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The Preliminary Study About the Involvement of Relaxine in Adverse Effect of Polychlorinated Biphenyls on Bovine Myometrial Contractions

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Abstract

Polychlorinated biphenyls (PCBs) are a group of synthetic xenobiotics, which were used in lots of industrial applications. Two individual congers (PCB 153 and PCB 77) belong to the most prominent environmental contaminations among PCBs, but they have opposite type of configuration and therefore they can exert a different way of action. Since PCBs increased the force of myometrial contractions, in this work, their effect on myometrial sensitivity on inhibitor of myometrial contractions (relaxine, RLX) was studied.

Bovine myometrial cells and strips, and luteal cell at 4-8 month of pregnancy, were obtained accidentally in commercial abattoir. They were treated by PCB 153 and PCB 77 and mRNA expression of RLX receptor in myometrial cells and contractions of myometrial strips as well as the secretion of progesterone (P4) and oxytocin (OT) from luteal cells, were measured under their effect.

Both studied congeners decreased ($P<0.05$) the mRNA expression of RLX receptor in myometrium. Admittedly, both PCB 77 and 153 increased ($P<0.05$) the basal force of contractions, while RLX showed any effect on myometrial contractions. Studied PCBs increased also ($P<0.05$) secretion of P4 and OT from luteal cells.

It is suggested that PCBs can decreased the sensitivity of myometrium on RLX but their direct effect on RLX function have to be further investigated.

Keywords: Endocrine Disruption; Pregnancy; Uterus

Introduction

Polychlorinated biphenyls (PCBs) are a group of synthetic, chlorinated aryl hydrocarbons, which were extensively used in lots of industrial applications, mainly in transformers, capacitors or as hydraulic fluids, heat exchange liquids as well as plasticizers and others [1]. Because of different degree of chlorination, there are possible up to 209 congeners of PCBs. Among them, 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) is one of the most abundant PCB congeners in human milk. This congener is a non-ortho chlorine substituted with coplanar configuration and appear to exert its toxic effects via interacting with the cytosolic aryl hydrocarbon receptor (Ahr). Hence is recognized also as dioxin-like PCBs [2]. Since PCB 77 can affect the cell also through estrogen [3] and glucocorticoid receptors [4], it is recognized as a significant environmental pollutant and endocrine disruptor [3] with a wide spectrum of way of action. Admittedly 2,2′,4,4′, 5,5′- hexachlorobiphenyl PCB 153 is also one of the most prominent environmental contaminants, but oppositely to PCB 77 is classified as di-ortho, non-coplanar, non-dioxin congener of PCBs [5] and is known to have rather estrogenic properties [6]. Even though PCBs production and use were banned in USA and Europe more than three decades ago, they are still measured in environment [7]. Due to their lipophilic nature and resistance for biodegradation, PCBs have been bio-accumulated in animal tissues [8], also in domestic animals [9,10]. Therefore PCBs have been also measured in food samples [11] and they can be mentioned as a serious health risk.

Since PCBs accumulation in pregnant uterine muscle is higher than placenta, maternal adipose tissue or fetal blood [12], exposure to PCBs has been followed by different reproductive disorders. Their affected length of menstrual cycle [13] or pregnancy [14,15] as well as PCBs even increased the frequency of miscarriages in women [16] or monkeys [17]. Next, it was stated that the PCBs treatment has been associated with the increase of frequency or force of myometrial strips contractions in vitro in rats [18] or cows [19], respectively. Finally, it was also suggested that oxytocin (OT) and prostaglandin (PG) F2, which both are the most important natural stimulators of myometrial contractions, are involved in the adverse effect of PCBs on myometrial contraction [20-23].

The proper regulation of uterine contractility is crucial to achieve the success in reproduction. The postovulatory increase of uterine and oviductal muscle contractions facilities transport of gametes and thereby enable fertilization as well support the labor. However, almost all length of pregnancy needs uterine quiescence [24,25]. It is reached by progesterone (P4) block of oxytocin (OT) receptors in the myometrium, [26] and relaxin (RLX), which directly inhibit the uterine contractions, as well it is involved in cervical dilation and relaxation of pubic symphysis [27]. Moreover, RLX induced suppression of OT release from corpus luteum in cow [28]. Bovine corpora lutea from late pregnancy contain active RLX, while there is markedly lower than in swine [29]. Since it was found previously that PCBs increased amount of stimulators of contractions (OT and PGF2), here it was hypothesized that PCBs can increase the uterine

contractions also by impair the function of inhibitor of myometrial contractility.

