

Ethoxyresorufin O-deethylase induction by TCDD, PeCDF and TCDF in ring-necked pheasant and Japanese quail hepatocytes: Time-dependent effects on concentration–response curves

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ABSTRACT

Ethoxyresorufin O-deethylase (EROD) activity was measured in primary cultures of ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) embryonic hepatocytes exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) for 12, 24, 36 and 48 h. In ring-necked pheasant hepatocytes there was a significant time-dependent increase in the EROD-inducing potency of TCDD, PeCDF and TCDF (i.e. decrease of the EC50). In Japanese quail hepatocytes there was no time-dependent change in the EROD-inducing potency of TCDD, PeCDF and TCDF. There was no time-dependent change in the relative potency of PeCDF and TCDF (i.e. compared to the potency of TCDD) in ring-necked pheasant hepatocytes and of PeCDF in Japanese quail hepatocytes. The results indicate that the relative potencies of these compounds at 24 h are representative of their relative potencies between 12 and 48 h. However, in Japanese quail hepatocytes, the relative potency of TCDF decreased in a time-dependent manner (up to 3.6-fold difference). These results suggest that the effect of time on the EROD-inducing potency of TCDD, PeCDF and TCDF in ring-necked pheasant and Japanese quail hepatocytes is compound- and species-specific, but experimental conditions could also be involved in the differences observed.

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1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) are environmentally persistent contaminants detected in abiotic and biotic samples in North America (Hebert et al., 1999; Kannan et al., 2008) and elsewhere (Fang et al., 2007; Turrio-Baldassarri et al., 2009). Because they bind to the aryl hydrocarbon receptor (AHR) and cause similar patterns of toxic and biochemical effects as those caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), PCDDs, PCDFs and some PCBs are referred to as dioxin-like compounds (DLCs). Subsequent to binding to the AHR, DLCs induce cytochrome P450 1A (CYP1A) enzymes (Mimura and Fujii-Kuriyama, 2003; Okey et al., 2005; Poland et al., 1976). CYP1A enzymes are enzymes of phase I metabolism involved in the oxidation of various endogenous and

exogenous substrates (Denison and Nagy, 2003; Nebert and Gonzalez, 1987). The metabolism of DLCs is influenced by their ring structure and the positioning of the chlorine atoms on their structures (Birnbaum, 1985). For example, chlorine in position 4 (adjacent to the oxygen atom) on PCDFs causes steric hindrance, thus interfering with metabolism (Brewster and Birnbaum, 1988). Indeed, the half-life of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is about 32-times longer than the half-life of 1,2,3,7,8-pentachlorodibenzofuran in rats (Brewster and Birnbaum, 1987, 1988). Differences in metabolism of DLCs were also observed in chicken (*Gallus gallus domesticus*) embryonic hepatocyte cultures. Between 24 and 72 h, there was a 90-fold decrease in the CYP1A-inducing potency of 3,3',4,4'-tetrachlorobiphenyl (PCB77), compared to a decrease lower than 10-fold for TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and PCB126 (Bastien et al., 1997). It was shown that the decrease in potency of PCB77 was due to the loss of the parent compound (Bastien et al., 1997).

To our knowledge, time-dependent effects on CYP1A-inducing potency of DLCs have not been assessed *in vitro* in hepatocytes from any avian species other than the chicken. Here we measured

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the induction of CYP1A catalytic activity (by use of the ethoxyresorufin *O*-deethylase (EROD) assay) in primary cultures of ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) embryonic hepatocytes exposed to TCDD, PeCDF or TCDF for 12, 24, 36 or 48 h. These species were chosen because they represent a range of sensitivity among avian species; while the chicken has a high sensitivity to DLCs, the ring-necked pheasant has a moderate sensitivity to DLCs and the Japanese quail has a low sensitivity to DLCs (Head et al., 2008). Most wild species have either a moderately or low sensitivity to DLCs (Head et al., 2008), therefore it is important to investigate time-dependent effects of DLCs on other species than chicken. This work is part of our on-going research to predict the sensitivity of avian species to the toxic effects of DLCs using *in vitro* approaches (Head et al., 2008; Herve et al., 2010; Kennedy et al., 1996, 2003; Sanderson et al., 1998).

2. Materials and methods

2.1. Source of eggs and incubation conditions

Fertile ring-necked pheasant and Japanese quail eggs were obtained from Couvoir Simetin (Mirabel, QC, Canada). Eggs from each species were incubated at 37.5 °C and 60% relative humidity until 1–3 days before hatch date. Ring-necked pheasant eggs were incubated for 23 days, and Japanese quail eggs were incubated for 16 days. Eggs were candled periodically and infertile eggs and eggs containing dead embryos were discarded.

