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Time-dependent transcriptomic and biochemical responses of 6-formylindolo[3,2-b]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are explained by AHR activation time

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ABSTRACT

6-Formylindolo[3,2-b]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are ligands of the aryl hydrocarbon receptor (AHR) and bind to the AHR with high affinity. Until recently, TCDD was considered to be the most potent AHR agonist, but several recent studies indicate that FICZ binds with greater affinity to the AHR than TCDD. To advance our understanding of the similarities and differences of the effects of FICZ and TCDD exposure in chicken embryo hepatocyte (CEH) cultures, we compared relative expression changes of 27 dioxin-responsive genes by the use of a chicken PCR array, porphyrin accumulation and ethoxyresorufin-*O*-deethylase (EROD) activity at different time points. In addition, an egg injection study was performed to assess the effects of FICZ on the developing chicken embryo. The results of the current study showed: (1) mean EROD-derived relative potency values for FICZ compared to TCDD changed as a function of time (i.e. 9, 0.004, 0.0008 and 0.00008 at 3, 8, 24, and 48 h, respectively) in CEH cultures; (2) FICZ exposure did not result in porphyrin accumulation in CEH cultures; (3) concordance between gene expression profiles for FICZ and TCDD was time- and concentration-dependent, and (4) no mortality or morphological abnormalities were observed in chicken embryos injected with 0.87 ng FICZ/g egg into the air cell. The results presented herein suggest that while FICZ and TCDD share similar molecular targets, transient versus sustained AHR activation by FICZ and TCDD result in differential transcriptomic responses. Moreover, rapid metabolism of FICZ in hepatocytes resulted in a significant decrease in the induction of EROD activity.

1. Introduction

The aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of receptors, mediates numerous toxic and biological effects subsequent to activation by a variety of exogenous and endogenous ligands [38,5]. 6-Formylindolo[3,2-b]carbazole (FICZ), a derivative of tryptophan, is an endogenous ligand of the AHR that binds to the receptor with high affinity, initiates AHR signaling at very low concentrations and is metabolized readily to inactive metabolites [44,57,48]. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental contaminant and carcinogen [19], is one of the most potent and studied ligands of the AHR. Unlike FICZ, TCDD and other dioxin-like compounds (DLCs) are metabolized very slowly [18,39,58]. Interestingly, FICZ has a higher AHR binding affinity than TCDD [44,45] and, in cells transiently transfected with avian AHRs, it induces AHR-mediated luciferase reporter gene activity with greater potency than TCDD (41- to 1534-fold more potent; [11]).

There are large differences in sensitivity of species to DLCs, and recent studies indicate that the sensitivity of avian species to toxic and biochemical effects of DLCs is associated with the structure of the AHR1 isoform, and more specifically, with the identity of amino acids at positions 324 and 380 within the ligand-binding domain (LBD) [13,10,12,25,32,60]. In contrast, avian AHR1 responsiveness to FICZ does not appear to be associated with the identity of amino acids at positions 324 and 380, suggesting a novel mode of interaction of FICZ with the avian AHR1 LBD that is distinct from the binding mode of the well-characterized DLC ligands [11]. Previous studies also reported different transcriptional properties and distinct effects for FICZ and TCDD [30,47,50,54]. For example, FICZ and TCDD exhibited distinct immuno modulatory properties [47,50].

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With the goal of advancing understanding of the differences and similarities between the effects of FICZ and TCDD in birds, we present the results of experiments conducted at the whole animal, biochemical and transcriptomic levels. The goals of this study were (1) to calculate the relative potency of FICZ at different time-points in chicken (*Gallus domesticus*) embryo hepatocyte (CEH) cultures by the use of the ethoxyresorufin-*O*-deethylase (EROD) assay, (2) to compare FICZ- and TCDD-induced porphyrin accumulation in CEH cultures, (3) to compare gene expression changes in CEH cultures exposed to FICZ and TCDD for 3 and 24 h by the use of a customized chicken polymerase chain reaction (PCR) array containing 27 TCDD-responsive genes, and (4) to determine *in ovo* toxicity of FICZ.

2. Materials and methods

2.1. Preparation of FICZ and TCDD solutions

A detailed description of the preparation of FICZ (Enzo Life Science, Farmingdale, NY, USA; 95% purity) and TCDD (a gift from Dow Chemical Co., Midland, Michigan, USA) solutions is provided elsewhere [11,16]. Serial dilutions of FICZ and TCDD were prepared in dimethyl sulfoxide (DMSO).