The aim of this study was to investigate the effect of PCB 77 and PCB 153 on myometrial reception of RLX signal during pregnancy, and therefore on (a) mRNA expression of receptor of RLX and (b) RLX-inhibition of myometrial strips contractions. Moreover, luteal cells were used as a controls for condition of endocrine function of reproductive system of studied cows (ratio OT:P4). While the effect of PCBs on secretory function of luteal cells was studied additionally. The preliminary study about the involvement of RLX in adverse effect of PCBs on bovine myometrial contractions was also prepared to develop the constructed model to study the effect of chlorinated xenobiotics on the function of reproductive system.

Material and Methods

Collection and preparation of material

Bovine uteri and ovaries at 4-8 month of pregnancy were collected accidentally in a commercial slaughterhouse. The stage of pregnancy was identified according to [30]. All materials used in these studies were purchased from Sigma-Aldrich (PL) unless otherwise stated. Each medium was supplemented with gentamycin (20 µg/ml), amphotericin (2 µg/ml) and antioxidants: ascorbic acid (20 µg/ml; Merck, USA), sodium selenite (5 ng/ml; ICN, USA) and transferrin (5 µg/ml). The media did not contain phenol red.

Myometrial cells were obtained by enzymatic dispersion after the separation of the myometrium from the perimetrium and endometrium. The tissue (7 g from each uterus) was minced with scissors and then digested (2 h at 38°C) in oxygenated (95% O_2+5% CO_2) medium (20 ml of M199 supplemented with 0.1% BSA) with collagenase IA (1.5 mg/ml) and dispase (0.2 mg/ml Gibco, GB), according to Wrobel & Kotwica [23]. Luteal cells were obtained by perfusion with collagenase according. Cell viability was estimated by exclusion of 0.04% trypan blue dye. Only cells showing viability above 80% were used for further studies. The cells suspensions were transferred into 6 or 48-well plates (Nunclon Δ-Surface, NUNC, NL) for measure the mRNA in myometrium (5×10^5/ml of cells) and for measure the OT and P4 level in luteal cells (2.5×10^5/ml of cells), respectively. The cells were precultured (95% air and 5% CO_2, 100% humidity, 38°C; Memmert INCO 180, D) for 96 h (myometrium) or 24 h (luteal cells) to allow them to attach to the bottom of the wells. Next, they were washed twice with M199 and the medium was replaced with DMEM/HAM-12 supplemented with 0.1% BSA.

Three strips (6-7 mm long and 3-4 mm wide) of longitudinal smooth muscle were dissected from each myometrium. The strips were immediately incubated (24 h, 95% air and 5% CO_2, 4°C) with treatments immersed in 2 ml of aerated (95% air and 5% CO_2) physiologic salt solution (116 mM NaCl, 4.6 mM KCl, 1.16 mM $NaH_2PO_4 \cdot H_2O$, 1.16 mM $MgSO_4 \times 7\ H_2O$, 21.9 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 11.6 mM dextrose, 0.03 mM CaNaEDTA; pH=7.4), and the force of myometrial strips contractions was measured according to [22].

Treatments

PCB 153 and 77 were dissolved in DMSO (HPLC purity grade) and studied doses (0.1-10 ng/ml) were affect neither myometrial [22] nor luteal cells [31] viability in cows. Final concentration of the DMSO in the culture media did not exceed 0.1%. Hence, 0.1% of DMSO was added to the control samples. While to study the inhibition of contractions, there were used two type of RLX. It was porcine RLX (30, 60 and 100 ng/ml, according to Kaczmarek et al. [32], it is 200-500 ng/ml) since it was also used in study in beef heifers in vivo [29,33]. Since signaling by Relaxin-2 human through its target receptors enhances the growth of pubic ligaments and ripening of the cervix during birth [34] it was also used in this study (10-400 ng/ml).

