COMPARATIVE GENE EXPRESSION ANALYSIS OF TCDD-, 4-PeCDF- AND TCDF-TREATED PRIMARY RAT AND HUMAN HEPATOCYTES

J. Craig Rowlands[1], Robert Budinsky[1], Bhaskar Gollapudi[1], Darrell Boverhof[1], Stephen Ferguson[3], Raymond F. Novak[2], Daniela Cukovic[2], Sridevi Salagrama[2], Alan Dombkowski[2]

[1]Toxicology and Environmental Research & Consulting, The Dow Chemical Company, Midland, MI; [2]Institute of Environmental Health Sciences, Wayne State University, Detroit, MI; [3]CellzDirect, Inc., Pittsboro, NC

Abstract

Primary human and rat hepatocytes were used as models to explore species- and congener-specific differences in gene expression profiles after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) at the EC_{50}'s for CYP1A1 mRNA induction. The results indicate that at equipotent doses significantly more genes (>2X) were altered in rat cells compared to human cells and there was little overlap between the genes altered by TCDD when compared to those altered by the furans. Moreover, only a very small number of common genes were altered between rat and human cells treated with TCDD (5 genes), TCDF (6 genes) or 4-PeCDF (5 genes) and consisted largely of well characterized AHR core battery genes. These results indicate that (1) TCDD and furans induce significantly different gene expression patterns in both humans and rats, and (2) significant differences exist in the response of rats vs. humans to TCDD and other dioxin-like chemicals.

Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the prototype for a class of environmental halogenated aromatic hydrocarbon (HAH) pollutants known as dioxin-like chemicals (DLCs) that include seven structurally related polychlorinated dibenzo-p-dioxins (PCDDs) and ten dibenzofurans (PCDFs). Given the complexity of performing a risk assessment on environmental mixtures of these PCDDs and PCDFs, regulatory authorities around the globe have adopted the toxic equivalency factor (TEF) approach for risk assessment. A TEF is the potency for a given DLC relative to TCDD and is determined from DLC studies of numerous different endpoints from multiple *in vitro* and *in vivo* experiments in different species.[10] The TEF approach is founded on the assumption that nearly all of the biological effects of DLCs are mediated through a common mode of action (MOA) that involves binding to and activation of the aryl hydrocarbon receptor (AHR) and this MOA is conserved between tissues and species. It is assumed that the effects of individual DLCs are additive with parallel dose response curves, and that AHR activation occurs in the same way leading to identical downstream biochemical responses.

The DLC, 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF) receives much attention from scientists and regulatory agencies due to its potential to bioaccumulate and its relative potency to TCDD. 4-PeCDF, with a recently adjusted TEF of 0.3,[10] can significantly contribute to the total toxic equivalents (TEQs) measured in many environmental and biomonitoring samples including background blood TEQ concentrations.[4,8] Furthermore, the interpretation of toxicological data are complicated by the tendency of 4-PeCDF to be dose-dependently sequestered in the liver and possibly other tissues by CYP1A2, an AHR regulated cytochrome P450 enzyme.[1,5,10] An accurate TEF estimate for 4-PeCDF is of major importance given the potentially significant role it can play in decisions related to environmental remediation initiatives. Unlike 4-PeCDF, 2,3,7,8-tetrachlorodibenzofuran (TCDF) is not sequestered in tissues, but rather it is one of the few PCDD/PCDF DLCs that is metabolized.[6] TCDF is not a major contributor to dioxin exposure via food and is not readily measured in biomonitoring studies,[4,7] which is not surprising considering that TCDF induces CYP1A1, the enzyme responsible for its own metabolism and ultimate clearance. Nevertheless, the WHO TEF committee has assigned TCDF a TEF of 0.1.[10]

The purpose for this study was to use gene array methodology to test the validity of TEF assumptions by (1) comparing profiles of TCDD-, TCDF- and 4-PeCDF-induced gene expression patterns, and (2) comparing these induced patterns between human and rat primary hepatocytes.

Experimental Design, Materials and Methods

Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was supplied by The Dow Chemical Company (Midland, MI). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Dexamethasone (DEX) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). All DLCs were dissolved in DMSO.

