LOWER CHLORINATED NON-DIOXIN-LIKE PCBs ACT AS PARTIAL AGONIST ON THE $GABA_A$ RECEPTOR AND INHIBIT AROMATSE ACTIVITY.

Antunes Fernandes EC, Daamen FEJ, van Kleef RGDM, van den Berg M, van Duursen MBM, Westerink RHS.

Institute for Risk Assessment Sciences (IRAS), Cellular and Molecular Toxicology, Utrecht University, P.O.Box 80177, NL-3508 TD Utrecht, The Netherlands

Abstract

The use of polychlorinated biphenyls (PCBs) has been largely prohibited since the 1980's, though PCBs are still common in the environment. Non-dioxin-like (NDL) PCBs are abundant in food and (human) tissue samples and previous studies have shown that NDL-PCBs act as endocrine disruptors by affecting steroidogenesis and are potentially neurotoxic. However, their toxicological properties are still poorly characterized. In the present study, human placental microsomes were used to assess the effect of 20 NDL-PCBs on aromatase activity, a key enzyme in the steroidogenesis pathway. Of these 20 congeners, only PCB28 inhibited aromatase activity (IC_{50} 1.3 μM). In subsequent experiments, the effects of four common NDL congeners (PCB28, 52, 153 and 180) on human $GABA_A$ receptors expressed in *Xenopus* oocytes were investigated using the two-electrode voltage clamp technique. None of the tested congeners had antagonistic effects. However, PCB28 and PCB52 (at 1 and 10 μM), were able to potentiate the GABA response under conditions of low receptor occupancy, indicating that these NDL-PCBs act as partial GABA agonist. GABA is not only a major inhibitory neurotransmitter in the brain, but also an important modulator of the endocrine system. These findings further add importance to the neurotoxic and endocrine disruptive potential of NDL-PCBs.

Introduction

Polychlorinated biphenyls (PCBs) comprise a group of 209 different congeners. Due to their non-flammability, chemical stability and high dielectric constants, PCBs have been widely used in a number of industrial and commercial applications such as fluids in transformers, capacitors, rubber products and plasticizers in paints[1]. Although production and use of PCBs has been prohibited in most countries since the 1980's, they are still present in electronics, plastics and building materials and can be found in the environment and the food chain. Consequently, laboratory studies have been performed to assess the neurobehavioral effect of PCBs, demonstrating that non-dioxin-like (NDL) PCBs, dioxin-like PCBs and PCBs mixtures interfere with motor activity, learning, memory and attention, responsiveness to aversive stimuli, neuromuscular development, and sensory function[2-8]. Additionally, animal studies with PCB mixtures demonstrated that PCBs decrease serum levels of thyroxine (T4) and triiodothyronine (T3), enlarge the thyroid glands and increase cytochrome P450 activities.[9-11]

NDL PCBs have been considered less toxic because of their *ortho*-substituted chlorines, which prevent interaction with the aryl hydrocarbon (Ah) receptor[12]. Nevertheless, several *in vivo* and *in vitro* studies have shown that NDL-PCBs have neurotoxic effects by interfering with Ca^{2+} homeostasis, or change brain neurotransmitter levels[13-16]. This is of particular interest because several of the studied parameters are thought to be related to modulation of motor activity, learning and memory, neural damage and abnormal brain development.

NDL-PCBs have also been reported to affect the endocrine system, including both estrogenic and anti-estrogenic effects. Studies have reported an increase in uterine weight and changes in estrogen and progesterone receptors[17]. Furthermore, other studies have shown that NDL-PCBs can interfere with the binding of testosterone to the androgen receptor[18].

Comparison of the toxicological properties of NDL-PCBs in feed and food has been hampered by the lack of consistency of the studied congeners, as their chemical purity was not always tested and the mixtures used were not relevant for human exposure situations. Therefore, in 2005 EFSA has released a risk assessment publication regarding the presence of NDL-PCBs in feed and food, based on 6 congeners. The sum of these six indicator congeners represents about 50% of all NDL-PCBs in food. Although some *in vivo* and *in vitro* studies have been performed with the referred congeners, a clear toxicological overview and risk assessment of the effects of NDL-PCBs is hampered by the limited amount of available data.

