EFFECT OF PCB3 AND ITS HYDROXYLATED METABOLITES ON ESTRADIOL SECRETION, CELL VIABILITY, AND CASPASE-3 ACTIVITY IN PORCINE SMALL FOLICLES.

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Introduction

In general, highly chlorinated PCBs are slowly metabolized and eliminated. Lower chlorinated PCBs on the other hand are hydroxylated *in vitro* and in vivo [1,2]. Surprisingly this does not necessarily mean that these hydroxylated PCBs are rapidly excreted, as recent findings of substantial amounts of hydroxylated PCBs in animal and human blood have shown [3]. Therefore it must be assumed that not only the PCBs themselves, but also their metabolites can participate in the toxic effects of PCBs. Indeed, some hydroxylated metabolites of PCBs (OH-PCBs) have significant estrogenic activity through binding to the estrogen receptors [4]. Surprisingly, PCB54 (2,2',6,6'-tetrachlorobiphenyl) has about 10% of the activity of 4-OH-PCB54 in the MCF-7 focus assay, but does not bind to the estrogen receptor, suggesting the possibility of an additional, yet unknown mechanism of estrogenicity [5]. We found that PCBs and their hydroxylated metabolites cause an increase in estrogen secretion from ovarian follicular cells *in vitro* [6], with PCB3 < 4-OH-PCB3 < 3, 4-OH-PCB3. The most sensitive follicles were those collected during the early stage of their development [7]. In the present study we used this type of follicles to answer the question whether this observed huge stimulatory action of PCB3 and/or its metabolites on estrogen release into the medium is due to the action on cells viability and cell apoptosis.

Materials and Methods

Reagents: PCB3 and its hydroxylated metabolites, 4-OH-PCB3 and 3,4-diOH-PCB3 (Fig. 1) were synthesized using the Suzuki coupling reaction as described earlier [8,9,10]. PCBs were dissolved in DMSO. The final concentration of DMSO in the medium was in each case 0.2%.

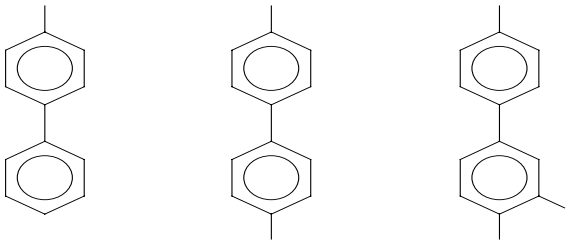

Figure 1: Structure of PCB3 and a mono- and di-hydroxy-PCB3

Cells cultures: Granulosa cells (Gc) and theca interna cells (Tc) from porcine small (<5mm) follicles were isolated and co-cultured according to the technique described by Stokłosowa and coworkers [11]. After a 24 h attachment period the culture medium (M199 supplemented with 5% calf serum) was replaced with new medium containing PCB3 or its metabolites at a concentration of 6 ng/ml or solvent alone. The choice of this concentration of the test compounds was based on results from our previous experiments [6]. Medium was collected and replaced with fresh medium containing test compounds every day for 4 days, i.e. at 24, 48, 72 and 96 hrs after the initiation of the experiment, and frozen for later determination of estradiol levels (immunoassay ELISA kits; IBL, Hamburg) and lactate dehydrogenase (LDH) activities (Cytotoxicity Detection Kit (LDH); Roche Applied Science, Mannheim, Germany). In a separate experiment theca and granulosa cells were cultured individually and after 24 hrs of exposure to the test compounds fixed for measurements of caspase-3 activities [12] as described previously [13].

Statistical analysis: Each treatment was repeated three times in quadruplicates and thus the total number of determinations was 12. Since the variations among the experiments were small, those 12 results were averaged and analyzed by analysis of variance followed by Duncan's new multiple range test. Each average (n = 12) is expressed as mean ± SEM.

