

For Western blot analysis, cells after 1, 3, 6, 24 and 48 hours of incubation were transferred into ice-cold lysis buffer (50mM Tris-HCl pH 7.5; 100mM NaCl; 0.5% Na-deoxycholate; 0.5% Nonidet NP-40; 0.5% sodium dodecyl sulfate and protease inhibitor EDTA-free). Total cell lysates were prepared and stored at –20°C.

For determination of CYP1A activity, cells were exposed to the test compound for 48 hours. Then media were removed and the cells were washed with cold phosphate buffered saline (PBS) and stored at –70°C for later determination of CYP1A1 activity.

Western blot analysis

The protein concentrations in lysates were determined with the Bradford reagent (Bio- Rad Protein, Bio-Rad Laboratories, Inc., CA, USA). Equal amounts of protein (20μg) from each treatment group were separated by SDS-PAGE and transferred to PVDF membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., USA). The blots were blocked overnight in 5% dry milk with 0.1% Tween-20 in 0.02M TBS buffer. Blots were incubated for 2 hours with antibodies specific to AhR (sc-8088), CYP1A1 (sc-9828) and ERβ (sc-8974) (all from Santa Cruz Biotechnology Inc., CA, USA), ERα (M7047) (DakoCytomation, Denmark) and β-actin (A5316)

(Sigma Chemical Co., MO, USA). After incubation with the primary antibody, the membranes were washed three times and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody: P0447 (DakoCytomation, Denmark) for ERα and β-actin; sc-2004 (Santa Cruz Biotechnology Inc., CA, USA) for ERβ; sc-2020 (Santa Cruz Biotechnology Inc., CA, USA) for CYP1A1and AhR. Immunopositive bands were visualized using the Amplified Opti-4CN Kit (Bio-Rad Laboratories, Inc., USA).

The EROD activity assay to measure CYP1A1 activity

Cells were lysed by removal from the freezer and thawing for 10 min. Ethoxyresorufin-O-deethylase (EROD), a specific measure of CYP1A activity, was determined as described by Kennedy and Jones (32). The fluorescence of resorufin generated by conversion of ethoxyresorufin by CYP1A was measured in 15 min intervals for up to 2 hours with a fluorescence plate reader (FLx 800, Bio-Tek, USA) using a 530nm excitation and 590nm emission filter. After 2 hours, the protein concentration in each well was determined using fluorescamine (Sigma Chemical Co. MO, USA). Results were calibrated against a resorufin standard curve (0-100nM) and BSA standard curve (0-1000μg).

Statistical analysis

Each treatment was repeated three times (n = 3) in quadruplicates. The average of the quadruplet values was used for statistical calculations. Statistical analysis was performed using Stastistica 6.0. Data were analyzed by 1-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 significantly different.

RESULTS

Time course of the effects of PCB3 and 17β-estradiol on CYP1A1 protein levels

Fig. 1a depicts CYP1A1 protein expression, measured by immunoblot analysis, in control cells and cells exposed to 6 ng/ml of PCB3 for 1 to 48 h. β-actin was used as control for equal loading of the lanes. The basal expression of CYP1A1 in the untreated cells was very low and slightly increased during the first six hours of exposure to PCB3. However, the protein blot shows a marked increase in the intensity of a protein immunorelated to CYP1A1 after 24 and 48 h of exposure to PCB3 and from 6 to 48 hours after exposure to 17β-estradiol (E2) (*Fig.1b*).

CYP1A1 enzyme activity (EROD assay) after PCB3 and 17β-estradiol treatment in the presence and absence of α-naphthoflavone (ANF) and 4-hydroxytamoxifen (TMX)

The basal EROD activity was observed (40.1 ± 1.7 pmol/100 μg protein/min). Both PCB3 and 17β-estradiol increased EROD activity in the same manner (55.5 ± 0.78 pmol/100 μg protein/min and 51.9 ± 2.3 pmol/100 μg protein/min after 48

