

TCDD and a Putative Endogenous AhR Ligand, ITE, Elicit the Same Immediate Changes in Gene Expression in Mouse Lung Fibroblasts

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The aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor, mediates toxicity of several classes of xenobiotics and also has important physiological roles in differentiation, reproduction, and immunity, although the endogenous ligand(s) mediating these functions is/are as yet unidentified. One candidate endogenous ligand, 2-(1'H-indolo-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), is a potent AhR agonist *in vitro*, activates the murine AhR *in vivo*, but does not induce toxicity. We hypothesized that ITE and the toxic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), may modify transcription of different sets of genes to account for their different toxicity. To test this hypothesis, primary mouse lung fibroblasts were exposed to 0.5 μM ITE, 0.2 nM TCDD, or vehicle for 4 h, and total gene expression was evaluated using microarrays. After this short-term and low-dose treatment, several hundred genes were changed significantly, and the response to ITE and TCDD was remarkably similar, both qualitatively and quantitatively. Induced gene sets included the expected battery of AhR-dependent xenobiotic-metabolizing enzymes, as well as several sets that reflect the inflammatory role of lung fibroblasts. Real time quantitative RT-qPCR assay of several selected genes confirmed these microarray data and further suggested that there may be kinetic differences in expression between ligands. These data suggest that ITE and TCDD elicit an analogous change in AhR conformation such that the initial transcription response is the same. Furthermore, if the difference in toxicity between TCDD and ITE is mediated by differences in gene expression, then it is likely that secondary changes enabled by the persistent TCDD, but not by the shorter lived ITE, are responsible.

Key Words: aryl hydrocarbon receptor (AhR); endogenous ligand; TCDD; microarray; mouse lung fibroblasts; gene expression.

Although the aryl hydrocarbon receptor (AhR) has been most extensively studied as a mediator of toxicity of several classes of environmental contaminants, it is also recognized as having important endogenous functions. For example, in its absence (in AhR^{-/-} mice), vascular differentiation is severely disrupted, reproductive ability is impaired, and liver and

immune abnormalities are observed (McMillan and Bradfield, 2007). It also has a role in regulation of inflammation (Baglole *et al.*, 2008; Thatcher *et al.*, 2007), in cell cycle (Elferink, 2003), and impacts numerous other pathways of cell signaling (Beischlag *et al.*, 2008). The many physiological roles identified for the AhR also support the presumed existence of endogenous ligands for this transcription factor, and much effort has been expended on the search for such molecules. Although no definitive endogenous ligand has yet been identified, several classes of naturally occurring compounds as well as physiological compounds have been found that bind the AhR with varying affinities and have varying abilities to activate or antagonize its transcriptional function (Denison and Nagy, 2003; Nguyen and Bradfield, 2008).

Among the stronger candidates for putative endogenous AhR ligands are various photoproducts and/or metabolic products of tryptophan, including 6-formylindolo[3,2-*b*]carbazole (FICZ) and 2-(1'H-indolo-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), as well as several arachidonic acid derivatives (Chiaro *et al.*, 2008a,b; Schaldach *et al.*, 1999). FICZ is a potent AhR activator and a good substrate for Cytochromes P450 (CYPs) 1A1, 1A2, and 1B1; sulfoconjugates of a hydroxylated metabolite of FICZ were recently identified in human urine samples (Wincent *et al.*, 2009). ITE was originally isolated and identified from porcine lung tissue and found to be a potent AhR activator (Song *et al.*, 2002). Compared with the prototypical toxic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), ITE binds the AhR and elicits *in vitro* DNA (aryl hydrocarbon response element, AhRE) binding with similar potency (Henry *et al.*, 2006). ITE injected *iv* into pregnant transgenic mice is able to cross the placenta to activate the AhR-dependent LacZ reporter in fetal tissues but does not induce the teratogenic effects typical of prenatal TCDD exposure such as cleft palate and hydronephrosis. Also unlike TCDD, ITE has no effect on the thymus after *in vivo* dosing of adult mice, whereas it induces thymic toxicity in fetal thymic organ culture. This difference in potency between TCDD and ITE *in vivo* is consistent with their relative chemical stabilities in tissues—unlike the

sustained presence and activity of TCDD, available data suggest that ITE is rapidly degraded (Bemis and Gasiewicz, unpublished data). Although the breakdown products have not yet been identified, it is likely that ITE is a substrate for cytochrome P450s as reported for FICZ (Wincent *et al.*, 2009). Indeed, it is expected that endogenous activators would be relatively short-lived, unlike the xenobiotic toxic ligands typified by TCDD.

Another possible explanation for the difference in toxicity is that the binding of different ligands to the receptor elicits subtly different protein conformations that may in turn differ qualitatively as well as quantitatively in their binding to DNA and interactions with other cofactors. This could result in some variation in the gene expression profile induced by the AhR and hence the ultimate toxicity or lack thereof. Such an hypothesis is supported by research on steroid-receptor complexes (McDonnell *et al.*, 1995; Wijayarathne and McDonnell, 2001), as well as by studies in which individual AhR-dependent genes and/or toxic responses are reported to differ between ligands (Gouedard *et al.*, 2004a,b; Matikainen *et al.*, 2001; Quintana *et al.*, 2008). The goal of the current study was to compare the changes in gene expression elicited by short-term exposure to ITE and TCDD to determine whether there was a disparity that could explain the difference in toxicity. We chose to use mouse lung fibroblasts for this study since they are a primary culture rather than a transformed cell line and because of previous data suggesting a major role for AhR in regulation of inflammation (Vogel and Matsumura, 2009), which in the lung is partially mediated by lung fibroblasts (Baglolle *et al.*, 2008). Our results indicate that the short-term response to ITE and TCDD is essentially identical. However, validation of several of the affected genes shows some differences in kinetics consistent with the more rapid degradation of ITE compared to the persistence of TCDD.

