INDUCTION OF CYP1A1 AND AhRR GENE EXPRESSION IN FEMALE MOUSE AND RAT LYMPHOCYTES BY TCDD, 4-PeCDF AND PCB126: UPTAKE VERSUS SYSTEMIC DOSE LEVELS

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Introduction

Chlorinated dioxins (PCDDs), furans (PCDFs) and biphenyls (PCBs) are persistent, widespread environmental contaminants, which can still be detected in humans at levels that might cause long-term health effects. Risk assessment of these dioxin-like compounds is based on the toxic equivalent factor (TEF) approach, endorsed by the World Health Organization (WHO). Each present TEF is derived from multiple relative effect potencies (REPs) obtained from mainly *in vivo* studies using oral uptake as route of administration. The actual value of the TEF is important to estimate the health risk for humans and animals for dioxins and dioxin-like compounds. This is done, by using an additive mixture toxicity model, the Toxic Equivalency (TEQ) concept. TEQ values are a sum of the products of a congener-specific toxic equivalency factor (TEF) and its concentration in a matrix, such as food or human plasma. However, a fundamental short coming in the present TEQ concept originates from the use of almost exclusive 'uptake' TEFs based on the oral dose levels applied *in vivo* experiments. Using these 'uptake' TEFs as a biomarker of effect may lead to misinterpretation of risk, because its validity has not sufficiently been proven for systemic (blood and tissue) levels[1-3].

Known sensitive target genes for dioxin exposure, are the cytochrome P450 1A1 (CYP1A1) enzyme and the aryl hydrocarbon receptor repressor (AhRR) protein. Both genes are regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway, which is ubiquitously expressed in mammalian tissues including peripheral blood lymphocytes (PBL). Therefore, expression of these genes in PBLs has frequently been proposed as biomarker for effect to dioxin and dioxin-like compounds. Yet, many studies, including those from our own institute, have questioned the applicability of this biomarker. A large human population study failed to show a correlation between total TEQ and CYP1A1 mRNA levels in blood lymphocytes[4]. Furthermore, an *in vitro* study with human PBLs showed a much lower REP for PCB126 (0.006) in inducing CYP1A1 expression[5]. Nevertheless, the use of PBL as biomarker of effect to dioxin-like compounds is interesting, because human PBLs can be obtained with a minimal evasive procedure and maybe a good representation of internal (systemic) exposure. Yet, a more accurate estimation of REP values in PBL is needed for a more accurate human risk assessment. For that, more knowledge about possible differences or similarities between 'uptake' and 'systemic' REPs among various dioxin-like compounds is essential.

In this study, gene expression of CYP1A1 and AhRR were investigated in freshly isolated PBLs from female C57Bl/6 mice and Sprague-Dawley rats exposed to various concentrations of TCDD, 4-PeCDF and PCB-126. REPs were calculated using administered dose (uptake) as well as plasma levels (systemic).

Materials and methods

Chemicals

2,3,7,8-tetrachlorodibenzodioxin (TCDD), 2,3,4,7,8,-pentachlorodibenzofuran (4-PeCDF), and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). The chemicals were dissolved and diluted in corn oil (Sigma-Aldrich, Stockholm, Sweden).

Animals

Female C57BL/6 mice and Sprague-Dawley rats were purchased at 9 weeks of age from Harlan laboratories (Venray, The Netherlands) and allowed to acclimate for 1,5 week.

The animals were housed in standard cages and conditions (temperature 23 ± 2°C, 50% to 60% relative humidity, 12-h dark and light cycle) with free access to food and water. The animals were randomly assigned to treatment groups (6 animals/group). Mice and rats received a single dose of TCDD (0, 2.5, 10, 25 or 100µg/kg body weight), 4-PeCDF (0, 25, 100, 250 or 1000µg/kg body weight) or PCB-126 (0, 25, 100, 250 or 1000µg/kg body weight). The chemicals were administered in corn oil by oral gavage at a dosing volume of 10ml/kg bw. Animals were sacrificed at day 3 by CO_2/O_2. Blood was obtained from the abdominal aorta directly after decease and collected in plastic whole blood tubes with spray coated K2EDTA (BD, Breda, the Netherlands). All animal treatments were performed with permission of the Animal Ethical Committee and according to Dutch law on Animal Experiments.

Plasma and lymphocyte isolation

Plasma and peripheral lymphocytes were isolated from fresh blood of each individual rat or 2 pooled mice by using Ficoll-Paque gradient according to manufacturer's instructions. Plasma samples were stored directly at -80°C until they were used to determine the plasma concentration of TCDD, 4-PeCDF and PCB-126. Isolated lymphocytes were lysed with RLT buffer (Qiagen, Venlo, the Netherlands) supplemented with 1/100 β-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) as described in the Qiagen RNAeasy kit protocol. The lysate were passed through 22-25 gauge needles to improve the RNA yield and stored until use at -80°C.

Chemical analysis

The blood plasma samples were cleaned up using a solid phase extraction with Chem-Elut and NaCl. After clean up the samples were analysed by GC-MS. To retain unique individual results, plasma samples were not pooled within the same treatment group but between similar exposure levels of TCDD, 4-PeCDF and PCB-126. The concentrations were calculated at lipid base.

