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Correlation between an in vitro and an in vivo measure of dioxin sensitivity in birds

Jessica A. Head · Sean W. Kennedy

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Abstract We describe a statistically significant correlation between two well-characterized responses to dioxin-like compounds in birds; induction of 7-ethoxyresorufin-*O*-deethylase (EROD) activity in cultured hepatocytes, and embryo mortality. Data were obtained from a review of the literature. EROD EC50 values for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 6 polychlorinated biphenyls (PCBs) were strongly correlated with LD50 values in chicken embryos ($r^2 = 0.93$, $P < 0.005$). Similarly, EROD EC50 values for TCDD and a potent dioxin-like compound, PCB 126, were correlated with embryonic LD50 values in different species of birds (chicken, ring-necked pheasant, turkey, double-crested cormorant, and common tern) ($r^2 = 0.92$, $P < 0.005$). Our findings contribute to a developing understanding of the molecular basis for differential dioxin sensitivity in birds, and validate the EROD bioassay as a useful predictive tool for ecological risk assessment.

Keywords EROD · Egg injection · Avian · Dioxin-like compound · EC50 · LD50

J. A. Head (✉)
Cooperative Institute for Limnology and Ecosystem Research, University of Michigan and NOAA's Great Lakes Environmental Research Laboratory, Ann Arbor, MI, USA
e-mail: jesshead@umich.edu

S. W. Kennedy
National Wildlife Research Centre, Environment Canada, Ottawa, ON, Canada

S. W. Kennedy
Department of Biology, Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Introduction

Dioxin-like compounds are lipophilic environmental contaminants that are toxic to most vertebrates. Sensitivity to the adverse effects of dioxin-like compounds can be extremely variable among species. This variability poses a challenge for ecological risk assessment since it is not practical to carry out toxicity testing in many species of wildlife. Consequently, a focus of our research has been the development of biochemical markers of dioxin sensitivity in avian models (Head et al. 2008; Karchner et al. 2000; Karchner et al. 2006; Kennedy et al. 1996a).

Toxicological effects of dioxin-like compounds occur through the aryl hydrocarbon receptor (AHR), a ligand-activated nuclear transcription factor. AHR activation results in a number of biochemical changes in the cell, including induction of cytochrome P4501A enzymes, commonly measured as 7-ethoxyresorufin-*O*-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) activity. Cytochrome P4501A induction is not generally considered to be overtly toxic in itself, but as an indicator of AHR activation it can be a powerful biomarker of both exposure to and effects of dioxin-like compounds (Okey et al. 2005).

Several examples of associations between biochemical aspects of the AHR response pathway and toxicological measures of dioxin sensitivity have been cited in the rodent literature. For example, it has long been known that the structure of a dioxin-like compound and the affinity with which it binds to the AHR is related to its toxic potency (Poland and Knutson 1982; Safe 1990). Experiments using the H4IIE rat hepatoma cell line demonstrate that −log EC50 values for induction of AHR-mediated AHH activity in vitro are correlated with −log ED50 values for in vivo endpoints such as AHH induction, body

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weight loss and thymic atrophy in rats (Safe 1987; Safe 1990). This finding has been useful for risk assessment because it establishes that the in vivo potency of a compound can be estimated using a high-throughput in vitro assay. EC50 values from the H4IIE assay have contributed to the derivation of mammalian toxic equivalency factors (TEFs) for a number of less commonly studied dioxin-like compounds (Van den Berg et al. 1998; Van den Berg et al. 2006).

In avian species, biochemical effects of dioxin-like compounds have been studied using a primary embryo hepatocyte culture model. EROD EC50 values assessed in cultured hepatocytes are available for over 30 dioxin-like compounds in chicken, and in 10 species of wild and domestic birds (Bosveld 1995; Kennedy et al. 1996a; Kennedy et al. 2003; Kennedy et al. 1996b; Lorenzen et al. 1997; Sanderson et al. 1998). These data have contributed to the derivation of avian-specific TEFs under the assumption that, analogous to the rat model, the in vivo potency of a compound can be predicted by its in vitro potency in birds (Van den Berg et al. 1998). Similarly, it has been hypothesized that EROD EC50 values might be useful for predicting in vivo dioxin sensitivity for individual species of birds (Kennedy et al. 1996a). More recently, molecular studies have provided a mechanistic basis for this hypothesis by identifying an association between the genetic sequence of the avian AHR and species differences in biochemical and toxicological responses to dioxin-like compounds (Head et al. 2008; Karchner et al. 2006).

