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In vitro Effects of TCDD, PCB126 and PCB153 on Estrogen Receptors, Caspases and Metalloproteinase-2 mRNA Expression in the Chicken Shell Gland*

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Among the environmental chemicals which disturb endocrine functions, dioxins and polychlorinated biphenyls (PCBs) are known as the most toxic. Numerous studies in mammals revealed that dioxins and PCBs disrupt functions of the uterus, delay implantation and increase embryo loss. The direct effect of these chemicals on the avian oviduct is not known. Therefore, in the study chicken shell gland tissues were used to examine the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), coplanar PCB126 and non-coplanar PCB153 on estrogen receptors (ERs), initiator caspase-1, executioner caspase-3 and metalloproteinase-2 (MMP-2) mRNA expression. Fragments of shell gland tissue isolated from the laying chicken were incubated for 24h with TCDD (100nM), PCB126 (100nM) or PCB153 (100 μM). Quantitative PCR analysis showed that: (1) TCDD increased ER beta (ERβ) mRNA expression, (2) PCB126 increased ER alpha (ERα), ERβ and caspase-1, and decreased MMP-2 mRNA expression, (3) PCB153 elevated the ERβ and caspase-1 expression levels and (4) expression of caspase-3 was not altered by any investigated xenobiotics. The results obtained using the shell gland explants model indicate that dioxins and PCBs have a direct effect on the chicken oviduct, especially the shell gland, by affecting the expression of genes involved in the function of this oviductal segment. It is suggested that coplanar PCBs such as PCB126, by changing cellular and extracellular regulators gene expression, may lead to disruption of shell gland activity and impair egg components formed in this organ.

Key words: ERs, caspases, MMP-2, TCDD, PCB, chicken shell gland.

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Among the environmental chemicals which disturb endocrine functions, dioxins and polychlorinated biphenyls (PCBs) are known as the most toxic. They are one of the ligands of the aryl hydrocarbon receptor (AhR) which induce a spectrum of developmental and toxic responses such as modification of gene expression, alteration in hormonal profiles and disruption of cell proliferation and differentiation (BOCK & KOHLE 2006). Since dioxins and PCB show structural similarities to estrogens and exert some estrogenic activity (see review FRYE *et al*. 2012) the main site of their actions are the reproductive organs.

Numerous studies in mammals revealed that dioxins and PCBs disrupt functions of the uterus (WANG *et al.* 1993; POHJANVIRTA & TUOMISTO 1994; KOTWICA *et al.* 2006; BRUNER-TRAN *et al.* 2008; BRUNER-TRAN & OSTEEN 2010), delay implantation and increase embryo loss (HUTT *et al.* 2008; see review FOWLER *et al.* 2012). The direct effect of these chemicals on the avian oviduct is not known. From among environmental chemicals only one dramatic effect, i.e. egg shell thinning caused by dichlorodiphenyldichloroethylene (DDE), a degradation product of dichlorodiphenyltrichloroethane (DDT) was reported (LUNDHOLM 1993, 1997; see review GIESY *et al.* 2003). Moreover, embryonic exposure to the synthetic estrogen ethynyloestradiol caused histological and functional disturbance in the shell gland in the adult quail (BERG *et al.* 2001; HOLM *et al.* 2001; HALLDIN 2005). Similar effects, as well as, the

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shell thinning, were observed in the domestic hen after synthetic estrogen exposure (BERG *et al.* 2004). These observations, and in addition the expression of AhR in the chicken shell gland (our unpublished data), indicate that the shell gland of the avian oviduct is a target site for the action of endocrine-disrupting chemicals.