The effect of PCBs on mRNA expression of RLX receptor in myometrial cells

Myometrial cells from cows (n=7) were incubated (24 h) with PCB 77 or PCB 153 (both at concentration 10 ng/ml) and with estradiol (E2; 10-7M). After incubation, the medium was removed, and the cells were covered with Phenozol (300 µl for each well; A&A Biotechnology, PL). The plates were stored at -70°C for subsequent real-time PCR analysis of the mRNA expression of RLX receptor. Total RNA was isolated using the Total RNA Kit (A&A Biotechnology, PL) according to the manufacturer's instruction. The concentration and purity of the isolated RNA samples were determined using a spectrophotometer (NanoDrop 1000; Thermo Scientific, USA). The absorbance ratio (A260:A280) for all samples was between 1.8 and 2. Total RNA (0.5 µg for each sample) was reverse transcribed (42°C for 1 h) using reverse transcriptase. The TATA box-binding protein (TBP) was used as the most stable housekeeping gene to normalise the gene expression in the bovine myometrium. The primer sequences (RLX receptor – predicted to Bos Taurus relaxin/insulin like family peptide receptor 1, variant X1, Accession no. XM 610789.8; Product size: 109; Forward: TCTGCAGTTACGTGCTTTGGA;

Reverse: CAGTCGGCACAGCAGAGAGA; TBP Accession no. NM_001075742; Product size: 194; Forward: CAGAGAGCTCCGGGATCGT;

Reverse: ACACCATCTTCCCAGAACTGAATAT), were synthesised (IBB PAN PL). Real-time PCR (25 µl volume) was performed using the APB Prism 7900 sequence detection system (Applied Biosystems, USA). The reaction mixture contained cDNA (5 µl; 200 ng/µl), SYBR Green PCR master mix (12.5 µl; mix-B, lot. 171011; A&A Biotechnology, PL), Hi-ROX (0.4 µl; lot. 41011; A&A Biotechnology, PL), both PCR primers (2.5 µl of each; 200 nM) for each studied gene and water (2.1 µl). The PCR reactions for each pair of primers were performed as follows: initial denaturation (95°C for 10 min) followed by 40 cycles of denaturation (95°C for 15 s) and annealing (60°C for 1 min for annealing and extension). Melting curves were set up using stepped increases from 60 to 95°C to ensure the specificity of the amplified product. The PCR products were electrophoresed on a 2% agarose gel to confirm their specificity.

The effect of PCBs on myometrial contractions

Myometrial strips (3 from each cow) were incubated (24 h, 4°C) with PCB 153 or PCB 77 (n=4 cows), each at the dose of 10 ng/ml, according to Wrobel et al. [22,23]. Next, the myometrial strips were individually placed into the chambers of a HSE Schuler Organbath apparatus (March-Hugstetten, D). Each chamber contained Krebs-Ringer's solution (KRS; pH=7.4; 10 ml) composed of NaCl (120.3 mM), KCl (5.9 mM), $CaCl_2$ (2.5 mM), $MgCl_2$ (1.2 mM), NaH_2PO_4 (1.2 mM), $NaHCO_3$ (15.5 mM) supplemented with glucose (11.5 mM). Each strip was attached to the base with a stationary hook and tied to the isometric contraction transducer (HSE Type 372) with surgical silk. The KRS was maintained at 38°C and oxygenated (95% O_2 and 5% CO_2). All preparations were allowed to equilibrate for 2 h. The force of the isometric contractions of the smooth muscle was measured every 2 s for 20 min before (basal contractions) and after OT (10-7M) and porcine (purified and gifted from Dr. O.J. Sherwood, IL, USA) or human RLX application. The track of contractions was recorded according to Wrobel et al. [22].