In spite of important applications to ecological risk assessment, the perceived association between biochemical and toxicological measures of dioxin sensitivity has never been tested in birds. Here, we review and analyse previously published data in order to evaluate statistical correlations between EROD EC50 and LD50 values for multiple compounds and in multiple species. To our knowledge, this is the first time such an analysis has been undertaken for birds and the first analysis to consider the relationship between EROD EC50 and LD50 among species. This work has important implications for ecological risk assessment and contributes to an emerging picture of mechanisms underlying variation in dioxin sensitivity in birds.

Data and methods

Data selection: LD50

Many studies report on toxicological effects of dioxin-like compounds in birds (reviewed in Barron et al. 1995; Bosveld and Van den Berg 1994; Hoffman et al. 1996). Experiments can vary with respect to contaminant, endpoint, developmental stage, route of exposure, and duration of exposure. Because it is difficult to compare data generated using different experimental approaches we only included mortality data derived from laboratory-based egg injection studies in our analysis. Experimental constraints involved in working with eggs favor a comparative approach because the timing and duration of the exposure are tied to the developmental stage of the embryo (since most exposures start early in development and are carried through until hatching). Moreover, the pool of data describing mortality in avian embryos resulting from egg injection is relatively large.

LD50 data were used to evaluate the sensitivity of chicken embryos to a variety of dioxin-like compounds. Chicken embryo mortality data for TCDD and 6 PCB congeners were taken from a series of experiments performed by Brunström and colleagues (Brunström 1990; Brunström and Andersson 1988; Brunström and Halldin 1998). Although embryonic LD50 values from other groups are available we chose to use this data set exclusively in order to reduce error associated with methodological differences between experiments. The PCB congeners were; 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105), 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169), 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 156), and 2,3,3′,4,4′,5′-hexachlorobiphenyl (PCB 157). In each study, the test compound was injected into the air cell of fertilized eggs on day 7 of incubation and embryos were monitored for 72 h following exposure.

We also evaluated the embryonic lethality of dioxin-like compounds in different species of birds. Due to limited data availability, this inter-species analysis involved comparison among studies with different, but similar, protocols. Relevant data from each study are summarized in Head et al. (2008). Source studies varied in some aspects of experimental design, but each had the following elements in common; (1) fertilized avian eggs were injected with either TCDD or PCB 126, (2) injection occurred during the first third of the incubation period, and (3) embryos were monitored until at least 1 day pre-hatch. All available LD50 data which met these criteria, and for which corresponding EROD EC50 values existed, were included in our analysis (the data used for the multi-compound analysis were not included because they do not meet criterion 3). For chickens, multiple egg injection studies met the above criteria, and so we used chicken LD50 mean reference values for TCDD and PCB 126 as defined in Head et al. (2008). For turkeys, the LD50 value was obtained from linear interpolation between the lowest observable adverse effect level (LOAEL), and the dose causing 100% mortality as reported by Brunström and colleagues (Brunström 1989).

Data selection: EROD EC50

EROD EC50 data were taken from a series of experiments performed in avian embryo hepatocyte cultures (Bosveld 1995; Kennedy et al. 1996a; Kennedy et al. 1996b; Lorenzen et al. 1997; Sanderson et al. 1998). All available EROD EC50 data with corresponding LD50 values were included with one exception: representative EROD EC50 values for TCDD and PCB 126 in chicken embryo hepatocytes were used (see below for details). Many aspects of experimental design such as the method of preparing cell cultures, the volume of solution used to dose cells, and the duration of exposure to test compounds were the same in all of the experiments reviewed. Two important exceptions are noted. First, the carrier solvent for the test compound was dimethyl sulfoxide (DMSO) for all species except for double-crested cormorant where isooctane was used. The EROD EC50 data presented for double-crested cormorant represent DMSO-normalized values as presented by the authors of the original study (Sanderson et al. 1998). Second, birds were euthanized at various developmental stages. Chickens, ring-necked pheasants, and turkeys were euthanized at 1–3 days pre-hatch (Kennedy et al. 1996a). Double-crested cormorants were euthanized within 24 h of hatching (Sanderson et al. 1998), and common terns were euthanized within 24 h of hatching (Bosveld 1995), or at 2–5 days pre-hatch (Lorenzen et al. 1997). This variability in experimental design was not ideal for comparisons between studies; EROD EC50 values have been shown to be higher in hepatocytes cultured from older embryos or hatchlings (Bosveld et al. 1997). We nevertheless chose to include raw data from each study, in part because variability between experiments appears to be more important than variability related to difference in developmental stage (Bosveld et al. 1997; Bosveld 1995). A more accurate predictive model would require multi-species EROD EC50 data from a single developmental stage.