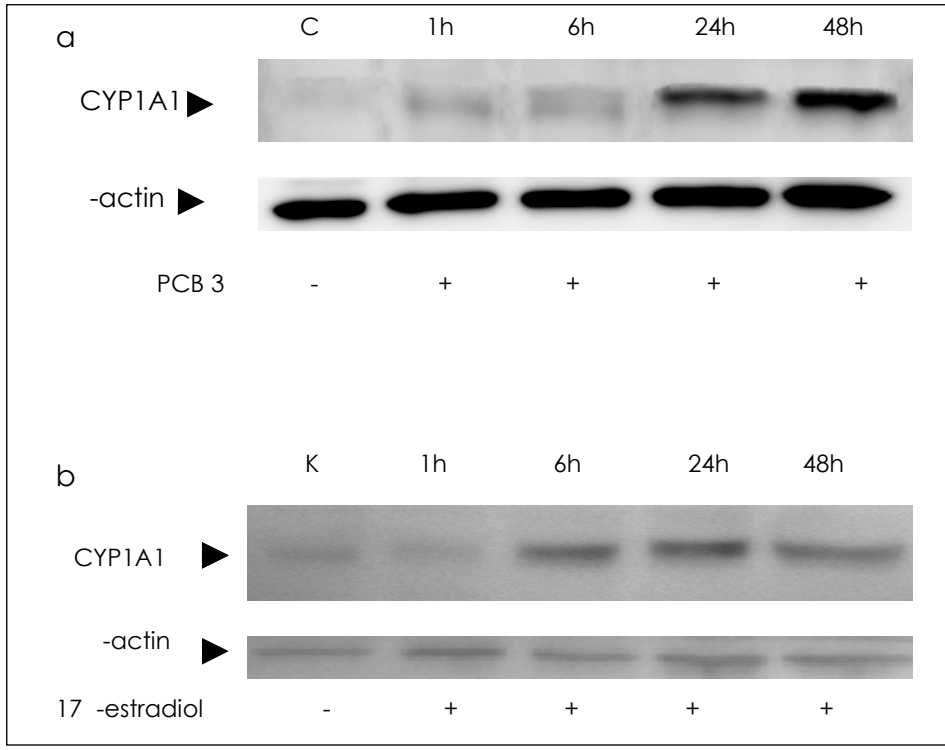

Fig. 1. Immunoblot of cytochrome P-450 1A1 from ovarian follicle cells treated for 1 to 48 hours with a) PCB3 (6ng/ml) and b) 17β-estradiol (6ng/ml). Control cells (C) were treated with 0.2% DMSO only. The protein load of each lane was 20 µg. β-actin protein levels were determined as a control for loading and transfer of cell protein.

h of exposure to PCB3 and E2, respectively), ($p<0.05$) (*Fig. 2*). Treatment of the cells with ANF reduced CYP1A1 enzyme activity (0.46 ± 0.29; 2.23 ± 0.35; 1.18 ± 0.71 pmol/100 µg protein/min in control culture, PCB3 and E2 treatment culture, respectively), ($p<0.05$). Treatment of cells with TMX resulted in 32% suppression of the basal CYP1A1 activity (27.17 ± 2.95 in treated cells *vs.* 40.11 ± 1.7 pmol/100 µg protein/min in control), ($p<0.05$). Similarly, TMX resulted in 46% and 42% suppression of CYP1A1 activity under the influence of PCB3 and E2, respectively. ($p<0.05$) (*Fig 2*). None of the treatments caused an increase in cell death, as determined by LDH level in the culture medium (data not shown).

Time course of the effects of PCB3 on AhR protein level

To examine the possibility that the increase in CYP1A1 protein level was due to a direct agonistic action of PCB3 on AhR, the effect of PCB3 on AhR expression level was examined using western blot analysis. An immunorelated

