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TCDD dysregulation of 13 AHR-target genes in rat liver

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ABSTRACT

Despite several decades of research, the complete mechanism by which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other xenobiotic agonists of the aryl hydrocarbon receptor (AHR) cause toxicity remains unclear. While it has been shown that the AHR is required for all major manifestations of toxicity, the specific downstream changes involved in the development of toxic phenotypes remain unknown. Here we examine a panel of 13 genes that are AHR-regulated in many species and tissues. We profiled their hepatic mRNA abundances in two rat strains with very different sensitivities to TCDD: the TCDD-sensitive Long–Evans (*Turku/AB*; L–E) and the TCDD-resistant Han/Wistar (*Kuopio*; H/W). We evaluated doses ranging from 0 to 3000 μg/kg at 19 h after TCDD exposure and time points ranging from 1.5 to 384 h after exposure to 100 μg/kg TCDD. Twelve of 13 genes responded to TCDD in at least one strain, and seven of these showed statistically significant inter-strain differences in the time course analysis (*Aldh3a1*, *Cyp1a2*, *Cyp1b1*, *Cyp2a1*, *Fmo1*, *Nfe2l2* and *Nqo1*). *Cyp2s1* did not respond to TCDD in either rat strain. Five genes exhibited biphasic responses to TCDD insult (*Ahrr*, *Aldh3a1*, *Cyp1b1*, *Nfe2l2* and *Nqo1*), suggesting a secondary event, such as association with additional transcriptional modulators. Of the 12 genes that responded to TCDD during the dose–response analysis, none had an ED_{50} equivalent to that of *Cyp1a1*, the most sensitive gene in this study, while nine genes responded to doses at least 10–100 fold higher, in at least one strain (*Ahrr* (L–E), *Aldh3a1* (both), *Cyp1a2* (both), *Cyp1b1* (both), *Cyp2a1* (L–E), *Inmt* (both), *Nfe2l2* (L–E), *Nqo1* (L–E) and *Tiparp* (both)). These data shed new light on the association of the AHR target genes with TCDD toxicity, and in particular the seven genes exhibiting strain-specific differences represent strong candidate mediators of Type II toxicities.

Introduction

The aryl hydrocarbon receptor (AHR) is a helix–loop–helix, ligand-activated transcription factor of the Periodic, AHR nuclear translocator, Single-minded (PAS) family. PAS proteins act in many metabolic and developmental pathways, including regulation of circadian rhythm, protection against hypoxia and regulation of neural development (Gu et al., 2000). The classic pathway of AHR action begins with its binding to a ligand, leading to heterodimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Okey et al., 2005). This heterodimer enters the nucleus and interacts with regulatory regions of target genes, altering mRNA abundances of hundreds to thousands of genes in a tissue and species-specific manner (Boutros et al., 2008, 2011b; Boverhof et al., 2005, 2006; Fletcher et al., 2005; Franc et al., 2008; Hayes et al., 2007; Ovando et al., 2006; Slatter et al., 2006; Tijet et al., 2006; Vezina et al., 2004).

The AHR binds to and is activated by a wide variety of halogenated and polycyclic aromatic hydrocarbons (Linden et al., 2010). Of these, the most potent activator and most toxic congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD has been formed as an undesirable product during the synthesis of fungicides and herbicides, low temperature incineration, electronics recycling and paper making (Ma et al., 2009; Ruokojarvi et al., 2000; Schecter et al., 2006; Wen et al., 2009). Currently, the main source of production is low temperature burning of organic compounds in the presence of chlorine-containing compounds, such as waste-dump fires (Costopoulou et al., 2010; Dyke et al., 1997). TCDD is extremely stable, remaining in the environment for decades. This persistence, in combination with poor metabolism due to its lipophilic nature, leads TCDD to bio-accumulate.

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It therefore represents an ongoing risk, particularly to animals at the top of food-chains (US-EPA, 2012; Wan et al., 2010).

TCDD has myriad toxic effects in mammals and these vary among species and even between strains within a species. Some outcomes of TCDD-insult, such as teratogenicity, immune dysfunction, thymic atrophy and carcinogenesis are essentially universal, although they vary in severity across species or strains (Bock and Kohle, 2006). Other outcomes may be species- or strain-specific. For example, one of the most notable outcomes of TCDD exposure in humans is chloracne whereas in laboratory animals, a rapid loss of body weight (called wasting syndrome) and acute lethality are more pronounced (Sorg et al., 2009; Sweeney and Mocarelli, 2000; Tuomisto et al., 1995, 1999a). The dose-sensitivity of TCDD-induced lethality in animals varies dramatically even among rodents: hamsters have an LD_{50} of 1000–5000 μg/kg body weight, while guinea pigs are at least 1000-fold more sensitive with an LD_{50} of 1–2 μg/kg (Pohjanvirta and Tuomisto, 1994). Other common animal models include the L–E rat (LD_{50} 17.7 μg/kg), the C57BL/6 mouse (LD_{50} 182 μg/kg) and the H/W rat (LD_{50} > 9600 μg/kg) (Pohjanvirta and Tuomisto, 1994; Pohjanvirta et al., 1999; Viluksela et al., 1996).

Much of this variability in response originates from variations in AHR structure. For example, the differential sensitivity of L–E and H/W rat strains is largely caused by a single nucleotide polymorphism in the H/W *Ahr* that leads to aberrant mRNA splicing that produces a protein with a modified transactivation domain (TAD) (Pohjanvirta et al., 1998; Tuomisto et al., 1999b). Although the AHR of H/W rats binds TCDD and alters transcription of many genes, the modified TAD allows H/W animals to tolerate enormous doses of TCDD (Boutros et al., 2011b; Pohjanvirta et al., 1999), while avoiding most – but not all – of the toxic outcomes observed in L–E rats (Okey et al., 2005; Pohjanvirta et al., 1989). Some of the toxicities that are similar in both strains (Type-I responses) include fetotoxicity, thymic atrophy and decreases in circulating thyroxine, while some of those specific to L–E include acute lethality, wasting syndrome and liver toxicity (Pohjanvirta and Tuomisto, 1994; Pohjanvirta et al., 1989, 1993). Phenotypic responses that are different between strains, including lethality, are considered Type-II responses (Simanainen et al., 2002, 2003). The most definitive evidence linking the AHR to toxic outcomes derives from studies of *Ahr* knockout mice (Birnbaum et al., 1990; Bunger et al., 2003; Chapman and Schiller, 1985; Fernandez-Salguero et al., 1996; Herlin et al., 2013; Mimura et al., 1997; Vorderstrasse et al., 2001), which are essentially unaffected by TCDD exposure. Similarly, mice engineered to produce AHR isoforms that cannot translocate into the nucleus (Bunger et al., 2003) or are unable to bind to aryl hydrocarbon response elements in DNA (Bunger et al., 2008) are largely refractory to TCDD. Lastly, mice hypomorphic for ARNT exhibited no measured phenotypic responses to TCDD (Walisser et al., 2004). These studies demonstrate that DNA-binding of the AHR:ARNT: TCDD complex is essential for toxicity, implicating AHR-regulated transcription as essential for pathogenesis. There are some lines of evidence implicating non-transcriptional activities of the AHR (Li et al., 2010; Matsumura, 2009), but the toxicological significance of these remain to be elucidated.

