# Ethoxyresorufin *O*-deethylase (EROD) Induction by Halogenated Aromatic Hydrocarbons (HAHs) in Chicken Embryo Hepatocyte Cultures: Time-Dependent Effects on the Dose-Response Curves

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## Abstract

Dose-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 3,3',4,4'-tetrachlorobiphenyl (PCB 77) on 7-ethoxyresorufin *O*-deethylase (EROD) activity in chicken embryo hepatocyte (CEH) cultures were determined after the cells were exposed to the compounds for 24, 48 or 72 hr. For all compounds, dose-response curves shifted to the right with exposure time. Between 24 hr and 72 hr, the dose that elicited a half-maximal response ($ED_{50}$) in EROD activity for TCDD, TCDF and PCB 126 increased 5-, 7- and 5-fold, respectively. Therefore, the potencies of TCDF and PCB 126 relative to TCDD (the relative potencies, or REPs) were similar at the different time points. There was a larger shift in the PCB 77 dose-response curve (the $ED_{50}$ increased approximately 90-fold between 24 hr and 72 hr), thus reducing its REP from 0.03 at 24 hr to 0.002 at 72 hr. PCBs 77 and 126 were extracted from the cell cultures at different time points and analyzed by gas chromatography. Only 7% of PCB 77 was recovered after 48 hr, and hydroxylated metabolites were detected. In contrast, there was no significant loss of PCB 126 with exposure time. We conclude that the large shift in the PCB 77 dose-response curve, was caused, in part, by metabolism of the parent compound to metabolites which are not EROD inducers.

## Introduction

Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and other halogenated aromatic hydrocarbons (HAHs) are widespread environmental contaminants that induce hepatic drug metabolizing enzymes. The potency of a particular HAH to induce cytochrome P4501A (CYP1A) can be a good indication of the toxic potency of that compound [1]. Once induced, CYP1A attempts to metabolize the HAH. As a rule, oxidative metabolism of PCBs occurs most readily when the PCB contains vicinal unsubstituted *meta-para* or *ortho-meta* positions and less than one *ortho*-chlorine [2].

Recent research in our laboratory has been directed toward determining the CYP1A inducing potencies of HAHs in avian hepatocyte cultures for two overall purposes: (*i.*) to predict the sensitivity of developing embryos to toxic effects of HAHs [3,4] and (*ii.*) to develop avian hepatocyte bioassays that can be used to determine TCDD-equivalent concentrations of complex

mixtures of HAHs in environmental mixtures [5].

Lorenzen *et al.* [6] studied time-dependent changes in immunodetectable CYP1A and CYP1A catalytic activity (using the EROD assay) in CEH cultures treated with PCB 77, and found an apparent decrease in the CYP1A-inducing potency of PCB 77 between 24 and 48 hours. A similar effect was reported by other investigators in rat hepatoma H4IIE cells exposed to PCB 77 or TCDF [7], and in mouse hepatoma Hepa-1 cells exposed 3-methylcholanthrene [8]. The time-dependent decrease in induction potency of PCB 77 in CEH cultures suggested to us that the concentration of PCB 77 was decreasing with time due to metabolism, but neither the concentrations of the parent compound or its metabolites were measured. The present study examined the changes in both EROD induction and concentration of PCB 77 in CEH cultures, and results were compared to those observed with TCDD, TCDF and PCB 126 at varying exposure times up to 72 hr.

## Experimental Methods

*Cell culture / EROD Assays*

Primary cultures of chicken embryo hepatocytes were prepared in 48-well plates as described previously [9]. Serial dilutions of HAHs in dimethyl sulfoxide (DMSO) were added to the plates (2.5 µl/well) and the plates were placed in a humidified incubator (37°C, 5% $CO_2$) for various times, ranging up to 72 hr. Medium was removed, and plates were frozen (-80°C) until EROD assays were carried out using the method described elsewhere[10]. Dose-response data were fitted to Gaussian curves, and $ED_{50}$ values were determined as described in Kennedy *et al.*[5].

*Chemical Extraction and Analysis*

Plates for analysis of PCB 77 and PCB 126 were prepared in the same manner as above. Only one 10 nM dose per plate was used in all of the wells. Instead of removing the medium and freezing the plates, the cells were scraped and the contents of the wells and the medium were pooled and extracted (Bastien *et al.*, in preparation) for analysis by gas chromatography using an electron capture detector (GC-ECD) or a mass spectral detector (GC-MSD). Experiments to measure metabolites of PCB 77 were carried out with hepatocytes that were cultured in 15 cm glass Petri dishes rather than in 48-well plastic plates. The cells were scraped, the contents of the plate were extracted and metabolite fractions were derivatized with diazomethane prior to analysis by GC-MSD.