2.2. Hepatocyte culture and dosing

A detailed description for preparing primary hepatocyte cultures from 19-day-old chicken embryos is described elsewhere [28]. In brief, embryos were euthanized by decapitation, and livers were pooled and digested with collagenase (Sigma–Aldrich, St. Louis, MO, USA). Hepatocytes were separated from erythrocytes by the use of Percoll (Amersham Bioscience, Uppsala, Sweden) density gradient centrifugation, and DNAse (Roche, Laval, Quebec, Canada) treatment was performed to avoid cell clumping. Cells were plated in 48-well culture plates in Medium 199 (Sigma–Aldrich) at 37 °C under 5% CO_2 for 24 h. For ethoxyresorufin-*O*-deethylase (EROD) activity and porphyrin accumulation assays, CEH cultures were treated in triplicate with DMSO (0.5% final concentration) or DMSO solutions of FICZ (ranging from 0.000001 to 1000 nM) or TCDD (ranging from 0.00001 to 100 nM). Following various incubation times (1, 3, 8, 24, or 48 h), medium was removed, plates were flash frozen on dry ice, and stored at −80 °C until analysis. Plates used for the EROD assay were rinsed with PBS–EDTA (200 μl/well) before they were flash frozen on dry ice.

2.3. Cell viability

ViaLight® Plus kits (Lonza Rockland Inc., Rockland, ME, UK) were used, according to the manufacturer's instructions, to compare the cell viability of CEH treated with FICZ or TCDD with the viability of untreated cells (used as a live cell control) and sodium hypochlorite (5%)-treated cells (used as a dead cell control). This assay is based upon the bioluminescent measurement of adenosine triphosphate (ATP) that is present in all metabolically active cells. Luciferase was utilized in this method to catalyze the formation of light from ATP and luciferin. CEH were lysed 24 h after dosing and the luminescence emitted from the ATP-dependent oxidation of luciferin was measured with a LuminoSkan Ascent luminometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4. EROD assay and porphyrin analysis

EROD activity and total porphyrin concentrations were determined as described previously [27]. Triplicate plates, containing three replicate wells of each FICZ or TCDD concentration, were analyzed. All reagents were obtained from Sigma–Aldrich (Oakville, Ontario, Canada). Briefly, hepatocytes were incubated at 37.5 °C in the presence of nicotinamide adenine dinucleotide phosphate (reduced) and 7-ethyoxyresorufin. EROD reactions were stopped after 7 min by the addition of cold acetonitrile containing fluorescamine. Resorufin and protein standard curves were prepared for each plate. Plates were analyzed for both EROD activity (excitation wavelength: 530 nm, emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm, emission wavelength 460 nm) using a Cytofluor 2300 fluorescence plate reader (Millipore, Bedford, MA). EROD results are expressed as pmol/min/mg protein.

Total porphyrin accumulation was measured shortly after the plates were analyzed for protein and resorufin concentrations according to a previously described method [27]. Briefly, hydrochloric acid (3N; 500 μl) was added to each well, and the plates were incubated for 30 min. The plates were scanned with a 400 nm excitation filter and a 645 nm emission filter with the Cytofluor 2300 fluorescence plate reader. Total porphyrin concentration is expressed as pmol/mg protein.

2.5. RNA isolation and complementary DNA synthesis

Total RNA was extracted from CEH using RNeasy 96 kits according to the manufacturer's protocol (Qiagen, Mississauga, ON), including the on-column genomic DNA contamination elimination step. Approximately 120 ng of the extracted RNA was reverse-transcribed using QuantiTect Reverse Transcription kits (Qiagen) according to the manufacturer's instructions with minor modifications described elsewhere [41].

2.6. Microarray data for TCDD-exposed hepatocytes

Microarray data for TCDD-exposed hepatocytes were generated in a previously described experiment [37], and can be downloaded from the NCBI Gene Expression Omnibus (GEO, accession number GSE33291). Briefly, cultured CEH cultures were exposed to a DMSO solvent control, 0.03 nM TCDD, or 1 nM TCDD (n = 5 per group) for 24 h. Complementary RNA (cRNA) was prepared from total RNA and labeled with cyanine 5 (Cy5). The cRNA was hybridized to Agilent 4 × 44 k chicken whole-genome arrays (design 015068; Agilent Technologies) against a reference pool of cRNA labeled with Cy3. After hybridization, arrays were scanned on an AgilentG2505B Microarray Scanner System (Agilent Technologies). Data were acquired using Agilent Feature Extraction software version 9.5.3.1.

Differentially expressed probes were identified with a blocked reference design using the MAANOVA package in R (http://www.R-project.org) as described elsewhere [37]. Briefly, signal intensities were normalized by the LOWESS method, the least-squares means were used to estimate the fold change for each pairwise comparison and p-values were estimated using the permutation method (30,000 permutations) followed by a false discovery rate (FDR) adjustment for multiple comparisons by the Benjamini and Hochberg Method [2].

2.7. PCR array

A custom-designed Profiler RT^2 PCR Array was built to our specifications by SABiosciences (Qiagen; CAPG12083) to analyze the expression of TCDD-responsive genes. Based on the microarray analysis (described above), a total of 27 TCDD-responsive genes were selected for inclusion on the PCR array. Only genes that met the following criteria in the high concentration treatment group (1 nM TCDD) were selected: (1) fold change (FC) > 2 and FDR p < 0.01 (24 genes), or (2) FC > 1.8, FDR p < 0.01 using the biomarker filter tool in Ingenuity Pathways Analysis (IPA; 3 genes).