# CYTOCHROME P4501A INDUCTION IN CHICKEN EMBRYO HEPATOCYTE CULTURES BY POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) AND TCDD - AN APPROACH FOR PREDICTING THE TOXIC POTENCY OF PAHs IN DEVELOPING AVIAN EMBRYOS

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants formed as byproducts of incomplete combustion of organic materials. Major anthropogenic sources of PAHs include leaching from creosoted products, spillage of petroleum, metallurgical and coking plants, and atmospheric deposition[1].

The acute toxicity of 24 PAHs in chicken embryos was investigated by Brunström *et al.*[2,3]. The most toxic PAHs were found to be the most potent cytochrome P4501A (CYP1A1) inducers, suggesting that some of the toxic effects of PAHs are mediated by the aryl hydrocarbon receptor (AhR). The majority of the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and structurally related planar halogenated aromatic hydrocarbons (PHAHs) are thought to be mediated by initial binding to the AhR, followed by synthesis of certain proteins, including CYP1A1[4]. Although it is unlikely that PHAHs and PAHs exert all of their toxic effects through similar mechanisms, several PAHs are known ligands to the AhR[5]. Thus, it is possible that some of the toxic effects of PAHs are mediated by the AhR.

Previous studies in our laboratory with PHAHs have demonstrated that CYP1A induction can be conveniently measured in cultured avian hepatocytes using the ethoxyresorufin-*O*-deethylase (EROD) assay[6]. EROD inducing-potency by PHAHs in cultured hepatocytes is predictive of the toxic potency of PHAHs in developing avian embryos[6]. The objective of the study summarized in this report was to determine whether EROD induction by PAHs in chicken embryo hepatocyte (CEH) cultures can also be used to predict *in ovo* sensitivity to the toxic potencies of PAHs. The EROD-inducing potencies of the PAHs were compared to the EROD-inducing potency of TCDD, the most potent AhR agonist in chicken embryos.

**Comment:** Previous studies in our laboratory have demonstrated that cultured avian hepatocytes are a useful tool for determining the sensitivity of wild birds to the *in ovo* toxic and biochemical effects of various PHAHs.

## Experimental Methods

PAHs were from Sigma (Sigma-Aldrich Canada, Ltd., Oakville, Canada) or EQ Laboratories (Atlanta, GA, USA). TCDD was a gift from Dr. J. Ryan (Health Canada, Ottawa, Ontario). All compounds were stated to be at least 99% pure by the supplier. Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO) as previously described[7]. Reagents for hepatocyte cultures were obtained from suppliers indicated elsewhere [7].

Chicken (*Gallus domesticus*) eggs were from a local supplier. Eggs were incubated at 37$^{O}$C and 60% relative humidity for 19 days. Primary cultures of embryo hepatocytes (CEH cultures) were prepared on Falcon 48-well plates as previously described[7]. After incubation for 24 hr at 37$^{O}$C in a humidified incubator with 5% $CO_2$, serial dilutions of TCDD or PAHs were

added to the plates and the cells were incubated for a further 24 hr. Cells were rinsed, flash frozen on dry ice and stored at -80$^{O}$C until analyzed. EROD induction was measured with a fluorescence plate reader (Cytofluor 2300, Millipore Ltd.) and the concentration-response data were fitted to a Gaussian curve as previously described [7]. Two relative potency estimates, the concentration causing a half maximal response (EC50) and TCDD-threshold dose (ECthr, defined as the concentration of inducer that produces an EROD response equal to 10% of TCDD, see Figure 1), were calculated as previously described[6].

**Results and Discussion**

Because the induction of CYP1A1 requires the presence of a functional AhR signal transduction pathway, induction of EROD activity provides a sensitive biomarker of exposure to compounds that have affinity for the AhR. Previous studies in our laboratory have demonstrated that cultured chicken embryo hepatocytes (CEH) possess a functional AhR signal transduction pathway, and respond with induction of CYP1A when challenged with TCDD and related PHAHs[6].

