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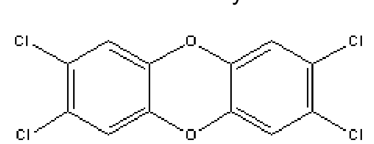
Next Generation Scientists—Next Opportunities

2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) Induces Organ-specific Differential Gene Expression in Male Japanese Medaka (*Oryzias latipes*)

overview

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD or dioxin) is a widely studied polychlorinated, tricyclic aromatic compound known to induce adverse affects in humans and wildlife, including cancer, reproductive and developmental effects, immunotoxicity, and cardiovascular disease. In recent years, unbiased genome-wide gene expression analysis has been exploited using *in vitro*- and *in vivo*-based mammalian models to uncover additional AhR-dependent or -independent TCDD-responsive genes. However, the majority of these studies have centered on gene expression analysis in TCDD-exposed hepatoma cells or liver. To this end, we utilized suppression subtractive hybridization (SSH) as an impartial screening tool to initially evaluate qualitative gene expression changes in male Japanese medaka (*Oryzias latipes*) organs (brain, liver, and testis) following intraperitoneal TCDD injection (10 µg-TCDD/kg-body weight) and exposure for 48 h. SSH analysis provides non-biased evaluation of mRNA-level differences between control and toxicant-exposed animal tissues, and relies on hybridization-dependent subtraction of equally abundant transcripts and selective PCR-amplification and enrichment of differentially expressed genes.

In this study, after identification of suspected differentially expressed transcripts based on SSH, expression of genes hypothesized to be strongly responsive to TCDD exposure was semi-quantified using organ-specific replicate nylon membrane cDNA arrays. Moreover, qualitative histopathologic evaluation was used to associate organ histopathology with gene expression patterns in male medaka brain, liver, and testis. Overall, we demonstrate that TCDD induces organ-specific qualitative and semi-quantitative gene expression differences in male medaka, and that these differences are associated with adverse histopathological changes. Based on these data, brain-, liver- and testis-specific mRNA-level targets in male medaka were identified as promising biomarkers of TCDD-induced toxicity for future investigations.



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

- Sources include incomplete combustion, industrial discharge, and biological processes.
- In the U.S. (1995), air emissions account for the majority of environmental TCDD release, where municipal solid waste incineration, backyard refuse barrel burning, and medical waste incineration contribute >70% of total dioxin releases (NCEA, ORD, U.S. EPA).
- Insoluble in water (19.3 ng/L at 25ºC) and relatively immobile and persistent on soil ($t_{1/2}$ = 1-3 yr) and sediment ($t_{1/2}$ > 1.5 yr) surfaces.
- Highly lipophilic (log K_{ow} = 6.80) and significant bioaccumulation and biomagnification potential (log BCF = 3.2-3.9 in fish) in ecological food chains, resulting in elevated levels in wild animals and commercial food supply.

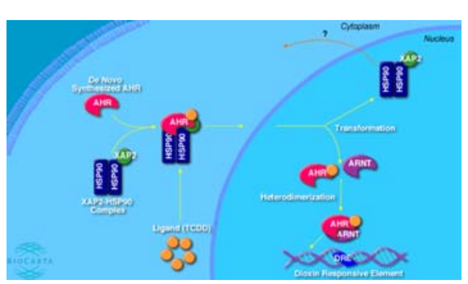


Figure 1. Upon entry into cellular cytosol, TCDD is highly specific for the aryl hydrocarbon (Ah) receptor (AhR). Following ligand binding, activated AhR enters the nucleus, dissociates from the XAP2-HSP90 complex, and forms a heterodimer with AhR nuclear translocator (ARNT); this transcription factor complex binds to dioxin response elements (DREs) in promoter regions of target genes, driving activation of the "Ah gene battery" and regulation of numerous yet unidentified dioxin-inducible genes. In general, these TCDD-induced signaling pathways elicit down-stream adverse effects such as oxidative stress, cell cycle arrest, and apoptosis. Pathway image from Biocarta (biocarta.com).

research findings

Table 1. Subtractive hybridization summary for TCDD-exposed male medaka organs.

	Medaka Organ			
	Brain	Liver	Testis	Total
Colonies screened	768	768	768	2304
True positives sequenced	184 (23.9%[a])	335 (43.6%)	261 (33.9%)	780 (33.9%)
Redundant genes	98 (12.8%)	258 (33.6%)	214 (27.9%)	570 (24.7%)
No significant homology	86 (11.2%)	77 (10.0%)	47 (6.1%)	210 (9.1%)
Non-redundant genes	58 (7.6%)	112 (14.6%)	165 (21.5%)	335 (14.5%)
Genes spotted on cDNA array	8 (1.0%)	22 (2.9%)	12 (1.6%)	42 (1.8%)

[a] All percentage values are relative to total colonies screened from each organ.