2.2. Preparation and dosing of cultured hepatocytes

A pool of primary hepatocyte culture was prepared from avian embryos of each species using methods described elsewhere (Kennedy et al., 1995) including subsequent modifications (Head and Kennedy, 2007). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Reagents were obtained from Sigma (St. Louis, MO, USA) unless another supplier is indicated. In brief, embryos were decapitated and livers were removed, pooled and digested with collagenase. Pools of ring-necked pheasant and Japanese quail livers were prepared from 50 and 120 embryos, respectively. Percoll (Amersham Bioscience, Uppsala, Sweden) was used to separate erythrocytes from hepatocytes and DNase (0.02 mg/ml; Roche, Laval, QC, Canada) treatment was carried out to prevent cell clumping (Kennedy et al., 2003). The resulting cell pellet was weighed and suspended in a volume of Medium 199 equal to 32-times its mass. Cells were plated in 48-well plates by adding 25 µl of the cell suspension to 500 µl of medium to each well. Medium 199 was supplemented with insulin (1 µg/ml), and thyroxine (1 µg/ml). Cells were incubated for 24 h at 37 °C in a humidified incubator with 5% CO₂, allowing them to form a monolayer of non-proliferating cells attached to the bottom of the wells (70–80% confluence). Duplicate or triplicate plates were used for each compound at each time point. In each plate the hepatocytes were treated (2.5 µl of chemical/well in dimethyl sulphoxide (DMSO) – the medium was not changed) in triplicate with in-well concentrations of TCDD, PeCDF or TCDF ranging from 0.0003 to 10 nM (ring-necked pheasant) and 0.0003 to 30 nM (Japanese quail). In-well concentration of DMSO was 0.5% for each treatment. Cells were incubated for 12, 24, 36 or 48 h, medium was removed, plates were rinsed with phosphate buffered saline–ethylenediaminetetraacetic acid (PBS–EDTA; 200 µl/well), flash frozen on dry ice, and stored in a freezer (–80 °C) until they were analyzed.

2.3. Preparation of TCDD, PeCDF and TCDF solutions

A detailed description of the preparation of TCDD, PeCDF and TCDF solutions is provided elsewhere (Herve et al., 2010). In brief, serial dilutions of TCDD, PeCDF and TCDF were prepared from DMSO stock solutions with nominal concentrations between 650 and 1635 µg/ml. Identification and quantification of TCDD, PeCDF and TCDF in stock solutions were determined by isotope dilution following EPA method 1613 (US EPA, 1994) by use of high-resolution gas chromatography–mass spectrometry. The actual concentrations of the stock solutions were 1132, 1212 and 1706 nM for TCDD, PeCDF and TCDF, respectively.

2.4. EROD assays

EROD assays were conducted as described previously (Head et al., 2006; Kennedy et al., 1995). Reagents were obtained from Sigma unless another supplier is indicated. Briefly, hepatocytes were incubated at 37.5 °C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethoxyresorufin for 7 min. The reaction was stopped by the addition of cold acetonitrile containing fluorecamine (0.15 mg/ml). Resorufin and protein standard curves were prepared on each plate (Kennedy et al., 1995). Plates were analyzed for both EROD activity (excitation wavelength: 530 and emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm and emission wavelength: 460 nm) using a fluorescence plate-reader (Cytofluor 2350, Millipore, Bedford, MA, USA).

2.5. Cell viability

Cell viability was determined using the calcein–AM assay (Invitrogen – Molecular Probes, Eugene, OR, USA). Vehicle (DMSO)-treated cells were included as a positive control, and 99% ethanol-killed cells were used as a negative control. A working solution was prepared by addition of 3 µl of calcein–AM (1 mg/ml) to 10 ml of PBS–EDTA (in-well concentration: 0.3 µg/ml). The culture medium was removed and 200 µl of the calcein–AM solution was added to each well. Plates were incubated in the dark for 45 min and fluorescence was measured using a Cytofluor 2350 fluorescence plate-reader (Millipore, Billerica, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.6. Calculation of relative potency (ReP) values

The concept of using ReP values to compare the potencies of DLCs to that of TCDD is well established (Van den Berg et al., 1998). In this study, ReP values were calculated for each time point and are defined as: (EC50 of TCDD in species A at time point h) ÷ (EC50 of the compound of interest in species A at time point h).