MATERIALS AND METHODS

Cell culture. Primary mouse lung fibroblasts from C57BL/6 mice were obtained and cultured as described previously (Baglolle *et al.*, 2005, 2008). Cells were grown in six-well plates in Modified Eagle's Medium (MEM) + 10% fetal bovine serum (FBS) and supplemented with pyruvate, glutamine, sodium bicarbonate, and gentamicin. Serum was removed for 24 h before treatment with ITE or TCDD when cells were nearing confluence. Ligands were added in dimethylsulfoxide (DMSO) vehicle (1 μ l/ml medium).

Western blot analysis. At the end of the exposure time, culture media were removed, cells washed twice with PBS, and lysed (Reporter Lysis Buffer, Promega, Madison, WI). Lysates were stored at -80°C . Lysate proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8.4% acrylamide resolving gel) and transferred to polyvinylidene fluoride (PVDF) membrane which was then blocked using 5% nonfat milk in wash buffer (50mM Tris base, 150mM NaCl, and 0.2% Tween 20, pH 7.5). Antibodies used were Cox-2 (rabbit polyclonal, Cayman Chemical), CYP1B1 (Santa Cruz Biotechnology), AhR (mouse monoclonal Rpt-1 and ascites prepared in our laboratory), and β -actin (rabbit polyclonal, Sigma, St Louis, MO). The appropriate horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Proteins were visualized by chemiluminescence using LumiGlo reagents (KPL, Gaithersburg, MD).

RNA extraction. RNA was prepared from individual wells using Qiashredder and RNeasy kits (Qiagen, Valencia, CA) with DNase treatment (RNase-free DNase [Qiagen]). Concentrations were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA was stored at -20°C until used.

Microarrays. RNA was prepared from five replicate wells for each treatment (DMSO, ITE, TCDD; 4 h exposure). Gene expression analysis was performed by the Functional Genomics Center at University of Rochester using the Affymetrix Mouse M430 2.0 array. RNA from each well was analyzed separately (i.e., five microarrays per treatment group). Instrumentation used was GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G. Background subtraction and quantile normalization of arrays was done with the GeneChip Robust Multiarray Average method (Wu and Irizarry, 2004). Probe sets showing no expression according to the Affymetrix MAS5 presence/absence algorithm were eliminated from further calculations. Fold induction by ITE or TCDD compared to DMSO was calculated for each gene probe. The Significance Analysis of Microarrays plug-in (www-stat.stanford.edu/~tibs/SAM/) was used to determine the false discovery probability (*q* values). Nominal *p* values were calculated by the Microsoft Excel program. Further examination of the overall patterns of gene expression in these data was done using Gene Set Enrichment Analysis (GSEA, Broad Institute, MIT) (Subramanian *et al.*, 2005). For this analysis, 1000 iterations, permuting by gene set, were used for comparing each pair of treatments (ITE vs. DMSO, TCDD vs. DMSO, and ITE vs. TCDD). Gene sets included in the analysis were from the curated collection in the Molecular Signature Database, MsigDB, maintained by Broad Institute.

Quantitative real-time PCR. RNA (250 or 500 ng of each sample) was reverse transcribed using Superscript III and oligo(dT) primers (Invitrogen, Carlsbad, CA). Applied Biosystems Gene Expression Assays (Taqman chemistry) and the iCycler (Bio-Rad, Hercules, CA) were used to quantify expression of selected genes. Relative expression of the several target genes was determined from the measured PCR efficiencies for each gene and the ΔCt values (DMSO-treated) and normalized to the equivalent expression of *Gapdh* in each sample, as described by Pfaffl (2001).

RESULTS

Optimization of Doses and Exposure Time

Initial experiments were performed to characterize the dose and time dependence of expression of selected AhR-responsive gene products in order to determine an appropriate dose and exposure time for subsequent microarray analyses. Cell lysates from mouse lung fibroblasts exposed to a range of exposure times and doses of ITE or TCDD were compared by Western blot (Fig. 1). Levels of AhR (which is rapidly degraded in response to agonist) were partially restored within 24 h after ITE, but not after TCDD (Fig. 1). Based on several experiments evaluating levels of AhR and products of two AhR-responsive genes, CYP1B1 and Cox-2, we determined that TCDD at 0.2nM and ITE at 0.5 μ M elicited comparable effects on these proteins. Short-term exposure (4 h) was chosen in order to focus on the primary transcriptional response to these two AhR ligands.

Short-Term Exposure to TCDD or ITE Induces the Same Changes in Gene Expression

RNA extracted from mouse lung fibroblasts exposed to ITE, TCDD, or DMSO vehicle for 4 h was analyzed for changes in

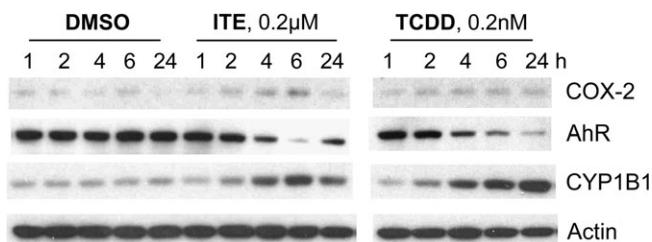


FIG. 1. Western blot of several proteins in mouse lung fibroblasts exposed to ITE (0.2 μ M) or TCDD (0.2nM). Cells were grown to near confluence, cultured for 24 h without FBS, and then treated with ligand or DMSO vehicle as indicated. Whole-cell extracts were separated by SDS-PAGE; proteins were identified by Western blot. Representative of results from three experiments.

overall gene expression using the mouse M430 2.0 array. Depending on the criteria used to determine significance (as indicated in Table 1), several hundred genes (probe sets) were identified as induced or repressed by ITE or TCDD compared with DMSO (Table 1, A and B). However, none of these showed a difference between ITE and TCDD at $q < 5\%$ (Table 1, C). This pattern is clearly illustrated by Figure 2 in which fold-change values for TCDD-treated samples are plotted against fold change by ITE for all the significantly changed genes. The correlation coefficient in each range of this plot was 0.98–0.99. This indicates that in these cells and under these conditions, there was essentially no difference between

TABLE 1
Summary of Microarray Results

(A) Filtered by ITE/DMSO	Total: 4521
Fold change	
> 2.5 or < 0.4	255
> 2.0 or < 0.5	482
> 1.5 or < 0.67	1271
(B) Filtered by TCDD/DMSO	Total: 3257
Fold change	
> 2.5 or < 0.4	269
> 2.0 or < 0.5	458
> 1.5 or < 0.67	1201
(C) Filtered by ITE/TCDD	21 ^a
Fold change	
> 2.0 or < 0.5	0
> 1.5 or < 0.67	0

Note. Microarray data were analyzed by Significance Analysis of Microarrays as described in Materials and Methods. In parts A and B, the total number of genes (probe sets) meeting the < 5% false discovery probability is shown in the first row of each table. These were further filtered by different magnitudes of fold change as shown. Calculated p -values were all < 0.015 at this estimated false discovery rate. In part C, criteria were relaxed as indicated.