The effect of PCBs on hormone secretion from luteal cells

Luteal cells from six cows were incubated (72 h, 38°C) separately with PCB 77 or PCB 153 (each at dose of 0.1, 1 or 10 ng/ml; n=5). Each treatment was performed in duplicate. The time of incubation was chosen, according to previous studies with polychlorinated biphenyls [4,31] and other chlorinated pesticides [23,35,36]. After incubation, the medium was collected into tubes containing 10 µl of 0.3 M EDTA in 1% acetylsalicylic acid [37] and stored at -20°C for subsequent determination of P4 and OT in cell cultures. The concentrations of both hormones were determined by EIA. Horseradish peroxidase-labelled P4 and the biotinylated OT were used as tracers. Antisera for P4 and OT, were used at final dilutions of 1:100000 and 1:25000, respectively. The standard curves for P4 and OT, ranged from 0.1 to 25 ng/ml and 7.8 to 2000 pg/ml, respectively. Finally, concentration of all hormones assigned in culture media was expressed per milligram of cellular protein measured by Bradford method (1976). Both hormone concentrations and protein measurements were performed using ELISA reader (Epoch BioTek, USA).

Statistical analysis

The mean (± SEM) values for contraction force were expressed in mN and calculated using all measurements collected every two seconds for 20 minutes. These measurements were compared by one-way ANOVA followed by the Newman-Keuls test. However, the effect of OT or RLX challenges was compared by parried t-test and the results were expressed in % to better extension of changes or lack of effect in comparison before and after theirs substitution. All other mean (± SEM) values were compared by one-way ANOVA for repeated measures followed by the Newman-Keuls test. The Prism 5 software (GraphPad Software, Inc., USA) was used to prepare all statistical analyses and figures. The real-time PCR Miner algorithm was used to analyse the relative mRNA quantification data [38].

Results with Discussion

Both studied congeners and E2 decreased (P<0.05) mRNA expression of RLX receptor in myometrium (Figure 1). In my knowledge, there is any others reports about the effect of chlorinated xenobiotics on mRNA expression of this receptor. It is worth to mentioned that high circulating 17β-estradiol is one of the factors which triggers the down-regulation in receptor of RLX during gestation in rats [39]. Hence, it was shown that both studied congeners exerts their estrogenic properties. Moreover, it can be suggested that studied estrogen-like PCBs diminish the receptivity of myometrium on inhibitor of myometrial contractions (it means RLX). Therefore, it is possible that the PCBs support increase of myometrial contractions, also in this indirectly way.

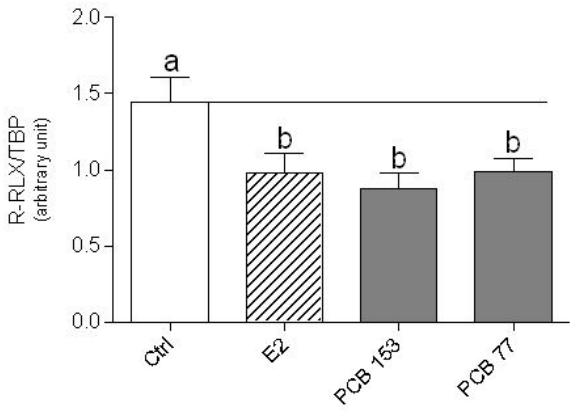

Figure. 1: The mean (±SEM) mRNA expression of receptor of relaxin (R-RLX) in myometrial cells after incubation (24 h) with PCB 153, PCB 77 (both at 10 ng/ml) and (E2, 10-7M). TBP was used as reference gen. a-b (P < 0.05).

Indeed, according to expectation, both, PCB 153 and PCB 77 increased (P<0.05) basal contractions of longitudinal myometrial strips, during late gestation (Figures 2A, 2D), similar to their effect during estrous cycle by Wrobel et al. and early (1-3 month) pregnancy in cows. Therefore, just PCB 153 and PCB 77 were used as a kind of control and start point to further studies about the involvement of RLX in the adverse effect of xenobiotics on myometrial contractions. The force of all control strips was increased (P<0.01) after OT challenge (Figures 2B, 2D), so they retain the ability to property motility and receptivity. Moreover, the similar stimulatory effect of OT was observed for strips pretreated with PCBs (Figure 2D). Unfortunately, neither porcine (Figures 2C, 2D) nor human RLX (data not showed) had effect (P>0,05) on contractions of control strips of longitudinal smooth muscle after 24 h of incubation separately or jointly with P4 (12 ng/ml, according to Mlynarczuk et al. [40]) (Figure 2C). Similarly, both RLXs had not effect on untreated circle smooth muscle (data not showed). While the accuracy of porcine RLX (100 ng/ml) was confirmed, since it decreased the contractions of myometrial strips from swine (Figure 2E). The lack of effect of porcine RLX on bovine myometrium has been surprised, since it had used to study effect on E2, OT and P4 secretion [34,41] and