As aromatase (CYP19) is one of the key enzymes in the steroidogenesis pathway, mediating the conversion of androgens into estrogens, in the present study the effects of NDL-PCBs on aromatase activity were investigated. Furthermore, as γ-aminobutyric acid (GABA) is known to play a role in the modulation of the endocrine system and is the main inhibitory neurotransmitter in the adult mammalian central nervous system, *in vitro* experiments were carried out to investigate the effects of NDL-PCBs on $GABA_A$ receptor, the main GABA receptor.

Materials and Methods

Chemicals

NDL-PCBs were synthesized and purified by Dr. Patrik Andersson (Chemistry Department, Ulmeä University, Sweden). The used PCBs were: PCB19, PCB28, PCB47, PCB51, PCB52, PCB53, PCB74, PCB95, PCB100, PCB101, PCB104, PCB118, PCB122, PCB128, PCB136, PCB138, PCB153, PCB170, PCB180, PCB190.

Human placenta microsome fraction

A human placenta was provided by Dr. Paul de Jong (Antonius Hospital, Nieuwegein, The Netherlands) and stored at -70˚C until analysis. In order to isolate the microsomal fraction from the human tissue, the samples were weighed and homogenized in 10 volumes of TRIS-HCl buffer using a plotter device, centrifuged for 25 minutes at 15,000 rpm at 4˚C and the supernatant was centrifuged again for 1.15 hr at 47,000 rpm at 4˚C. Then, the supernatant was decanted and the pellet was resuspended in sucrose solution (0.25M). The microsome suspension was frozen in aliquots at -70˚C and stored until use. Protein content was measured according to methods described earlier[19]. Protein levels were calculated from a standard curve using bovine serum albumin (Sigma A7030).

Aromatase assay

The catalytic activity of aromatase was determined in human placental microsomes based on the tritiated water-release method of Lephart and Simpson (1991)[20] with modifications as described by Sanderson et al. (2001)[21]. The aromatization of the substrate [1β-^{3}H]-androstenedione produces ^3H_2O (tritiated water), which is measured by this method. The specificity of the aromatase assay was verified using 4-hydroxyandrostenedione, an irreversible inhibitor of the catalytic activity of aromatase[21]. The 20 different congeners were tested at a high (10 μM) and low (1 μM) concentration. The PCBs which showed an inhibition of the aromatase activity were also tested in a concentration range of 10 nM to 25 μM.

All experiments were done in triplicate, and within an individual experiment each concentration was tested in quadruplicate. All results are presented as mean ± SD (*n*=3). IC_{50} calculations were done using Prism 3.0 (GraphPad Software Inc. San Diego, CA, USA).

Expression of $GABA_A$ receptor in Xenopus Laevis oocytes

Xenopus laevis were anaesthetized by submersion in 0.1% MS-222 and ovarian lobes were surgically removed. Oocytes were defolliculated manually after treatment with 1.5 mg/ml collagenase type I in Ca^{2+}-free Barth's solution for 1.30 hr at room temperature. Plasmids coding for the human α1, β2 and $\gamma 2_L$ subunits of neuronal $GABA_A$ receptors, dissolved in distilled water at a 1:1:1 molar ratio, were injected into the nuclei of stage V or VI oocytes (~1 ng of each plasmid; total injection volume of ~18 nl/oocyte). After injection oocytes were incubated at 21˚C in modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 50 μg/ml neomycin (pH 7.6 with NaOH). Experiments were performed on oocytes after 3-6 days of incubation[22].

All experiments were approved by the Utrecht University Ethical Committee for Animal Experiments, and in accordance with Dutch law.

Electrophysiology recording

Oocytes were voltage clamped using two microelectrodes (0.5-2.5 MΩ) filled with 3 M KCl and a custom-built voltage clamp amplifier with high-voltage output stage according to the methods described by Stühmer (1992)[23]. The external saline was clamped at ground potential by means of a virtual ground circuit employing an Ag/AgCl reference electrode and a Pt/Pt-black current-passing electrode. Membrane current was measured with a current-to-voltage converter incorporated in the virtual ground circuit. The membrane potential was held at -60 mV. All

experiments were performed at room temperature (22-24˚C). Oocytes, placed in a teflon tube with an inner diameter of 4 mm, were continuously perfused (superfusion rate ~30 ml/min) with saline solution containing (in mM) 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH). GABA and PCB-containing solutions were prepared freshly before each experiment. PCBs were applied by switching the superfusate between control and PCB-containing saline using a servomotor-operated valve.