2.7. Data analysis

Fluorescence data were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) for curve fitting. Concentration–response data were fit to a modified Gaussian curve as explained elsewhere (Kennedy et al., 1993). For each treatment, two or three EROD curves were generated from data originating from separate cell culture plates. EC50s and maximal values are presented as the mean values from two or three plates ± standard error.

Statistical differences among EC50 and maximal induction were tested using a one-way analysis of variance (ANOVA) with a Bonferroni correction. Significance was set at $p < 0.05$ for all tests.

3. Results

3.1. Shapes of EROD concentration–response curves

TCDD, PeCDF and TCDF increased EROD activity in a concentration-dependent manner. Maximal EROD activity was followed by a decrease in activity at greater concentrations (Fig. 1). This decrease was not caused by overt toxicity as measured by the calcein-AM assay (data not shown), but is likely caused by a competition between the substrate (ethoxyresorufin) and the inducer (DLC) for the CYP1A enzyme (Petrulis and Bunce, 1999).

3.2. Time-dependent effects on EROD EC50 values and relative potencies

3.2.1. Ring-necked pheasant

In ring-necked pheasant hepatocytes, the concentration–response curves were shifted to the left with exposure time for all three compounds (Fig. 1), and there was a statistically significant time-dependent decrease of the EC50s (Table 1). The EC50s decreased with exposure time (12 h compared to 48 h) from 0.13 to 0.019 nM for TCDD, 0.046 to 0.0064 nM for PeCDF, and 0.15 to 0.021 nM for TCDF (Table 1). Maximal EROD activity at 12 h for each compound was significantly lower than at the other time points (Table 1). The ReP values of PeCDF were greater than those of TCDD at all time points; they ranged from 1.9 to 3.0. In contrast, the ReP values of TCDF were lower than those of TCDD at all time points; they ranged from 0.4 to 0.9 for TCDF (Table 1).

3.2.2. Japanese quail

In Japanese quail hepatocytes, there was no statistically significant difference in EC50s or maximal responses among time points when exposed to TCDD, PeCDF or TCDF (Table 1 and Fig. 1). The ReP values of PeCDF were greater than those of TCDD at all time points;

they ranged from 7.2 to 14.9. In contrast, the ReP values of TCDF were either lower or greater than those of TCDD; they ranged from 0.5 to 1.8 (Table 1).

4. Discussion

The present study determined the concentration- and time-dependent effects of TCDD, PeCDF and TCDF on EROD activity in primary cultures of ring-necked pheasant and Japanese quail hepatocytes. Ring-necked pheasant hepatocytes were more sensitive (EC50 values were lower) than Japanese quail hepatocytes to EROD induction by TCDD, PeCDF and TCDF at 24 h, which is in agreement with a previous study (Herve et al., 2010). The EC50 values for the three compounds were also lower in ring-necked pheasant hepatocytes than in Japanese quail hepatocytes at 12, 36 and 48 h.

In ring-necked pheasant hepatocytes, there was a significant time-dependent increase in the potency (i.e. decrease of the EC50) of the three compounds, and at 12 h, the maximal EROD activity of all compounds was decreased compared to the other time points. For the three compounds, the EROD-inducing potency seemed to reach a maximum between 36 and 48 h (less than 1.5-fold change). This suggests that, in ring-necked pheasant hepatocytes, the compounds need about 36 h to reach their maximal EROD-inducing potency. The reason for this is not known, but it is likely that the CYP1A4 protein, which catalyzes the EROD reaction, reached its maximal induction after 36 h. In Japanese quail hepatocytes, there was no statistically significant time-dependent change in EROD-inducing potency (i.e. no change of EC50s) of TCDD, PeCDF or TCDF between 12 and 48 h. However, even if it was not statistically significant, there was a time-dependent decrease in the EROD-inducing potency (i.e. increase in the EC50s) of TCDF. The EC50 of TCDF was 1.5- and 4.1-fold greater at 36 and 48 h than at 24 h, respectively. This trend suggests that in Japanese quail hepatocytes exposed to TCDF, there could be a loss of