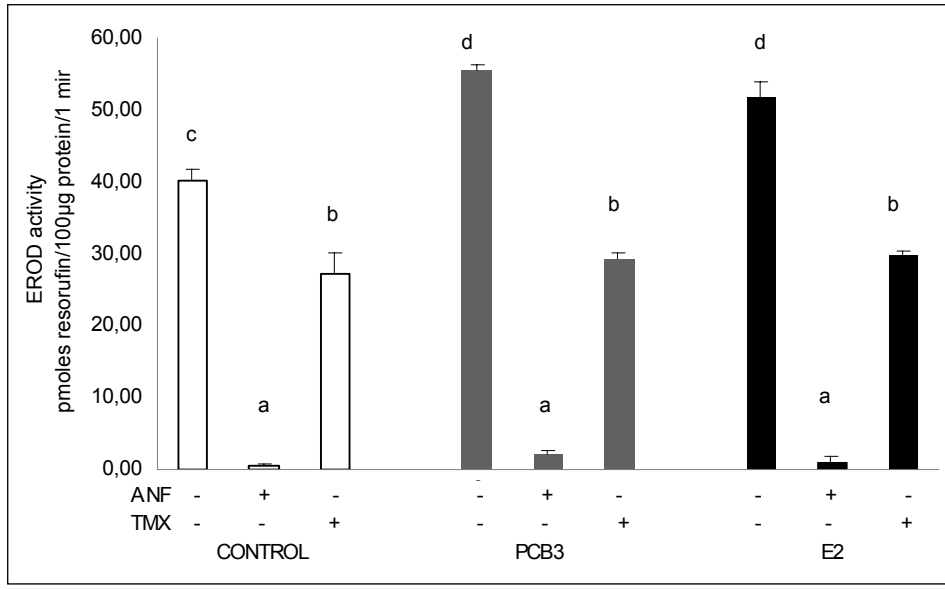

Fig. 2. Effect of PCB3 and 17β-estradiol, alone or combined with α-naphthoflavone (ANF, 10µM) and hydroxytamoxifen (TMX, 10µM), on CYP1A1 activity (EROD assay) after 48 hours of treatment. Control cells obtained in 0.2% DMSO only. Values are mean ± SEM. All means marked with different letters are significantly ($p < 0.05$) different from the respective control.

protein for AhR was detected in untreated control cells. Treatment with PCB3 did not affect AhR protein levels during 6 hours of exposure (*Fig. 3*).

Time course of the effects of PCB3 on ERα and ERβ protein levels

To investigate whether the PCB3 action was mediated by ERα and/or ERβ, the effect of PCB3 treatment on the protein levels of these hormone receptors in ovarian cells was examined by western blot. In control cultures of theca- and granulosa cells, the expression of ERα protein was very low (*Fig 4a*), whereas the expression of ERβ protein was high (*Fig. 4b*). A strong decrease of ERβ was visible in PCB3 exposed cells at the earliest time point tested (1 hour exposure to PCB3), which persisted during the 6 hour observation period (*Fig 4b*). PCB3 treatment had no effect on ERα expression at any time point tested (*Fig. 4a*).

DISCUSSION

To our knowledge, this study is the first to show constitutive expression of CYP1A1 at the background level by immunoblot analysis in porcine prepubertal follicle cells. The presented results indicate that the prepubertal ovary has some constitutive xenobiotic metabolic capacity, although at a relatively low level. This

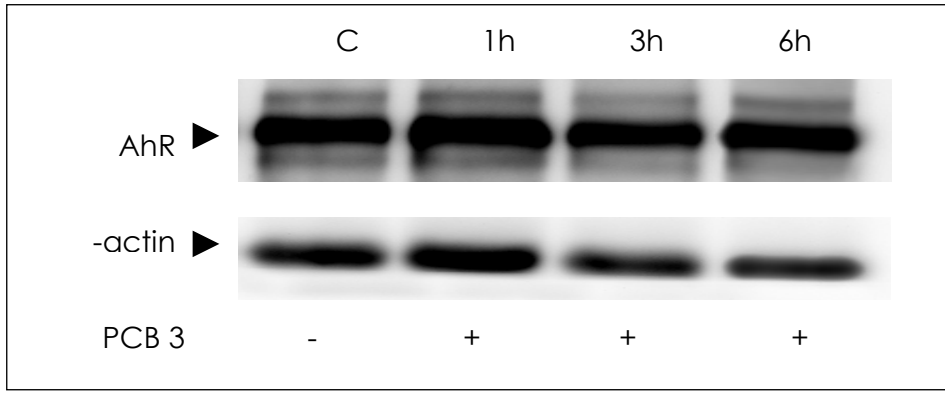

Fig. 3. Immunoblot of Ah receptor protein from ovarian follicle cells treated with 0.2% DMSO alone (C) or PCB3 (6ng/ml) for 1 to 6 hours. β-actin protein levels serve as a control for equal protein loading per lane (20 µg each).