Biological Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/Ham's F-12 (DMEM/F-12) was purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel® and ITS+ (Insulin, Transferrin, and Selenium) and collagen-coated cell culture plates (BioCoat®) were purchased from BD Biosciences (Bedford, MA). RNA isolation reagents were purchased from Applied Biosystems (Foster City, CA). Other supplies and molecular reagents were obtained from the sources indicated below.

Primary Culture of Rat and Human Hepatocytes

Primary hepatocytes were isolated from three female adult (10 weeks of age) Sprague Dawley®™ rats (Harlan, Indianapolis, IN) and three adult women (Table 1), as previously described.[3] Following isolation, 7.2 million viable hepatocytes were plated onto individual 24–well plates coated with type I collagen. Cultures were maintained in DMEM/ F-12 culture medium supplemented with ITS+ (6.25 μg/mL insulin, 6.25 μg/mL transferrin, and 6.25 ng/mL selenium) and 0.1 μM DEX for 48 h prior to exposure to inducers. Hepatocytes were treated with vehicle (final concentration was 0.1% DMSO) alone or test compounds (containing 0.1% DMSO final concentration) for 24 h (n = 4 per treatment group per donor). Hepatocytes were treated with TCDD (0.3 nM, 0.01 nM), TCDF (3.0 nM, 1.0 nM), or 4-PeCDF (0.3 nM, 0.3 nM) for human and rat hepatocytes, respectively. These doses represent the EC_{50} concentrations for CYP1A1 mRNA induction as determined in a dose response study (data not shown).

Table 1: Characteristics of the Human Donors

Donor	Sex	Age (years)	Race	Weight (lbs)	Height
Hu0489	F	68	Caucasian	143	5'7"
Hu0572	F	77	Caucasian	115	5'1"
Hu0579	F	44	Caucasian	174	5'5"

RNA isolation

Total RNA was originally isolated from cultures of human hepatocytes using an ABI 6100 Prepstation with optional on-column DNAse digestions (Absolute, ABI). RNA quantity and relative purity was assessed with a NanoDrop spectrophotometer. For determination of the EC_{50}s for induction of CYP1A1 mRNA, a portion of the RNA samples was reverse transcribed and analyzed via quantitative RT-PCR (qRT-PCR) assays (TaqMan®). Upon completion of the qPCR assays, the remaining RNA samples from the four replicates per treatment per donor were pooled, and further purified with the RNeasy Mini-prep (Qiagen, Valencia, CA, USA) to exchange the buffer to RNAse-free water and to concentrate the RNA samples for subsequent microarray analysis. Pooled, concentrated RNA was eluted in water and subsequently quantified on a NanoDrop spectrophotemter, and assessed for quality using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) prior to use in the microarray assays.

Global gene expression profiles were determined using Agilent® rat and human whole genome 4x44K oligonucleotide microarrays that contain more than 41 000 60-mer oligo probes. The microarrays were processed using a one-color format. For each sample, Alexa555-labeled cRNAs was generated from 500 ng of total RNA using the Epicentre TargetAMP 1-Round Aminoallyl-cRNA Amplification Kit per the vendor's protocol. After the Aminoallyl-cRNA is produced it was purified using Qiagen RNeasy Mini Elute Kit. On each microarray, 1.65 ug of labeled cRNA was hybridized for 17 hours and subsequently washed per the Agilent protocol. Microarrays were scanned using an Agilent® dual laser DNA microarray scanner, model G2565AA, with 5 μm resolution.

Microarray Data Normalization and Analysis

Microarray image analysis was performed with Agilent® Feature Extraction software, version 9.5.1 using spatial detrending to adjust for surface background effects. Intensity data were imported into Rosetta Resolver (version 5.1) for analysis. Transcripts were determined to be "present" by using a statistical test based on spot (feature) intensity, pixel variance, and background intensity. Transcripts with a p-value ≤ 0.001 were specified as present. For each comparison between treated samples and DMSO controls, transcripts absent in both treated and DMSO controls were excluded from subsequent analysis. One-way analysis of variance (ANOVA) was performed between each treatment and DMSO controls to identify transcripts with a statistically significant difference in expression. Benjamini & Hochberg multiple test correction was used with the false discovery rate set at 5%. Intensity data were normalized through intra-array scaling and variance stabilization prior to ANOVA analysis. Inter-species comparisons were accomplished using the following NCBI databases: Homologene build #53, Unigene rat build #159, and Unigene human build #202.