RNA isolation and cDNA synthesis

Total RNA extraction was performed using a Qiagen RNAeasy kit. Purity and concentration of the isolated RNA was determined by measuring the absorbance ratio at 260/280nm and 230/260nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA sythesis Kit, according to the manufacturer's instructions (Bio-Rad, Veenendaal, the Netherlands).

Real time PCR

Quantitative real-time PCR (RT-PCR) analyses were performed using the iQ Real-Time PCR Detection System (Bio-rad, Veenendaal, the Netherlands). The PCR primers CYP1A1, AhRR, β-actine (reference gene mouse) and ARBP (reference gene rat) were designed with the Primer designing tool (NCBI) for rat and mouse. The PCR master mixture contained SYBR Green supermix, 0,4µM forward and reverse primer, and 66,7µg/ml cDNA in a total volume of 25µl. The following program was used for denaturation and amplification of the cDNA: 3 min at 95°C, followed by 40 cycles of 15s at 95°C and 1min at 60°C. Data were analysed using iCycler iQ Optical System Software (Bio-Rad, Veenendaal, the Netherlands).

Results and discussion:

In mouse peripheral blood lymphocytes, *in vivo* exposed to TCDD, 4-PeCDF or PCB-126, CYP1A1 gene expression was dose-dependently increased (figure 1A). The lowest dose with a significant increase in CYP1A1 mRNA levels was, 10µg/kg bw for TCDD, 100µg/kg bw for 4-PeCDF and 1000µg/kg bw for PCB-126 with 6-, 13- and 27-fold induction over vehicle-control treated animals, respectively. Although none of the compounds reached their maximum response, the magnitude of the induction at the highest dose tested varied substantially between the compounds, with 4-PeCDF having the highest induction of 165-fold over control.

In contrast with mouse lymphocytes, CYP1A1 gene expression could not be detected in rat peripheral blood lymphocytes, indicating clear interspecies differences in response. Despite this discrepancy, the AhRR gene, another biomarker for AhR activation, could be detected. mRNA levels of the AhRR gene dose-dependently increased by the three compounds tested, although the maximum fold induction was considerably lower then found for CYP1A1 in mouse lymphocytes (figure 1C). The lowest dose to induce a significant increase in AhRR mRNA levels was comparable to the mouse data for TCDD and 4-PeCDF with respectively 2.2- and 2.5-fold induction. However, PCB126 was more potent in inducing AhRR expression in rat PBL compared with CYP1A1 induction in mouse PBL. A significant increase of AhRR mRNA was observed at 100µg PCB126/kg bw with a 1.6-fold induction. TCDD approached a maximum response at an administered dose of 25µg TCDD/kg bw. Comparable to the mouse data, 4-PeCDF had the greatest induction of 6.5-fold over control at the highest dose tested.

The potency of 4-PeCDF and PCB126 were calculated relatively to TCDD. These relative potencies were calculated using the EC20 of TCDD as benchmark effect (EC_{20TCDD}). This method was chosen since the compounds did not reach a maximum response at the highest dose tested. The EC_{20TCDD} values calculated for CYP1A1 expression in mouse PBL were 22.41 µg/kg bw, 117.16 µg/kg bw and 383.65 µg/kg bw for TCDD, 4-PeCDF and PCB126, respectively for administration dose levels (uptake) and 0.056 µg/g lipid, 0.047 µg/g lipid and 1.087 µg/g lipid for TCDD, 4-PeCDF and PCB126, respectively for plasma concentration levels (systemic). The EC_{20TCDD} values calculated for AhRR expression in rat PBL were 9.26 µg/kg bw, 52.54 µg/kg bw and 137.80 µg/kg bw for TCDD, 4-PeCDF and PCB126, respectively for administration dose levels and 0.027 µg/g lipid, 0.008 µg/g lipid and 0.301 µg/g lipid for TCDD, 4-PeCDF and PCB126, respectively for plasma concentration levels.

REP values, calculated using the $EC_{20TCDD,}$ are summarized together with the WHO-TEFs[6] in table 1. REPs calculated based on administered dose levels were comparable with the WHO-TEFs for both mouse and rat. However, recalculating the REP values of 4-PeCDF based on systemic plasma levels showed a remarkable 4- and 12-fold higher REP value for mouse and rat, respectively when compared to the WHO-TEF.