While AHR-regulated transcription is a requirement for toxicity, it is unclear which of the hundreds to thousands of regulated genes are responsible for the toxic phenotypes. It has been suggested that species-specific gene expression profiles mediate species-specific toxicities of TCDD (Forgacs et al., 2013). Conversely, genes which are TCDD-regulated in many species and tissues may be responsible for toxic outcomes common to many species, such as cancer and other hepatic toxicities. To identify candidates for causation of common toxicities, we focused on a group of genes that are regulated by TCDD in a wide range of species, which we refer to as "AHR-core" genes. These genes are members of the [ah] gene battery, AhR-Nrf2 gene battery and other genes that have been shown to be TCDD regulated in multiple species (Boutros et al., 2008; Forgacs et al., 2013; Nebert et al., 2000; Tijet et al., 2006; Yeager et al., 2009). Here, we evaluate whether these "AHR-core" genes play a role in Type-II liver toxicity by comparing their RNA abundance changes in TCDD-sensitive L–E and TCDD-resistant H/W rats, using both time course and dose–response experiments.

Methods and materials

Animal handling. Male H/W and L–E rats, 10–12 weeks of age, were grown in breeding colonies of the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. Rats were housed in groups of four (an entire treatment group per cage) in suspended stainless-steel wire-mesh cages with pelleted R36 feed (Lactamin, Stockholm, Sweden) and tap water available *ad libitum*. The temperature in the animal room was maintained at 21 ± 1 °C, relative humidity 50 ± 10% and a lighting cycle of 12/12 h light/dark. Study plans were approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government.

For the dose–response study, liver was harvested between 8:30 and 11:00 from rats treated by gavage with a single dose of TCDD or with corn oil vehicle for 19 h. Four experimental (TCDD-treated) rats were used for each dosage (0.001, 0.01, 0.1, 1, 10, 50, 100, 1000 or 3000 μg/kg, Supplementary Fig. 1).

For the time course study, animals were treated with a single dose of 100 μg/kg TCDD in corn oil and the liver was harvested at the appropriate time points following treatment. This dose is approximately five times the LD_{50} of L–E rats, but is non-lethal for H/W rats (Pohjanvirta et al., 1993). L–E animals were harvested at 3, 6, 10, 19, 96 and 240 h post-TCDD treatment (n = 4, 4, 4, 4, 4, 5) and H/W animals were harvested at 1.5, 3, 6, 10, 19, 96, 240 and 384 h after TCDD treatment (n = 3, 4, 4, 4, 4, 5, 5, 4), Supplementary Fig. 1). All experimental time points were prior to the onset of lethality and the treated animals displayed weight loss consistent with TCDD exposure (Supplementary Fig. 2, Tuomisto et al., 1999a). In all instances, tissues were snap frozen in liquid nitrogen as quickly as possible and stored at temperatures no higher than −80 °C. One vehicle control animal (H/W 19 h) was excluded from the final analysis since it was determined to be an outlier (Dixon Q test, 99% confidence level) with high levels of *Cyp1a1* and *Aldh3a1*. Fold change values were determined by dividing the normalized counts for the specific gene/treatment by the mean determined for the 19 hour corn oil treated animals, for that gene/treatment (n = 4). The 19 hour corn oil time point was used because it was the baseline for most of the treatments and statistical analysis of the corn oil treated animals indicated that there was no significant difference in fold change values regardless of the corn oil treated animal group used to calculate fold change (Supplementary Fig. 3).

RNA isolation. RNA was extracted from rat liver using an RNeasy Mini kit (Qiagen, Mississauga, Canada) following the manufacturer's recommended protocol. RNA was quantified using a NanoDrop spectrophotometer. Integrity of the RNA was verified by electrophoresis on an Agilent 2100 Bioanalyzer, using an RNA Nano 6000 total RNA assay. All samples had an RNA integrity number greater than 8.5, indicating minimal or no sample degradation (Supplementary File 1).

RNA analysis. RNA abundance was measured using the NanoString nCounter system, which provides direct counts of mRNA within a single 100 ng aliquot of total RNA. It was selected because it provides a direct count of the transcripts of interest, avoids bias that may be introduced during cDNA synthesis or PCR amplifications, and requires simple sample preparation reducing the likelihood of technical errors (Prokopec et al., 2013; Waggott et al., 2011).

RNA was diluted to a concentration of 50 ng/μL and 50 μL of each sample was loaded into one well of a 96-well plate and sent to the University Health Network Microarray Centre (Toronto, ON) on dry

ice for analysis on a NanoString nCounter. Desired mRNA targets were submitted in advance and the required CodeSet (color-coded probes) was designed and synthesized by NanoString prior to RNA analysis. Probe sequences were verified by BLAST analysis (Johnson et al., 2008) against the *Rattus norvegicus* nr/nt database to ensure each probe identified a single gene (June, 2010). The CodeSet and all raw and pre-processed data have been deposited in the NCBI's Gene Expression Omnibus (Edgar et al., 2002) as GSE43251. The resulting data consists of direct counts for each specific molecule of interest present in the sample. These data were compiled in Microsoft Excel using NanoString's Raw Code Count Collector Tool for the initial data normalization for experimental variability according to the manufacturer's recommendations. Positive spike-in RNA control counts were summed for each lane and the average across all lanes was taken to produce a normalization reference. A normalization factor was calculated for each lane using the sum of the positive spike-in RNA control counts divided by the normalization reference. The remaining experimental and control code counts were multiplied by this factor to account for hybridization efficiency (NanoString Technologies, Seattle, WA). Normalized data were then loaded into the R statistical environment (v2.15.2) and counts were further normalized to account for variation in the concentration of RNA in the initial sample following the manufacturer's data analysis guidelines. The geometric mean of the code counts for the reference genes *Eef1a1*, *Gapdh*, *Hprt1*, *Ppia* and *Sdha* (Pohjanvirta et al., 2006) for each lane was calculated and the average of these across all lanes was used as the normalization reference. A normalization factor was then calculated and applied as above. All pre-processing methods are available within the NanoStringNorm (v0.9.4) package for the R statistical environment (Waggott et al., 2011).