Results and Discussion

Evaluation of species differences in gene expression responses to three DLCs with varying TEF values was conducted using whole genome expression analysis of DLC treated primary rat and human hepatocytes treated with TCDD, TCDF or PeCDF. Comparison of the 24-h induction profiles within a species revealed that the furans altered a different set of genes when compared to TCDD as there was little overlap between altered responses (Table 2). This response was observed in both rat and human cells. When comparing congener responses for homologous genes across species, more genes were altered by TCDD in rats (≥2X) than in humans and a similar response was observed for TCDF (~3X more in rats) and 4-PeCDF (~3.5X more in rats) (Table 3).

Table 2: Number of Differentially Expressed Genes Across Treatments

Species[a]	Filter[b]	TCDD[a]	TCDF[a]	4-PeCDF[a]	Common
Human	0	132	147	181	27
	1.5	92	91	126	17
	2.0	62	55	86	13
Rat	0	320	479	466	77
	1.5	251	331	308	51
	2.0	154	192	178	26

[a]EC_{50} based on CYP1A1 mRNA induction in a separate study (data not shown). EC_{50}s for humans, 0.3 nM TCDD, 0.3 nM 4-PeCDF, 3.0 nM TCDF; EC_{50}s for rats, 0.01 nM TCDD, 0.3 nM 4-PeCDF, 1.0 nM TCDF.

[b]the number of significantly altered genes in each treatment were determined induced/repressed at and beyond the indicated fold level.

To identify genes that are commonly differentially expressed in both species in response to each congener, a rat-human homolog database was created and used to cross reference induced human genes to induced rat genes. The common gene responses across species for each congener were:

- TCDD: 5 genes common: CYP1B1, CYP1A1, CYP1A2, TIPARP, IL17RB
- TCDF: 6 genes common: CYP1B1, CYP1A1, CYP1A2, IL17RB, SERPINA5, ALDH3A1
- 4-PeCDF: 5 genes common: CYP1B1, CYP1A1, CYP1A2, IL17RB, TIPARP

In all treatments, IL17RB was induced in humans and repressed in rats. Also, with the exception of TIPARP, the induced levels for each gene were quite different between the two species. The majority of these genes are known to be TCDD-regulated (CYP1B1, CYP1A1, CYP1A2, ALDH3A1, TIPARP). These results in both rats and humans suggest that there are important differences in gene expression responses to DLCs that may be explained by differences in congener activation of the AHR both within species and between humans and rats. Moreover, this initial examination has revealed that only a very

small number of TCDD and DLC affected genes are in common between rats and humans indicating the AHR pathway may not be well conserved between rats and humans beyond the regulation of the well characterized AHR core gene battery, which has important implications for extrapolating TCDD- and DLC-induced effects from rats to humans. Work is under way to include additional human and rat donors to the analysis and affirm these results by quantitative qRT-PCR analysis.

Table 3: Number of Differentially Expressed Genes with a Rat-Human Homologue Across Treatments

Treatment[a]	Filter[b]	Rat	Human	Common
TCDD	0	147	71	5
	1.5	114	45	4
	2.0	69	27	3
TCDF	0	238	87	6
	1.5	158	53	4
	2.0	89	29	3
4-PeCDF	0	262	75	5
	1.5	170	45	5
	2.0	97	29	4

[a]EC_{50} based on CYP1A1 mRNA induction in a separate study (data not shown). EC_{50}s for humans, 0.3 nM TCDD, 0.3 nM 4-PeCDF, 3.0 nM TCDF; EC_{50}s for rats, 0.01 nM TCDD, 0.3 nM 4-PeCDF, 1.0 nM TCDF.
[b]the number of significantly altered genes in each treatment were determined induced/repressed at and beyond the indicated fold level.

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