Studies of 24 PAHs by Brunström *et al.*[2] demonstrated that several of the PAHs were embryotoxic when injected into the air cells of 7 day old chicken embryos, inducing 100% mortality at 300 µg/g-egg. Good correlations were found between the EROD inducing potency of several PAHs and their toxicity in chicken embryos.

In the present study, seventeen PAHs were investigated for their EROD inducing potencies in cultured chicken embryo hepatocytes, and the results were compared against their mortality rates in chicken embryos (Table 1). The concentration dependent effects of TCDD and the four most toxic PAHs (Group 1, Table 1) are shown in Figure 1. The PAHs in Group 1 caused 100% mortality at 300 µg/g-egg. The rank order in EROD inducing potency in CEH cultures for the Group 1 PAHs was benzo[k]fluoranthene (B[k]F) > dibenz[ah]anthracene (D[ah]A) > benz[a]anthracene (B[a]A) > benzo[b]naphtho[2,3-d]thiophene (BNT). The corresponding LD50s for these PAHs were 56, 140, 349 and 350 pmol/g-egg, respectively[2]. A linear plot (not shown) of the LD50 versus the EC50 and ECthr for the four Group 1 PAHs resulted in $r^2$ values of 0.763 and 0.632, respectively. The strong correlation between the EC50 and the LD50 suggests that the CEH boassay may be useful for predicting the toxic potencies of PAHs *in ovo*.

Comparisons between the Brunstr□m *et al.*[2] LD50 data and our EROD potency estimates are complicated by the varying EROD activities expressed by chicken embryos at different stages of development[8]. Bosveld *et al.*[9] demonstrated that the EROD EC50 of a variety of halogenated compounds (TCDD, TCDF, PCB 126 and PCB 118) from 14 day old embryos was significantly different from hepatocyte cultures from 19 day old embryos and 1 day old hatchlings. This confounding factor may affect the present comparisons since the embryos used by Brunstr□m *et al.*[2] were 7 days old at the time of injection, while the hepatocytes in our *in vitro* bioassay were from 19 day old embryos.

PAHs in Group 2 displayed EC50 values intermediate to those of Group 1, ranging from 112 to 268 nM. These PAHs had intermediate embryotoxic potencies, with mortality rates ranging from 55% to 75% at a dose of 300 µg/g-egg (Table 1). Indeno[1,2,3-cd]pyrene was unique amongst the PAHs in this study in that it was the third most potent EROD inducer in CEH cultures, but it caused only 65% mortality i*n ovo* at 300 µg/g-egg. PAHs in Group 3 displayed little or no EROD activity, and did not exhibit embryotoxicity at the doses administered.

The dose response curves of the PAHs in Figure 1 are shifted several orders of magnitude to the right relative to TCDD. As the curves shift to the right, the maximal EROD activity

decreases. There may be several reasons for the decrease in EROD activity, including inhibition of CYP1A1 catalytic activity by high concentrations of residual inducer[10]. Since the EC50 may overestimate the potency of agonists exhibiting lowered maximal EROD activities, alternative methods of estimating potency, including the ECthr, have been introduced. The

**Table 1**. *In ovo* toxicity of 17 PAHs compared to EROD-inducing potency in cultured embryo hepatocytes from White Leghorn chicken. In general, those PAHs exhibiting EC50 values less than 500 nM, are toxic to developing embryos. TCDD-EQ is defined as EC of TCDD / EC of PAH for EC50 or ECthr values.