The most important hormones regulating development and function of the chicken oviduct including egg white protein synthesis and secretion and mobilization of calcium for egg shell formation are estrogens. Estrogenic-like endocrine disruptors have the potential to disturb natural hormone synthesis and processes by different mechanisms. Besides AhR, their effects are exerted through action on estrogen and androgen receptors, where they can have an agonistic or an antagonistic effect (FOWLER *et al.* 2012). Thus, in the present study the *in vitro* effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), coplanar 3,3',4,4',5-pentachlorobiphenyl (PCB126) and non-coplanar 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) on estrogen receptor alpha (ERα) and beta (ERβ) expression in the shell gland explants are evaluated.

One of the most important processes involved in avian oviduct function and remodeling is apoptosis. This process in the chicken oviduct is regulated by estrogens (MONROE *et al.* 2002). Numerous genes are involved in the decision of a cell to undergo apoptosis, providing potential targets of endocrine disruptors to affect the cell death pathways. Since the activation of caspase cascade is one of the most important way leading to apoptosis, in the present study the *in vitro* ability of TCDD, PCB126 and PCB153 to regulate mRNA expression of initiator caspase-1 and executor caspase-3 expression is examined.

It has been well-established that proper functioning of several organs including the avian oviduct depends also on extracellular matrix (ECM) components, which in turn influence basic cellular processes such as proliferation, differentiation, adhesion and cell death (see review NY *et al.* 2002; PAGE-MCCAW *et al.* 2007). Because of the key role in ECM remodeling plays matrix metalloproteinase (MMP) system it is reasonable to investigate the effect of TCDD and PCBs on metalloproteinase-2 expression in the chicken shell gland explants.

Material and Methods

Chemicals

BSA, DMSO, antibiotic-antimycotic solution (100x; Sigma, St. Louis, USA); TCDD (CIL Inc., USA); PCB126, PCB153 (Dr. Ehrenstorfer GmbH, Augsburg, Germany); Eagle's medium (Laboratory of Sera and Vaccines, Lublin, Poland); TRI-reagent (MRC Inc., Cincinnati, USA), RevertAid M-MuLV Reverse Transcriptase, Ribonuclease inhibitor, dNTP mix, buffers, (Fermentas, Vilnius, Lithuania), primers, oligo-dT_{18} (IBB, Warsaw, Poland), SYBR Green Master Mix, Eukariotic 18S rRNA Endogenous Control (Applied Biosystems, Foster City, USA). All other reagents were obtained from ICN Biomedicals (USA) or POCH (Poland).

Birds and experimental procedure

All procedures were approved by the Local Animal Ethics Commission in Kraków, Poland (No. 31/2010).

The experiment was performed on laying Hy-Line hens (n=5) at the age of 25 weeks purchased from Drobeco commercial farm (Palowice, Poland). The birds were housed individually under a photoperiod of 14L:10D with free access to commercial food and water. Individual lay patterns were monitored daily. Hens were decapitated 2h after oviposition and the oviduct was removed. From each oviduct four fragments of the shell gland wall weighting ~ 50 mg were dissected and immediately placed in ice-cold PBS and rinsed twice with PBS containing 20 IU/ml penicillin, 20 μg/ml streptomycin and 50 ng amphotericin B/ml. Shell gland explants were dispersed to wells in 24-well plates containing 1ml of Eagle's medium supplemented with 0.05% bovine serum albumin and 2 μl antibiotic antimycotic solution (20 IU penicillin, 20 μg streptomycin and 50 ng amphotericin B). Tissues were cultured for 24h at 38°C in humidified atmosphere of 95% air and 5% CO_2 in the presence of 100 nM TCDD or PCB126 or 100 μM PCB153 or vehicle i.e. DMSO (control). The final concentration of DMSO in medium was below 0.6%. The concentrations of chemicals were established taking into account the value of the indicator for the TEF toxicity of these compounds in birds (VAN DEN BERG *et al.* 1998) and the dose-response carve from preliminary experiments.