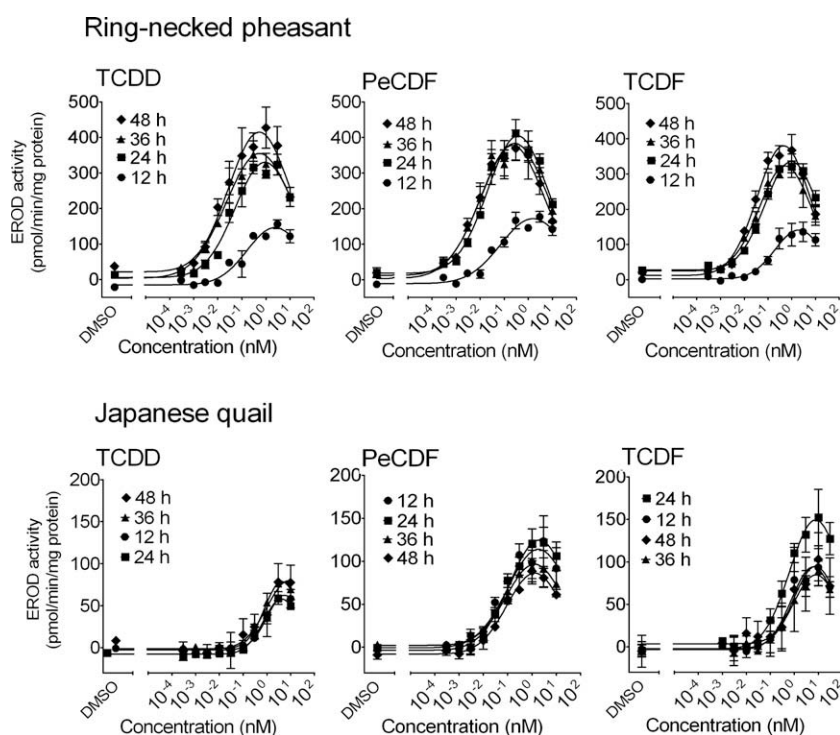


Fig. 1. EROD concentration–response curves for ring-necked pheasant and Japanese quail hepatocytes exposed to TCDD, PeCDF, and TCDF, for 12 h (●), 24 h (■), 36 h (▲), or 48 h (◆). Points represent mean EROD activity from two or three replicate cell culture plates; values before the axis break indicate EROD activity observed for control (DMSO-treated hepatocytes). Bars represent standard errors.

Table 1
EC50 values, maximal responses and relative potency values (RePs) for EROD data obtained in ring-necked pheasant (R-n pheasant) and Japanese quail (J.quail) hepatocyte cultures exposed to TCDD, PeCDF, or TCDF for 12, 24, 36, and 48 h.

Species	Chemical	Time point (h)	EC50 (nM)	Max EROD activity (pmol/min/mg protein)	RePs
R-n pheasant	TCDD	12	0.13 ± 0.06 ^a	150 ± 13 ^a	1.0
R-n pheasant	TCDD	24	0.036 ± 0.006 ^{a,b}	332 ± 23 ^b	1.0
R-n pheasant	TCDD	36	0.013 ± 0.003 ^b	354 ± 32 ^b	1.0
R-n pheasant	TCDD	48	0.019 ± 0.004 ^b	417 ± 61 ^b	1.0
R-n pheasant	PeCDF	12	0.046 ± 0.01 ^a	173 ± 14 ^a	2.8
R-n pheasant	PeCDF	24	0.013 ± 0.002 ^b	406 ± 29 ^b	2.8
R-n pheasant	PeCDF	36	0.0068 ± 0.001 ^b	385 ± 39 ^b	1.9
R-n pheasant	PeCDF	48	0.0064 ± 0.0006 ^b	378 ± 47 ^b	3.0
R-n pheasant	TCDF	12	0.15 ± 0.04 ^a	143 ± 30 ^a	0.9
R-n pheasant	TCDF	24	0.050 ± 0.009 ^b	337 ± 12 ^b	0.7
R-n pheasant	TCDF	36	0.029 ± 0.002 ^{b,c}	327 ± 28 ^b	0.4
R-n pheasant	TCDF	48	0.021 ± 0.002 ^c	370 ± 23 ^b	0.9
J.quail	TCDD	12	0.79 ± 0.05 ^a	63 ± 10 ^a	1.0
J.quail	TCDD	24	0.53 ± 0.005 ^a	121 ± 51 ^a	1.0
J.quail	TCDD	36	0.60 ± 0.08 ^a	79 ± 4 ^a	1.0
J.quail	TCDD	48	0.88 ± 0.4 ^a	83 ± 16 ^a	1.0
J.quail	PeCDF	12	0.068 ± 0.02 ^a	117 ± 20 ^a	11.6
J.quail	PeCDF	24	0.074 ± 0.007 ^a	125 ± 16 ^a	7.2
J.quail	PeCDF	36	0.055 ± 0.004 ^a	96 ± 20 ^a	10.9
J.quail	PeCDF	48	0.059 ± 0.004 ^a	86 ± 9 ^a	14.9
J.quail	TCDF	12	0.43 ± 0.1 ^a	90 ± 13 ^a	1.8
J.quail	TCDF	24	0.42 ± 0.04 ^a	150 ± 25 ^a	1.3
J.quail	TCDF	36	0.65 ± 0.1 ^a	86 ± 11 ^a	0.9
J.quail	TCDF	48	1.73 ± 0.87 ^a	103 ± 32 ^a	0.5