^aFor all but one of these, the q value was 14–42%; p values were all \leq 0.0016.

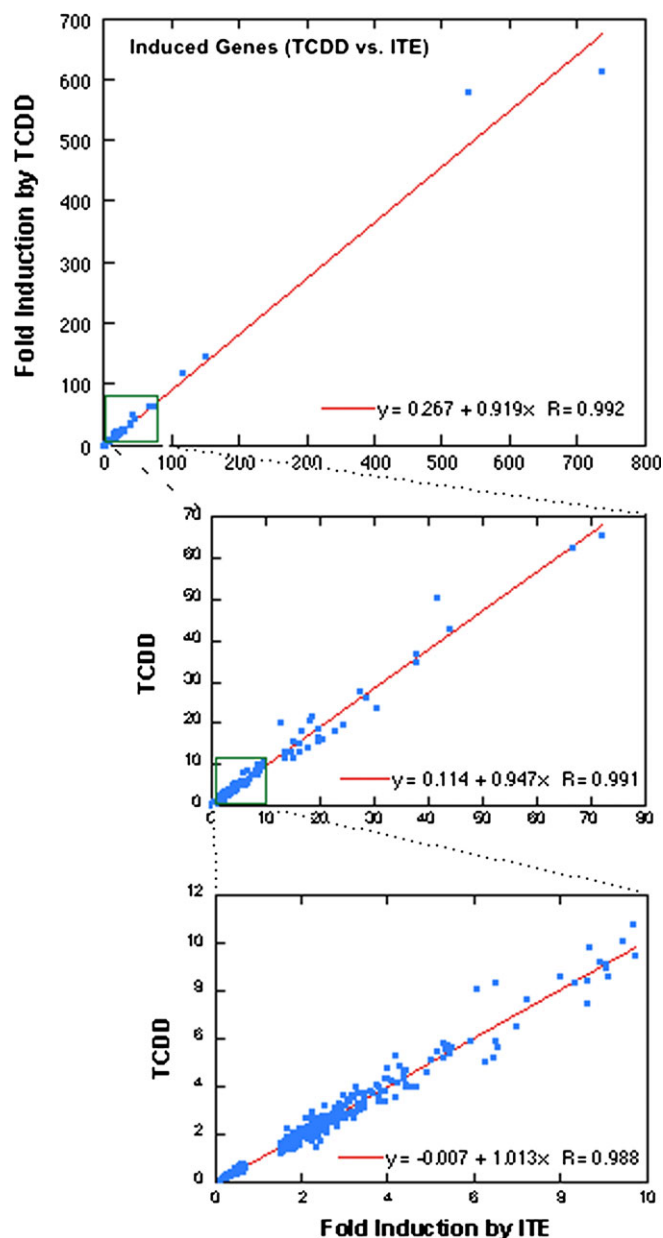


FIG. 2. Gene induction by ITE and TCDD is highly correlated. Calculated mean fold induction/decrease by TCDD compared with DMSO is plotted against mean fold induction/decrease by ITE for all genes that were significantly changed (> 1.5-fold or < 0.67-fold, q value < 5%). Linear regression and correlation coefficients are shown for each range of the plot.

the genes affected at 4 h after treatment with ITE compared with TCDD. In Table 1, the numbers in each category differ slightly between ITE and TCDD; this reflects the fact that there were some probe sets in each case for which (1) a similar mean fold change relative to DMSO ($n = 5$) was observed for both ligands, but for one or the other treatment, the p value or q value did not quite meet the criterion value or (2) the fold change by one ligand exceeded the indicated cutoff value but for the other ligand was slightly less than this cutoff.

TABLE 2
The Most Highly Induced/Repressed Genes Identified by Microarray

Relative expression				
Symbol	ITE/DMSO	TCDD/DMSO	ITE/TCDD	Description
Aldh3a1	735.916	611.531	1.203	Aldehyde dehydrogenase family 3, subfamily A1
Cyp1a1	538.552	577.663	0.932	Cytochrome P450, family 1, subfamily a, polypeptide 1
Megf10	150.877	146.849	1.027	Multiple EGF-like domains 10
Ahrr	116.436	119.109	0.978	AhR repressor
Tac1	72.179	65.510	1.102	Tachykinin 1
Nsg2	66.602	62.499	1.066	Neuron-specific gene family, member 2
Tnfrsf19	43.960	42.908	1.025	Tumor necrosis factor receptor superfamily, member 19
St8sia1	41.767	50.470	0.828	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
Slc1a2	37.978	37.087	1.024	Solute carrier family 1 (glial high-affinity glutamate transporter), member 2
Slc2a6	37.937	34.988	1.084	Solute carrier family 2 (facilitated glucose transporter), member 6
Nsg2	29.508	39.405	0.749	Neuron-specific gene family, member 2
Retn	28.617	26.067	1.098	Resistin
Ccnu	27.563	27.670	0.996	Cyclin U
Tgfb1	24.449	19.460	1.256	Transforming growth factor, beta induced
Nmnat2	22.617	17.992	1.257	Nicotinamide nucleotide adenylyltransferase 2
Mpp4	19.698	18.798	1.048	Membrane protein, palmitoylated 4 (MAGUK p55 subfamily, member 4)
Tgfb1	19.584	16.833	1.163	Transforming growth factor, beta induced
Tiparp	18.753	21.690	0.865	TCDD-inducible poly(ADP-ribose) polymerase
St8sia1	18.274	20.822	0.878	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
Nr4a3	17.642	14.125	1.249	Nuclear receptor subfamily 4, group A, member 3
Rgnef	16.680	17.953	0.929	Rho-guanine nucleotide exchange factor
Olfm1	16.109	15.298	1.053	Olfactomedin 1
Slc1a2	16.099	13.291	1.211	Solute carrier family 1 (glial high-affinity glutamate transporter), member 2
Slc14a1	15.117	11.633	1.300	Solute carrier family 14 (urea transporter), member 1
Lrp4	14.549	13.108	1.110	Low-density lipoprotein receptor-related protein 4
Prss35	13.718	11.407	1.203	Protease, serine, 35
Tspan33	13.631	12.905	1.056	Tetraspanin 33
St8sia1	12.878	20.101	0.641	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
Afp	12.311	13.211	0.932	Alpha fetoprotein
Nos3	9.832	12.234	0.804	Nitric oxide synthase 3, endothelial cell
Cpox	9.690	10.815	0.896	Coproporphyrinogen oxidase
Tiparp	9.428	10.120	0.932	TCDD-inducible poly(ADP-ribose) polymerase
Nqo1	9.113	8.614	1.058	NAD(P)H dehydrogenase, quinone 1
Bmpr1b	0.268	0.210	1.279	Bone morphogenetic protein receptor, type 1B
Crct1	0.267	0.307	0.869	Cysteine-rich C-terminal 1
Kcnh1	0.247	0.243	1.019	Potassium voltage-gated channel, subfamily H (eag related), member 1
Chst1	0.242	0.357	0.680	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
Fgfr2	0.240	0.254	0.947	Fibroblast growth factor receptor 2