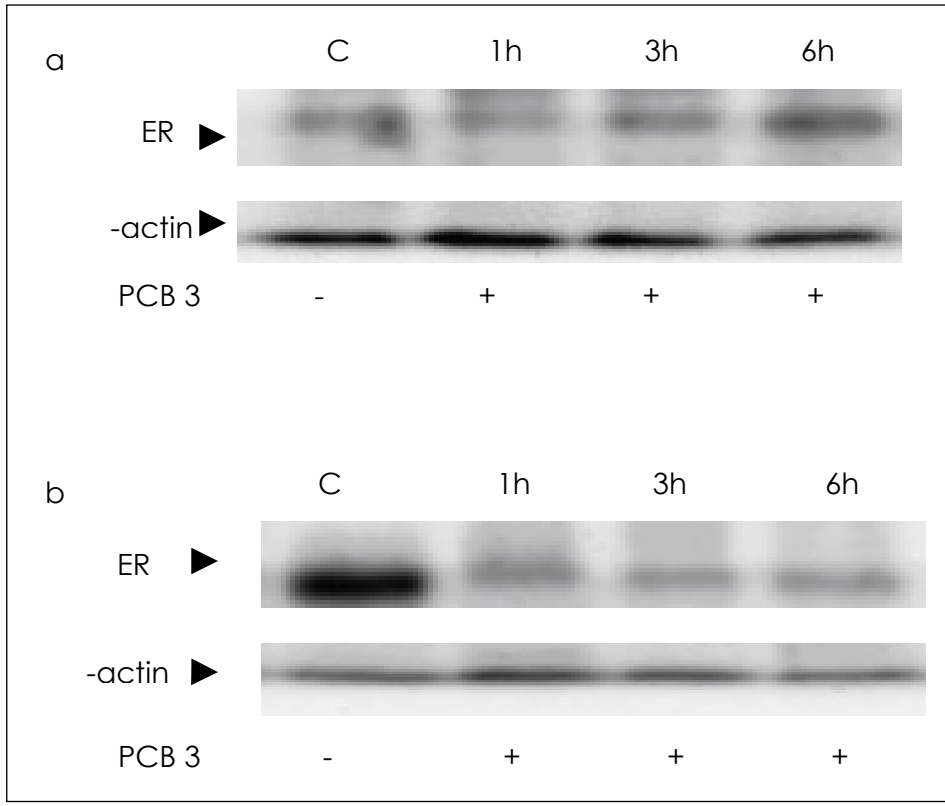

Fig. 4. Immunoblot of a) ERα and b) ERβ in ovarian follicle cells in culture. Cells were treated with PCB3 (6ng/ml) for 1 to 6 hours or 0.2% DMSO only (controls, C). The protein load per lane was 20µg. β-actin was used as a control for equal protein loading.

finding is in agreement with Leighton and coworkers (10), who described CYP1A1 expression at the mRNA level in porcine ovarian granulosa cells, and with Nestler *et al.* (21), who reported that CYP1A1 is expressed in porcine cumulus-oocyte complexes after *in vitro* maturation. In the mouse, constitutive CYP1A1 expression has been described in the ovary and oocytes (17). In addition, a constitutive expression of CYP1A1 in the fertilized ovum and basal and inducible expression of CYP1A1 in the early embryo of the mouse were reported (22). In bovines, constitutive expression of CYP1A1 mRNA at a background level was observed in immature oocytes, but not in the surrounding cumulus cells. However, a significant increase in CYP1A1 expression in both oocytes and cumulus cells was seen after *in vitro* maturation (IVM) (23).

The present findings demonstrate the induction of CYP1A1 protein and EROD activity in porcine prepubertal ovaries after exposure to 17β-estradiol and PCB3. It is well known that endogenous estrogens can be hydroxylated at multiple positions by cytochrome P450 enzymes. A major metabolite of estradiol, 2-hydroxyestradiol, is mainly catalyzed by CYP1A2 and CYP3A4 in the liver, and by CYP1A1 in extrahepatic tissues (33). This 2-hydroxylation of estradiol has been observed in the breast, uterus, placenta, ovary, brain and pituitary (9, 34).