EROD EC50 values for TCDD or PCB 126 in chicken embryo hepatocytes were presented in many of the studies referenced in this paper. We chose to use representative values taken from a single study (Kennedy et al. 1996a) because these data were the most consistent in addressing the variations in methodology noted above; i.e. DMSO was used as the vehicle, embryos were euthanized at an intermediate developmental stage (1 day pre-hatch), and hepatocytes were cultured from a large pool of embryos with multiple replicates analyzed. Moreover, these values were very close to mean values calculated by taking data from all referenced papers into consideration.

Analysis

LD50 values from each study were converted to molar concentrations of test chemical and expressed as nmol/kg egg. Pearson correlation was used to evaluate the relationship between log EROD EC50 and log LD50 values ($P < 0.005$). All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

Results and discussion

The ability to predict in vivo sensitivity to environmental contaminants based on molecular parameters is valuable for ecological risk assessment. Here, we provide evidence that a well-characterized biochemical measure of the potency of dioxin-like compounds (EROD EC50 in hepatocyte cultures) is significantly correlated with a toxicological measure of dioxin sensitivity (LD50) in birds.

A survey of the literature produced EROD EC50 and embryonic LD50 values for 7 dioxin-like compounds in chicken. A highly significant correlation between log EROD EC50 values and log LD50 values was observed for TCDD and 6 PCB congeners (Fig. 1, $r^2 = 0.93$, $P < 0.005$). A similar result has been reported previously for the H4IIE rat hematoma cell line. Linear correlations between −log AHH EC50 values assessed in H4IIE cells and in vivo measures of dioxin sensitivity (thymic atrophy ($r = 0.92$), and body weight loss ($r = 0.93$)) were observed for 22 dioxin-like compounds. Cytochrome P4501A-based bioassays have also been used to estimate the potency of dioxin-like compounds in fish models (Clemons et al. 1994; Hahn et al. 1996; Henry

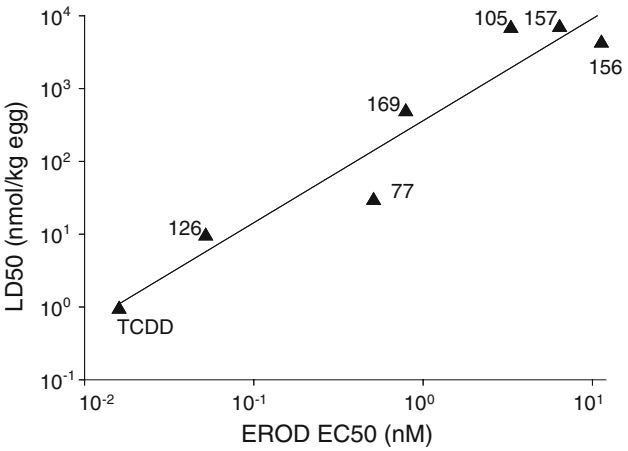

Fig. 1 Relationship between in vitro and in vivo potency of TCDD and 6 PCB congeners in chicken. EROD EC50 values for each compound were determined in chicken embryo hepatocyte cultures (Kennedy et al. 1996a; Kennedy et al. 1996b). LD50 values were assessed in chicken embryos over 72 h of exposure starting on embryonic day 7 (Brunström 1990; Brunström and Andersson 1988; Brunström and Halldin 1998). EROD EC50 values were significantly correlated with LD50 values ($r^2 = 0.93$, $P < 0.005$)

et al. 2001). In rainbow trout, TEFs derived from EC50 values for cytochrome P4501A mRNA induction in a gonadal cell line were correlated with TEFs derived from early life stage mortality ($r^2 = 0.66$) (Zabel et al. 1996).

Based on previous findings (Kennedy et al. 1996a), we hypothesized that the relationship between EROD EC50 and LD50 that was observed for different compounds (Fig. 1) would also apply to differences in sensitivity among species. Because the amount of data available for any single compound was insufficient for testing this hypothesis, we included results for two different test compounds, TCDD and PCB 126, in our analysis. Log EROD EC50 values for TCDD and PCB 126 were significantly correlated with log LD50 values in different species of birds (Fig. 2, $r^2 = 0.92$, $P < 0.005$). TCDD and PCB 126 are both potent dioxin-like compounds that are resistant to metabolism. Based on results from the rat H4IIE cell line, more readily metabolized compounds might not be expected to exhibit the same linear correlation (Safe 1987). Further analysis of the relationship between EROD EC50 and LD50 among species will require additional data for multiple compounds in multiple species of birds. It remains to be seen whether the correlation we observed would be replicated for a single test compound in multiple species, or for other less potent dioxin-like chemicals.