TABLE 2—Continued

Relative expression				
Symbol	ITE/DMSO	TCDD/DMSO	ITE/TCDD	Description
Edn1	0.240	0.240	1.000	Endothelin 1
Ivl	0.227	0.207	1.097	Involucrin
Ccdc85a	0.219	0.271	0.810	Coiled-coil domain containing 85A
Sprr1a	0.184	0.185	0.997	Small proline-rich protein 1A
Klhl29	0.180	0.203	0.888	Kelch-like 29 (<i>Drosophila</i>)

For all genes shown, $q < 5\%$. Omitted from this list were several probe sets identified only by riken no. or expressed sequence no. and two additional *Tgfb1* (they are included in the Supplementary table 1).

The complete list of significantly altered genes (probability of false discovery [q value] of $< 5\%$) and their fold induction by ITE and TCDD is included in Supplementary table 1. The cutoff for inclusion in this table was 1.5-fold (up- or downregulated). In this table are included those genes that may meet the above criteria for one compound but not for the other (e.g., 1.55-fold induced by ITE but only 1.45-fold by TCDD or *vice versa*). Such genes are generally found in the borderline range of 1.5- to 2-fold change in expression. Sorting the list of genes according to magnitude of either ITE- or TCDD-induced expression yielded very similar order, as expected from the very high correlation shown in Figure 2. Among the most highly induced genes (Table 2) are the expected AhR-dependent drug-metabolizing enzymes, including *Aldh3a1* and *Cyp1a1* (> 500 -fold), *Nqo1* (ninefold), *Cyp1b1* and *Adh* (fourfold to fivefold; not included in Table 2), as well as *AhRR* (116-fold).

Evaluation of Altered Gene Pathways

The major objective of this study was to determine differences (or lack thereof) between the spectrum of genes responding to ITE compared with TCDD; clearly, the initial transcriptional activation by these two ligands was very similar in these cells. Since this is the first time to our knowledge that

gene array studies have been reported for mouse lung fibroblasts, we evaluated the patterns of alterations induced by these AhR ligands in more detail. Simply focusing on the most highly up- or downregulated genes (such as listed in Table 2 or in Supplementary table 1) does not necessarily provide much information regarding the whole spectrum of biological pathways that may be affected (e.g., pathways in which many individual components may not be highly changed). A number of tools have been developed to analyze genome-wide expression data to extract more physiological information. We chose to use GSEA (Subramanian *et al.*, 2005). This technique is designed to determine whether observed patterns of gene expression show evidence of alterations in groups of genes that share biological functions or pathways.

A summary of the GSEA results is presented in Table 3. In this analysis, 1442 gene sets were included (containing 14–500 genes per set). Comparing ITE or TCDD data with the DMSO control data showed nominal enrichment of a large number of gene sets. However, of these, only 13 and 9 showed enrichment in ITE and TCDD samples, respectively, at a false discovery rate (FDR) of $< 25\%$ (Table 3) (an FDR of 25% indicates a three in four chance that the result is valid; this level is considered appropriate for the stated purpose of hypothesis generation from GSEA [Subramanian *et al.*, 2005]). The

TABLE 3
Summary of GSEA

	ITE versus DMSO (DMSO vs. ITE)	TCDD versus DMSO (DMSO vs. TCDD)	ITE versus TCDD (TCDD vs. ITE)
# Gene sets upregulated (downregulated)	676 (766)	714 (728)	524 (918)
# Gene sets significantly enriched at FDR $< 25\%$	13 (0)	9 (0)	0 (0)
# Gene sets enriched at nominal $p < 1\%$	21 (22)	21 (29)	2 (10)

Microarray data were subjected to GSEA as described in “Materials and Methods” section using the three indicated comparisons (ITE vs. DMSO, TCDD vs. DMSO, and ITE vs. TCDD); 21,891 genes (after collapsing the 45,101 array features into gene symbols since many genes are represented by two or more probe sets on the arrays); 1442 gene sets were included in analysis (14–500 genes per set); analyses performed with 1000 iterations.