To establish a GABA dose-response curve and, therefore, to determine EC_{20} and EC_{80} values, i.e., the concentrations producing 20% and 80% of the maximal response, oocytes expressing $GABA_A$ receptors were exposed to a GABA concentration range of 1 μM to 3 mM (Fig 2A). To determine antagonistic or agonistic effects, NDL-PCBs were applied for 20s, either alone (1 μM or 10 μM), or co-applied with GABA-containing saline with EC_{20} and EC_{80} concentrations of GABA (see Fig 2B for example recording). A washout period of 2-5 min was allowed between each PCB or GABA application to allow the receptors to recover from desensitization.

Amplitudes of ion currents were measured and normalized to the amplitude of GABA-induced control responses (1 mM) to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitude over time. The percentage of PCB-induced potentiation of the GABA-induced ion current was calculated from the quotient of the maximum amplitude of the co-application response (during 20 sec) of the PCB and that of the control response at the same time point. Concentration-effect curves were fitted to the data obtained in separate experiments using GraphPad prism (version3.0) and data represent mean ± SD. For each experiment 3-5 oocytes were used.

Results and Discussion

NDL-PCBs effects on aromatase activity:

The possible inhibitory effect of 20 different NDL-PCBs on aromatase activity was studied using human placental microsomes. Of the 20 tested congeners, only PCB28 showed a significant inhibition of aromatase activity at 1 and 10 μM (Fig 1A). This PCB was further tested at concentrations ranging from 10 nM to 25 μM, to obtain a dose-response curve. At the highest concentration tested (25 μM), PCB28 inhibited aromatase activity by approximately 70%, with an estimated IC50 value of 1.3 μM (Fig 1B). This suggests that PCB28 interferes with aromatase activity via catalytic inhibition, indicating a potential interaction of this PCB in steroidogenesis. Further studies will be performed to determine the possible potentiating effects of NDL-PCBs on aromatase activity and expression levels in the H295R human adrenocarcinoma cell line

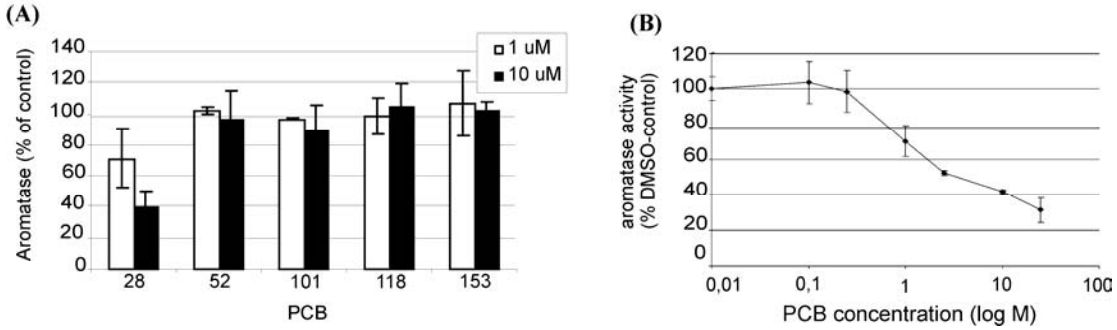

Fig 1. Effects of different NDL-PCNs on aromatase activity (A) and dose-response curve of PCB28-induced inhibition of aromatase activity in human placental microsomes (B). Values represent mean ± S.D.

NDL-PCB effects on $GABA_A$ receptor activation

Oocytes expressing human α1, β2 and $\gamma 2_L$ $GABA_A$ receptors were voltage clamped at -60 mV and superfused with external solution containing the agonist GABA. The amplitudes of the resulting ion currents were dependent on the agonist concentration. Peak amplitudes of the GABA-induced currents were measured, normalized to the response obtained with 1 mM GABA, and plotted against GABA concentration in each experiment for fitting a concentration-effect curve according to the Hill equation (Fig 2A).