Differential disposition and hepatic sequestration of 4-PeCDF and PCB126 has been described earlier by De Vito *et al.*[2] Most likely this hepatic sequestration appears to be caused by high affinity binding of these compounds to the CYP1A2 protein[7]. In our study a large difference was observed when comparing the plasma levels of 4-PeCDF with those of PCB126. Although the administered doses of both compounds were similar, the blood plasma levels of 4-PeCDF were between 3- to12-fold lower than those found for PCB126 in mouse and rat. Although low plasma levels were measured for 4-PeCDF, the responses in gene expression were relatively high and comparable to TCDD, resulting in a higher REP value compared to the WHO-TEF. In contrast, the systemic REPs calculated for PCB126 were comparable with the WHO-TEF for rat and 2-fold lower in mice. Similar finding were described by Harper *et al.*[8], who studied the dose dependent immunosuppressive activity of PCDDs, PCDFs and PCBs in splenic lymphocytes of C57BL/76 and DBA/2 mice, and calculated REP values varying in the range from 0.58–4.0 for 4-PeCDF and 0.18–0.11 for PCB126. Thus, substantial species- and organ- differences in susceptibility toward dioxin exposure exist due to variation in pharmacokinetics and pharmacodynamics. Taking into account tissue concentrations as opposed to administered dose, might clearly overcome many of these differences. Extending this knowledge to other compounds and tissues will contribute to the accuracy and applicability of the current WHO-TEFs, resulting in a better human risk assessment for dioxin and dioxin-like compounds.

Table 1: Relative Effect Potencies (REPs) for CYP1A1 and AhRR gene expression in mouse and rat peripheral blood lymphocytes calculated using administered dose (uptake) and blood plasma levels (systemic). REPs were calculated using the EC20 of TCDD.

	Uptake REP		Systemic REP		
	Mouse	**Rat**	**Mouse**	**Rat**	**WHO-TEF[6]**
TCDD	1	1	1	1	1
4-PeCDF	0,2	0,2	1,2	3,5	0,3
PCB 126	0,06	0,07	0,05	0,09	0,1

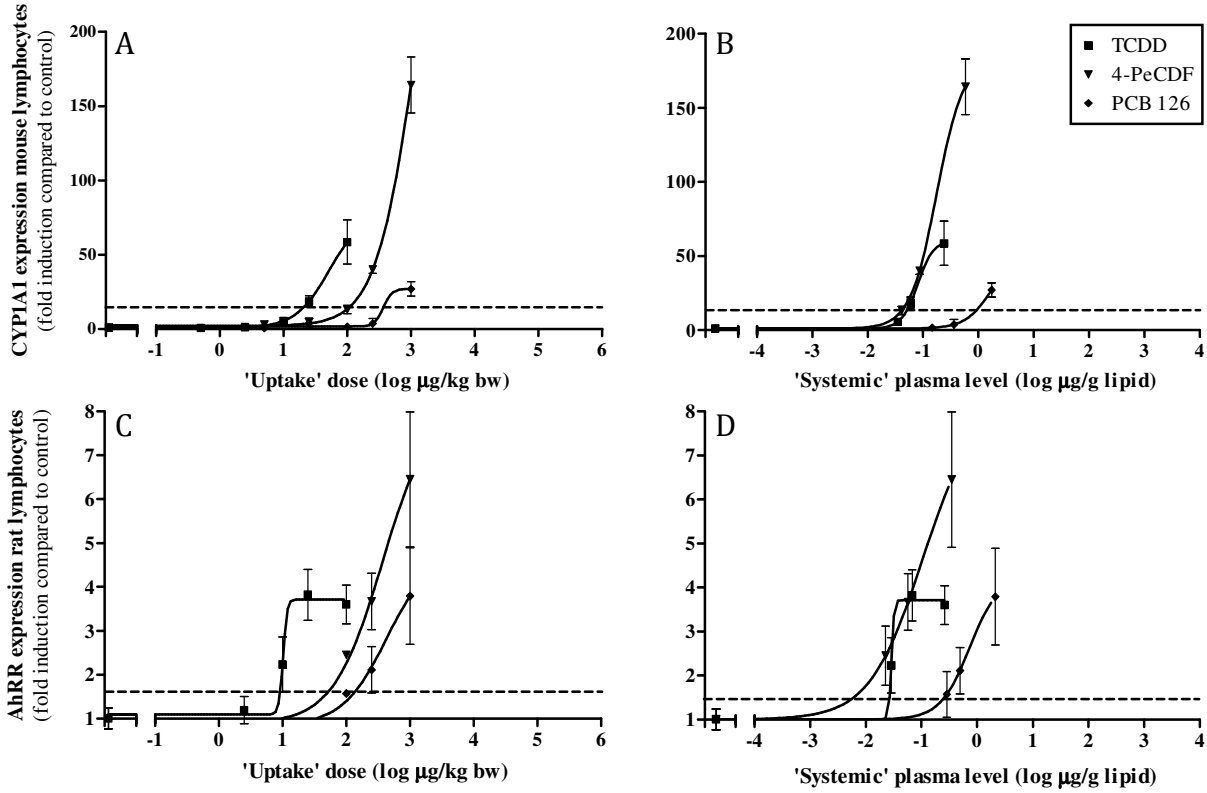

Figure 1: CYP1A1 gene expression in mouse peripheral blood lymphocytes (A&B) and AhRR gene expression in rat peripheral blood lymphocytes (C&D) three days after a single oral dose of TCDD, 4-PeCDF or PCB126. Dose-response curves are calculated using administered dose (uptake) or plasma levels (systemic). EC_{20TCDD} is indicated with a black dotted line. Data are represented as mean ± SD (N=6).

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