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was extracted from the tissues using TRI-reagent according to the manufacturer's recommendation. Two μg of total RNA from each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U) and oligo-dT_{18} primers (0.5 μg). Non-transcribed tissue RNA (reverse transcriptase omitted) was used as a negative control. Two μl of cDNA (10x diluted samples after the RT) were amplified in a 96-well thermocycler (StepOne Plus; Applied Biosystems,

Table 1

Characteristic of primers used in this study

Gene	GenBank No.	Primer sequence	PCR product
ERα	X03805	F: 5'-GTGCCTTAAGTCCATCATCCT-3' R: 5'-GCGTCCAGCATCTCCAGTAAG-3'	300 bp
ERβ	AB036415	F: 5'-TGATATGCTCCTGGCCATGAC-3' R: 5'-CTTCATGCTCAGCAGATGCTC-3'	304 bp
Caspase-1	AF031351.1	F: 5'-GATACGTGACTCCATCGACCC-3' R: 5'-CTTCTTCAGCATTGTAGTCC-3'	313 bp
Caspase-3	AF083029	F: 5'-AGCAAGCGAAGCAGTTTTGT-3' R: 5'-TGCGTTCCTCCAGGAGTAGT-3'	300 bp
MMP-2	U07775.1	F: 5'-AGCTGCACCGTCACCAATCAT-3' R: 5'-CCTGCATCTGTGCAGCTGTTG-3'	668 bp

USA) according to the recommended cycling program:10 min at 95°C initial denaturation followed by 95°C/15 s; 60°C/60 s (40 cycles). The singleplex real time qPCR reactions were performed in 10 μl of volume containing 5 μl of SYBR Green Master Mix, 0.1 μmol of sense and antisense primers and water. The specific set of primer pairs already used by HRABIA & JANKOŚ (2011) for ERs and caspases, and HRABIA & LEŚNIAK (2012) for MMP-2 are shown in Table 1. As a reference gene 18S ribosomal RNA was chosen. Each sample was run in duplicate. No template control was included in any of the runs. A relative quantification (RQ) of examined genes was calculated after normalization with an 18S rRNA transcript and the expression in the control group as the calibrator using the $2^{-\Delta\Delta Ct}$ method. Quantification was performed using Step- One integrated software.

Statistical analysis

Results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences of values were considered to be significant at $P<0.05$. The data are presented as means ± SEM.

Results

The mRNA expression of all examined genes was found in cultured explants of the chicken shell gland. The relative expression (RQ) of ERα was significantly elevated by PCB126 from 0.88 ± 0.045 to 1.36 ± 0.140 (Fig. 1A). The relative expression of ERβ was significantly increased from 0.88 ± 0.740 in control to 1.38 ± 0.217, 2.01 ± 0.342 and 1.82 ± 0.355 by TCDD, PCB126 and PCB153, respectively (Fig. 1B).

In respect to caspase-1, it was not affected by TCDD whereas PCB126 and PCB153 increased expression levels of this caspase from 0.90 ± 0.049

