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The Transcriptional Signature of Dioxin in Human Hepatoma HepG2 Cells

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ABSTRACT. We have used a high density microarray hybridization approach to characterize the transcriptional response of human hepatoma HepG2 cells to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We find that exposure to 10 nM TCDD for 8 hr alters by at least a factor of 2.1 the expression of 310 known genes and of an equivalent number of expressed sequence tags. Treatment with TCDD in the presence of 20 μg/mL of cycloheximide blocked the effect on 202 of these genes, allowing us to distinguish between primary effects of TCDD exposure, which take place whether cycloheximide is present or not, and secondary effects, which are blocked by inhibition of protein synthesis. Of the 310 known genes affected by TCDD, 30 are up-regulated and 78 are down-regulated regardless of cycloheximide treatment, and 84 are up-regulated and 118 are down-regulated only when protein synthesis is not inhibited. Functional clustering of genes regulated by TCDD reveals many potential physiological interactions that might shed light on the multiple biological effects of this compound. Our results, however, suggest that arriving at a sound understanding of the molecular mechanisms governing the biological outcome of TCDD exposure promises to be orders of magnitude more complicated than might have been previously imagined. BIOCHEM PHARMACOL **60**;8:1129–1142, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. TCDD; Ah receptor; microarray hybridization; transcriptional program; signal transduction

TCDD,† the prototype dioxin, causes a large number of apparently unrelated biological effects. In humans, TCDD and many other chlorinated phenolic agents cause chloracne, a long-lasting skin disease characterized by the hyperkeratinization of follicular sebocytes [1, 2]. Epidemiologic studies in accidentally exposed populations have also established a link between high doses of TCDD and certain types of cancer [3–6] and cardiovascular disease [7, 8]. Recent studies in Japan confirm the observations in primates and rodents [9, 10] that TCDD may also be responsible for environmentally induced endometriosis in humans [11]. In mice, TCDD exposure during embryogenesis causes developmental abnormalities [12] including hydronephrosis and cleft palate [13, 14], whereas exposure of adult rats results in an elevated incidence of hepatic carcinoma and pulmonary and skin tumors [15–17]. Exposure of developing lower vertebrates to TCDD causes disturbances of lipid metabolism, cardiovascular and craniofacial teratogenesis [18, 19], and immunotoxic [20], reproductive and endocrine effects [21–24], some of which also appear to be present in exposed humans [25–27]. TCDD is a powerful endocrine disruptor in rodents and in human cells, inhibiting multiple estrogen-induced responses, including development or growth of human mammary and endometrial cancer cells, carcinogen-induced mammary cancer in rats, and mammary cancer in mice bearing breast cancer cell xenografts (reviewed in [28]).

Effects at the cellular level are just as diverse. TCDD inhibits estrogen-dependent proliferation of human breast cancer cells [29], but it induces proliferation of human keratinocytes [30] and rat hepatocytes [31] and causes a decrease in rat hepatocyte proliferation rates [32–34]. In human keratinocytes, TCDD has also been shown to induce terminal differentiation [35–38], whereas immature thymocytes from rats and mice treated with TCDD *in vivo*, but not *in vitro*, may show increased apoptosis [39–41]. TCDD has also been reported to induce apoptosis [31] and to inhibit UV-induced apoptosis [42] in rat hepatocytes.

At a molecular level, most of the effects of TCDD exposure have been known for many years to result from the activation of the AHR, a ligand-activated transcription factor. AHR dimerization with ARNT (HIF-1β) is respon-

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† *Abbreviations:* AHR, aryl hydrocarbon receptor; AhRE, AHR response element; ARNT, aryl hydrocarbon receptor nuclear translocator; CX, cycloheximide; DEPC, diethyl pyrocarbonate; E2F, (adenoviral) E2A (promoter)-(binding) factor; EST, expressed sequence tag; IL, interleukin; IP_3, inositol 1,4,5-triphosphate; HIF, hypoxia-inducible factor; PAI, plasminogen activator inhibitor; PDCD, programmed cell death, PI, phosphatidylinositol; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; RB, retinoblastoma; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transforming growth factor; VAMP, vesicle-associated membrane protein; VEGF, vascular endothelium growth factor; VLDL, very low density lipoprotein; TNF, tumor necrosis factor; and PCR, polymerase chain reaction.

sible for the up-regulation of genes in the *Ah* gene battery [43], which comprises several well-characterized genes in the cytochrome P450 CYP1A family and several Phase II detoxification genes (reviewed in [44, 45]). It is generally believed that up-regulation of gene expression by the TCDD-activated AHR/ARNT complex results from transactivation through promoter domains containing AhRE (also termed XRE, DRE) motifs; however, dioxin effects also include transcriptional repression, as determined for TGF-β2 [35] and fibrinogen γ chain and plastin mRNAs [46], an observation that cannot be explained by invoking a direct transactivation mechanism. It is unclear whether the effect of dioxin on other targets, such as the genes for PAI-2 and IL-1β [47], the *FOS* and *JUN* immediate-early gene families [48, 49], *COX-1* and *COX-2* [50–56], and TNF-α [57–60], is a primary response, a secondary response, a combination of the two, or a higher-order response resulting from interactions between effectors elicited in different tissues of an exposed organism.