*Equipment*

The GC-ECD was a Hewlett Packard Model 5890 (HP5890) equipped with a $^{63}Ni$ ECD detector, an HP 7673A automatic injector and a DB-5MS column (30m, .25mm i.d., 25 µm film). The injector and detector temperatures were 250 °C and 300 °C respectively. Injections were 2 µL made in splitless mode. GC-MSD (electron impact, EI) analysis was performed on a HP5890 chromatograph equipped with a 5970 series mass selective detector, an HP 7673A automatic injector and a DB-5MS column (30m, 0.25 mm i.d., 25 µm film). Injections were 2 µL made in splitless mode. The injector and detector temperatures were 250 °C and 280 °C, respectively. Both systems were controlled by an IBM-compatible computer and Hewlett Packard Chemstation software. The GC temperature program for both methods of detection was as follows: initial temperature held for 2 min at 80°C, 10°C /min to 150°C and 8°C /min until 280 °C. The

fluorescence multiwell plate reader was a Cytofluor Model 2350 from Millipore Ltd. It was equipped with a Hamamatsu R928 red sensitive photomultiplier and controlled by an IBM-compatible computer. Multiwell plates were from Falcon.

*Reagents*

PCB 77 and PCB 126 standards of $\geq$ 99% purity were from Ultra Scientific (North Kingstown, RI, USA). TCDD was provided by Dr. J. J. Ryan (Health Canada, Ottawa, Ontario, Canada). TCDF was from Cambridge Isotopes (Andover, MA, USA). The standards were dissolved in DMSO obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA). Concentration and purity of all standards were confirmed using GC-ECD and/or GC-MSD. Organic solvents used for extractions (n-hexanes, iso-octane and methyl-tert-butylether ether) were from BDH (Toronto, Ontario, Canada). Reagents used for culturing cells and EROD assays were obtained from the suppliers indicated elsewhere[10].

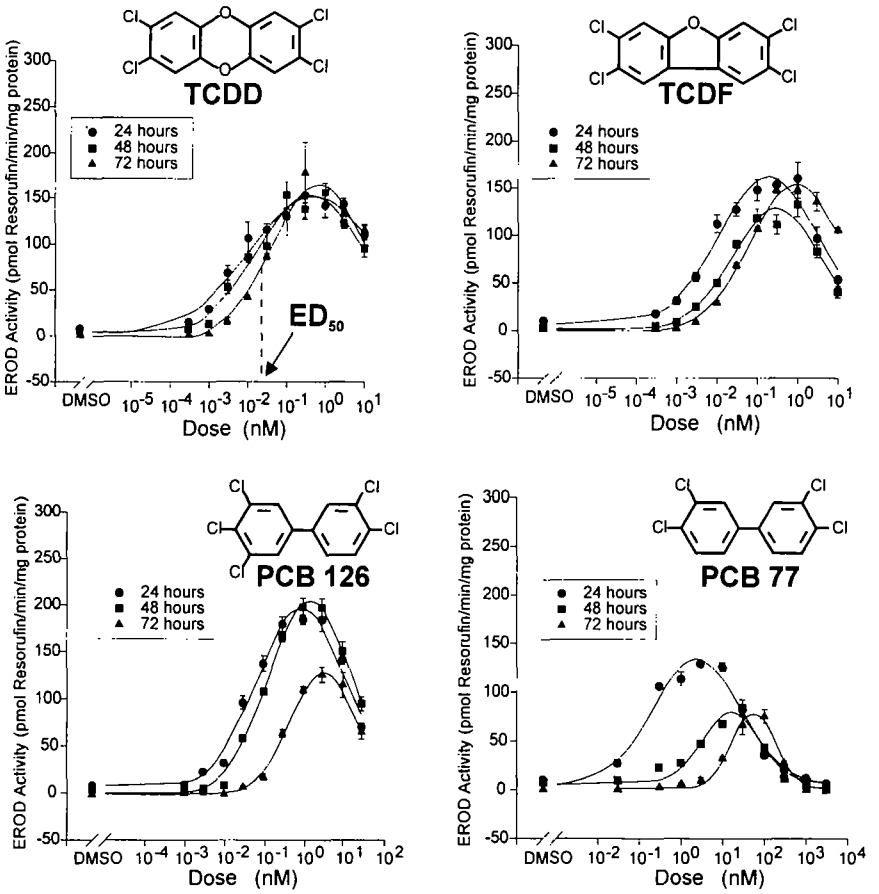


**Figure 1.** Effect of exposure time on the dose-dependent effect of TCDD, TCDF, PCB 126 and PCB 77 on EROD activity in CEH cultures. Each point on the dose-response curves represents the mean responses of triplicate doses; bars represent standard errors.

**Results and Discussion**

*EROD Dose-Response Curves*

Dose-dependent effects of TCDD, TCDF, PCB 126 and PCB 77 in CEH cultures exposed to the compounds for 24, 48 and 72 hours are shown in Figure 1. For all compounds, dose-response curves shifted to the right with exposure time, resulting in time-dependent increases in $EC_{50}$s (Table 1). The mean $EC_{50}$s of PCB 77 at 24, 48 and 72 hours were 0.17 nM, 3.1 nM and 15 nM, respectively. The $EC_{50}$s of PCB 126, TCDF and TCDD also increased with exposure time but to a much lesser degree (all less than 7-fold).