Statistical analysis. Data were analyzed in the R statistical environment (v3.0.1) (Ihaka, 1996). ED_{50} values with 90% confidence intervals were determined using the drc package (v2.3-7). Response curves were fit using a four-parameter log-logistic model ($f(x) = c + [\{d-c\}/1+ \exp(b(\log(x)-\tilde{e}))]$); where b = slope at the inflection point, c = lower limit, d = upper limit and $\tilde{e} = \log(ED_{50})$. Differences in ED_{10} and ED_{50} parameter values were determined between strains and p-values generated by means of approximate t-tests (Ritz and Streibig, 2012). Inter- and intra-strain differences in mRNA abundance at each time point or TCDD dose were determined using unpaired Student's t-tests. Calculated p-values were corrected for multiple testing using the false-discovery rate adjustment (Storey and Tibshirani, 2003). Differences (between strains or from basal levels) were considered significant if two consecutive points in the time course (normalized expression levels, not fold change) were statistically significant at $p < 0.10$, resulting in a joint-probability of $p < 0.01$. ED_{10} and ED_{50} differences were considered significant for $p < 0.05$. To determine if any genes had an ED_{50} value that was statistically equivalent to *Cyp1a1*, the ED_{50}s were compared using inferential confidence intervals with $\Delta = 2 \times ED_{50}$ 90% confidence range for *Cyp1a1* (Beckstead, 2008; Tryon and Lewis, 2008). Supplementary Files 2–9 contain the values used for and the results of the statistical tests used for data analysis.

Visualization. The data were visualized in the R statistical environment (v3.0.1) with the lattice (v0.20-15) and latticeExtra (v0.6-24) packages. Error bars on all plots represent standard error of the mean.

Results

We focused on 13 "AHR-core" genes that change mRNA abundance in response to TCDD exposure in most species and tissues (Table 1). We profiled the hepatic mRNA abundances of these genes following TCDD treatment in TCDD-sensitive L–E and TCDD-resistant H/W rat strains. Both dose–response (at 19 h) and time course (at 100 μg/kg) studies were performed (Supplementary Fig. 1).

We first compared the broad trends between sensitive and resistant rats at both time course (Fig. 1A) and dose–response levels (Fig. 1B). Twelve of the 13 genes responded to TCDD treatment in at least one strain, while *Cyp2s1* did not respond (did not have two consecutive times significantly different from corn oil treated animals, $p < 0.1$) to the 100 μg/kg dose of TCDD used for the time course series (Fig. 1, Supplementary Fig. 4). Similar results were observed for the dose–response, with *Cyp2s1* being nonresponsive to all TCDD doses administered. *Fmo1* was responsive to the various doses of TCDD, however the range of responses for the dose curve was lower than that observed for later times in the time course (Supplementary Fig. 5). This suggests that the ED_{50} for *Fmo1* may be better determined using a later time when the response is maximal. Summary information for each gene is given in Table 1; detailed per-gene abundance dose–response and time course profiles are given in Supplementary Figs. 4–16.

Genes unchanged between sensitive and resistant rats

We defined a gene as being significantly altered between strains if there were at least 2 consecutive time points that differed significantly ($p < 0.1$, see Methods and materials) or if the inter-strain ED_{50}s were significantly different ($p < 0.05$). Of the 13 genes examined, five did not differ significantly between the strains: *Ahrr*, *Cyp2s1*, *Inmt*, *Tiparp* and *Ugt1a1* (Supplementary Figs. 4, 6–9). There have been reports of large intra-strain variability in mRNA abundance in basal levels; however, our results show remarkably little variability in basal levels and in the TCDD responsiveness of the "AHR-core" genes (*i.e.* small error bars, Supplementary Fig. 17, Fig. 1). These small error bars suggest that the basal levels and the TCDD-responsiveness of genes analyzed in this study are essentially identical from animal to animal (Boutros et al., 2011a).

Strain-specific differences in abundance

Several genes display clear differences in abundance when the normalized mRNA counts are compared; however, when converted to fold-change from basal levels, the differences are often masked or lost. This is evident when considering *Cyp1b1*, where abundance differences of very small magnitude in untreated animals leads to complete loss of the statistically significant differences identified when considering normalized counts (Fig. 2A compared to Fig. 2B, Supplementary Fig. 10 panel A compared to B). Since it has not been clearly shown that fold-change values are more physiologically relevant than absolute changes in mRNA abundance, and since that each may yield unique information, it may be informative to consider both. Other studies have suggested that absolute mRNA abundance measurement may be more relevant than fold-change for mRNA species with widely varying basal levels (Ruiz-Laguna et al., 2006). As a reference, fold-change for each gene following TCDD treatment is shown in Supplementary Fig. 18.

Four genes differed significantly in absolute abundance, beginning at very early time points and, in most cases, continued throughout the time course (*Cyp1b1*, *Cyp2a1*, *Cyp1a2* and *Nqo1*, Fig. 3, Supplementary Figs. 10–13). *Cyp1a2* has statistically significant inter-strain abundance differences at the earliest time points. However, later time points and the dose–response curve essentially overlap between the strains, except for the abundance at the 240 hour time point, which is much lower in L–E animals.

Three genes were observed to deviate in abundance between strains at 10 h or later (*Aldh3a1*, *Fmo1*, *Nfe2l2*; Fig. 3, Supplementary Figs. 5,14,15). Genes with later responses may be changing due to toxicity, rather than as a direct effect of TCDD. *Cyp1a1* and *Cyp1b1* (Fig. 4, Table 1, Supplementary Fig. 19) displayed statistically significant inter-strain differences in their ED_{50} values, indicating inter-strain differences in sensitivity to TCDD. However, when ED_{10} was considered, there were no significant inter-strain differences ($p < 0.05$, Supplementary File 7, Supplementary Fig. 20).

Table 1
Response of "AHR-core" genes to TCDD treatment.

