AhR- AND ER-MEDIATED ACTIVITIES IN HUMAN BLOOD SAMPLES COLLECTED FROM PCB-CONTAMINATED AND BACKGROUND REGION IN SLOVAKIA

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Introduction

Endocrine disruption mediated through activation of aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) by polychlorinated biphenyls (PCBs) and other persistent organic pollutants (POPs) has been studied extensively both in vivo and in vitro[1]. Non-ortho- and mono-ortho-substituted polychlorinated biphenyls (PCBs) are potent AhR agonists[2]; therefore, increased dioxin-like activity of complex blood samples might reflect an increased exposure to PCBs. The induction of expression of CYP1A1 and CYP1B1 in different tissues, including lymphocytes, also depends on activation of AhR and it could be useful as a potential biomarker of exposure to dioxin-like compounds[3].

Using various in vivo and in vitro models, the exposure to PCBs or hydroxy-PCBs has been reported to lead to either induction of ER-mediated activity or to an antiestrogenic effect associated with a suppression of estradiol-induced ER-dependent gene expression[1, 4-7].. Nevertheless, relative (anti)estrogenic potencies of a large set of prevalent environmental PCBs have not been yet compared in a single bioassay. A cross-talk between AhR and ER has been suggested to lead to a suppression of ER-mediated gene expression[8]. Therefore, presence of dioxin-like compounds in blood could potentially suppress the ER-mediated activity. Additionally, AhR-dependent induction of CYP1A1 and especially CYP1B1, two enzymes involved in oxidative metabolism of estradiol and other estrogens, might enhance the metabolism of

estradiol and it has been suggested to cause a potential depression of estrogen levels in the body[9].

The aim of the present study was to determine dioxin-like, estrogenic and antiestrogenic activities in human blood samples collected in two Eastern Slovakia regions differently polluted with PCBs using established in vitro bioassays. We also studied mRNA expression of CYP1A1 and 1B1 in lymphocytes and the genotypes of CYP1B1 as possible biomarkers of exposure for PCBs and related compounds. The biological data obtained were compared with analytical data on concentrations of major classes of POPs.

Materials and Methods

Blood sampling and chemical analysis of POPs: In this study, 300 individual male and female blood samples were collected in two areas of Eastern Slovakia, which are differently contaminated with PCBs, namely in the Michalovce district, where the commercial PCB mixtures were produced for a number of years, and in the Stropkov district, which was selected as a background area with low PCB contamination. The whole blood was collected from fasting subjects in S-Monovette vacutainer tubes without anticoagulants, immediately centrifuged at 3000 rpm for 15 min and stored in glass tubes at -70°C.

Determination of effects associated with AhR activation: The CYP1A1 and CYP1B1 mRNA levels in human peripheral lymphocytes were determined by RNA extraction and a quantitative RT-PCR method using TaqMan technology[10]. The CYP1B1 genotype (Val432Leu polymorphism) was determined by a PCR-RFLP method adapted after Tang etal.[11]. First, an RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI, USA). Reactions were performed with 750 ng RNA in a 25 µl reaction volume containing 200 µM dNTPs, 1.5 mM $MgSO_4$, 25 pmole of a forward primer (5'-CTGCCAACACCTCTGTCTTG-3') and 25 pmole of a reverse primer (5'-CTGAAATCGCACTGGTGAGC-3'). Subsequently, 10 µl PCR product was digested with 10 units *Eco*57I (Fermentas Inc., Hanover, MD, USA) according to the manufactor's instructions. 4.5 µg lambda DNA (Fermentas Inc., Hanover, MD, USA) was used as positive control for endonuclease restriction. The unrestricted PCR product and the restricted PCR product were loaded on a 2.5% non-denaturing agarose gel containing ethidium bromide (0.05 ug/ml). The bands were visualized on a UV light and polymorphisms were determined by visual inspection of the restriction fragments. Endonuclease digestion of the PCR product (271 bp) yielded two DNA fragments (166 and 105 bp) when the mutated allele (G→C transition) was present.
The in vitro potencies of POPs present in blood serum to activate AhR were measured in sulfuric acid/silica treated extracts by a luciferase reporter gene assay (DR-CALUX, BioDetection Systems, Amstersdam, The Netherlands) as described previously[12].