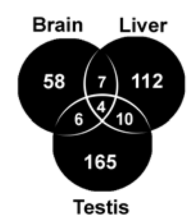


Figure 2. Numerical distribution of non-redundant genes identified in adult male medaka brain, liver, and testis following a 48-h 10 µg-TCDD/kg-body weight exposure. Of 335 total genes identified, only 2-3% of TCDD-responsive genes in all three libraries were shared between any two libraries, suggesting a high degree of organ specificity at the level of gene expression in response to TCDD exposure.

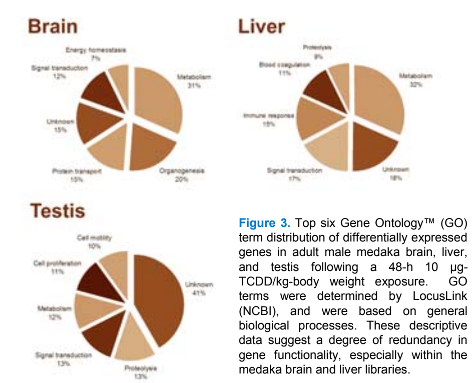


Figure 3. Top six Gene Ontology™ (GO) term distribution of differentially expressed genes in adult male medaka brain, liver, and testis following a 48-h 10 µg-TCDD/kg-body weight exposure. GO terms were determined by LocusLink (NCBI), and were based on general biological processes. These descriptive data suggest a degree of redundancy in gene functionality, especially within the medaka brain and liver libraries.