Gene symbol	TCDD response	H/W[a]ED_{50} (μg/kg)	L–E[a]ED_{50} (μg/kg)	Strain-specific difference	[c,j]Percent difference	[d,j]Absolute difference	Change to/from near zero	Entrez Gene ID	[e]Rat-mouse
Ahrr	Induced biphasic[j]	0.093 0.008[h]/1.08[i]	0.56[f] 0.15[h]/2.06[i]	None[j]	39.9	64	Yes	498999	No
Aldh3a1	Induced biphasic[j]	0.48[j] 0.17[h]/1.33[i]	1.13[f] 0.73[h]/1.75[i]	L–E higher[j]	59.3	28567	Yes	25375	No
Cyp1a1	Induced[j]	0.013 0.008[h]/0.021[i]	0.035[b] 0.024[h]/0.051[i]	ED_{50}[j]	12.1	14429	Yes	24296	Yes
Cyp1a2	Induced[j]	0.088[f] 0.058[h]/0.132[i]	0.16[i] 0.115[h]/0.216[i]	Variable/ED_{50}[j]	48.0	334367	No	24297	Yes
Cyp1b1	Induced biphasic[j]	7.77[j] 4.14[h]/14.60[i]	1.55[i,b] 0.86[h]/2.78[i]	L–E higher/ED_{50}[j]	61.5	19296	Yes	25426	Yes
Cyp2a1	Induced[j]	ND[f]	6.20[f] 2.40[h]/16.04[i]	L–E higher[j]	82.5	45985	No	24894	No
Cyp2s1	None[j]	ND[g]	ND[g]	None[j]	58.1	19	No	308445	No
Fmo1	Repressed[j]	ND[g]	ND[g]	L–E lower[j]	39.6	526	No	25256	Yes
Inmt	Repressed[j]	6.80[f] 3.28[h]/14.10[i]	12.48[i] 5.13[h]/30.35[i]	None[j]	48.2	267	Yes	368066	Yes
Nfe2l2	Induced biphasic[j]	2.35 0.20[h]/27.12[i]	0.73[i] 0.21[h]/2.59[i]	L–E higher[j]	50.3	4371	No	83619	Yes
Nqo1	Induced biphasic[j]	0.12 0.02[h]/0.71[i]	0.60[e] 0.14[h]/2.54[i]	L–E higher[j]	76.3	18896	Yes	24314	Yes
Tiparp	Induced[j]	1.44[f] 0.33[h]/6.32[i]	5.54[f] 1.65[h]/18.60[i]	None[j]	60.5	1260	Yes	310467	Yes
Ugt1a1	Repressed[j]	5.61 0.06[h]/476.20[i]	0.39 0.03[h]/5.57[i]	None[j]	37.4	1046	No	24861	No

[a] ED_{50} values calculated with a 4-parameter logistic model.
[b] Statistically significant difference between H/W and L–E, $p < 0.05$.
[c] Absolute difference value as a percentage of the maximum normalized counts for either strain during time course.
[d] Difference between strains in normalized counts for time-point with the maximal difference.
[e] Whether gene was identified as differentially expressed in both H/W and L–E (Boutros et al., 2008).
[f] Significantly lower sensitivity (ED_{50}) than *Cyp1a1* ($p < 0.05$).
[g] Not determined or 90% confidence interval is very broad.
[h] 90% confidence interval lower limit.
[i] 90% confidence interval upper limit.
[j] Determined using normalized expression values.

Unexpected time course profiles

Time course profiles for five of the genes had an unexpectedly different shape than the prototypic AHR-regulated gene, *Cyp1a1*. These genes had an inflection point 10 h after TCDD treatment or later, representing an exaggeration of the original response (Table 1, denoted by "biphasic" in the response column). Most of these genes (*Aldh3a1*, *Cyp1b1*, *Nfe2l2* and *Nqo1*, Fig. 1, Supplementary Figs. 10, 13–15) also had hepatic inter-strain abundance differences in H/W and L–E rats (Table 1, Strain Specific Difference column). *Ahrr* was the only gene in this study that had a biphasic abundance pattern but was not differentially abundant between the time courses of the two rat strains (Fig. 1, Supplementary Fig. 6).

Genes changed from/to near undetectable levels and genes with differential TCDD sensitivity

Following TCDD exposure, seven genes (Fig. 1A) displayed rapidly changed mRNA counts from very low (<500 counts) to high (*Ahrr*, *Aldh3a1*, *Cyp1a1*, *Cyp1b1*, *Nqo1* and *Tiparp*, Supplementary Figs. 6,8,10,13,14,16) or from high to very low (*Inmt*, Supplementary Fig. 7). Interestingly *Cyp1b1* is markedly less sensitive to TCDD treatment than the other two *Cyp1* genes studied, *i.e.* the dose–response curve for *Cyp1b1* is shifted to the right and the ED_{50} value is at least an order of magnitude higher than that for *Cyp1a1* or *Cyp1a2*, and is more similar to *Cyp2a1* (Table 1). When compared with our most sensitive gene, *Cyp1a1*, no genes were equivalently sensitive (Supplementary File 7), while nine genes were significantly less sensitive to TCDD in at least one strain including *Ahrr*, *Aldh3a1*, *Cyp1a2*, *Cyp2a1*, *Cyp1b1*, *Inmt*, *Nfe2l2*, *Nqo1* and *Tiparp* (Table 1, significantly less sensitive ED_{50} indicated with [e], Supplementary File 8).

Discussion

We hypothesize that genes responsible for L–E specific TCDD-induced toxicities will show differential responses between the TCDD-sensitive L–E rat and the TCDD-resistant H/W rat. To identify genes that may be involved in toxicities common to many species we examined a panel of "AHR-core" genes, which are TCDD-regulated in a wide variety of species. The proteins produced by these genes and their reported functions are outlined in Supplementary Table 1. We considered an extensive range of TCDD doses and time points to comprehensively profile mRNA abundances both in terms of fold-changes relative to vehicle control and, exploiting the NanoString platform, of absolute abundances. Genes which display similar TCDD-induced alterations in sensitive rats and mice (Boutros et al., 2008), but divergent ones in resistant rats (Type-II responses) represent strong candidates to mediate major forms of Type-II TCDD toxicity, particularly hepatotoxicities. Previous studies have examined AHR regulation of many of these genes in a variety of species (Boutros et al., 2008, 2011b; Boverhof et al., 2005, 2006; Dere et al., 2011; Forgacs et al., 2013; Franc et al., 2008; Moffat et al., 2010; Yao et al., 2012), however this is the first comprehensive dose–response and time course study to compare the absolute amount of liver mRNA for "AHR-core" genes from resistant and sensitive strains.