to cause a cervical dilatation [34,**42]** in beef heifers in vivo, as well as cow RLX preparations gave reactions of identity with the porcine RLX [43]. Therefore, it is suggest that RLX from swine and human is not appropriate to use as a support of in vitro model to study the effect of xenobiotics on myometrial contractions. The next evaluation of this part of study has to be done with bovine RLX or even relaxin-like factor (RLF), since it may be functionally substituting for RLX in ruminants such as the sheep and cow [44].

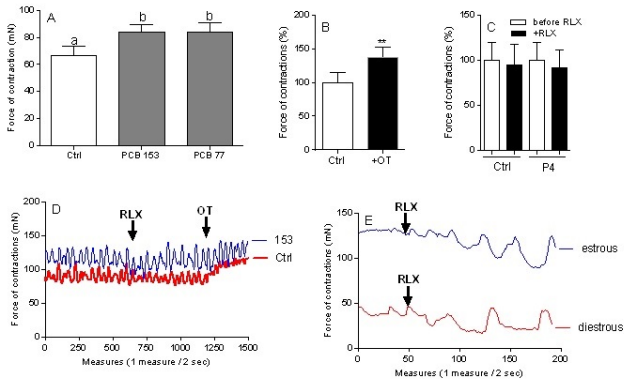

Figure. 2: (A) The mean (±SEM) basal force of myometrial strips contractions after incubation (24 h) with PCBs (10 ng/ml). a-d ($P < 0.05$). (B) The effect of OT (10-7M) challenge ** ($P < 0.01$). (C) The effect of porcine RLX (100 ng/ml) challenge on force of contraction after incubation (24 h) with P4. (D) Individual chart of contractions of myometrial strips (control and treated with PCB 153) from one cow before and after porcine relaxin (RLX; 100 ng/ml) and oxytocin (OT; 10-7M) challenge (arrow). (E) Individual chart of contractions of myometrial strips from two swines (at estrous and diestrous) before and after porcine relaxin (RLX; 100 ng/ml) challenge (arrow).

If the changes in direct effect of bovine RLX or RLF on myometrium pre-treated with PCBs will be confirmated, the check the effect of PCBs on amount of RLX or RLF secreted at from luteal cell cultures has to be done also. Here, the control luteal cells secreted a large amount P4 in comparison with OT, so it suggested that the P4 block was ability at studied cows. Moreover, both congeners increased ($P<0.05$) secretion of P4 and OT from these cell culture (Figure 3). Similarly, in previous study, both congeners increased OT secretion during estrous cycle [4] and PCB 77 also at early (1-3 months) pregnancy [41]. Moreover, PCB 77 increased P4 secretion during estrous cycle [41]. Therefore, this experiment was rather a supplement of previous showed effect of PCBs and control of reproductive system function of studied cows.

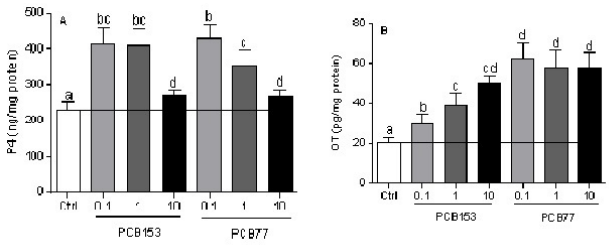

Figure. 3: The mean (±SEM) secretion of P4 (A) and OT (B) from luteal cells at late gestation, after incubation (72 h) with PCB 153 or PCB 77 (each at 0.1, 1 and 10 ng/ml). a-d ($P < 0.05$).

In summary PCBs can decreased the sensitivity of bovine myometrium on RLX and they changed the secretory function (P4 and OT) of luteal cells at late gestation. But the direct involvement of RLX in adverse effect of polychlorinated biphenyls on bovine myometrial contractions was not confirmated. Neither porcine nor human RLX use not developed the model to study the adverse effect of PCBs on myometrial contractions. However, the interaction between PCBs and RLX and the study about their direct effect on RLX function has to be further evaluated.

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