MIXTURE INTERACTIONS IN THE *IN VITRO* CYP1A1 INDUCTION BIOASSAY USING CHICKEN EMBRYO HEPATOCYTES

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1. Introduction

Risk assessment of mixtures of polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) is generally performed using the toxic equivalency factor (TEF) concept[1,2]. Each congener has been assigned a potency relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). By multiplying the individual congener concentrations with their respective TEFs, the toxic equivalency (TEQ) concentration of the mixture is calculated. Using this approach, the concentrations of all different PHAHs have to be determined by gas chromatography combined with mass spectrometry. As an alternative approach, TEQ concentrations can be determined using a bioassay in which an Ah-receptor mediated effect of the mixture is compared with the effect of TCDD. The advantage of risk assessments based on this approach is the fact that unidentified Ah-receptor active compounds and/or possible mixture interactions are included in the outcome. Cytochrome P450 (CYP) 1A1 associated ethoxyresorufin *O*-deethylase (EROD) induction is such an Ah-receptor mediated effect and is suggested for use in various bioassays measuring the total TEQ (bioTEQ) content of a sample. Recently assays based on either primary hepatocyte cultures or hepatoma cellines have been developed and validated for use[3,4]. However, a common phenomenon of these *in vitro* assays is the biphasic dose-respons relationships which shows a dose related increase of the EROD induction up to a maximal activity, followed by a dose-related decrease at higher concentrations. In most studies this biphasic respons remained unexplained or is assigned to cytotoxicity or competition for the CYP1A1 catalytic binding site between the administered PHAHs and 7-ethoxyresorufin (7-ER), which serves as a substrate for the EROD activity measurement. Studies by Gooch and co-workers[5] have shown that competitive inhibition can play a role when isolated microsomal fractions of rat liver are used and when 3,3',4,4'-TeCB (PCB 77) is added to the reaction mixture for EROD measurement. However, to our knowledge compititive inhibition have never been studied in detail in CYP1A1 *in vitro* induction assays using primary hepatocytes or hepatoma cellines. When dose-respons relationships are influenced by cytotoxicity, competitive inhibition of EROD activity, or other non Ah receptor related mechanisms that induce a biphasic dose-response relationship, it can be expected that measured bioTEQ concentrations might show a discrepancy with the "real" TEQ concentrations. In order to elucidate the mechanism behind the biphasic dose respons relationship we studied 1) the influence of TCDD on cell viability along the full biphasic effect range, 2) the effect of additional TCDD in the reaction mixture on the 7-ER metabolism by CYP1A1, and 3) the effect on the EROD activity

when 2,2',4,4',5,5'-HxCB (PCB 153), a non Ah receptor active PCB, is coadministrated with TCDD.

2. Materials and methods

2.1 *EROD Induction assay*

In the assay, primary hepatocyte cultures from 19 d old White Leghorn chicken embryos were used. Egg incubation, cell culturing, dosing, and EROD activity measurements were performed according to Kennedy *et al.*[4]. The cells were cultured in 48 wells plates from Costar (Costar Inc. Cambridge, MA, USA). After 24 hours of preincubation the cells were dosed with TCDD or PCB 153. DMSO was used as a carrier. The concentrations in the medium ranged from $1*10^{-4}$ to $3.3*10^{2}$ nM for TCDD and from $5*10^{-1}$ to $1.6*10^{3}$ nM for PCB 153. Controls received DMSO alone and blancs received no treatment. After 24 hours of incubation the medium was removed and plates were stored at -70°C until EROD analysis. EROD activity was measured using a Cytofluor 2300 platereader[a].

2.2 *Viability tests*

The viability of TCDD dosed cells was determined with two seperate parameters; lactate dehydrogenase (LDH) activity and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) metabolism. LDH is a cytosolar enzyme wich is unable to cross the intact cell membrane. The ratio between LDH inside the cells and in the medium is a measure for membrane disruption and associated loss of viability. The LDH test was performed basicly according to Bergmeijer[5] and adapted for use with primary hepatocytes in 48 wells plates. In the test pyruvate is metabolized to lactate. This reaction is catalized by LDH and NADPH is added as a cofactor. The reaction was quantified by the decrease of NADPH, measured spectrophotometrically at 340 nm. The ratio $LDH_{cell}/LDH_{cell+medium}$ in PCB exposed cells was compared to DMSO exposed controls. In a seperated experiment the reduction of MTT to formazane was measured as an index for mitochondrial respiration and associated cellviability. Cells were incubated with MTT for 45 min at 37 °C. Cellular MTT metabolism was quantified by measuring the isopropanol extractable formazane spectrophotometrically at 560 nm. Cellular MTT metabolism activity in exposed cells was compared to a DMSO exposed control. Detailed descriptions of both methods will be published elswhere.

2.3 *Competition assays*

The influence of the precence of TCDD as a competitive inhibitor of the metabolism of 7-ER was studied using two TCDD dosed plates. TCDD concentrations in the medium ranged from $3.3*10^{-3}$ to $3.3*10^{2}$ nM. Each concentration was dosed in sixfold. After the incubation of the cells with TCDD, and prior to EROD measurements, an additional $1.1*10^{2}$ nmol TCDD/l was added to three of the six wells of each of the seven concentrations. The remaining wells with similar initial TCDD concentration received DMSO (carrier) only. Following a preincubation with the additional TCDD, the EROD activity was measured in each well and seperated dose-response curves were fitted for TCDD dosed cells with and without additional TCDD added to the EROD reaction mixture.