TCDD and the AHR have also been shown to regulate more complex biological processes. TCDD causes sustained inhibition of intercellular communication in mouse hepatoma cells [61] and induces extensive oxidative damage in cultured cells [62], in mice [63], and in female rats [64]. Perhaps as a consequence of increased oxidative damage, TCDD has also been found to induce genomic instability by promoting intrachromosal recombination [65]. Activation of the Ah receptor by TCDD has recently been shown to block estrogen-induced proliferation of MCF-7 cells by inhibiting cyclin-dependent kinase 2 (cdk)-, cdk4-, and cdk7-dependent kinase activities and estradiol-induced hyperphosphorylation of RB [28]. TCDD-dependent [66, 67] and -independent [68] effects on cell cycle have been observed, suggesting that the antiproliferative action of TCDD is not limited to estrogen-dependent proliferation. In this context, the TCDD-activated AHR has been shown to induce $p27^{kip1}$ transcription and to inhibit proliferation of rat hepatoma 5L cells and fetal thymocytes [69], to induce cdc2, cdk2, and $P21^{Waf1}$ levels in mouse liver [70, 71], and to bind to hypophosphorylated RB [72, 73] and potentiate RB-mediated active repression of E2F-dependent transcription and entry into S-phase [73]. AHR content, independently of ARNT, modulates aspects of ceramide-induced apoptosis in mouse hepatoma 1c1c7 cells [74].

A complete signature of transcriptional regulatory mechanisms affected by TCDD, which might shed light on the mechanisms responsible for its many biochemical, physiological, and biological effects, has never been obtained. We have used commercially available high density DNA microarrays to begin the development of a database of genes whose expression is affected by TCDD. We have used human hepatoma HepG2 cells in culture, cognizant of the fact that many effects taking place in the whole organism or in a different cell lineage or tissue will not be observed in these cells and that, vice versa, effects in these cells may not occur in other tissues or lineages. Our results reveal many possible clusters of interacting gene functions that may provide an explanation for the multiple effects of TCCD and serve as the basis to propose novel experimental hypotheses.

MATERIALS AND METHODS

Cells, Growth Conditions, and Treatments

Human hepatoma HepG2 cells were grown to 80% confluence in alpha minimal essential medium (α-MEM) (GIBCO BRL Life Technologies) containing 10% fetal bovine serum and 1% antibiotics. Four sets of experimental conditions were analyzed. One set consisted of cells treated for 8 hr with 10 nM TCDD dissolved in methanol; the second set was a control for the previous one and was treated with the same concentration of methanol as the TCDD-treated set. A third set received 20 μg/mL of CX 1 hr prior to being treated with 10 nM TCDD for an additional 4 hr. The fourth set was a control for the third and was treated with 20 μg/mL of CX for 5 hr.

RNA Isolation

RNA was isolated using TRI reagent (MRC, Inc.) followed by purification of polyadenylated RNA using 2 consecutive cycles of Oligotex™ (Qiagen) chromatography. The final PolyA(+) RNA preparations were dissolved in DEPC-treated water at a concentration of 50 ng/μL.

Fluorescent Labeling of Target cDNAs

Labeling of cDNAs, preparation of microarrays, and hybridization reactions were performed as a custom service by Incyte and are briefly described here for informative purposes. Detailed descriptions may be found at the Incyte Web site, http://www.incyte.com. Fluorescently labeled cDNAs were prepared from the polyadenylated RNA preparations using the protocols devised for labeling and hybridization to proprietary gene expression microarrays. Target cDNAs from control RNA samples were labeled by reverse transcription using 5′ Cy3-labeled random 9-mer primers and those from TCDD-treated samples were similarly labeled using Cy5-random 9-mers.

High Density Microarray Hybridization

The Incyte human UNIGEM V microarray was used for these experiments. As of this date, this array consists of 9182 sequence-verified PCR products of human cDNA clones, representing 8635 total unique genes, of which 5686 are annotated UniGene human database clones and 2949 are unannotated ESTs. The hybridization probes were therefore these cDNA PCR products each affixed in a 100-nm diameter spot to polylysine-treated microscope slides. The hybridization targets were the paired Cy3- and Cy5-labeled control and test cDNAs, which were mixed in approximately equal proportions and applied to the mi-

croarray for hybridization under high stringency conditions. After hybridization and washing of unhybridized targets, Cy3 (green) and Cy5 (red) fluorescent channels were simultaneously scanned with independent lasers at 10-μm resolution.