Abundance of *Cyp2s1* mRNA, a cytochrome P450 enzyme reported to be AHR regulated in human, rat and mouse did not change following TCDD insult; therefore, *Cyp2s1* should not be considered an "AHR-core" gene (Rivera et al., 2007; Saarikoski et al., 2005). This was unexpected since *Cyp2s1* had previously been shown to be TCDD responsive in Sprague Dawley rat liver (Deb and Bandiera, 2010). Interestingly, *Cyp2s1* was nonresponsive to the AHR agonists 3,3′,4,4′,5-pentachlorobiphenyl and B-naphthoflavone in Sprague Dawley rats (Wang et al., 2011). Similarly, it was surprising that TCDD treatment of both H/W and L–E animals led to reduced levels of *Ugt1a1* mRNA in the liver, as validated by qPCR (Supplementary Fig. 21). Other studies examining the abundance of *Ugt1a1* in rat liver after TCDD-insult observed increased abundance (Munzel et al., 1994; Okey et al., 2005).

Several genes displayed what we term "biphasic" time course profiles, with a modest initial early response to TCDD treatment, followed by an inflection point and a second phase of exaggerated response. This pattern may indicate a different mechanism for regulation or delayed recruitment of additional transcription factors to the promoter, resulting in an altered transcription rate (Hankinson, 2005)

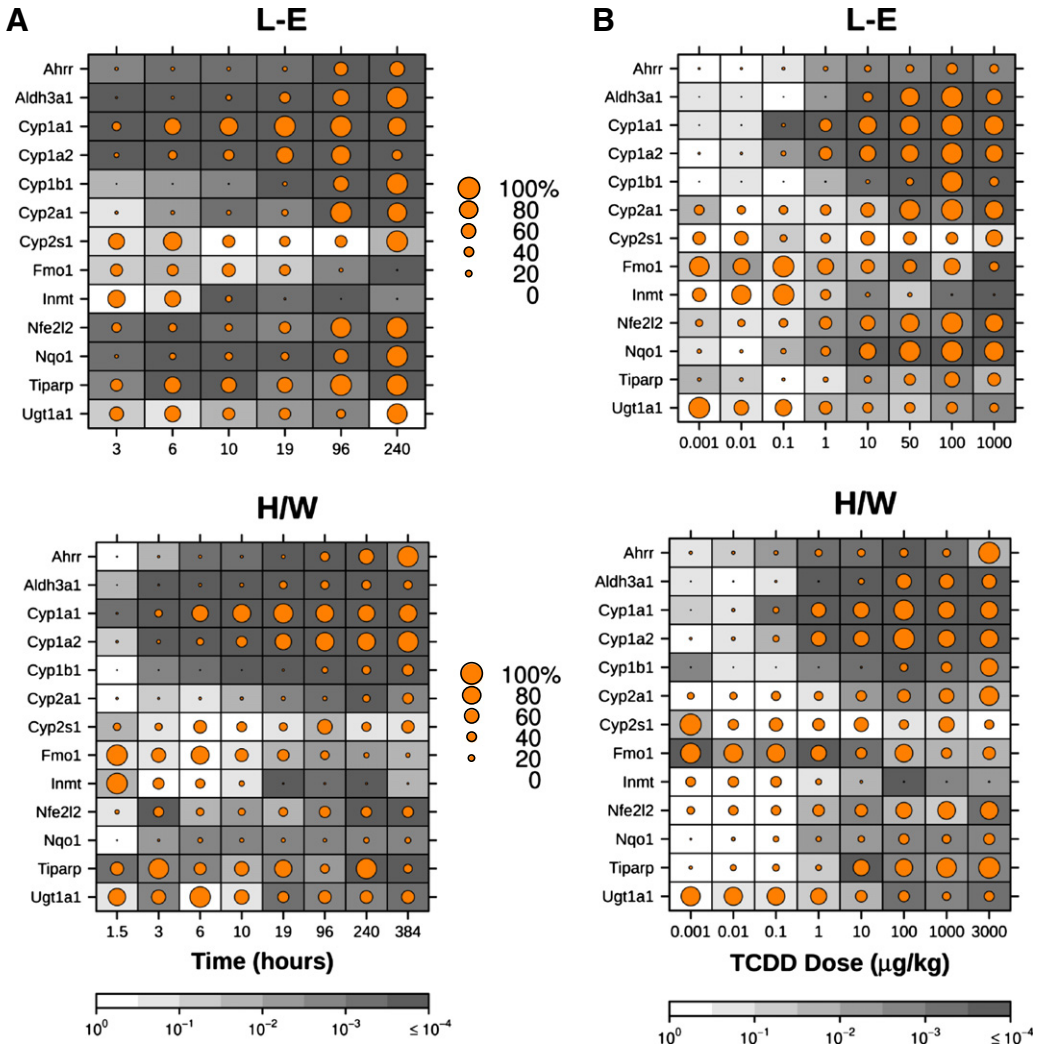

Fig. 1. Summary of "AHR-core" gene mRNA abundance changes as a percent of maximal normalized expression level following TCDD treatment. Increasing dot size indicates the magnitude of change as a percent of the maximal normalized expression level for that gene in either H/W or L–E (whichever strain has the highest expression level for that mRNA). The left panels (A) are data from the time course and right panels (B) are data from the dose–response experiments with the species being displayed indicated by the panel title. Shading of individual squares represents the FDR adjusted p-value for an unpaired Student's *t*-test comparing TCDD induced expression to the 19 hour vehicle control. Differences from basal levels were considered significant if two consecutive points in the time course (normalized expression levels, not fold change) were statistically significant at $p < 0.10$, resulting in a joint-probability of $p < 0.01$.

or by altered mRNA stability. Since this biphasic pattern occurs in both H/W and L–E animals, it may not be directly AHR-regulated, or may involve AHR regions conserved between the strains.

Several genes displayed a time-dependent, rapidly changing mRNA abundance, altered from extremely low levels in untreated animals to significantly increased levels or alternatively, from detectable mRNA abundances to background levels following TCDD-treatment (Table 1). *Cyp1a1* and *Inmt* are prototypical examples of this behavior: *Inmt* mRNA decreased at least 64-fold in L–E rats following TCDD exposure, representing a decrease from about 0.22 molecules per cell to almost zero. In rodents not exposed to AHR-agonists, *Cyp1a1* and *Cyp1b1* are usually expressed in the liver at very low levels or not at all. Upon treatment with TCDD, the mRNA for both of these genes becomes highly expressed in rodent liver. This rapid and prolonged alteration in the tissue specific transcriptional program may indirectly play a role in the onset of toxic outcomes. Since these changes represent a drastic shift in the hepatic metabolic program, they could sensitize the liver to the toxic effects of TCDD-responsive genes essential for toxicity. Indeed male *Cyp1a1*$^{-/-}$ mice have attenuated responses to TCDD (Uno et al., 2004).

