Sensitivities of Chicken and Pheasant Embryos and Cultured Embryonic Hepatocytes to Cytochrome P4501A Induction and Porphyrin Accumulation by TCDD, TCDF and PCBs

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Abstract

Experiments were conducted to determine the sensitivities of primary cultures of embryonic hepatocytes and livers of intact embryos from two avian species towards various halogenated aromatic hydrocarbons (HAHs) including: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169; IUPAC nomenclature), 3,3',4,4'-tetrachlorobiphenyl (PCB 77), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) and Aroclor 1254 (a commercial mixture of PCBs). The species studied were the chicken (*Gallus domesticus*, white leghorn) and the ring-necked pheasant (*Phasianus colchicus*). Both the cultured hepatocytes and intact embryos of each species were exposed to similar concentrations of HAHs for 24 hours. Hepatocytes were analyzed for total porphyrin accumulation and P4501A induction - measured as ethoxyresorufin-O-deethylase (EROD) activity and immunodetectable P4501A. Liver microsomes prepared from embryos treated *in ovo*, were similarly analyzed for P4501A induction. Results of the cell culture study indicated that the EROD inducing-potencies of the various HAHs, relative to TCDD, were consistent between the two species with the notable exceptions of PCB 118 and Aroclor 1254 which had higher relative potencies in the pheasant than in the chicken. All HAHs tested, except PCB 118 and Aroclor 1254, were approximately 5 times less potent in the pheasant than in the chicken on a molar basis, whereas the concentrations of PCB 118 and Aroclor 1254 required to elicit P4501A induction were similar between the two species. Compared to chicken hepatocytes, pheasant hepatocytes accumulated only minor amounts of porphyrins. Treatment of intact embryos with various HAHs resulted in significant P4501A induction only for TCDD and TCDF in both the chicken and pheasant, despite administration of these compounds at concentrations and at a developmental stage similar to those of the cultured hepatocytes. Lack of sensitivity of the intact embryos may have been due to pharmacokinetic factors (rates of absorption, distribution, elimination) affecting the concentration of HAH actually reaching the embryonic liver.

Introduction

A major objective of this laboratory is to develop a method which can accurately predict the susceptibility of wild birds towards environmental HAH contamination. Previous experiments conducted in this laboratory compared the P4501A-inducing potencies (measured as EROD activity) of six different HAHs applied to cultured hepatocytes derived from the

embryos of eight species or breeds of birds [1]. The results of th s work indicated that the method routinely used for the culture of chicken embryo hepatocytes was applicable to other species or breeds of birds. Furthermore, it became clear that the effective concentrations required for half-maximal (EC50) P4501A induction and the maximal levels of EROD activity varied considerably between the species, or breeds, of birds tested.

The present study was undertaken to further investigate the pattern of P4501A induction observed for chicken and pheasant embryo hepatocytes, and to compare this pattern to that obtained from liver microsomes prepared from embryos of these species treated *in ovo*. Secondly, total porphyrin accumulation was measured in both chicken and pheasant hepatocytes to evaluate the applicability of this endpoint as an indicator of HAH exposure.

Materials and Methods

Chemicals. Chemical suppliers and purity were identical to those described previously [2].

Cell culture experiments. Preparation, culture, HAH treatment, EROD and porphyrin assays of hepatocytes were carried out as described previously [2]. Total protein was determined using the fluorescamine protein assay modified for fluorescence plate readers [3]. EROD and total porphyrin assays were conducted on the same multi-well plate.

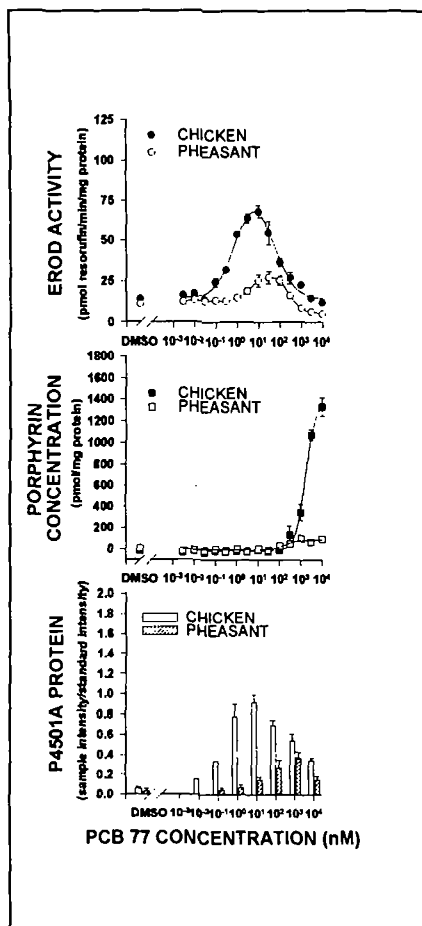
Egg injection experiments. The methods used for the egg injection study were similar to those described previously [4]. Briefly, two days prior to hatching, eggs were candled and divided into five treatment groups of triplicate eggs for each HAH to be tested. Each treatment group was injected into the air sac with test compound, or solvent (DMSO), to obtain a range of concentrations within 0.01 to 100 ppb (µg HAH/kg egg). After a further 24 hours incubation, the embryos were sacrificed and the livers were removed and pooled for each treatment group. Liver homogenization, preparation of S9 fractions and microsomes were carried out as described previously [4]. Assay of microsomes for EROD activity and total protein was conducted according to the method of Kennedy and Jones [5] after determining optimal reaction time, microsomal protein, substrate and co-factor concentrations for both the chicken and pheasant.