Note: Means are derived from two or three replicate cell culture plates ± standard error. Superscript letters indicate significant differences among time points ($p < 0.05$) within each treatment. EROD data were fit to a modified Gaussian curve.

the parent compound with time. This is in agreement with what was observed in Japanese quail embryos injected with TCDF (unpublished data). In chicken hepatocytes, Bastien et al. (1997) observed a time-dependent decrease in the potency of TCDD (2.2-fold) and TCDF (2.9-fold) between 24 and 48 h. Compound- and species-specific differences in metabolism (Bastien et al., 1997; Murk et al., 1994) could explain the difference seen within and among the two studies. Differences in experimental conditions, such as the uptake of the compound by the cell or medium or compound attachment to the plastic dish, could also be partly responsible for the variations observed in time-dependent effects on the EROD-inducing potency (Bastien et al., 1997).

In vitro ReP values are usually measured after 24 h of exposure (Herve et al., 2010; Kennedy et al., 1996; Sanderson et al., 1998). It was of interest to investigate if the ReP value of PeCDF and TCDF at 24 h was similar to the ReP values at 12, 36 and 48 h. In ring-necked pheasant hepatocytes exposed to PeCDF or TCDF and in Japanese quail hepatocytes exposed to PeCDF, the variation in ReP values was lower than 2.5-fold and was not time-dependent. This indicates that ReP values at 24 h are representative of ReP values between 12 and 48 h. In ring-necked pheasant hepatocytes, ReP values were 1.9–3.0 for PeCDF and 0.4–0.9 for TCDF, which is in agreement with what was observed in a previous study (Herve et al., 2010). In Japanese quail hepatocytes, ReP values of PeCDF were 7.2–14.9, which had also been observed before (Herve et al., 2010). Unlike PeCDF and TCDF in ring-necked pheasant hepatocytes and PeCDF in Japanese quail hepatocytes, there was a time-dependent decrease in the ReP values of TCDF in Japanese quail hepatocytes. This is in agreement with the observation that TCDF has a shorter half-life than TCDD in other species (Birnbaum et al., 1980; Rose et al., 1976). However, in chicken hepatocytes, the relative potency of TCDF stayed stable (less than 1.5-fold change) between 24 and 72 h. In this study, ReP values of TCDF in Japanese quail hepatocytes were 0.5–1.8, while an earlier study reported ReP values between 0.1 and 0.3 (Herve et al., 2010). The

reason for this difference is not known, but it could be due to differences in the sensitivity of the embryos used in the two different studies or to experimental conditions.

Longer exposure times could allow observation of greater changes in the potency of DLCs and measurement of the parent compounds and metabolites would add information about metabolism. Greater variation in the ReP values could also be observed if the compounds were not metabolised at the same rate. However, longer exposure times would necessitate the replacement of the medium due to depletion of nutrients over time. The distribution of the compound and metabolites in the cells, in the medium, and on the plastic of the well is in continuous change and replacing the medium would modify this dynamic. For this reason, careful design of experiments with prolonged exposure time in primary cultures of avian hepatocytes is required.

In this study time-dependent effects on the EROD-inducing potency of DLCs were determined to be both species- and compound-specific. The results of the present study and those of a previous study (Bastien et al., 1997) indicate that relative potencies of certain compounds, in certain species, can vary as a function of exposure time. Therefore, when measuring relative potencies of DLCs, the exposure time has to be selected carefully. It has been shown that the relative EROD-inducing potencies of DLCs in primary cultures of avian hepatocytes exposed for 24 h is predictive of their potency to cause embryoletality (Head and Kennedy, 2010; Kennedy et al., 1996). Therefore, a 24 h exposure time appears to be an appropriate time point to measure relative potencies.

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