Differential Expression Analyses

We analyzed analog electrical signals from the scanner using the Incyte GemTools™ algorithm to determine differential expression levels of each of the cDNAs detected. Not all cDNA probes hybridize to the target; our criteria for inclusion of a cDNA in the analyses was that the fluorescent signal exceeded a ratio of 2.5 over background and that it covered 40% or more of its grid location on the microarray. Of the 9182 PCR products in the array, only 4076 met these criteria and were included in our analyses. The criteria for inclusion of a cDNA in a group as either induced or repressed expression was whether the balanced differential expression (the ratio of the normalized Cy3/Cy5 fluorescence for that grid location) was greater than 2.1 in either direction, such that, if Cy3/Cy5 $\geq$ 2.1 the gene was considered to be repressed by TCDD and if Cy5/Cy3 $\geq$ 2.1, the gene was considered to be induced. Based on the estimated variability in the system, the 2.1-fold threshold is an optimal choice for balancing the specificity and the sensitivity of the analysis. That is, in addition to maintaining the chance of falsely concluding that a gene is differentially expressed at 6%, it also gives us an 80% chance of detecting any 3-fold or higher differentially expressed gene. To minimize the effect of differentially expressed genes on the estimate of the standard deviation, a standard deviation of 0.4 for log-transformed differential expressions was estimated using the interquartile range-based robust estimator. Assuming a Gaussian distribution for log-transformed differential expressions and based on this estimated value for the standard deviation, 94.0% of balanced differential expressions were expected to be less than 2.1. That is, 6% of genes are expected to have differential expression of at least 2.1 in a single experiment, 0.36% in two experiments, 0.0216% in three experiments, and so on. Accordingly, 28 of the 310 affected genes could have shown expression changes by chance alone, although only 1 or 2 of the genes affected by TCDD both in the presence and the absence of CX would belong in this group. Based on experimental evidence from Incyte, 99% of the cDNAs display $<$1.4 differential expression when compared with themselves and can be considered to be differentially expressed when they change by a ratio greater than 1.7-fold [75].

RESULTS AND DISCUSSION

Treatment of HepG2 cells with 10 nM TCDD resulted in gene expression changes in 310 genes from a total of 5686 annotated gene sequences and an additional 400 ESTs from 2949 unannotated sequences; only 4076 PCR products on the slide gave fluorescent signals that met the inclusion criteria (see the Materials and Methods Section), suggesting that many of the genes included in the array might not be expressed in HepG2 cells. Of the known genes, 114 were up-regulated and 196 down-regulated. Thirty genes were up-regulated and 78 down-regulated whether or not cells had been treated with CX, suggesting that expression of these genes was directly regulated by TCDD-dependent processes. Eighty-four genes were up-regulated and 118 down-regulated only in the absence of CX, suggesting that the effect of TCDD on their regulation was secondary to primary effects and required protein synthesis (Table 1). It must be emphasized that the observed changes reflect differences in mRNA accumulation, which can be due either to transcriptional regulatory mechanisms or to post-transcriptional events involving mRNA stability.

TABLE 1. mRNA changes caused by TCDD in HepG2 cells

	Number of known genes	
Treatment	**Induced**	**Repressed**
10 nM TCDD, 8 hr	84	118
10 nM TCDD + 20 μM CX, 5 hr	30	78
Total	114	196

Approximately 150 TCDD-affected genes code for proteins with distinct functions in interrelated cellular processes, thus allowing us to cluster these genes in groups with related functions. Below and in the following tables, we address each of these groups separately. We include accession numbers, extent of effect by TCDD in the presence and absence of CX, a short description of the gene function and one reference for each gene to a recent review on the function of the corresponding gene as it may pertain to the assigned cluster.

Genes Involved in Ras/MAPK Related Signaling Pathways

Genes in this cluster include *KRAS2* and the guanine nucleotide exchange factor *SOS*, both induced at high levels and blocked by CX by only 50%, suggesting that they are primary TCDD responders (Table 2). Activation of the RAS signaling pathway by other Ah receptor agonists has been documented previously in vascular smooth muscle cells, although in those cells *HRAS1*, not *KRAS2*, was activated [76, 77]. *GAP1* and *MEK5* were also up-regulated by TCDD, but only in the absence of CX. Several genes involved in phosphorylation and dephosphorylation of RAS, RAC, RAY, and RAF were also disregulated, as were PI-3K and the IP_3 receptor. Changes in the expression of these genes are likely to have major consequences in the regulation of cell growth and responses to external stimuli, and may contribute significantly to the carcinogenic potential of TCDD.