The three *Cyp1* family members examined here each have been identified as similarly abundant in two TCDD sensitive species (Boutros et al., 2008). *Cyp1a1* and *Cyp1a2* are separated by approximately 14 kbp and are in a head-to-head orientation on chromosome 8 in the rat genome, whereas *Cyp1b1* is on chromosome 6. The abundance of mRNA from all three *Cyp1* genes was rapidly increased, consistent with primary TCDD-induced transcriptional up-regulation via the AHR (Harrigan et al., 2006). There were some differences, however. *Cyp1a1* was similarly abundant in L–E and H/W rat across all time points studied; a statistically significant inter-strain difference in the ED_{50} was observed, however, with H/W rats responding to lower doses of TCDD. This is opposite to the response observed for CYP1A1/2 activity (Sand et al., 2010), where L–E rats were slightly more sensitive. This may indicate that the difference in ED_{50} observed between H/W and L–E rat is not physiologically relevant. *Cyp1a2* was significantly different in abundance between L–E and H/W rats at three non-consecutive time points; the most physiologically relevant of these likely being the difference at the 240 hour time point, which displayed a decreased abundance in L–E (Fig. 3). This very large late decrease probably reflects a response to overt toxicity, but is notably absent in *Cyp1a1* and *Cyp1b1*. In contrast *Cyp1b1* displayed inter-strain differences at all time-points (Fig. 3). It is interesting that while the L–E rats had approximately three times more *Cyp1b1* mRNA counts than H/W rats at the 240-hour time point, there was no difference observed between the two

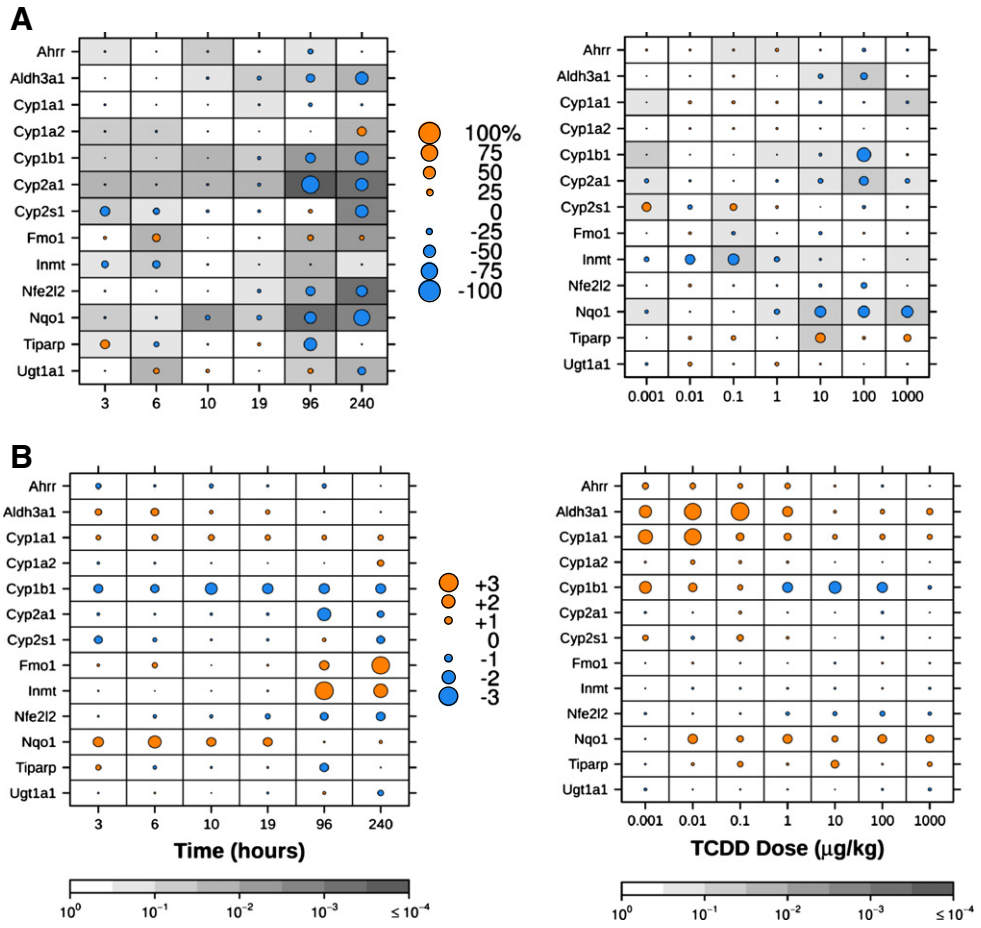

Fig. 2. Summary of "AHR-core" gene inter-strain mRNA differences following TCDD treatment. The upper panels (A) are L–E percent change–H/W percent change values. The lower panels (B) are inter-strain differences displayed as fold-change from the level of corn oil-treated control animals (19 h) in $\log_2$ space (fold-change L–E–fold-change H/W, Supplementary Fig. 11). Shading of individual squares represents the FDR adjusted p-value for an unpaired Student's *t*-test comparing the inter-strain differences. None of the inter-strain fold-change values had a p-value < 0.1.

rat strains when considering the data as fold-change from basal levels (Supplementary Fig. 10, panel B). The higher number of mRNA molecules may lead to considerably higher absolute amounts of CYP1B1 protein in the liver. It has been reported that TCDD treatment increases CYP1B1 and CYP1A1 protein levels in Sprague Dawley rat liver (Walker et al., 1998) and the magnitude of increased protein has been reported to correlate with increasing levels of mRNA in other mammalian species (Hirakawa et al., 2007). *Cyp1b1* expression has been correlated with increased cancer risk (Sissung et al., 2006) and reducing *Cyp1b1* expression may be protective (Hayes et al., 1996; Shimada et al., 1996).

Aldh3a1 has higher abundance in the TCDD-treated L–E liver at time points after 19 h post treatment. ALDH3A1 can play a cyto-protective role, detoxifying aldehydes such as the products of lipid peroxidation. Elevated *Aldh3a1* levels in L–E rats may be a response to TCDD-induced oxidative stress and lipid peroxidation (Canuto et al., 1994; Korkalainen et al., 1995; Pohjanvirta et al., 1990), and *Aldh3a1* may be regulated by mechanisms distinct from canonical AHR signaling (Dunn et al., 1988; Korkalainen et al., 1995). Metabolism of compounds such as 4-hydroxyl-2-nonenal by ALDH3A1 promotes cell proliferation by removal of these toxic inhibitory compounds (Canuto et al., 1994; Muzio et al., 2012). ALDH3A1 regulates proliferation, development and maintenance of stem cells and cancer stem cells (Ma and Allan, 2011), and its activity is increased in cancer cells (Canuto et al., 1994; Patel et al., 2008). In addition, ALDH3A1 inhibition reduces cancer cell proliferation (Moreb et al., 2008). Taken together, dysregulation of *Aldh3a1* by TCDD exposure may promote liver carcinogenesis.