ER-mediated activity and determination of 17beta-estradiol (E2) in male blood samples: In 150 male serum samples, ER-CALUX bioassay was performed using human breast carcinoma T47D.Luc cell line[13]. The ER-mediated activity was determined in the cells treated with either total serum hexane/diethyl ether extracts or with a fraction of POPs obtained by a consequent sulfuric

acid/silica fractionation of total samples. Antiestrogenicity of POPs fractions was determined as a decrease in response to E2 in the cells co-treated with the POPs fraction. Concentrations of E2 were determined by a commercial ELISA kit (ADVIA Centaur Estradiol-6 III assay, Bayer HealthCare, Torrytown, NY) in 60 samples selected according to stratified PCB levels. Estrogenic and antiestrogenic activities of 18 prevalent PCB congeners were determined by the ER-CALUX assay. Cytotoxicity of extracts or individual PCBs was determined by a neutral red uptake assay after 24 h exposure.

Chemical analysis of POPs: Chemically derived TEQs were calculated from HPGC/MS data on blood concentrations of PCDD/Fs and non-ortho- and mono-ortho-chlorinated PCB. Concentrations of other prevalent (noncoplanar) PCB congeners and p,p‘-DDE were determined by GC/MS method[14]. Sum of PCBs used in the correlation and multivariate statistical analysis was based on the data on concentrations of both indicator PCBs and mono-ortho-chlorinated PCB congeners.

Data analyses: All calculations were performed with Microsoft Excel, SlideWrite 3.0 for Windows or Statistica 6.1 for Windows. Nonparametric statistical analyses (Kruskal-Wallis analysis of variance and the Mann-Whitney U test) were used for data analysis. The relationships among biological and chemical data were determined by the correlation analysis and multivariate principal component analysis (PCA). The correlations among the compared parameters were assessed using nonparametric Spearman’s rank coefficient (Rs). For the correlation and PCA analyses, all the data were normalized using the transformation log (X+1).

Results and Discussion

Dioxin-like activities and levels of CYP1A1/CYP1B1 mRNA expression were determined in 300 serum and lymphocyte samples of male and female blood. No significant correlations were found between total PCB concentrations and CYP1A1 or CYP1B1 mRNA expression. No relationship was also observed when CYP1B1 Val432Leu polymorphism was taken into account (data not shown). In addition, there were no significant differences between mRNA levels and total PCB levels among the Val/Val, Val/Leu and Leu/Leu genotypes. No association was found either when at least one CYP1B1 Leu432 allele was present. Average TEQ values were not different in the samples from two regions under study; however, a stratified analysis showed that TEQ values were associated with CYP1A1 expression in men living in the contaminated area. A weak correlation was found between PCB levels and TEQ values obtained in the DR-CALUX bioassay ($Rs = 0.387$, $p < 0.001$). A significant increase of TEQ values was found in the upper quartile of the serum samples stratified according to PCB content (Figure 1A).
The total male serum extracts (containing both non-persistent compounds including hormones and xenoestrogens and POPs) elicited ER-mediated activity corresponding to 15 - 45 pg E2 equivalents per ml. A significant decrease of overall estrogenic responses was observed in the upper quartile of the serum samples stratified according to PCB content (Figure 1B); the ER-mediated activity was associated with serum concentrations of E2 ($Rs = 0.510$, $p < 0.001$). Weak negative correlations were also found between ER-CALUX and DR-CALUX data ($Rs = -0.227$, $p < 0.1$) and between ER-CALUX data and levels of CYP1A1 mRNA ($Rs = -0.241$, $p < 0.05$). The PCA revealed association between the ER-mediated activities and E2 concentrations and negative associations between ER-inducing potencies and expression of CYP1A1 or CYP1B1 mRNA (Figure 2A).

Both estrogenic and antiestrogenic activities of the fractions of POPs were identified in a part of samples. The POPs fractions from the background area elicited ER-mediated activity at significantly higher incidence than the samples from the PCB-polluted area; in contrast, the antiestrogenic activity was found more frequently in the samples from contaminated region. Associations between antiestrogenic potencies of POPs fractions, expression of CYP1A1 and CYP1B1 mRNA, dioxin-like activities and sum of PCBs were found by multivariate PCA (Figure 2B).