| | | *In Ovo* Toxicity[a] | | EROD Induction (CEH) | | | |
|---|---|---|---|---|---|---|---|
| | | % Mortality | | EC50 | ECthr | TCDD-EQ | |
| Group | PAH | 300 | 100 | (nM) | (nM) | $EC_{50based}$ | $EC_{thrbased}$ |
| 1 | benzo[k]fluoranthene | 100 | nt[b] | 26.3 | 4.6 | 0.001 | 0.0003 |
| | dibenz[ah]anthracene | 100 | nt | 69.6 | 16.2 | 0.0004 | 0.00008 |
| | benz[a]anthracene | 100 | nt | 213 | 126 | 0.0001 | 0.00001 |
| | benzo[b]naphtho-[2,3-d]thiophene | 100 | nv[c] | 422 | 371 | 0.00006 | 0.000003 |
| 2 | chrysene | 75 | 25 | 261 | 173 | 0.0001 | 0.000007 |
| | Indeno[1,2,3-cd]pyrene | 65 | 5 | 112 | 32 | 0.0002 | 0.00004 |
| | benzo[a]pyrene | 55 | 0 | 268 | 191 | 0.0001 | 0.000007 |
| 3 | dibenzothiophene | 5 | nt | nr[d] | nr | — | — |
| | 1-methylpyrene | 0 | nt | 3164 | nr* | 0.000008 | — |
| | anthracene | 0 | nt | nr | nr | — | — |
| | benzo[e]pyrene | 0 | nt | 1500 | nr* | 0.00002 | — |
| | Coronene | 0 | nt | 3000 | nr* | 0.00001 | — |
| | Fluoranthene | 0 | nt | nr | nr | — | — |
| | Fluorene | 0 | nt | nr | nr | — | — |
| | Phenanthrene | 0 | nt | nr | nr | — | — |
| | Perylene | 0 | nt | nr | nr | — | — |
| | Pyrene | 0 | nt | nr | nr | — | — |

[a]Percent mortality at 300 µg/g-egg and 100 µg/g-egg; from Brunström *et al.*, 1991.
[b]not tested at this dose; [c]no value given, but tested at this dose; [d]no response
*maximal EROD activity below 10% of TCDD; cannot calculate ECthr

ECthr has the advantage of being insensitive to inhibitory effects of inducers of EROD activity. The ECthr approach results in a wider range of values than the EC50. With the EC50 approach, TCDD is almost three orders of magnitude more potent than B[k]F, while there was only a 16 fold difference in EC50s between B[k]F and BNT. However, with the ECthr approach, TCDD is approximately 3500 times more potent than B[k]F, and the difference between B[k]F and BNT increases to 80 fold. Consequently, the ECthr provides a broader range of values than the EC50 suggesting that the ECthr approach has a greater ability to discriminate the toxic potencies of

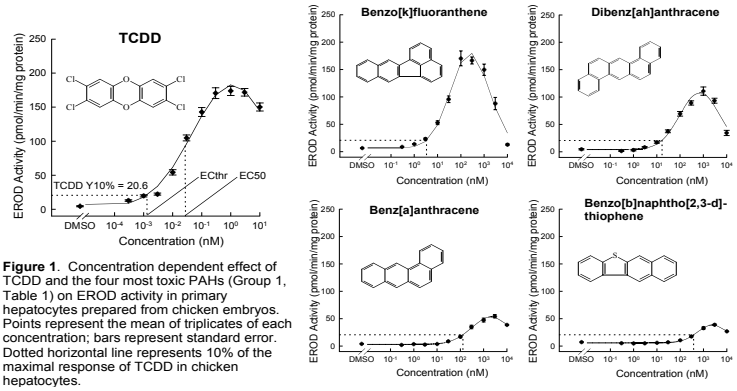


**Figure 1**. Concentration dependent effect of TCDD and the four most toxic PAHs (Group 1, Table 1) on EROD activity in primary hepatocytes prepared from chicken embryos. Points represent the mean of triplicates of each concentration; bars represent standard error. Dotted horizontal line represents 10% of the maximal response of TCDD in chicken hepatocytes.

of AhR agonists than the EC50. Western blot analyses of immunodetectable CYP1A protein are planned to determine whether the ECthr approach is valid for PAHs. Overall, these results suggest that the CEH bioassay may be a useful tool for predicting the toxic potencies of PAHs in avian embryos.

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