TABLE 4
Gene Sets Enriched in ITE Samples (vs. DMSO)

Rank ITE ^a	Rank TCDD ^b	Gene set name	Description ^c	# Genes in set ^d	FDR <i>q</i> value ^e	Nominal <i>p</i> value
1	6	Dendritic cell pathway	ANPEP, several interleukins, TLRs, interferons, and CD molecules	21 (2)	0.047	0.000
2	3	Arachidonic acid metabolism	PTGS1,2; carbonyl reductases; GGT; PTGES, PTGDS; phospholipases, Cyp2U1 and 2E1; and GPXs, . . .	40 (10)	0.138	0.002
3	4	IDX TSA DN cluster1	Genes downregulated 48–96 h during 3T3-L1 fibroblast differentiation into adipocytes with IDX (insulin, dex, and isobutylxanthine)	40 (12)	0.094	0.000
4	25	γHexachlorocyclohexane degradation	Acid and alkaline phosphatases, Cyp3a's, and paraoxonases	14 (4)	0.118	0.000
5	2	Fatty acid metabolism	AcylCoA dehydrogenases, oxidases, synthetases; ADHs, AldHs, many P450s, . . .	60 (10)	0.101	0.000
6	5	Metabolism of xenobiotics by cytochrome P450, . . .	Many AldHs, Cyps, GSTs, epoxide hydrolase, and UDPGTs	34 (7)	0.116	0.002
7	1	Prostaglandin and leukotriene metabolism	Lipoxygenases, Cyp4F's, carbonyl reductases, PTGES, and phospholipases, . . .	28 (7)	0.193	0.000
8	15	Greenbaum E2A DN	E2A target genes in lymphocyte development	25 (8)	0.170	0.004
9	9	Eicosanoid synthesis	PG synthases, arachidonate lipoxygenases, PTGS1,2	16 (7)	0.153	0.004
10	10	Butanoate metabolism		27 (8)	0.231	0.006
11	43	Pomeroy desmoplastic versus classic MD_DN	Genes expressed in classic medulloblastomas	37 (2)	0.236	0.002
12	13	Nicotinate and nicotinamide metabolism		22 (8)	0.241	0.006
13	14	Valine, leucine, and isoleucine degradation	Transferases, dehydrogenases, . . .	35 (10)	0.225	0.007
(6) ^f	(11)	TGFβ signaling pathway	TGFβs, TGFβRs, LTBP1; bone morphogenic proteins, Id1–4; MAPK1,3; protein phosphatase 2's; CDK inhibitor 2B	82 (27)	0.510	0.002

Note. Listed are the gene sets which were identified by GSEA to be enriched in ITE-treated cells compared with DMSO treated, with FDR < 25%. ADHs, alcohol dehydrogenases; AldHs, aldehyde dehydrogenases; ANPEP, alanyl aminopeptidase; GGT, *g*-glutamyl transpeptidase (or gamma-glutamyl transpeptidase); GPXs, glutathione peroxidases; GSTs, glutathione S-transferases; MAPK, mitogen-activated protein kinase; PG, prostaglandin; PTGS, prostaglandin endoperoxide synthase; PTGES, prostaglandin E synthase; PTGDS, prostaglandin D synthase; TLR, toll-like receptor; UDPGTs, UDP-glucuronyltransferases.

^aRank within the enriched gene sets, based on normalized enrichment score (ITE vs. DMSO). Rank differs among repeated runs of GSEA due to random permutation process of analysis.

^bRank within enriched gene sets (TCDD vs. DMSO).

^cSome of the genes/classes of genes included in indicated gene set.

^dTotal number of genes in set (number individually induced in ITE samples).

^eFDR, estimated probability that set represents a false positive.

^fOne of the sets downregulated by ITE and TCDD relative to DMSO; FDR > 25% (as it is for all downregulated sets).

13 gene sets that met the 25% FDR criterion for the ITE versus DMSO comparison are identified and described in Table 4. Among these enriched sets are the expected “metabolism of xenobiotics by cytochrome P450,” as well as several that reflect the inflammatory role of lung fibroblasts (“prostaglandin and leukotriene metabolism,” “arachidonic acid metabolism,” and “eicosanoid synthesis”). Consistent with the high correlation between ITE and TCDD results noted above for individual genes, the top-ranked gene sets for TCDD samples were largely the same ones as for ITE. Note that the rank order as well as the differences in rank order between ligands are not considered to be biologically meaningful. Indeed, since the

analysis involves random permutation of phenotype or gene set labels in order to determine significance of enrichment results, each analysis produces slightly different ordering. However, the fact that out of > 1400 gene sets ITE and TCDD samples were found to contain a similar group of affected sets based on these calculations is considered to strongly support the conclusion that these ligands act very similarly. Additionally, GSEA identified no gene sets that were significantly enriched in the ITE compared with TCDD datasets (Table 3).

Generally, within each enriched gene set, not all the member genes are actually significantly induced/repressed. The analysis looks at the distribution of all the genes in the set

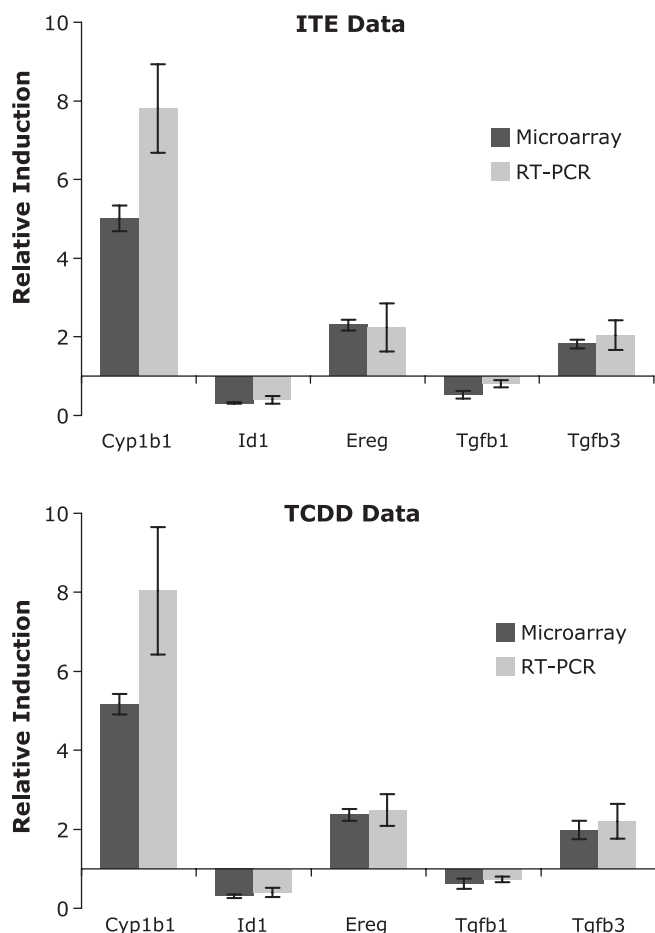


FIG. 3. RT-qPCR confirms gene expression data from microarrays. Fold induction by ITE and TCDD relative to DMSO as calculated from microarrays and RT-qPCR using the same RNA samples is shown for selected genes. *Cyp1a1* also showed equivalent induction by RT-qPCR and microarray, but actual values are off this scale and are not shown. All values are normalized to expression of *Gapdh* in the same samples; mean \pm SD ($n = 5$).