TABLE 2. TCDD-induced fold changes in expression of genes involved in Ras/MAPK-related signaling pathways

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
AI002781	Guanine nucleotide exchange factor	7.1	3.1			Ras activation	[86]
L00049	Ki-ras2 proto-oncogene	4.5	2.3			MAPKKK activation	[86]
AB002329	Semaphorin	2.3	2.1			Plexin ligand; PI-3K activator	[87]
D26070	Inositol 1,4,5-triphosphate receptor	2.9	1.3			Calcium mobilization	[88]
U25278	MEK5 (MAP/ERK5)	2.7	1.7			Ser/Thre MAP kinase	[89]
L33075	Ras GAP-1	2.5	1.3			Ras activation	[90]
AI765074	Advillin			2.4	2.6	Downstream Rac effector	[91]
U29171	Casein kinase 1 delta			2.1	2.6	A-Raf, Mos kinase	[92]
AL022729	RAY1			2.3	1.1	Rab1A and Rab1B homolog	[93]
AL034562	SHP-2 tyrosine phosphatase			2.2	1.7	Mediates mitogen-activation of Ras	[94]
U26424	MST2 serine/threonine kinase			2.2	1.2	Signaled to by Rac, Cdc42, and Ras	[95]
X83368	Phosphoinositide 3-kinase			2.1	1.1	Multiple signal transduction pathways	[96]
Z46973	Phosphoinositide 3-kinase			2.1	1.1		

Genes Involved in Calcium Regulation

Calcium level disregulation is one of the major consequences of TCDD exposure in cell culture [50, 78]. Not surprisingly, expression of many genes involved in calcium regulation or in downstream calcium effects was affected by TCDD (Table 3). The IP_3 receptor and phospholamban were affected in different directions, which, given the role that each of these genes has in gating intracellular calcium stores, suggests a major effect of TCDD on intracellular calcium levels. TCDD up-regulated calmodulin expression and down-regulated three different calcium-binding proteins, suggesting that the balance of TCDD exposure would favor protein dephosphorylation and decrease phosphorylation. It is also noteworthy that two phospholipases, A2 and D, are directly or indirectly—through phosphatase 2B—affected by TCDD treatment.

Genes Involved in Receptor-Associated Kinases, Phosphatases, and Their Effectors

This is one of the largest clusters of genes affected by TCDD treatment. In addition to several of the genes

TABLE 3. TCDD-induced fold changes in expression of genes involved in calcium-dependent regulation

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
M21054	Phospholipase A2	2.8	2.4			Arachidonic acid hydrolysis	[97]
D26070	Inositol 1,4,5-triphosphate	2.9	1.3			Calcium mobilization	[88]
AF062075	Calcium-modulating cyclophilin	2.2	1.3			Activates Ca^{2+} influx signaling	[98]
AF068179	Calmodulin	2.1	1.7			Regulation of Ca^{2+}-dependent phosphatases	[99]
AI203843	Neurogranin (PKC substrate)			2.1	2.4	Binds to the Ca^{2+}-free form of calmodulin	[100]
AI743140	Phosphatidic acid phosphatase			2.1	2.3	Phospholipase D-mediated signaling	[101]
AA583574	S100 calcium-binding protein A7			4.1	1.1	Ca^{2+}-dependent protein phosphorylation	[102]
D83664	S100 calcium-binding protein			3.5	1.5		
M80563	S100 calcium-binding protein A4			2.5	1.6		
U00803	FRK, fyn-related kinase			2.5	1.6	Intracellular tyrosine kinase	[103]
M29551	Calcineurin A			2.5	1.6	Phosphatase 2B catalytic subunit	[104]
F02034	Visinin-like factor 1			2.4	1.4	Calcium sensing	[105]
M63603	Phospholamban			2.3	1.7	SERCA regulation	[106]
M19879	Calbindin 1			2.3	1.1	Regulation of intracellular free Ca^{2+}	[107]
AA677417	Spindle pole body protein			2.1	1.7	Binds calmodulin in the absence of calcium	[108]

TABLE 4. TCDD-induced fold changes in expression of genes involved in receptor-associated kinases and phosphatases