Immunoblotting. Based on the original western blot method developed by Towbin *et al.* [6], 15 µg of hepatocyte or microsome samples were separated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE, 9% acrylamide) and electrophoretically transferred to Rad-Free membranes (Schleicher & Schuell, Keene, NH). Aroclor 1254-induced rat liver microsomes (prepared from commercially available postmitochondrial supernatant - Molecular Toxicology Inc., Annapolis, MD) were used as standards. Immunodetection of CYP1A was performed using a mouse monoclonal antibody prepared against scup cytochrome P4501A1 which recognizes CYP1A in all taxonomic groups of vertebrates examined so far [7,8]. The secondary antibody was a goat anti-mouse IgG linked to alkaline phosphatase. Immunoreactive proteins were detected by chemiluminescence (Rad-Free, Schleicher & Schuell, Keene, NH) and the light intensities of the immunoreactive protein bands were quantified by video imaging densitometry (UMP Gel Documentation System 7500, San Gabriel, CA).

Results and Discussion

Figure 1 shows plots of typical data obtained for EROD, total porphyrin and P4501A immunoblotting assays for chicken and pheasant hepatocytes treated with PCB 77. Table 1 summarizes similar data from all HAHs tested in each species. A similar dose-response pattern was observed for P4501A induction whether measured as EROD activity or immunodetectable P4501A protein. Minor amounts of porphyrins accumulated in pheasant hepatocytes when compared to chicken hepatocytes for all HAHs tested.

The data summarized in Table 1 indicates that the EROD-inducing potencies



HAH	SPECIES	EROD EC50 (95% CI)	RELATIVE POTENCY EC50 TCDD / EC50 HAH
TCDD	chicken	0.01 nM (0.006-0.02)	1
	pheasant	0.05 nM (0.03-0.09)	1
TCDF	chicken	0.01 nM (0.008-0.02)	1
	pheasant	0.07 nM (0.03-0.13)	0.7
PCB 169	chicken	0.7 nM (0.5-1)	0.01
	pheasant	4 nM (2-8)	0.01
PCB 77	chicken	0.5 nM (0.4-0.7)	0.02
	pheasant	3 nM (1-8)	0.02
PCB 118	chicken	27 nM (18-38)	0.0004
	pheasant	12 nM (4-38)	0.004
PCB 153	chicken	+∞	-∞
	pheasant	+∞	-∞
Aroclor 1254	chicken	0.02 µg/ml (0.01-0.022)	0.0002
	pheasant	0.01 µg/ml (0.003-0.05)	0.002

Table 1. Summary of hepatocyte EROD data and ranking of the HAHs by their relative potencies. EROD data was fitted to a Gaussian curve, and curve parameters were used to obtain EC50 values (defined as the lower concentration of HAH where EROD activity is midway between basal and maximal values).

Figure 1. EROD (top), total porphyrin (middle)and P4501A protein (bottom) data from pheasant and chicken embryo hepatocytes treated with PCB 77.

of the various HAHs, relative to TCDD, were consistent between the two species with the notable exceptions of PCB 118 and Aroclor 1254 which had higher relative potencies in pheasant embryo hepatocytes than in chicken embryo hepatocytes. *All HAHs tested, except PCB 118 and Aroclor 1254,* were approximately 5 times less potent on a molar basis in pheasant embryo hepatocytes than in chicken embryo hepatocytes. The concentrations of PCB 118 and Aroclor 1254 required to elicit P4501A induction were similar between the two species.

Treatment of intact embryos with various HAHs resulted in significant P4501A induction (assayed as either EROD activity or immunodetectable P4501A protein) only for TCDD and TCDF. EC50s obtained after fitting Gaussian curves to the data were for the chicken embryo: TCDD 0.1 (0.06-0.2) ppb, TCDF 0.4 (0.3-0.4) ppb; and for the pheasant embryo: TCDD 0.3 (0.1-1) ppb, TCDF 1 (0.6-1.6) ppb. Based on these results, *in ovo* exposure resulted in a 4 and 2.5 times greater sensitivity of the chicken embryo to TCDD and TCDF, respectively, when compared to the pheasant embryo. Furthermore, the relative potencies of TCDF were 0.25 for the chicken embryo and 0.40 for the pheasant

embryo which are somewhat lower than those observed in the hepatocyte study.

The EC50 of 0.3 ppb TCDD for *in ovo* EROD induction observed for pheasant embryos in the present study is in good agreement with the EC50 (312 pg TCDD/g egg) determined in a previous study for EROD induction in 1 day old pheasant chicks exposed to graded doses of TCDD for the entire duration of incubation [9]. Chick embryos exposed to graded doses of TCDD or TCDF, under conditions similar to those used in the present study, also gave EC50 values for EROD induction (approximately 0.2 ppb) very similar to those reported here [4]. Although not observed in the present study, others have observed significant induction of EROD activity in chick embryos after exposure of the embryos to doses of PCB 77 and PCB 169 in the same concentration range as that used here [10]. It is likely that pharmacokinetic factors may be responsible for this disparity, since the embryos examined in the previous study were exposed for a time period which was 3 times as long as that used here.

In summary, this study describes an initial attempt to validate the use of avian hepatocyte cultures as a surrogate for *in ovo* injection studies to determine the sensitivities of various species of birds to HAHs. Although the data are preliminary and incomplete, the results indicate that HAH-mediated effects observed in avian embryo hepatocytes may mimic those observed after *in ovo* treatment. In terms of feasibility of assessing many species of birds (including those that are rare and endangered) for their sensitivities to HAHs and other pollutants, it is likely that culture and assay of avian hepatocytes may become the technique of choice.

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