within the list of all genes on the array (ranked by magnitude of change relative to control) to determine whether they are distributed randomly throughout or concentrated within the upper or lower extremes of the ranked list. Not all that are listed as showing “core enrichment” in a particular enriched gene set are actually identified as significantly affected based on simple fold change by ITE (or TCDD) compared to DMSO as calculated based on individual mean values and shown in Supplementary table 1.

There is some overlap of gene members among sets, not surprisingly, since few biomolecules are involved in only one unique pathway, and therefore some genes that are induced are members of multiple gene sets. In this experiment, relatively few genes determined the calculations that yielded the conclusion that certain gene sets are significantly induced. For example, *Aldh3a1*, *Adh7*, *Ptgs1* and 2, *Cyp1a1*, and other *Cyps* are in several of the enriched gene sets. Many more genes that were individually up- or downregulated are members of

one or more gene sets in the database, but those gene sets were not determined to be “enriched” in this experiment or were not included in this analysis (14–500 genes per set). Notably, one of the most highly induced genes (*Ahrr*) happens not to be in any of the induced gene sets, including metabolism of xenobiotics by CytP450, perhaps because the function of AhRR is not yet fully understood.

Quantitative-PCR Confirmation of Expression Changes of Selected Genes

Several genes identified by microarray to be affected by ligand treatment were chosen for validation of results using real-time PCR quantitation. Genes chosen represented highly induced (*Cyp1a1* and *Cyp1b1*), modestly induced (epiregulin [*Ereg*] and *Tgfb3*), and decreased (*Id1* and *Tgfb1*) according to microarray analysis. *Ereg* is a recently identified AhR target gene, is a member of the epidermal growth factor family of growth regulators, and its promoter includes an AhRE sequence (Patel *et al.*, 2006). The cytokine transforming growth factor β (TGF β) impacts many cellular processes, and its cross talk with AhR signaling has been widely reported, though not yet well understood (reviewed by Gomez-Duran *et al.*, 2009). In the present experiment, *Tgfb1* and 2 were modestly downregulated, whereas *Tgfb3* was upregulated; promoters of all three forms are reported to contain AhRE sites (Sun *et al.*, 2004). The *Id* (inhibitor of DNA binding) genes were among the more prominently downregulated genes in our cells, as well as in human dermal fibroblasts (Akintobi *et al.*, 2007). Using the same RNA as analyzed on the microarrays, the correlation between microarray and RT-qPCR was very strong for these genes (Fig. 3). Induction of *Cyp1a1* also was highly correlated between RT-qPCR and microarrays and between ITE and TCDD but is not shown in Figure 3 because its magnitude of induction is far off the scale used for the other genes (inductions of > 500-fold in all cases).

Since responses to ITE and TCDD were so similar at 4 h, and yet clearly these ligands have different toxicity (at least in mice), it is possible that divergent patterns of gene expression would be detectable after longer exposure periods. Therefore, these selected genes were also quantified by RT-qPCR analysis of RNA extracted from separate cultures of mouse lung fibroblasts exposed over a range of time intervals or to a range of doses to determine time-course and dose-response of these changes (Fig. 4). These data were consistent with the microarray results, although over the dose range used, a clear dose-response pattern was not observed for all the tested genes. Between 2 and 24 h after ITE treatment, *Ereg*, *Cyp1a1*, and *Cyp1b1* and *Tgfb3* induction diminished over time, whereas these levels were maintained by TCDD up to 24 h. The downregulation of *Id1* was maintained over the 24 h by both ligand treatments; the modest decrease in *Tgfb1* appeared to be more transient.