Relative in vitro estrogenic and antiestrogenic potencies of individual 18 prevalent PCB congeners in the ER-CALUX assay were determined. Lower-molecular-weight PCBs 28, 52, 66, 74, 99, and 105 induced a significant ER-mediated activity at micromolar concentrations; IEF50 values relative to E2 ranged from 10^{-7} to 10^{-8}. The most prevalent PCBs 138, 153, 170, 180 as well as PCB congeners Nos. 101, 156, 187, 194, 199, and 203 significantly inhibited E2 response. The inhibitory potencies, relative to model antiestrogenic compound ICI 182,780, were from 10^{-5} to 10^{-6}. Interestingly, mono-ortho-chlorinated PCB 118 and non-ortho-chlorinated PCB 126, both known as strong AhR agonists, elicited no estrogenic or antiestrogenic activity; the antiestrogenic potency of dioxin-like PCBs, suggested by other authors[4, 8], was not found in the T47D.Luc cells.

In summary, only weak associations between concentrations of PCBs and potential biomarkers of dioxin-like toxicity and estrogenicity were found in this epidemiological study. Prevalent PCBs might contribute to a decrease of overall estrogenic activity in human male blood and it seems reasonable to conclude that these compounds were responsible for the weak antiestrogenic activity observed in the POPs fractions of blood. Nevertheless, a major part of total estrogenic activity was elicited by E2. Therefore, modulations of levels of E2 (e.g. by perturbations in its biosynthesis or catabolism) could be a more significant endocrine-disrupting effects of POPs than the direct modulations of ER-mediated activity, as suggested by negative association between CYP1A1/1B1 expression and antiestrogenic activity.

Figure 1. AhR- and ER-mediated activities of male human blood samples. Median values of total dioxin-like (A) or estrogenic (B) activities of samples arrayed into quartiles according to PCB concentrations, p = 0.002 and p = 0.02, respectively.

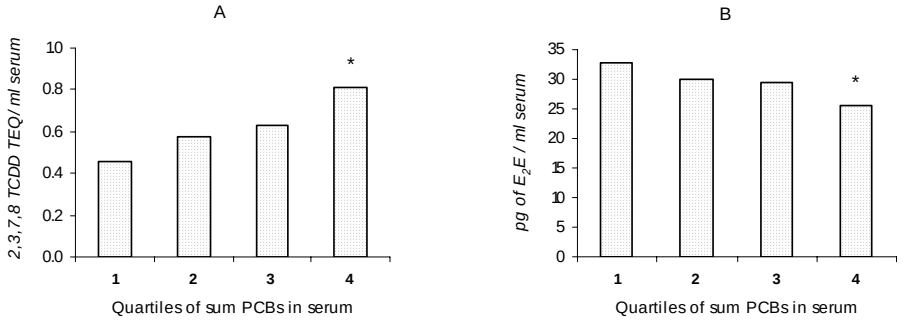
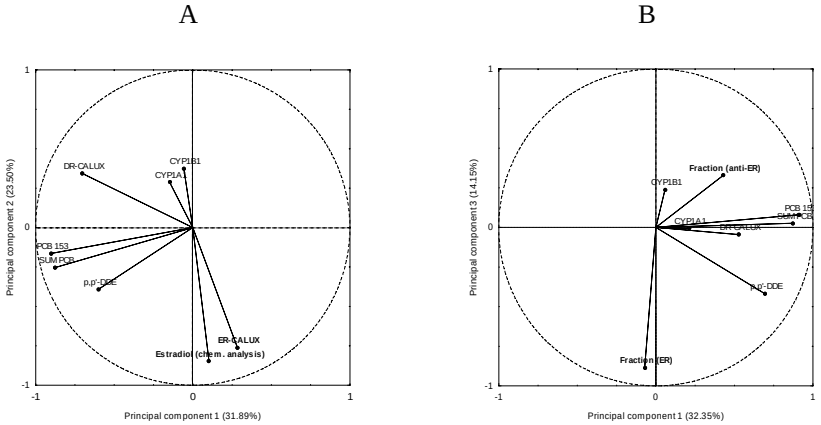


Figure 2. Multivariate PCA of the chemical and in vitro toxicological data including overall ER-inducing activity (A) and (anti)estrogenicity of POPs fraction (B).



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