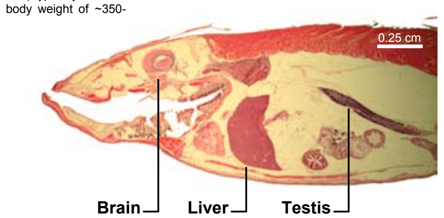
Table 2. Array data summary of gene expression changes in male medaka organs following 48-h exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Gene Name (in alphabetical order)	Origin[a]	Accession ID[b]	Gene Ontology™	Brain TRT/CTL[c]	Brain P[d]	Liver TRT/CTL	Liver P	Testis TRT/CTL	Testis P
Actin, beta	Testis	AJ630123	Cell motility	0.951	0.811	1.116	0.319	**0.684**	0.035**
Acyl-coenzyme A dehydrogenase	Brain	AAH45911	Fatty acid metabolism	1.049	0.819	1.157	0.209	**0.764**	0.079*
Alpha-2-HS-glycoprotein	Liver	CAC27520	Acute phase response	0.921	0.709	1.097	0.577	**0.652**	0.011**
Amylase, alpha	Testis	AF416651	Carbohydrate metabolism	1.032	0.897	**1.189**	0.077*	**0.683**	0.017**
Apolipoprotein A-1	Liver	AAP20157	Lipid transport	1.029	0.884	1.000	0.995	**0.730**	0.009***
Apolipoprotein B	Liver	AAP97376	Lipid transport	0.920	0.753	1.068	0.456	**0.808**	0.034**
Apolipoprotein C-I	Liver	Q9XSN5	Lipid transport	1.279	0.418	**1.244**	0.096*	0.990	0.922
Apolipoprotein E	Brain	AAP20154	Lipid transport	1.006	0.979	1.143	0.188	**0.786**	0.008***
Apolipoprotein E	Liver	CAB65356	Lipid transport	2.245	0.421	1.069	0.557	**0.849**	0.052*
Bile salt-activated lipase	Testis	AAN63868	Cholesterol metabolism	1.011	0.961	1.146	0.226	**0.742**	0.057*
Carboxypeptidase B	Testis	AB099302	Proteolysis	1.023	0.918	1.166	0.123	**0.716**	0.073*
C-myc binding protein	Testis	XP_216518	Mitosis	1.052	0.842	**1.187**	0.093*	**0.710**	0.013**
Coagulation factor XIIIb, precursor	Liver	XP_355235	Blood coagulation	0.957	0.844	1.170	0.158	**0.773**	0.012**
Complement C2	Liver	D84063	Complement activation	1.025	0.904	**1.370**	0.037*	**0.744**	0.045**
Complement C3-1	Liver	AB025575	Complement activation	1.039	0.853	1.001	0.993	**0.771**	0.023**
Complement C3-2	Liver	AB025576	Complement activation	0.956	0.829	0.963	0.779	**0.707**	0.013**
Complement C4	Liver	AB025577	Complement activation	1.071	0.751	1.096	0.267	**0.739**	0.012**
Cytochrome P450 1A	Brain	AY297923	Drug/toxicant metabolism	1.120	0.588	**1.613**	0.006***	0.886	0.292
Cytochrome P450 1A	Liver	AY297923	Drug/toxicant metabolism	**1.585**	0.055*	**2.538**	0.069*	1.009	0.958
Elastase 1, precursor	Testis	AB029755	Proteolysis	1.048	0.837	1.002	0.982	0.853	0.126
Elastase A, precursor	Testis	AB029756	Proteolysis	1.051	0.830	1.122	0.265	0.803	0.103
Ependymin	Liver	AAB40063	Cell adhesion	0.977	0.908	1.295	0.307	**0.723**	0.031**
Fibrinogen, alpha	Liver	AAH41754	Blood coagulation	1.008	0.973	1.077	0.318	**0.746**	0.006***
Fibrinogen, gamma	Liver	AJ310418	Blood coagulation	1.053	0.821	1.114	0.224	0.823	0.150
Glyceraldehyde 3-phosphate dehydrogenase	Brain	BAB62812	Glycolysis	0.964	0.880	1.039	0.791	**0.701**	0.026**
Hepcidin precursor	Liver	AAS66305	Immune response	0.969	0.891	**1.465**	0.074*	**0.587**	0.003***
High choriolytic enzyme 1, precursor	Testis	AAL40376	Unknown	0.915	0.575	1.177	0.132	**0.645**	0.009***
Male sex-determining protein	Testis	AY129241	Sex differentiation	1.224	0.487	**1.450**	0.056*	**0.768**	0.087*
MHC Class I	Liver	AB073376	Immune response	0.874	0.391	**1.258**	0.047**	**0.715**	0.027**
Mitochondrial DNA	Testis	AP004421	Unknown	0.935	0.759	0.977	0.831	**0.843**	0.045**
Myo-inositol oxygenase	Liver	AF401311	Myo-inositol catabolism	1.046	0.848	1.171	0.119	0.756	0.112
NADH dehydrogenase	Brain	NP_739815	Fatty acid biosynthesis	1.040	0.888	1.078	0.572	**0.760**	0.006***
Nucleoside diphosphate kinase	Brain	AF266216	Nucleoside metabolism	1.128	0.662	1.202	0.260	0.867	0.206
Plexin A2	Brain	AY358496	Development	0.935	0.625	1.255	0.122	**0.726**	0.016**
Precerebellin-like protein	Liver	AAF04305	Neurogenesis	0.993	0.971	1.134	0.659	**0.745**	0.002***
Protamine	Testis	D63796	Spermatogenesis	1.015	0.940	1.104	0.286	**0.681**	0.059*
Tributyltin-binding protein	Liver	BAB83525	Unknown	1.054	0.807	**1.279**	0.025**	**0.767**	0.007***
Transferrin	Liver	D64033	Iron ion transport	0.949	0.779	1.116	0.133	**0.725**	0.010***
Trypsinogen 1	Testis	AB029750	Proteolysis	1.004	0.988	1.155	0.281	**0.671**	0.020**
Tubulin, alpha	Brain	AAP89018	Microtubule movement	0.930	0.721	**1.479**	0.046**	**0.686**	0.003***
UDP-glucoronosyl transferase	Liver	AJ249082	Drug/toxicant metabolism	1.091	0.730	**1.355**	0.035**	**0.759**	0.009***
Warm-temperature-acclimation-related-65 kDa-protein	Liver	AB075198	Acute phase response	1.022	0.914	0.913	0.413	**0.729**	0.019**
Control (*Arabidopsis* Cab1)	N/A	X56062	N/A	0.984	0.515	1.021	0.466	1.006	0.777

[a] Original organ-based subtracted cDNA library where gene was identified.
[b] Accession IDs are from GenBank (http://www.ncbi.nlm.nih.gov) and identify sequences with significant homology (E<1X10[illegible]) to subtracted cDNAs in this study.
[c] Bold ratios denote significant gene expression differences based on least square means of normalized array density values.
[d] P values based on a mixed linear model considering fixed- and random-effects parameters; P<0.10*, P<0.05**, P<0.01***.