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
M21054	Phospholipase A2	2.8	2.4			Arachidonic acid hydrolysis	[97]
X04476	p56 Lck (LSTRA)	2.8	2.1			T-cell receptor-associated tyrosine kinase	[109]
X51602	FLT1, fms-related tyrosine kinase	2.2	2.1			VEGF receptor-associated tyrosine kinase	[110]
X06318	Protein kinase C-beta	2.1	2.2			Vascular dysfunction	[111]
D26070	Inositol 1,4,5-triphosphate receptor	2.9	1.3			Calcium mobilization	[88]
Z15108	Protein kinase C-zeta	2.8	1.7			Ras and ceramide downstream effectors	[149]
M69043	IκB-alpha	2.6	1.2			NF-κB inhibitor	[112]
M35198	Integrin beta 1	2.5	1.7			Cell adhesion; receptor phosphorylation	[113]
M35999	integrin beta 3	2.1	1.7				
X62654	CD63 antigen (integrin-associated)	2.1	1.6			Cell adhesion; receptor phosphorylation	[114]
AI272117	PKA regulatory subunit			2.5	2.3	cAMP-dependent signaling	[115]
L36151	Phosphatidylinositol 4-kinase			2.1	3.7	Polyphosphoinositide metabolism	[116]
AI743140	Phosphatidic acid phosphatase type			2.1	2.3	Phospholipase D-mediated signaling	[101]
L20321	Serine/threonine kinase 2			2.1	2.1	Stress-activated protein kinase pathways	[117]
AI679827	Thy-1 cell surface antigen			2.9	1.1	T-cell receptor signaling	[169]
AA583574	S100 calcium-binding protein A7			4.1	1.1	Ca^{2+} regulation of protein phosphorylation	[102]
D83664	S100 calcium-binding protein A12			3.5	1.5		
M80563	S100 calcium-binding protein A4			2.5	1.6		
U00803	FRK, fyn-related kinase			2.5	1.6	Intracellular tyrosine kinase	[103]
M29551	Calcineurin A			2.5	1.6	Phosphatase 2B catalytic subunit	[104]
AB002309	A-kinase anchor protein			3.4	1.4	PKA compartmentalization	[118]
M90359	A-kinase anchor protein			2.5	1.5		
X03884	CD3E (T-cell receptor complex)			2.5	1.2	T-cell activation and signaling	[119]
AL034562	SHP-2 tyrosine phosphatase			2.2	1.7	Mediator of Ras and MAPK activation	[94]
U26424	MST2 serine/threonine kinase			2.2	1.2	Signaled to by Rac, Cdc42, and Ras	[95]
X83368	Phosphoinositide 3-kinase			2.1	1.1	Multiple signal transduction pathways	[96]
Z46973	Phosphoinositide 3-kinase			2.1	1.1		

already discussed in previous sections, it includes at least two different members of the PKC family, two cell receptor-associated tyrosine kinases, and several integrins and integrin-associated proteins with functions in cell adhesion and receptor kinase phosphorylation (Table 4). These proteins are induced, but others with similar functions, including several serine/threonine and tyrosine kinases, are repressed, suggesting that one of the main consequences of TCDD exposure might be a derailing of posttranslational mechanisms regulating protein activity.

TABLE 5. TCDD-induced fold changes in expression of genes coding for transcription factors

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
AF000670	E74-like Ets-domain transcription	2.5	1.4			Angiogenesis; vascular morphogenesis	[120]
J04076	Egr-2 transcription factor	2.4	1.6			Gene expression in the nervous system	[121]
AI272010	Egr-1 transcription factor	2.4	1.4				
L06895	MAD (MAX dimerization partner)	2.4	1.1			Repression of MYC-regulated genes	[122]
L08895	MADS box transcription enhancer			4.3	1.2	MYC coregulator	[123]
AB017365	Frizzled (*Drosophila*) homolog			3.2	1.6	Wnt receptor/transcription/development	[124]
U13219	FREAC-2 forkhead-like protein			2.1	1.6	Lung embryonic development	[125]

TABLE 6. TCDD-induced fold changes in expression of genes involved in cardiovascular and pulmonary function

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
X04744	Plasminogen activator inhibitor 1	2.6	2.1			Fibrinolysis inhibitor, t-PA inhibitor	[126]
X51602	FLT1, fms-related tyrosine kinase	2.2	2.1			VEGF receptor-associated tyrosine kinase	[110]
X06318	Protein kinase C-beta	2.1	2.2			Vascular dysfunction	[111]
X07897	Troponin C	3.1	1.4			Cardiac muscle contractility	[127]
D26070	Inositol 1,4,5-triphosphate receptor	2.9	1.3			Calcium mobilization	[88]
X73029	Inducible NO synthase	2.7	1.6			NO-mediated hepatocyte injury	[128]
Y12476	G protein-coupled endothelin	2.5	1.7			hypertension, heart failure	[129]
AF000670	E74-like Ets-domain transcription	2.5	1.4			Angiogenesis; vascular morphogenesis	[120]
M93718	Endothelial NO synthase	2.3	1.7			NO production in blood vessels. Ischemia	[130]
M19481	Follistatin	2.3	1.3			Produced in atherosclerotic lesions	[131]
AF068179	Calmodulin	2.1	1.7			Ca^{2+}-regulated kinases and phosphatases	[99]
AI292063	FGL2, prothrombinase			5.4	3.7	Coagulation	[132]
L25615	Arginine vasopressin receptor 1A			4.1	2.9	Antidiuretic response; hypertension	[133]
X52195	5-Lipoxygenase-activaging protein			2.8	2.3	5-Hydroxyeicosatetraenoic acid generation	[134]
AA063086	Midkine			2.7	3.2	Positive regulator of tumor angiogenesis	[135]
D16494	Very low density lipoprotein receptor			3.5	1.6	Cholesterol homeostasis. Atherosclerosis	[136]
M11723	Coagulation factor XII (Hageman)			3.3	1.7	Coagulation; up-regulated by estrogen	[137]
M84755	Neuropeptide Y receptor Y1			2.9	1.2	Modulation of blood pressure homeostasis	[138]
AJ000185	Vascular endothelium growth factor			2.6	1.3	Angiogenesis	[139]
AI190041	Multiple exostoses 2 (EXT2)			2.3	1.6	Matrix biosynthesis; antithrombotic	[140]
AI128100	Dermatan sulphate proteoglycan			2.2	1.1	Anticoagulant, antithrombotic	[141]
L01131	Decorin			2.3	1.2	Proteoglycan in atherosclerotic lesions	[142]
M16552	Thrombomodulin			2.2	1.1	Blood vessel anticoagulant mediator	[143]