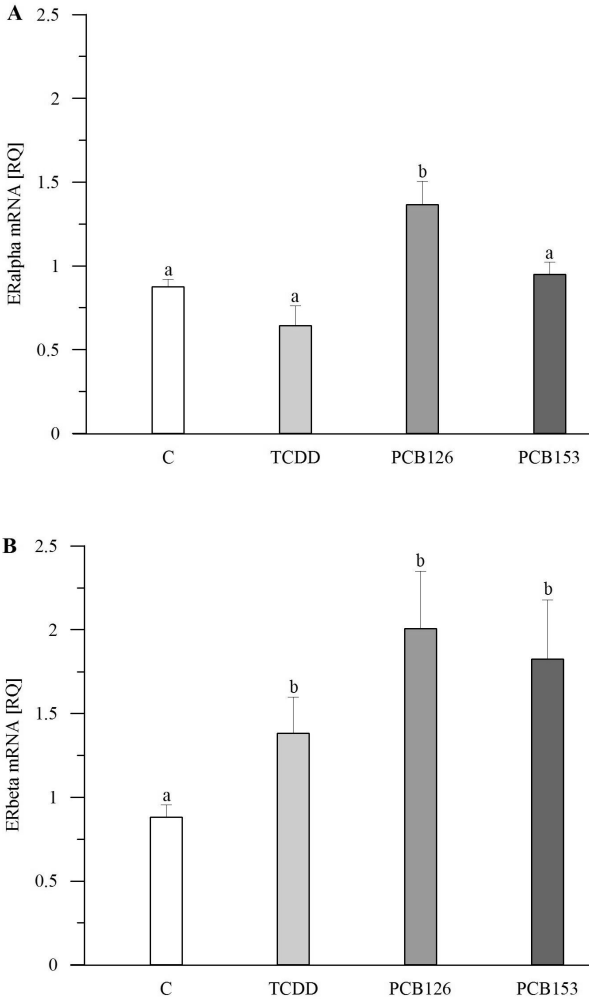

Fig. 1. Effect of TCDD (100 nM), PCB126 (100 nM) and PCB153 (100 μM) on ERα (A) and ERβ (B) gene expression in the chicken shell gland explants after 24h exposure. The data represent the mean of relative quantity (RQ) ± SEM from five birds standardized to the control treatment. Values with different superscripts (a, b) differ significantly at $P<0.05$.

in control to 1.54 ± 0.194 and 1.63 ± 0.107, respectively (Fig. 2A). Relative expression of caspase-3 was not changed by added dioxin and PCBs (Fig. 2B).

Expression of MMP-2 in the shell gland was significantly decreased by PCB126 from 1.07 ± 0.103 in the control group to 0.55 ± 0.126 in the experimental one (Fig. 3).

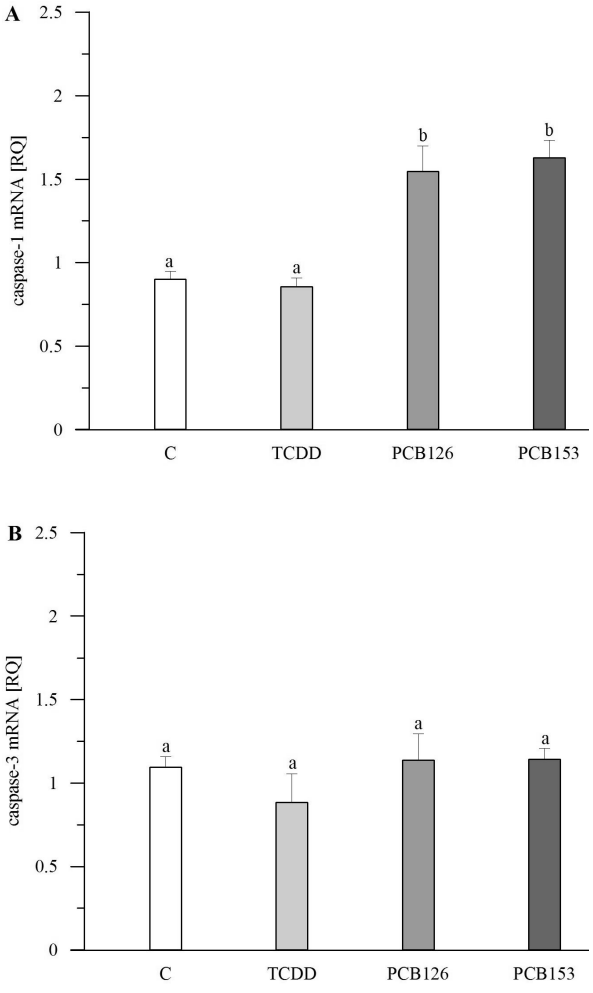

Fig. 2. Effect of TCDD (100 nM), PCB126 (100 nM) and PCB153 (100 μM) on caspase-1 (A) and caspase-3 (B) gene expression in the chicken shell gland explants after 24h exposure. For further explanations see Figure 1.