In subsequent experiments, possible antagonistic and agonistic effects of four common NDL-PCBs (PCB28, PCB52, PCB153 and PCB180) on $GABA_A$ receptor activation were studied. NDL-PCBs, diluted in saline, were applied for 20 sec to assess possible agonistic effects on the $GABA_A$ receptors. At the used concentrations of 1 μM and 10 μM none of the NDL-PCBs appeared able to activate $GABA_A$ receptors. Further, co-application of these PCBs (1 μM and 10 μM) with GABA at the EC_{80} concentration demonstrated that none of these PCBs had antagonistic properties at these concentrations. Finally, effects of co-application of these PCBs (1 μM and 10 μM) with GABA at the EC_{20} concentration were studied to reveal possible potentiating effects of the tested PCBs.

PCB28 (1 μM) potentiated the GABA-induced inward ion current by 89 ± 13 % when co-applied with GABA at EC_{20} concentration. The amount of potentiation increased to 159 ± 61 % at the high PCB concentration (10 μM) (Figure 2B and 2C). Comparable results were obtained for PCB52, although PCB52 was less potent in potentiating the GABA-induced ion current at EC_{20} concentration. The amount of potentiation at 1 μM PCB52 amounted only 13 ± 5.4 %, whereas 10 μM PCB52 potentiated the response with 28± 4.4 % (Fig 2B and 2C). PCB153 and PCB180 were unable to potentiate the GABA-induced ion current at EC_{20} concentration (Fig 2B).

Though these findings clearly indicate that PCB28 and PCB52 act as partial agonists of the $GABA_A$ receptor, further experiments are required to determine the full dose-response curve of the described potentiating effects of these NDL-PCBs. Nonetheless, these findings can be of considerable importance as studies have shown that lower chlorinated NDL-PCBs (such as PCB28 and PCB52) were detected more often and in higher concentrations in plasma or blood samples, after human exposure to several PCBs via indoor air[23]. Moreover, GABA is not only the main inhibitory neurotransmitter in the central nervous system but also plays a major role in the modulation of the endocrine system[26, 27]. Consequently, the observed inhibition of aromatase activity as well as the potentiation of $GABA_A$ receptor activation adds to the neurotoxic and endocrine disruptive potential of lower chlorinated NDL-PCBs.