Figure 4. Artistic rendition of an adult male medaka (right) and whole-body sagittal section (6-8-µm) (below) of a control adult male medaka ip-exposed to vehicle (DMSO) for 48 hr. Medaka is a small (~3-4 cm) egg-laying freshwater fish native to Japan, Korea, and eastern China. Adult males (6-8-months-old) typically have an average body weight of ~350-400 mg.

Adult male medaka (3-4 cm long)



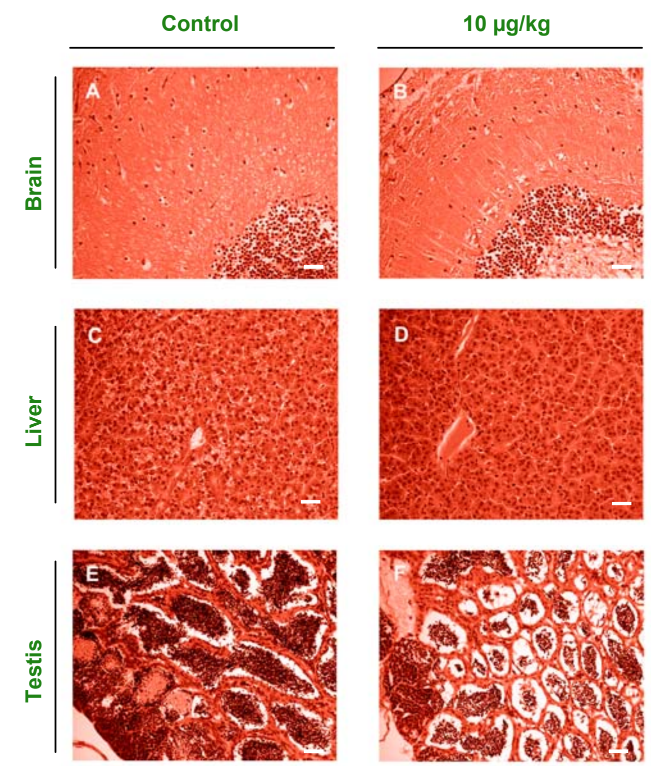


Figure 5. Histopathology of adult male medaka following a 48-h 10 µg-TCDD/kg-body weight exposure. Serial 6-8-µm whole-body sagittal sections were mounted on glass slides and stained with hematoxylin and eosin. All slides were imaged at 40X objective. Scale bar = 10 µm. (A) Brain from vehicle (DMSO) control medaka; (B) brain from TCDD-exposed medaka; (C) liver from vehicle (DMSO) control medaka; (D) liver from TCDD-exposed medaka; (E) testis from vehicle (DMSO) control medaka; and (F) testis from TCDD-exposed medaka.

conclusions

suppression subtractive hybridization

- 780 total cDNAs were sequenced: 210 (27%) sequences had no significant homology and 98, 258, and 214 sequences from the brain, liver, and testis respectively (570 total) sequences had significant homology to known sequences in NCBI databases.
- 58, 112, and 165 total distinct genes for the brain, liver, and testis respectively; although the same number of colonies were screened, the frequency of one or more genes within the library affected the library gene diversity and number of non-redundant genes.
- 2-3% of TCDD-responsive genes in all three libraries were shared between any two libraries, and only 4 genes were identified in all three libraries, suggesting a high degree of organ specificity at the level of gene expression in response to TCDD exposure.
- 31% (13 genes) and 32% (26 genes) of genes identified in the brain and liver library are involved in metabolism, and 41% (50 genes) of identified genes in the testis library have no known function.

gene arrays & organ histopathology

- Of 42 transcripts screened, CYP1A mRNA was the only transcript significantly higher in TCDD-exposed brain, whereas 12 transcripts (including CYP1A) were significantly higher in TCDD-exposed liver and 34 transcripts were significantly lower in TCDD-exposed testis.
- Minimal histopathological changes observed in brain; glycogen depletion and mild hepatocyte hypertrophy observed in liver; and disruption of primary spermatocytes and interstitia observed in testis.
- Based on gene expression and histopathological data, male medaka testis is highly sensitive to TCDD exposure, and mechanisms of reproductive toxicity are currently being pursued.
- Based on these data, promising organ-specific biomarkers of acute TCDD-induced toxicity include:
  - *Brain:* CYP1A (xenobiotic metabolism)
  - *Liver:* α-Amylase (glycogen metabolism), Hepcidin (immune defense), Tributyltin-binding protein (unknown function)
  - *Testis:* Male sex-determining protein (sex differentiation), Protamine (spermatogenesis)

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