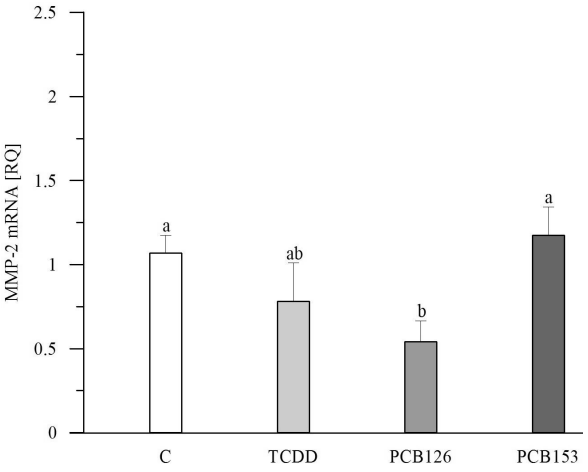

Fig. 3. Effect of TCDD (100 nM), PCB126 (100 nM) and PCB153 (100 μM) on metalloproteinase-2 (MMP-2) gene expression in the chicken shell gland explants after 24h exposure. For further explanations see Figure 1.

Discussion

To our knowledge this is the first study demonstrating the direct effects of TCDD, coplanar PCB126 and non-coplanar PCB153 on ERs, the chosen caspases and MMP-2 mRNA expression in the chicken shell gland. Changes in relative expression of the examined genes as a response to TCDD or PCB126 or PCB153 treatment support the suggestion that the chicken shell gland is a target organ of these chemicals as it was previously shown for DDE (LUNDHOLM 1993, 1997; see review GIESY *et al.* 2003).

The results of the present investigation clearly show that relative expression of ERα was affected only by PCB126 (it increased by 55%), whereas expression of ERβ was elevated by all the examined compounds, i.e. TCDD, PCB126 and PCB153, respectively by 57%, 128% and 107%. Partly, similar data which show an increase in ERα expression was obtained in mammary glands of postpubertal ovariectomised rats following *in utero* and lactational exposure to TCDD (LEWIS *et al.* 2001). On the other hand, no effect of *in ovo* TCDD exposure was observed on hepatic ER concentration in chicken and pigeon embryos. However, significantly elevated concentration of hepatic ER in TCDD-treated female pigeons was observed at hatch (JANZ & BELLWARD 1996). Downregulation of hepatic and uterine ER concentration was also reported in adult mammals (DEVITO *et al.* 1992). Stimulation of ERs expression especially ERβ by TCDD and chosen PCBs observed in the current study may be one mechanism by which these agents influence shell gland functions in chickens.

To better understand how dioxins and dioxin-like compounds affect avian oviduct functioning, the expression of the chosen proapoptotic genes was investigated in shell gland tissues exposed to TCDD, PCB126 and PCB153. Some molecular events involved in programmed cell death are largely mediated by caspases (see review JOHNSON 1996; JOHNSON & BRIDGHAM 2002). The caspase family includes initiator caspase (-1, -2, -8, -9, -10) and effector caspase (-3, -6, -7) and it was found that caspase-1, -2 and -3 play the essential role in the regression of chicken reproductive tissue (ANISH *et al.* 2008; SUNDARESAN *et al.* 2008). Thus, to ex-