Results

The amount of estradiol in the medium of PCB3 - treated cells was 446% and 569% of control after 24h and 48h, respectively, followed by a further 2.3 fold increase of estradiol levels to about 1350% of control after 72h and 96h of culture (Fig.2, open bars). The same tendency was noted under the influence of 4-OH-PCB3, where estradiol secretion increased to about 630% of control after 24h and 48h of treatment with an additional 4.5 and 8.8-fold increase at the 72h and 96h time points, respectively. The highest stimulatory action was noted after 3,4-diOH-PCB3-exposure. Estradiol levels of nearly 2500% and 1500% of control were measured after 24 and 48 hrs of culture and estradiol levels above 6000% and 8600% of control were reached at the 72h and 96h time point.

The increase of estradiol secretion under the influence of PCB3 and its metabolites noted during first 72 hrs of culture was not accompanied by a parallel loss in cell viability as measured by LDH activity in the media (Fig. 2, diamond and line graph). LDH levels in PCB-treated cultures were between 50-100% of control levels during the first 72 h of the experiments. However,

prolonged (96 h) treatment with PCB3 or its metabolites caused a 2-3 fold increase in LDH activity compared to control. (Fig. 2)

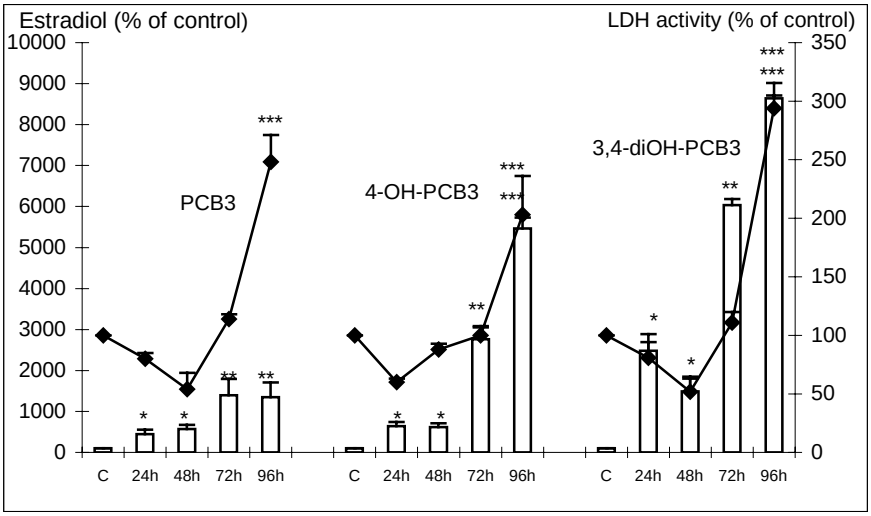

Figure 2: Time dependent effect of PCB3, 4-OH-PCB3 and 3,4-diOH-PCB3 on estradiol levels (open bars) and LDH activity (diamond and line graph) in culture medium. * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$.

We measured caspase-3 activity in cells after 48h of exposure to determine whether the loss of cell viability might be caused by apoptosis. As depicted in Fig 3, we noted a significant increase in caspase-3 activity in PCB-treated granulose (but not theca) cells, with PCB3 (125% of control) > 4-OH-PCB3 (113% of control) > 3', 4'-diOH-PCB3 (107% of control).