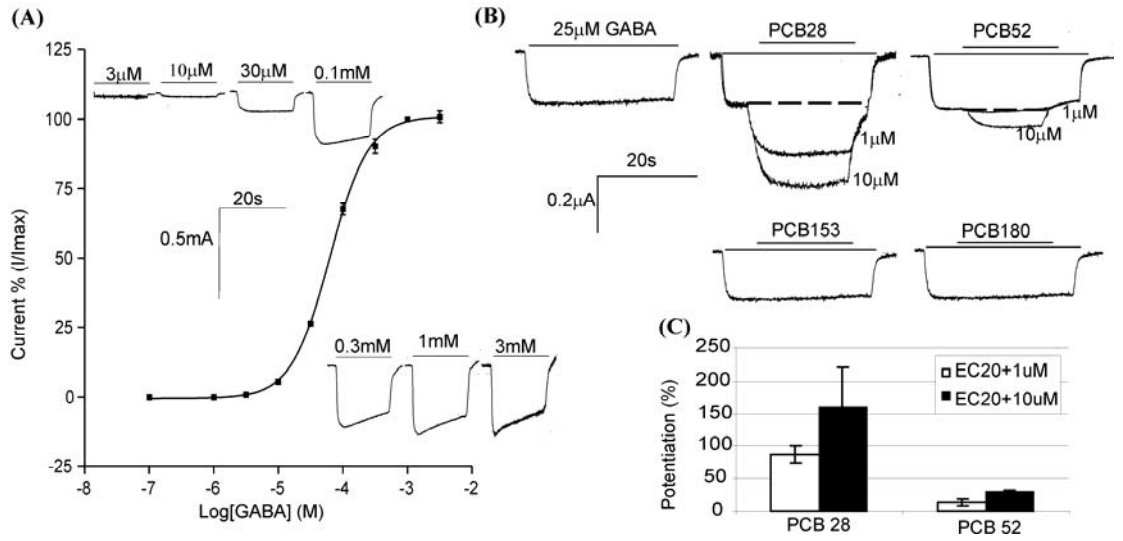

Fig 2. Agonist dose-response curve of human $\alpha1\beta2\gamma2_L$ $GABA_A$ receptor expressed in Xenopus oocytes with example inward Cl^- currents evoked at different GABA concentrations (A). Concentration-dependent potentiating effects of PCB28 and PCB52, in comparison to PCB153 and PCB180, which showed no effect. The traces illustrate the co-application of GABA and PCB (B). Potentiating effects of PCB28 in comparison to PCB52 (C). Data are mean ± S.D. (*n*=3 -5 oocytes)

Acknowledgements

This work was financially supported by Euopean Union project (FOOD-CT-2006-022923 ATHON). The authors would like to thank Dr. Patrik Andersson for providing the NDL-PCBs, Dr. Paul de Jong for providing the human placenta and Ing. Aart de Groot for excellent technical assistance.

References

1. Hutzinger O, Safe S, Zitko V. *The chemistry of PCBs*. 1974.
2. Hany J, Lilienthal H, Roth-Härer A, Ostendorp G, Heinzow B, Winneke G. *Neurotoxicology and Teratology* 1999; 21;147
3. Lilienthal H, Winneke G.*Fundamental and Applied Toxicology* 1991; 17; 368
4. Daly H B, Hertzler D R, Sargent D M. *Behavioral Neuroscience* 1989;103, 1356
5. Schantz S L, Seo BW, Wong PW, Pessah IN. *Neurotoxicology* 1997; 18; 457
6. Bernhoft A, Skaare J U, Wiig Ø, Derocher A E, Larsen H J S. *Journal of Toxicology and Environmental Health* 2000; 59; 561
7. Crofton K M, Rice D C. *Neurotoxicology and Teratology* 1999; 21, 299
8. Goldey E S, Tilson H A, Crofton K M. *Neurotoxicology and Teratology* 1995; 17;313
9. Byrne J J, Carbone J P, Hanson E A. *Endocrinology* 1987; 121; 520
10. Zoeller R T, Dowling A L, Vas A A. *Endocrinology* 2000; 141; 181
11. Tryphonas L, Arnold D L, Zawidzka Z, Mes J, Charbonneau S, Wong J. *Toxicologic Pathology* 1986b; 14;1
12. Bushnell P J, Rice D C. *Neurotoxicology and Teratology* 1999; 21, 381
13. Tilson H A, Kodavanti P R, Mundy W R, Bushnell PJ. *Toxicology Letters* 1998; 102; 631
14. Kodavanti P R, Tilson H A. , *Neurotoxicology* 1997; 18; 425
15. Yang J-H, Derr-Yellin E C, Kodavanti P R S 2003. *Molecular Brain Research* 2003; 111;123
16. Seegal R F , Bush B, Brosch KO. *Toxicology* 1991; 66;145
17. Li M H, Hansen L G. *Bulletin of Environmental Contamination and Toxicology* 1995; 54; 494
18. Schrader T J, Cooke G M. *Reproductive Toxicology* 2003; 17; 15
19. Lowry C C, Kraeft N H, Hughes F A. *The American Journal of Surgery*, 1951; 81; 676
20. Lephart E D, Simpson E R. *Methods in Enzymology*, 1991; 206; 477
21. Sanderson J T, Letcher R J, Heneweer M, Giesy J P, van den Berg M. *Environmental Health Perspectives* 2001; 109;1027
22. Zwart R, Vijverberg H P, Molecular Pharmacology 1997; 52; 886-95
23. Stühmer W. *Methods in Enzymology* 1992; 207; 309
24. Peper M, Klett M, Morgenstern R. *Environmental Health: A Global Access Science Source* 2005; 22
25. Drexler H, Kersher G, Liebl B, Angerer J. *gesundheitswesen* 2004; 66; 1
26. GladkevichA, Korf J, Hakobyan VP, Melkonyan KV. *Autonomic Neuroscience* 2006; 124; 1
27. Wiens S C, Trudeau V L. *Comparative Biochemistry and Physiology* 2006;144; 332