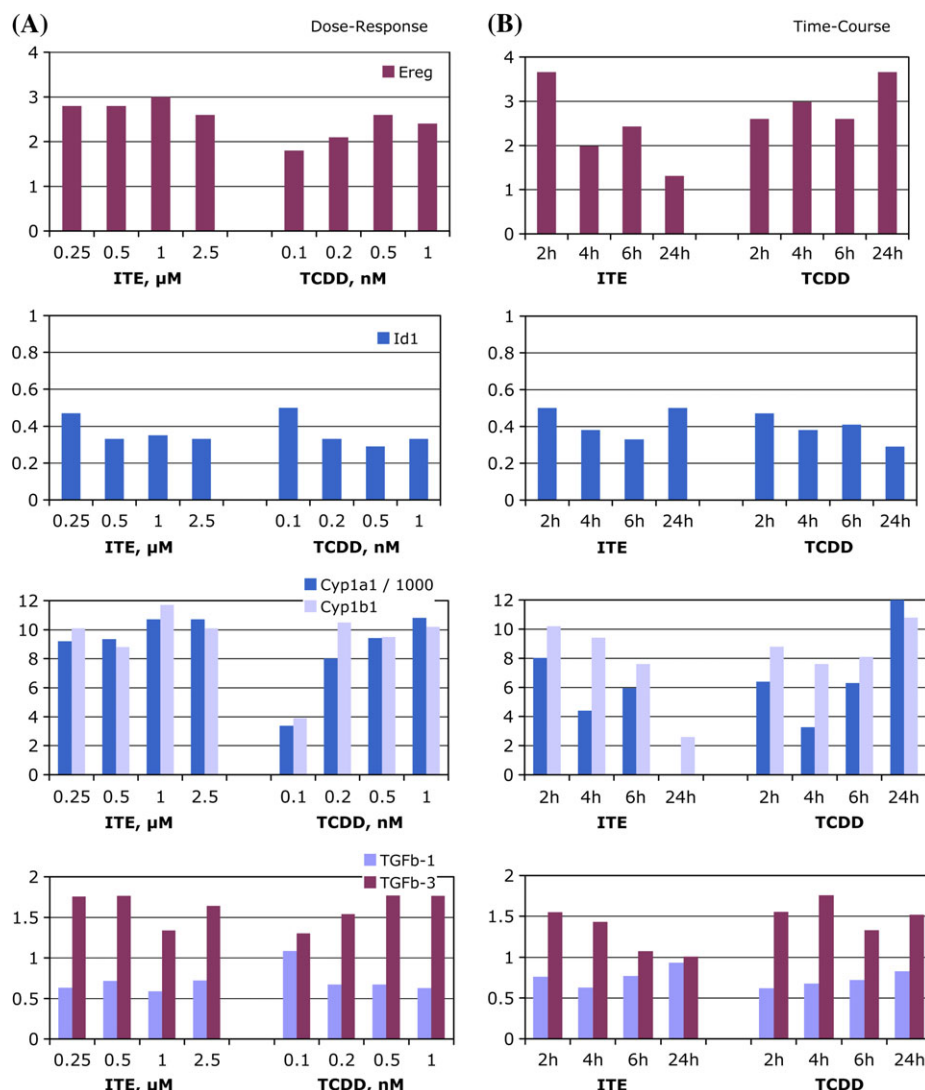


FIG. 4. Time-course and dose responsiveness of transcription of selected genes. Mouse lung fibroblasts were grown and treated as described in “Materials and Methods” section, with a range of concentrations of ITE or TCDD or with DMSO vehicle for 4 h (A), or were treated with DMSO, 0.5 μ M ITE, or 0.5nM TCDD for 2–24 h (B). Total RNA extraction and RT-qPCR were performed as described. Expression levels shown are relative to DMSO and are normalized to *Gapdh* assayed concurrently in the same samples. Representative of at least two experiments.

DISCUSSION

These results show a very close similarity in short-term gene expression changes in response to ITE and TCDD. At least in these lung fibroblast cells in culture, we find no evidence that the two ligands elicit divergent patterns of gene expression that could account for their very different *in vivo* toxicity. Since our original hypothesis was that when AhR is activated by different ligands a different spectrum of genes may be induced/repressed, we used a short exposure period (4 h) for microarray analysis so that observed gene expression changes can be considered to reflect a primary ligand-induced transcriptional response. This primary response was remarkably similar between ligands, both qualitatively and quantitatively, strongly

suggesting that ITE and TCDD elicit the same, or at least a sufficiently similar, conformational change in the AhR to initiate the similar transcriptional responses. The specific genes that respond to AhR ligand treatment are expected to differ among cell type and developmental stage at time of exposure, particularly *in vivo*, since the role of the AhR differs among cells/tissues and with their state of differentiation. However, we predict that the overall similarity in the total spectrum of initial gene expression changes in response to equivalent AhR-activating levels of ITE and TCDD would be observed consistently.

These data further suggest that the disparity in toxicity between these two high-potency AhR ligands is more likely related to their different chemical stability (*in vivo*) and

resultant difference in kinetics. This is consistent with earlier findings that whereas ITE and TCDD have similar AhR-binding affinity and similar AhR-DRE-binding potency *in vitro*, ITE is several orders of magnitude less potent than TCDD in inducing reporter gene activity in treated cells or mice (Henry *et al.*, 2006). The preliminary RT-qPCR time-course results in Figure 4 suggest an analogous kinetic difference in ligand-induced transcription of several genes. Furthermore, the fact that certain TCDD-sensitive genes remain up- or downregulated while ITE effects are less persistent suggests that the spectrum of later secondary gene expression likely differs substantially between ligands. The combination of more prolonged primary gene changes elicited by TCDD and subsequent secondary gene changes (due to prolonged occupancy and activation of the AhR that would not be produced by ITE) likely lead to its toxic actions. Additional studies would be needed to verify such an hypothesis, but much evidence has accumulated to indicate markedly different sequelae from sustained versus transient AhR activation (e.g., Mitchell and Elferink, 2009). Relevant to this concept, the present data also imply that in order to identify those gene expression changes that may be associated with or mediate TCDD toxicity, longer term exposure must be included in the experimental design.

Since the goal of this study was to compare the transcriptional effects of ITE and TCDD, the identity of individual affected genes is considered less important than the remarkable similarity of the whole spectrum of changes elicited by the two ligands. Indeed, the subset of genes whose expression is detectably altered by any particular treatment would depend on the cell type studied and the specific conditions (dose and duration) of exposure. Nonetheless, it is interesting to consider our findings in perspective with published microarray data from other cell types and *in vivo* data on AhR activity. Another putative endogenous ligand, indirubin, has been compared with TCDD with respect to gene expression and metabolism in HepG2 cells (Adachi *et al.*, 2004). In that study, both ligands were at 10nM for 8 h, and the microarray used was limited to 1176 genes relevant to "toxicology." Few genes were affected by either TCDD or indirubin; of the 28 listed as changed and that were also present on our microarrays, only *Cyp1a1* induction was in common with our results. Similar to ITE, *Cyp1a1* induction by indirubin was transient, and indirubin was degraded by CYP1A1 (Adachi *et al.*, 2004). In a recent study comparing human and mouse AhR, it was reported that the human AhR (hAhR) expressed in transgenic mouse liver binds indirubin (and quercetin) with higher affinity than the C57(wild-type) mouse AhR (mAhR) and that indirubin activates the hAhR more potently than the mAHR (Flaveny *et al.*, 2009). Although we found that ITE showed a similar dose-response and time-course of reporter gene induction in cultured human (HepG2) and mouse (Hepa1c1c7) cells (Henry and Gasiewicz, 2006, unpublished data), the data of Flaveny *et al.* (2009) suggest an interesting species-specific ligand selectivity that must be kept in mind when evaluating AhR ligands.