2.4 *Mixture interactions*

Three plates were dosed with TCDD alone and with a mixture of TCDD and PCB 153 at a ratio of 1:10 000 (seven concentrations each, all dosed in triplicate). For both TCDD and TCDD + PCB 153 dosed cells dose-respons relationships were fitted to the TCDD concentrations. Y_{max}, d_{max}, and EC_{50} of the TCDD + PCB 153 dosed cells were compared to Y_{max}, d_{max}, and EC_{50} of the TCDD dosed cells of the same plate.

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[a] Dose-effect curves were fitted according to the relationship: $y_d = Y_{basal} + (Y_{max} - Y_{basal}) \cdot C^{*(\ln(d) - \ln(d_{max}))^{exp2}}$, where $C = \ln(2) / [\ln(EC_{50}) - \ln(d_{max})]^2$, and y_d=the EROD activity at TCDD concentration d; Y_{basal}=the basal EROD activity; Y_{max}=the maximal EROD activity; d_{max}=the TCDD concentration when EROD activity is maximal; EC_{50}=EROD activity midway basal and maximal activity.

3. Results and discussion

3.1 *Viability*

Both LDH and MTT tests showed no dose related alterations in viability in relation to LDH leakage or MTT metabolism in cells exposed to increasing doses of TCDD (Fig. 1). These results suggest that the decrease in EROD activity as observed in the chicken embryo hepatocytes assay at the highest concentrations is not due to cytotoxic activity of TCDD.

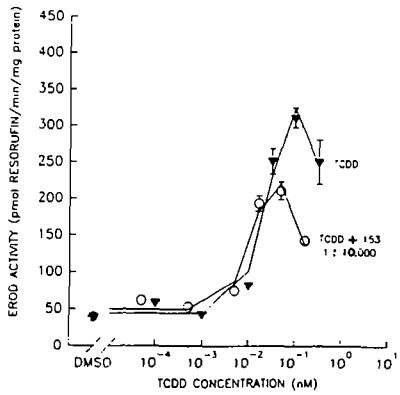

Fig. 1. EROD activity (▾), MTT metabolism (% of control) (O), and LDH leakage (activity in medium as % of activity in cells (●) in TCDD dosed cells.

3.2 *Competition assay*

Administration of TCDD during the EROD measurement did not influence the dose related respons of the EROD activity (Fig. 2). These results indicate, that competition between TCDD and 7-ER, as substrates for the CYP1A1 catalytic binding site, do not influence the EROD activity under the conditions used in this assay. As a consequence it is concluded that the biphasic respons observed in this *in vitro* EROD assay is *not* caused by competitive binding of PHAHs to the CYP1A1 catalytic site.

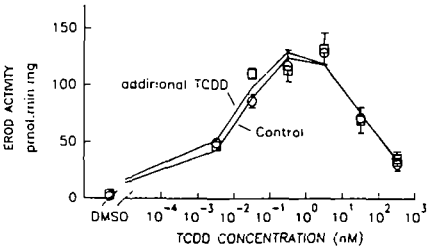

Fig. 2. Competitive effect of TCDD (ratio TCDD:7-ER = 1:4.7) administered during the EROD assay. $1.1*10^{2}$ nmol TCDD/l added prior to EROD assay (□). Controls; only 0.8% DMSO added (O).

3.3 *Mixture interactions*

Coadministration of PCB 153 with TCDD significantly reduced the Y_{max}, d_{max}, and EC_{50} values of the dose-response relationship between TCDD concentration and EROD activity. A typical dose-respons relationship is shown in Fig. 3. On average there was a 57±4% reduction in EC_{50} values for TCDD when PCB 153 was coadministred. Given the relatively high amounts of PCB 153 in biota[7], our results suggest that when extracts of environmental samples are tested in a hepatocyte CYP1A1 induction assay, the estimated TEQs in the extract might be *overestimated* when PCB 153 or related compounds are present. TEQs are calculated using the ratio $EC_{50}[TCDD]/EC_{50}[extract]$. Thus, the PCB 153 induced decrease of the EC_{50} value of the extract causes an overestimation of the TEQs. The mechanism for PCB induced decrease of EROD activity was recently studied by Tysklind and co-workers[8]. In their study an inverse relationship was found between EROD activity and uroporphyrin accumulation for a wide range of PCBs. These results suggest that inhibition of EROD activity is somehow related to porpherin accumulation.

4. Conclusions

CYP1A1 dependent EROD induction assays using primary hepatocyte cultures or hepatoma cell lines show biphasic dose-respons relationships. Results of our studies show that this eventual decrease in EROD activity is not due to cytotoxicity or competitive binding to the catalytic binding site of CYP1A1. It was found that coadministration of PCB 153 and TCDD significantly reduces the EC_{50} value, compared to administration of TCDD alone. These results suggest that bioTEQ measurements in these bioassays could lead to an overestimation of the actual TEQs if the tested environmental mixtures comprise of a mixture of PCDDs, PCDFs, and PCBs. As such these interactive effects are of direct consequence for risk assessment procedures when using these CYP1A1 based bioassays.

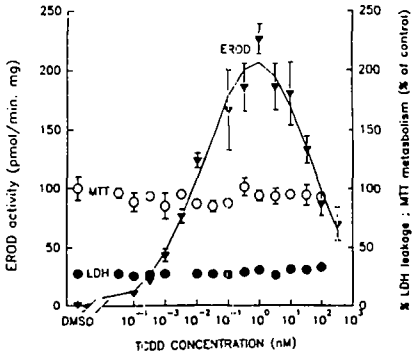

Fig. 3. Typical dose-respons curve for EROD activity in cells dosed with TCDD alone or a combination of TCDD and PCB 153 (1:10.000).

5. References

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