Between 24 hr and 72 hr, the relative potency of PCB 77 decreased from 0.03 to 0.002. In other words, after 24 hr PCB 77 appeared to be 33 times less potent than TCDD and after 72 hours it appeared to be 500 times less potent than TCDD. In contrast, the relative potencies of TCDF and PCB 126 did not change significantly at the different time points.

| | $EC_{50}$ (nM) (standard deviation), n=3 | | | Relative Potency ($EC_{50}$ of TCDD/$EC_{50}$ of compound) | | |
|---|---|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 24 hr | 48 hr | 72 hr |
| 2,3,7,8-TCDD | 0.0050 (0.0007) | 0.011 (0.004) | 0.025 (0.003) | 1.0 | 1.0 | 1.0 |
| 2,3,7,8-TCDF | 0.0070 (0.0002) | 0.020 (0.004) | 0.046 (0.004) | 0.7 | 0.5 | 0.5 |
| PCB 126 | 0.061 (0.04) | 0.093 (0.05) | 0.31 (0.04) | 0.08 | 0.11 | 0.08 |
| PCB 77 | 0.17 (0.04) | 3.1 (0.7) | 15.0 (2) | 0.03 | 0.003 | 0.002 |

**Table 1.** $ED_{50}$ values for TCDD, TCDF, PCB 126 and PCB 77 in CEH cultures at 24, 48 and 72 hours after exposure. Triplicate dose-response curves were obtained for each compound at each time point. Standard deviations are in parentheses. Potencies relative to TCDD were calculated by dividing the $ED_{50}$ of TCDD by the $ED_{50}$ of the compound at any given exposure time

*Chemical Analysis*

The concentration of PCB 77 that was added at an initial concentration of 10 nM at various time points is shown in Figure 2. After 6, 18, 24 and 48 hr, the amount of PCB 77 recovered was 87%, 81%, 58% and 7%, respectively. PCB 126 was extracted 1 and 48 hr after exposure, and there was no significant difference in the amount recovered (data not shown).

The dramatic shift in the EROD dose response curves for PCB 77 compared to the other compounds appeared to be related to the loss of parent compound with time (Figure 2). Three hydroxy-metabolites of PCB 77 were detected by GC-MSD, but these accounted for only a small percentage of the amount of parent compound that decreased with time. Further studies that will attempt to completely account for the time-dependent decrease in PCB 77 in the CEH cell culture system are planned, and these studies will attempt to identifiy phase two metabolites. Others have reported metabolism of PCB 77 *in vitro* by microsomes of rats[11-13], wild species[11,14] and *in ovo* by chick embryos[15].

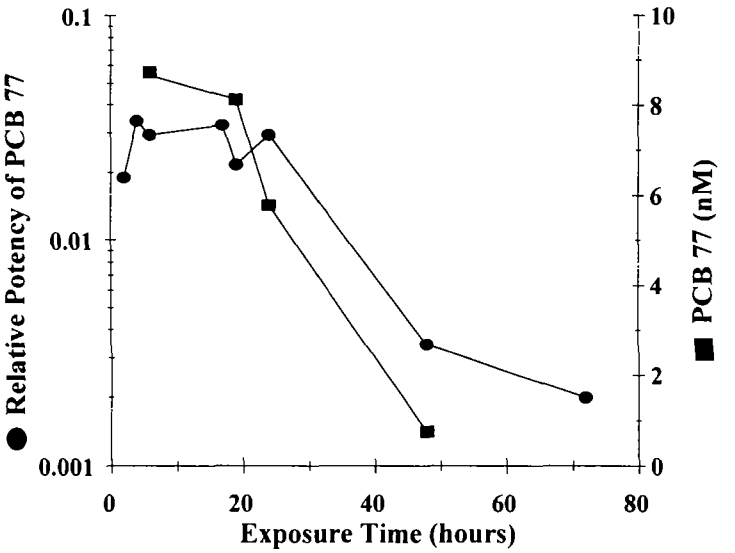


**Figure 2.** Time-dependent effect on PCB 77 concentration ( ■ ) and its relative potency ( ● ). in CEH cultures. PCB 77 was added at an initial concentration of 10 nM.

During this study an attempt was made to determine the distribution of the dosed compound within the environment of the cell culture plate. Preliminary results with PCB 77 showed that although most of the compound could be found in the cellular fraction, a significant amount was extracted from the medium and from the plastic. Others have reported PCB 77 adhering to culture plates and distributed in the medium[16] so that the actual dose in the cells can be much less than expected. It would seem logical that the concentration of the compounds distributed within the wells is continuously being changed due to uptake by the cells, plastic and medium. This might explain, in part, the slight changes in EROD dose-response curves for the other compounds used in this study, which are metabolised more slowly than PCB 77. This study analyzed homogenates of the entire content of the wells. The issue about how much compound actually gets into the cells, combined with differences in rates of metabolism can dramatically effect calculations of relative potency. Accounting for these parameters may allow for better extrapolations between *in vitro* and *in vivo* systems.

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