Moreover, the present study demonstrates the more rapid induction of the CYP1A1 protein by PCB3 (from 1 to 48 h), than by E2 (from 6 to 48 h). In addition, PCB3 produced a significant increase in CYP1A1 activity, slightly above the increase produced by E2. This is of importance since CYP1A1 may not only increase the metabolism of endogenous compounds, like estradiol, but also of PCB3 itself. An analogous situation was reported by McLean *et al.* (35), that five mono- and three di-hydroxy metabolites were formed in rat liver microsomes by PCB3. Moreover, they showed that, among the different CYPs, CYP1A metabolized PCB3 most efficiently. It must also be pointed out that the change in the expression level of CYP1A1 not only alters the intensity of the effects of PCB3, but also alters the effects of estrogen in ovary.

An inhibitory study using α-naphtoflavone (ANF) and hydroxy-tamoxifen (TMX) was carried out to better understand the induction effect of PCB3 on CYP1A1 activity. α-Naphtoflavone (ANF) is a cytochrome P450 inhibitor and antagonist of AhR (36). Hydroxy-tamoxifen (TMX) is a partial agonist of ERα, but is a pure antagonist for ERβ (37).

The inhibitory effects of α-naphtoflavone on the induction of EROD by PCB3 and E2 suggested an AhR mediated mechanism. Similarly, Ramadass *et al.* (38) demonstrated that treatment of vascular endothelial cells with ANF alone or with ANF plus PCB77 inhibited EROD activity and CYP1A1 expression. However, PCB3 did not show an effect on AhR protein levels in porcine ovary cells. Together, this indicates that PCB3 may induce CYP1A1 by a mechanism that does not involve direct binding to AhR. Also, literature reports suggest that lower chlorinated PCBs are not AhR agonists (19). Similar observations were reported with human primary hepatocytes and HepG2 cells after treatment with

primaquine (an antimalarial), which was capable of inducing a dose dependent increase in both EROD activity and CYP1A1 mRNA. However, neither primaquine nor its metabolites were able to bind to AhR (39). Thus, another signal transduction pathway, other than AhR, may be responsible for induction of CYP1A1 activity by PCB3 and other compounds like estradiol.

We observed that co-treatment with TMX resulted in suppression of EROD activity in both PCB3 and E2 treated cells. So, we postulate that the estrogen receptor is also involved in the activation CYP1A1. Similar effects were observed when a pure anti-estrogen (ICI 182 780) caused the dose dependent decrease and eventual block of the stimulatory effect of E2 on TCDD-induced EROD activity (40). This raises the question as to whether PCB3 has an effect on the protein levels of ERα and/or ERβ. In control cultures, expression of ERα protein was very low, whereas the ERβ protein was highly expressed. These results are in agreement with studies showing the expression of this receptor in the human (41), murine (42) and porcine ovary (43). After treatment of porcine ovary cells in culture with PCB3, a rapid decrease in immunoreactive ERβ protein was observed, indicating binding of PCB3 to ERβ. In contrast, PCB3 had no effect on ERα. Similarly, Alarid *et al.* (44) demonstrated, using Western analysis, a rapid loss of total ER protein content in response to estrogens. Using chase analysis, they also found that estrogen induces degradation of ER protein and shortens its half-life from greater than 3 hours to 1 hour. Some ER ligands can also induce the ubiquitination of the receptor, provoking its rapid degradation via the proteosomal pathway (down regulation) (44, 45). Our results suggest that this pathway of enhanced ER destruction is also induced through PCB3 binding to the ER and that PCB3 possesses estrogen receptor binding activity. Moreover, the present findings demonstrate that PCB3 binds to ERβ, which is predominantly expressed in the ovary. Indeed, it is now believed that the PCBs react with the estrogen receptor, thereby changing intracellular signaling. A PCB study using the modified yeast estrogen receptor assay demonstrated full agonist activity for PCB3 (46). Routledge *et al.* (47), using a GST pull-down assay, observed that differences exist in the binding affinity of the 4'-hydroxy-metabolites of PCB61 (2,3,4,5-tetrachlorobiphenyl) for the two ER subtypes. ERβ had a greater receptor binding affinity for all of the xenoestrogens tested, including HO-PCB61, as compared to ERα.

In conclusion, results of this study indicate that: first, PCB3 acts as agonist of the ERβ receptor but not the Ah receptor in the ovarian follicles. Second, PCB3 is not only an efficacious inducer of CYP1A1 expression and activity, but also a substrate for this enzyme. Third, inhibitory studies suggest that these effects are due to possible positive cross-talk between AhR and ERβ signaling. However, the induction mechanism has not been clarified yet and future studies are necessary to confirm this hypothesis.

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