Genes Coding for Transcription Factors

Activation and repression of transcription factors and the ensuing changes in gene regulation is one of the hallmarks of the molecular mechanisms of TCDD action [79, 80]. Table 5 lists the factors that we found to be affected in our experiments, including transacting factors involved in angiogenesis, MYC proto-oncogene activity, and lung development. None of the genes in this cluster are directly activated by TCDD, but rather their activation seems to be secondary to primary drug effects.

Genes Involved in Cardiovascular and Pulmonary Function

Dioxins and other ligands of the Ah receptor have been epidemiologically associated with ischemic heart disease [7, 8], with disturbance of regulatory mechanisms of gene expression in vascular endothelium [18, 81] and cardiac myocytes [78], and with induction of PAI-2 [47]. In addition, cross-talk between the AHR/ARNT complex and the HIF-1α/HIF-1β(ARNT) can cause the repression of hypoxia-inducible genes by induction of Ah receptor-mediated responses. Many genes involved in cardiopulmonary function were affected by TCDD (Table 6). Among these, PAI-1, a second member of the tissue plasminogen activator inhibitor family, was induced as a primary TCDD response, as well as *FLT1*, a VEGF receptor-associated tyrosine kinase. Troponin C, the endothelin receptor, and both the inducible and endothelial forms of NO synthase were induced only in the absence of CX. VEGF, the VLDL receptor, and several enzymes involved in coagulation and anticoagulation, blood pressure, hypertension, and angio-

TABLE 7. TCDD-induced fold changes in expression of genes involved in cell cycle regulation, differentiation, and apoptosis

		Increased		Decreased			
Accession	Gene name	TCDD	TCDD + CX	TCDD	TCDD + CX	Function	Reference
AA283172	Granzyme A (serine protease)	2.5	2.3			T-lymphocyte apoptosis	[144]
N87720	Cyclin B2	2.2	2.2			Cell cycle regulation	[145]
U35735	Human RACH1	2.2	2.1			Cell cycle, apoptosis	[146]
AA953246	Matrilin 2	2.1	2.3			Extracellular matrix, differentiation	[147]
AI161010	Lamin B receptor	2.1	2.2			Nuclear organization, differentiation	[148]
Z15108	Protein kinase C-zeta	2.8	1.7			Ras and ceramide effectors	[149]
U25804	Caspase-4	2.7	1.2			Apoptosis	[150]
M57230	Interleukin-6	2.5	1.5			Acute-phase response; inflammation	[151]
AI401549	PDCD2 (Programmed cell death)	2.5	1.5			Expressed during apoptosis	[152]
X65019	Caspase-1 (ICE)	2.4	1.3			IL-1-beta and IL-18 activation	[153]
L06895	MAD (MAX dimerization partner)	2.4	1.1			Repression of MYC-regulated genes	[122]
D86042	Tumor necrosis factor receptor	2.3	1.3			Apoptosis	[154]
L14812	p107, RB-related protein	2.2	1.1			G1 to S transition, cell cycle regulation	[155]
AI624501	TNF superfamily, member 10			3.6	2.3	Apoptosis activation	[156]
U08137	TNF superfamily, member 6			3.1	2.1		
AF019047	TNF superfamily, member 8			2.6	2.1		
U03398	TNF superfamily, member 9			2.5	2.3		
L33801	Glycogen synthase kinase 3 beta			2.9	2.5	Regulation of hepatocyte cell fate	[157]
U69161	CC3			3.1	1.7	Metastasis suppression. Apoptosis	[158]
L13698	Growth arrest-specific GAS-1 gene			2.8	1.6	Overexpression blocks cell proliferation	[159]
AA583574	S100 calcium-binding protein A7			4.1	1.1	Ca^{2+}-dependent regulation of proliferation	[102]
D83664	S100 calcium-binding protein A12			3.5	1.5		
M80563	S100 calcium-binding protein A4			2.5	1.6		
U29700	Anti-Mullerian hormone receptor			2.5	1.2	Regulation of male gonadal differentiation	[160]
AI073657	Heat-shock protein hsp40			2.3	1.4	Cell cycle control	[161]
AA677417	Spindle pole body protein			2.1	1.7	Binds calmodulin; essential for cell cycle	[108]
AA923277	NEK-2 Ser/Thre kinase			2.1	1.3	Cell cycle-regulated kinase; G2/M transition	[162]