The expected induction of xenobiotic-metabolizing enzymes (including *Cyps*, *Aldh*, and *Nqo1*), as well as *Ahrr*, was prominent in these mouse lung fibroblasts (Table 2), and the GSEA indicated enrichment of such a gene set (Table 4). Several other defined sets that were enriched reflect the inflammatory role of lung fibroblasts (Table 4). These sets share a number of genes that are markers of inflammation, including prostaglandin synthases, phospholipases, and carbonyl reductases. These sets are of particular interest since arachadonic acid derivatives such as eicosanoids may serve as endogenous AhR ligands (Chiaro *et al.*, 2008a,b). Also included in Table 4, although it did not meet the 25% FDR criterion, is the gene set "TGF β signaling pathway," which was downregulated compared with DMSO. Of the 82 member genes, 27 were listed with core enrichment and were downregulated, including *Tgfb1*, *Tgfb2*, *Tgfbr1*, *Id1-4*, *Mapk3*, *Bmp 4* and *6*, among many others. *Tgfb3* is also in this set but was induced. In contrast to the downregulation of a TGF β gene set, one of the most highly induced genes in our study (but not included in this TGF β gene set) was *Tgfbi*, a ubiquitously expressed protein induced by TGF β (type unspecified) that is a component of extracellular matrix, and loss or downregulation of which is associated with tumor cell lines and primary tumor specimens. The arrays also included several probe sets for *Ltbp* (latent TGF-binding proteins), but none were affected by ITE or TCDD. The fact that many TGF β pathway genes were enriched is interesting given the known interaction between AhR and TGF β both in cell culture and *in vivo* and both in the presence and absence of xenobiotic ligand (Gomez-Duran *et al.*, 2009; Haarmann-Stemmann *et al.*, 2009; Puga *et al.*, 2005). TGF β 3 is also the only member of the AHRE-II gene battery listed by Boutros *et al.* (2004) that was changed by ITE/TCDD in the current study. The *Id* proteins (inhibitor of DNA binding, *Id1-4*) were all repressed by ITE and TCDD and contributed to the enrichment of this TGF β gene set. These helix-loop-helix (HLH) proteins dimerize with other HLH proteins, thereby inhibiting their transcriptional activities. TCDD was also reported to downregulate *Id1* and *Id3* in human dermal fibroblasts (Akintobi *et al.*, 2007).

Based on observations that IL1R receptor (IL1R) and tumor necrosis factor receptor 1 and 2 (TNFR1,2) knockout mice are resistant to some types of TCDD hepatotoxicity and liver inflammation, though still show *Cyp* induction, hydropic degeneration, and hepatomegaly, Pande *et al.* (2005) concluded that IL1R-like cytokines mediate some (though not all) responses to TCDD. Since the cells we used are involved in inflammation, it is notable that several IL1R- and TNF receptor-related genes on the array were significantly affected by ITE and TCDD (Table 5). (The curated database of gene sets currently used in the GSEA, however, did not include a set containing this grouping of genes, although some of the individual genes were present in a few sets that were not enriched in this experiment.)

From our study, in which a large number of genes are induced or repressed, it is not possible to say how many of

TABLE 5
Some IL1R- and TNFR-Related Genes Are Altered by
ITE/TCDD

Gene	Gene name	Fold induction by ITE ^a
Il1rap	IL1R accessory protein	0.65
Irak2	IL1R receptor-associated kinase 2	1.83
IL33 (IL-1F11)	Interleukin 33	0.345
IL1rl2	IL1R-like 2	(1.2)
Tnfrsf19	TNF receptor superfamily, member 19	44
Tnfrsf18 GITR	TNF receptor superfamily, member 18	2.18
Tnfrsf12a	TNF receptor superfamily, member 12a	0.5
Traf5	TNF receptor-associated factor 5	2.86
Clqtnf3	Clq- and TNF-related protein 3	8.9

^aNumbers shown are fold induction by ITE relative to DMSO from microarray analysis.

these changes are biologically significant or would be observed consistently or in different cell types. Only 8 out of 22 genes with defined, functional AhREs (as listed in a recent review, Gasiewicz *et al.*, 2008) and on the arrays were affected by ITE and TCDD. Most of these eight are in the xenobiotic-metabolizing family of genes. Many more of the genes identified as up- or downregulated (Supplementary table 1) are obviously involved in a wide variety of basic cellular pathways, which is consistent with the very extensive spectrum of genes that was recently reported to comprise AhR-binding targets as determined by a combination of genetic and *in silico* investigations (Sartor *et al.*, 2009). The promoter regions of many of these targets (including *Tgfb1*, 2, and 3, *Tgfb1*, and *IL1rap*) have AhRE sequences determined computationally but not as yet verified to be functional (Sun *et al.*, 2004).

Microarrays, as utilized for this study, assess the genomic route of response to AhR ligands. There is also evidence for AhR-dependent but nongenomic effects (Matsumura, 2009). Some of these effects occur more rapidly than genomic responses, for example, increased intracellular Ca⁺⁺ is reported to occur within 15–30 min of TCDD addition in a variety of cell types. Calcium influx is proposed to be the initiator of inflammatory responses such as cytosolic phospholipase A2 and Cox-2 induction. These putative nongenomic responses can occur faster because they are mediated by processes such as protein phosphorylation and dephosphorylation rather than the more complex transcription/translation/posttranslational modification. It seems plausible that endogenous AhR ligands such as ITE would also mediate such nongenomic responses, and there may be differences among ligands in ability to initiate such effects. To our knowledge, these signals have not been investigated following treatments with ligands other than TCDD or related chemicals.

In summary, we conclude that the putative endogenous AhR ligand, ITE, and the prototypical xenobiotic ligand, TCDD,

despite their disparate chemical structures, likely elicit an analogous change in AhR conformation such that the initial transcriptional response is essentially the same (as analyzed in mouse lung fibroblasts). If differences in gene expression do mediate the difference in toxicity of these ligands, then it must be later, secondary changes in gene transcription that are enabled by the persistence of TCDD compared with the more short-lived ITE.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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