Our analysis of inter-species variability in dioxin sensitivity may have been affected by the quality of the available data as well as the quantity. Some of the LD50 values reported in Fig. 2 were affected by high mortality in the vehicle group or incomplete dose–response curves (Brunström 1989; Nosek et al. 1993; Powell et al. 1998). EROD data were not always reproducible as demonstrated by the almost 4-fold difference in EC50 values for PCB 126 in two common tern studies (Table 1). Furthermore, differences in design between experiments made direct comparisons somewhat problematic (see Data and methods section). In spite of these limitations, EROD EC50 appeared to be a strong predictor of LD50 over the large range of dioxin sensitivities observed. Improvements to the EROD bioassay and egg injection techniques may lead to more reproducible values, refinements of the model, and novel insights.

Our findings establish a correlation between EROD EC50 values for dioxin-like compounds assessed in cultured cells, and LD50 values assessed by egg injection experiments in birds. This finding validates the EROD bioassay as a useful method for evaluating the relative sensitivity of dioxin-like compounds in avian species, reducing the need for extensive in vivo toxicity testing. Furthermore, by establishing a correlation between an AHR-mediated biochemical endpoint (EROD) and in vivo toxicity, our results add weight-of-evidence to an emerging

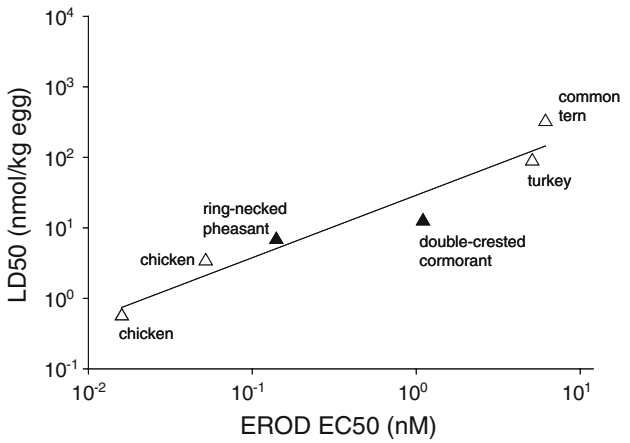

Fig. 2 Relationship between in vitro and in vivo potency of dioxin-like compounds in avian species. EROD EC50 and LD50 values for TCDD (*filled triangle*) or PCB 126 (*open triangle*) in five avian species are shown. EROD EC50 values were determined in hepatocytes cultured from each species. LD50 values were derived from egg injection studies which injected the test compound within the first third of incubation and monitored the embryos until at least 1 day pre-hatch. Source data and references are presented in Table 1. EROD EC50 values were significantly correlated with LD50 values for TCDD and PCB 126 in multiple species ($r^2 = 0.92$, $P < 0.005$)

Table 1 In vitro and in vivo measures of species sensitivity to TCDD and PCB 126 (references for Fig. 2)

Species	Compound	EROD EC50 (nM)	LD50	
			µg/kg egg	nmol/kg egg
Chicken	TCDD	0.016[a]	0.18[e]	0.56
Ring-necked pheasant	TCDD	0.14[a]	2.2[f]	6.8
Double-crested cormorant	TCDD	1.1[b,j]	4[g]	12.4
Chicken	PCB 126	0.052[a]	1.1[e]	3.4
Turkey	PCB 126	5.1[a]	28.75[h,l]	88.1
Common tern	PCB 126	2.5[c], 9.8[d,k]	104[i]	318.6

[a] Kennedy et al. (1996a)
[b] Sanderson et al. (1998)
[c] Bosveld (1995)
[d] Lorenzen et al. (1997)
[e] Head et al. (2008)
[f] Nosek et al. (1993)
[g] Powell et al. (1998)
[h] Brunström (1989)
[i] Hoffman et al. (1998)
[j] Normalized to account for different vehicle
[k] Mean of two common tern values is presented in Fig. 2
[l] Turkey LD50 value was derived by linear interpolation between the LOAEL (20 µg/kg; 36% mortality), and the LD100 (60 µg/kg; 100% mortality)

picture of the importance of AHR activation in differential biochemical responses to dioxin-like compounds in birds (Head et al. 2008; Karchner et al. 2006).

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