Nfe2l2, also known as NRF2, is a transcription factor which regulates genes whose products work to protect against damage from reactive oxygen species. The abundance profile for *Nfe2l2* is essentially identical between the two strains until the 19 time-point, when L–E animals display a strong secondary induction of *Nfe2l2*, which is substantially attenuated in H/W animals. As a result the mRNA abundance of *Nfe2l2* in L–E rats increases approximately two-fold more than in H/W rats. This higher mRNA level may reflect a higher level of oxidative damage caused by TCDD treatment (Pohjanvirta et al., 1990). This biphasic response was observed for several genes that displayed strain-dependent differences in regulation (Table 1). In addition to its essential dimerization partner, ARNT, the activated AHR may recruit additional transcription factors to genes that exhibit biphasic responses, subsequent to the initial activation. NRF2 is required to achieve complete activation of several AHR-regulated genes, including *Nqo1* (Yeager et al., 2009), and may be involved in some of the secondary biphasic responses. While mRNA levels are up-regulated by AHR activation, NRF2 abundance is tightly regulated: it is only activated in the presence of ROS (Kohle and Bock, 2007). Since *Nqo1* mRNA abundance increases only in the presence of active NRF2, increased mRNA counts in L–E rats may reflect increased ROS formation in the sensitive L–E rat. *Nqo1* mRNA abundance also increased in H/W rat, peaking in 10 h at much lower absolute amounts than for L–E. This is surprising, as ROS have not been detected in H/W rat at time-points as early as 10 h (Pohjanvirta et al., 1990).

The *Fmo1* gene product is a flavin containing monoxygenase that is involved in the metabolism of any soft nucleophile which can gain

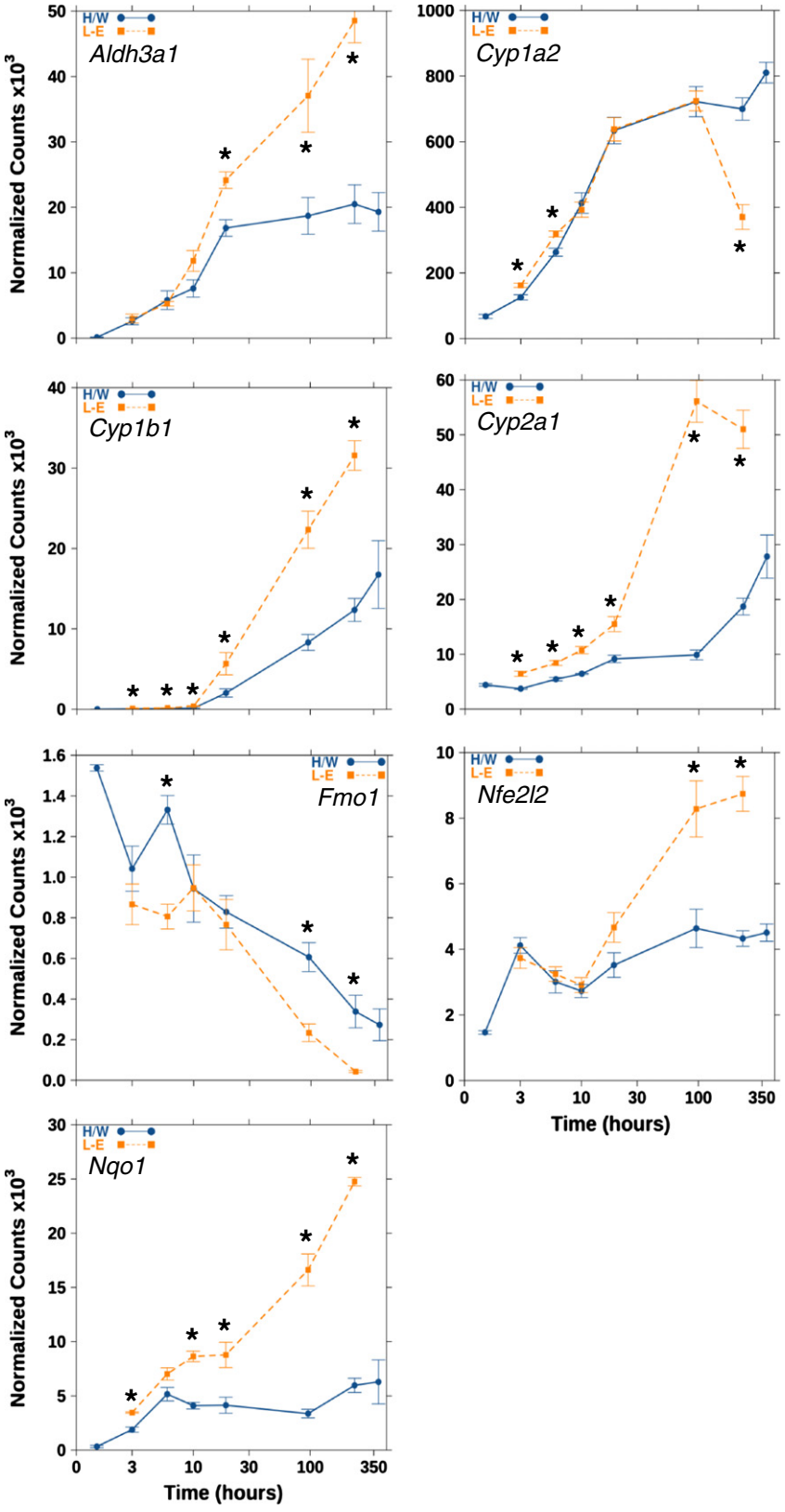

Fig. 3. Profiles for genes with statistically significant differential abundance during the time course. *Indicates $p < 0.1$ as determined by Student's *t*-test with FDR correction. Genes were not considered significantly changed unless they had two or more consecutive time points with $p < 0.1$, resulting in a joint-probability of $p < 0.01$.

access to the enzyme's active center. FMO1 is found in the endoplasmic reticulum as an activated enzyme containing a highly reactive C(4a) hydroperoxide derivative of FAD. *Fmo1* is a member of a family of flavin monoxygenases in vertebrate species (Shephard and Phillips, 2010). FMO1 is not expressed in adult human liver; however, it is present in adult kidney and fetal liver, which may be involved in the outcomes of prenatal exposure. Fmo1 is present in the adult liver of mice and rats (Shephard et al., 2007). In mice, TCDD treatment leads to increased