genesis were repressed. TCDD effects on the VLDL receptor have long been known to take place [82].

Genes Involved in Cell Cycle Regulation, Differentiation, and Apoptosis

As indicated in the introductory section, the Ah receptor plays an important role in cell cycle progression, differentiation, and apoptosis resulting from exposure to xenobiotic ligands as well as from constitutive receptor activation. Induction of cyclin B2 and p107 and repression of spindle pole body protein, NEK2, and several calcium-binding proteins (Table 7) suggest that the activated Ah receptor might have a major role in the inhibition of cell cycle progression, a conclusion independently confirmed in recent experiments from our laboratory [73]. Induction of granzyme A, several caspases, TNF receptor, and PDCD2 support the previous findings of induction of apoptosis by TCDD; however, TCDD also repressed CC3 and four TNF family members, in agreement with the dual role of TCDD in apoptosis observed in many laboratories and briefly summarized in the introduction.

Genes Involved in Development, Cell Adhesion, Cancer, and Metastasis

The functions of several of the genes in this group are intimately connected to many of the observed biological effects of TCDD exposure. Such is the case of genes involved in metastasis and focal adhesion, such as the

TABLE 8. TCDD-induced fold changes in expression of genes involved in development, cell adhesion, cancer, and metastasis

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
AB002329	Semaphorin	2.3	2.1			PI-3K activator; invasiveness, metastasis	[87]
AI553872	Breakpoint cluster region	2.2	2.1			Philadelphia chromosome; CML	[163]
AA953246	Matrilin 2	2.1	2.3			Extracellular matrix, differentiation	[147]
AI079858	LIM domain	4.1	1.1			Focal adhesion	[186]
M65030	Alpha (1,3) fucosyltransferase	2.4	1.7			Marker of chronic liver disease and hepatoma	[187]
M35198	Integrin beta 1	2.5	1.7			Cell adhesion; phosphorylation of receptor kinases	[113]
M35999	Integrin beta 3	2.1	1.7				
X62654	CD63 antigen	2.1	1.6				
Z34918	Translation initiation factor 4	2.1	1.3			Regulates overall rate of translation	[164]
U07664	Homeobox HB9			6.4	3.3	Neuronal, pancreatic, lymphoid development	[165]
S69369	Homeobox Pax3			3.3	2.5	Cell adhesion, morphogenesis	[166]
AF009801	Bagpipe homeobox homolog			2.3	2.1	Vertebral column, cranium bone development	[167]
D14582	Epimorphin			3.5	1.7	Hair follicle morphogenesis	[168]
AB017365	Frizzled (*Drosophila*) homolog			3.2	1.6	Wnt receptor/transcription and development	[124]
U69161	CC3			3.1	1.7	Metastasis suppressor/apoptosis activation	[158]
AI679827	Thy-1 cell surface antigen			2.9	1.1	T-cell receptor signaling	[169]
U29700	Anti-Mullerian hormone receptor			2.5	1.2	Regulation of male gonadal differentiation	[160]
AI491762	CD47 antigen			2.2	1.4	Integrin-associated; cell adhesion	[170]

integrins, CD63, semaphorin, and matrilin, all of which are induced by TCDD (Table 8). On the other hand, three homeobox genes are strongly repressed, even in the presence of CX. Repression of these genes may be involved in the teratogenic and developmental effects of TCDD [13]. We also find that the anti-Mullerian hormone receptor, a receptor involved in regulation of male gonadal differentiation, is repressed by TCDD, a finding that might explain the effects of TCDD on the male reproductive system of rats [83, 84] and possibly other vertebrates.