amine whether TCDD and PCBs affects cell apoptosis in the chicken shell gland the initiator caspase-1 and executioner caspase-3 mRNA expression was determined. We found that both PCB126 and PCB153 increased the mRNA expression of caspase-1 by 72% and 81%, respectively, whereas all applied toxicants did not change the mRNA expression of caspase-3. Our findings suggest that PCB but not TCDD may initiate apoptosis in the shell gland of the chicken, however increase in caspase-1 mRNA may be insufficient to realize apoptosis, process which required activation of several genes. It is well established that in the initiation of apoptotic cell death by the death receptor pathway caspase-8 is involved whereas in mitochondrial death pathway caspase-9. Participation of proteins from Bcl-2 family is also pivotal in induction of apoptotic cell death. In the other hand, eventual PCBs-induced apoptosis in the shell gland may not be regulated by changes in caspase-3 mRNA expression but by increase in caspase-3 activity, similarly as it was previously suggested for induction of atresia of mouse ovarian follicles after exposure to methoxychlor (BASAVARAJAPPA *et al*. 2012). Moreover, it should be considered that caspase-1 appears to be involved in inflammatory responses (CASANO *et al.* 1994; DENES *et al.* 2012), thus it is possible that PCBs stimulate inflammatory processes in the shell gland, especially when executor caspase-3 is not influenced. The observation that caspase-1 is transcriptionally stimulated by PCBs while caspase-3 is not, may suggest that initiator caspase is differently regulated than executioner caspase in the chicken shell gland. These observations support the previous suggestion of MONROE *et al.* (2002) in respect to estrogen regulation of caspase expression in the chicken oviduct. On the other hand, it also may indicate different mechanisms of TCDD and PCB action.

Another interesting finding of the present study is decreased MMP-2 mRNA expression in the shell gland explants treated with PCB126. Taking into consideration that MMP-2 is a main regulator of ECM turnover during remodeling of several types of tissues including reproductive ones, changes in its expression could impair shell gland activity. The main function of the shell gland is adding water and electrolytes to the egg white, formation of the egg shell and expulsion of the egg. Therefore, disruption of shell gland activity may be related to changes in prostaglandin synthesis, calcium deposition and/or protein secretion, and finally may result in a decrease in egg quality. These may be speculated, since some studies demonstrated that PCB congeners significantly stimulate uterine contractility in mammals at least in part by increasing intracellular concentrations of Ca^{2+} in myometrial cells and PGF2α secretion from endometrial cells (LOCH-CARUSO 2002; KOTWICA *et al.* 2006; WRÓBEL *et al.* 2009; MŁYNARCZUK *et al.* 2010). To our knowledge there is no information regarding the effect of PCBs on the MMP system in the mammalian uterus or avian oviduct, whereas exposure of human endometrium to TCDD promotes secretion of MMPs even in the presence of progesterone, which normally suppresses the expression of these proteins at both the mRNA and protein levels (BRUNER-TRAN *et al.* 1999). Moreover, in addition to alteration in expression of remodeling enzymes i.e. MMPs, it is strongly suggested that TCDD affects production of proinflammatory growth factors or cytokines such as IL-1, IL-6, TNFα and INFγ. Thus, TCDD and dioxin-like compounds via chronic stimulation of the expression and activity of the proinflammatory cytokines, involved in the cyclic regulation of endometrial remodeling, proliferation and cell death may promote pathogenesis of endometrial tissue such as endometriosis (RIER & FOSTER 2002). In women with endometriosis, peritoneal leukocytes are activated and secrete increased levels of TNFα and IL-6 (HALME 1989). We observed a slight but not significant decrease in MMP-2 mRNA expression in the shell gland explants treated with TCDD. It should be noted that in the present study the effects of TCDD and PCBs were analyzed after short (24h) incubation and in many cases, especially in natural conditions, effects of examined chemicals are observed after long periods. That is why longer exposition may cause more pronounced changes in oviductal functions. In order to explain the long-lasting effect of examined endocrine disruptors on processes occurring in the chicken oviduct additional *in vitro* and *in vivo* experiments are necessary.

In conclusion, the result obtained clearly shows for the first time the direct effects of TCDD, PCB126 and PCB153 on ERs, caspases and MMP-2 gene expression in chicken shell gland tissue. The TCDD was the least potent whereas the coplanar PCB126 had the most pronounced effect. It is suggested that coplanar PCBs such as PCB126 may lead to disruption of shell gland activity by changes in gene expression of cellular and extracellular matrix regulators and subsequently impair egg components formed in this organ.

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