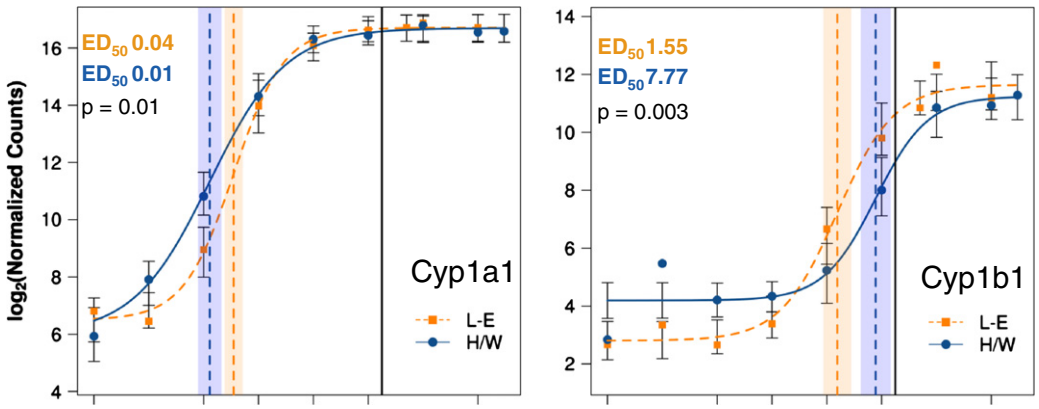

Fig. 4. Dose–response profiles for genes with statistically significant inter-strain ED_{50} differences. Genes are identified by the label in the panel; shaded bars represent 90% confidence intervals for the calculated ED_{50}, denoted by the dotted vertical line. L–E data are in yellow, with the mean values for each dose indicated by squares and the fitted values shown by the sigmoidal line. H/W data are in blue and the mean values are indicated by circles, with the line representing the fitted curve. The vertical solid black line represents the LD_{50} for TCDD in the L–E rat (17.7 μg/kg).

hepatic *Fmo1* mRNA in males but not in females (Boutros et al., 2008; Celius et al., 2008). In rat, treatment with TCDD leads to a decrease in hepatic abundance, occurring more rapidly in L–E animals, with statistically significant inter-strain differences in abundance at 96 h and later. While it is uncertain if the reduced levels of *Fmo1* have physiological relevance in the context of TCDD toxicity, *Fmo1*$^{-/-}$ mice develop and behave normally, although with defects in imipramine metabolism by N-oxidation (Hernandez et al., 2009).

Of the 13 genes considered in this study, five (*Ahrr*, *Cyp2s1*, *Inmt*, *Tiparp* and *Ugt1a1*) were similarly abundant in H/W and L–E rats in liver samples from both time course and dose–response analyses following TCDD treatment (Fig. 1, Supplementary Figs. 4, 6–9). We expect that genes with an essential role in L–E specific toxicity would have a different abundance profile between these strains, and thus the 5 similarly-abundant genes are not likely to play a direct role in the onset of L–E-specific pathological liver changes resulting from TCDD exposure. Five genes (*Ahrr*, *Aldh3a1*, *Cyp1b1*, *Nfe2l2* and *Nqo1*) displayed unexpected changes in abundance we considered to be biphasic responses, displaying a response to TCDD at early time points which is subsequently enhanced (Supplementary Figs. 6, 10, 13–15). In most cases this secondary response was greater in the L–E animals, suggesting mechanistic differences in regulation or effects of toxicity. In addition, some genes displayed switch-like changes, indicating radical alterations in the hepatic transcriptional program (*Ahrr*, *Aldh3a1*, *Cyp1a1*, *Cyp1b1*, *Inmt*, *Nqo1* and *Tiparp*, Supplementary Figs. 6–8, 10, 13, 14, 16). None of these "AHR-core" genes had an ED_{50} value significantly equivalent to or lower than that of *Cyp1a1*. Nine genes had lower sensitivity to TCDD treatment in at least one strain, responding to TCDD at doses significantly higher than those required to stimulate the most sensitive gene in this study, the prototypic AHR-regulated gene, *Cyp1a1* (Table 1, *Ahrr* (L–E), *Aldh3a1* (both), *Cyp1a2* (both), *Cyp1b1* (both), *Cyp2a1* (L–E), *Inmt* (both), *Nfe2l2* (L–E), *Nqo1* (L–E) and *Tiparp* (both). ED_{50} values for these genes were 10–100 folds higher than for *Cyp1a1*, suggesting differential mechanisms of AHR regulation.

While the goal of this paper was to identify genes which are candidates for Type-II toxicity in L–E rat liver, candidate genes identified herein may play a role in common TCDD toxicities in many species and organs. For instance, several cytochrome P450s have been implicated in TCDD-induced increases in eicosanoid levels in mice. It is probable that our panel of genes plays a role in this effect (Bui et al., 2012). It will be interesting to determine whether our candidate genes play a role in the variations observed in developmental toxicities (Huuskonen et al., 1994), including those for cardiovascular development (Wang et al., 2013).

In summary, we identify 7 genes that display strain-specific, time-dependent changes in response to TCDD (Fig. 3, *Aldh3a1*, *Cyp1a2*, *Cyp1b1*, *Cyp2a1*, *Fmo1*, *Nfe2l2* and *Nqo1*). Two genes show significant inter-strain differences in dose–response (Fig. 4, *Cyp1a1* and *Cyp1b1*). These genes form a complex, interconnected web, involved in metabolism of xenobiotic compounds and steroid hormones, responses to reactive oxygen stress and proliferative pathways. Each of these has previously been shown to be altered by TCDD exposure; however, the specific genes mechanistically involved in the observed metabolic dysregulation leading to toxicity are unknown. By identifying genes differentially abundant in TCDD-sensitive and -resistant rats, this study takes a step toward identification of specific genes underlying toxic outcomes in laboratory species. Future work will determine if these mRNA abundance changes lead to altered protein abundance, enzyme activity and sub-cellular localization. It will also be interesting to explore the possibility that these genes may be involved in TCDD-related toxicities in other organs and during other developmental stages.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.12.004.

Conflict of interest statement

ABO has served as a paid consultant to The Dow Chemical Company as a member of their Dioxin Scientific Advisory Board. All other authors declare that they have no conflicts of interest.

Supporting information

Plots of vehicle control values, per-gene abundance changes, dose–response profiles (with confidence intervals) and files containing p-values are available as supplementary material. Raw data has been deposited to the GEO repository with accession number GSE43251.

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