TABLE 9. TCDD-induced fold changes in expression of genes involved in protein traffic and membrane integrity

Accession	Gene name	Increased		Decreased		Function	Reference
		TCDD	TCDD + CX	TCDD	TCDD + CX		
AI424266	Phosphatidylinositol glycan, class H			3.5	3.1	Protein glycosylation	[171]
N69061	Phosphatidylinositol glycan, class K			3.2	2.2		
AI697591	Phosphatidylinositol glycan, class C			2.4	2.4		
L19711	Dystrophin-associated glycoprotein 1			3.4	2.5		
D63998	Mannosidase alpha type II			2.3	2.1	Glycoprotein biosynthesis	[172]
AA526368	VAMP 8			2.2	2.1	Docking of synaptic vesicles	[173]
AA223608	VAMP-associated protein			2.1	2.2	Endosomal trafficking	[173]
L36151	Phosphatidylinositol 4-kinase, catalytic			2.1	3.7	Cytoskeleton lipid–protein interactions	[116]
AI093196	Ankyrin 2, neuronal			4.1	1.7	Protein trafficking	[174]
AI521872	Gamma-butyrobetaine hydroxylase			3.7	1.7	Dioxygenase. Carnitine deficiency	[175]
D14582	Epimorphin			3.5	1.7	Hair follicle morphogenesis	[168]
AA039929	Membrane fatty acid desaturase			2.5	1.6	Maintenance of biological membranes	[176]
AI190041	Multiple exostoses 2 (EXT2)			2.3	1.6	Cellular matrix biosynthesis	[140]

TABLE 10. TCDD-induced fold changes in expression of genes involved in drug metabolism and DNA stability

		Increased		Decreased			
Accession	Gene name	TCDD	TCDD + CX	TCDD	TCDD + CX	Function	Reference
K03191	Cytochrome P450 CYP1A1	11.9	8.9			Electron transfer	[43]
U92322	Sulfotransferase 2B family member	2.6	1.7			Sulfation of phenolic procarcinogens	[177]
AA857130	*N*-methylpurine-DNA glycosylase	2.4	1.7			DNA adduct repair; base excision	[178]
AA191633	Thioredoxin peroxidase	2.3	1.5			Induced by ROS and electrophiles	[179]
AA707038	3-Hydroxybutyrate dehydrogenase			4.3	2.3	Fatty acid oxidation	[180]
AI359974	Suppressor of Ty homolog			3.2	2.5	Chromatin structure and remodeling	[181]
AA448993	Estrogen sulfotransferase			2.5	2.6	Estradiol source in breast cancer tiss	[182]
AI521872	Gamma-butyrobetaine hydroxylase			3.7	1.7	Dioxygenase. Carnitine deficiency. Fa	[175]
AI888396	Mammalian mutS homolog			3.6	1.7	Mismatch repair; chromosome instabil	[183]
X79444	Endonuclease G			2.4	1.6	Mitochondrial DNA oxidative damage	[184]
AI702009	Cytochrome c-1			2.4	1.5	Mitochondrial electron transfer	[185]

Genes Involved in Protein Traffic and Membrane Integrity

The most interesting aspect of this cluster is that all the genes included in it are repressed by dioxin (Table 9). Several phosphatidylinositol glycans involved in protein glycosylation are repressed even in the presence of CX, as well as genes coding for ankyrin, VAMP and VAMP-associated protein, epimorphin and others. The proteins encoded by these genes are likely to be involved in the well-known effect of TCDD on cellular membrane disruption [85].

Genes Involved in Drug Metabolism and DNA Stability

We have included two independent groups of genes in this cluster (Table 10). Predictably, the cytochrome P450 *CYP1A1* is the gene with the highest induction level. Several other genes involved in electron transfer, detoxification of phenolic carcinogens, and oxygen metabolism are also included in this group. In addition, *N*-methylpurine-DNA glycosylase, a gene involved in DNA adduct repair, is also induced, and this might explain the observation that TCDD induces formation of oxidized bases [62, 63]. On the other hand, the human *mut*S homolog and endonuclease G, two genes involved in mismatch repair and oxidative damage repair, respectively, are repressed, suggesting that their absence may be responsible for the induction of intrachromosomal recombination by TCDD and other Ah receptor agonists [65].

CONCLUSIONS

Functional clustering of genes regulated by TCDD reveals multiple physiological interactions that might explain many of the biological consequences of TCDD exposure, yet no unambiguous picture emerges from these results. For example, in most cases in which cellular processes such as apoptosis or angiogenesis are involved, some of the different genes with related roles are induced, whereas others are repressed with no apparent underlying rationale. Furthermore, although the majority of genes affected are involved in signal transduction or signal perception mechanisms and lend themselves to attractive speculation, their role in the biology of dioxin effects has never been assessed. Notwithstanding, most of the genes analyzed have proven functions that connect them to the plethora of biological effects resulting from TCDD exposure; hence, we expect that our results will serve as a springboard for the testing of hypotheses designed to bridge the gap between the functions of individual TCDD-disregulated genes in a cluster and the physiological response to exposure. Our results suggest that the establishment of such a connection might be orders of magnitude more complicated than previously imagined.

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