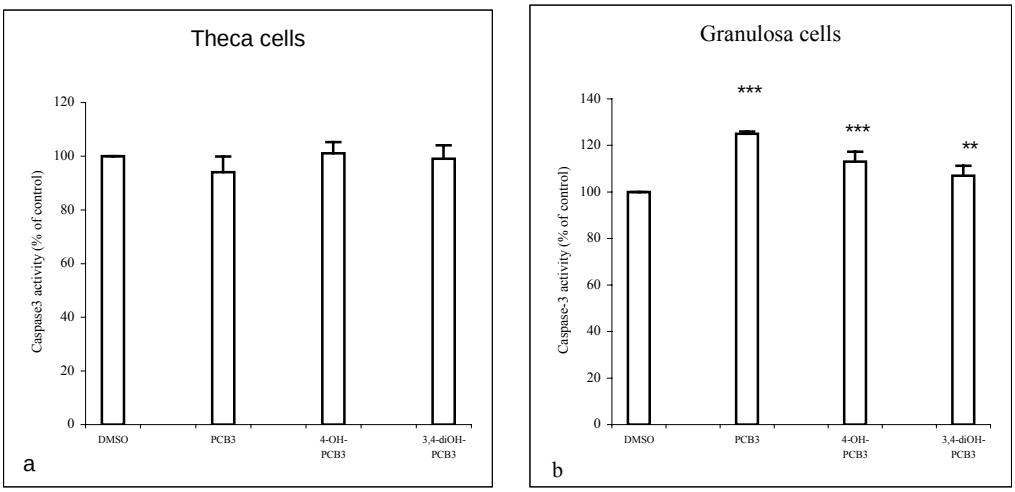

Figure 3. Effect of PCB3 and its metabolites 4-OH-PCB3 and 3,4-diOH-PCB3 on caspase-3 activity in theca and granulosa cells. ** $p< 0.01$, *** $p<0.001$.

Discussion

These results clearly show that the strongly increased estrogen release from PCB-treated ovarian follicle cells in culture after short term (24 and 48h) and even longer (72h) exposure is not due to cytotoxicity. Instead PCBs may influence follicular steroidogenesis, possibly by increasing aromatase activity, thereby causing the increased estrogen secretion into the medium. Prolonged treatment (96 h), however, did result in increased LDH activity in the medium, indicative of cytotoxicity and damage to the cell membranes. We therefore suggest that changes in cell permeability due to loss of membrane integrity caused the efflux of steroids into the medium after long treatment with PCB. This mechanism would be in agreement with results from Rogers et al.[14] concerning Aroclors action in a hamster ovary cell line and our previous data with TCCD in ovarian follicles [15]. Interestingly this steep, more than 3-fold, increase in estrogen secretion between 72 to 96 h was only seen with hydroxylated PCBs, although PCB3 itself was similarly cytotoxic. This raises the question whether the cytotoxicity was caused in part by different mechanisms, like apoptosis vs necrosis. We therefore examined the effect of PCB3 and its metabolites on caspase-3 activity, a mediator in the apoptotic pathway [16]. Our results suggest that indeed PCB3 does trigger pro-apoptotic caspase-3 activity as early as 24 h after exposure in granulosa cells, the major estrogen producing cell type, but not in theca cells, and that this effect is reduced following oxidative metabolism of PCB3. Interestingly we observed an inverse relationship for estrogen secretion and caspase-3 activity and an equal cytotoxic activity (LDH activity) of the test compounds. This could indicate, *a.* that these three effects are mediated by independent mechanisms or *b*. that the stronger pro-apoptotic effect is a reason for the lower increase in estrogen secretion in PCB3-treated cultures and/or *c.* that hydroxylated PCB3 metabolites may induce cytotoxicity by a mechanism (for example production of oxidative stress?) that is or is not related to their activity to induce estrogen secretion.

Conclusions

The results of these studies show that 1) the increased estrogen secretion from ovarian follicle cells after short term exposure to PCB3 and its metabolites is not caused by membrane damage, whereas 2) long term (96 h) exposure *in vitro* to these compounds is cytotoxic, probably causing some of the enhanced efflux of steroids into the medium, and 3) PCB3 and metabolites have pro-apoptotic activity, whereby increased oxidation of PCB3 decreases this pro-apoptotic activity, but increases the effect on estrogen secretion in these follicle cells *in vitro*. This inverse relationship between estrogen secretion and caspase-3 activation by equal overall cytotoxicity (LDH release) of the compounds opens new questions about the mechanisms of these effects that need further examination.

Acknowledgements

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