Differential Expression Profiling of the Hepatic Proteome in a Rat Model of Dioxin Resistance

CORRELATION WITH GENOMIC AND TRANSCRIPTOMIC ANALYSES*[S]

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One characteristic feature of acute 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity is dramatic interspecies and interstrain variability in sensitivity. This complicates dioxin risk assessment for humans. However, this variability also provides a means of characterizing mechanisms of dioxin toxicity. Long-Evans (*Turku/AB*) rats are orders of magnitude more susceptible to TCDD lethality than Han/Wistar (*Kuopio*) rats, and this difference constitutes a very useful model for identifying mechanisms of dioxin toxicity. We adopted a proteomic approach to identify the differential effects of TCDD exposure on liver protein expression in Han/Wistar rats as compared with Long-Evans rats. This allows determination of which, if any, protein markers are indicative of differences in dioxin susceptibility and/or responsible for conferring resistance. Differential protein expression in total liver protein was assessed using two-dimensional gel electrophoresis, computerized gel image analysis, in-gel digestion, and mass spectrometry. We observed significant changes in the abundance of several proteins, which fall into three general classes: (i) TCDD-independent and exclusively strain-specific (*e.g.* isoforms of the protein-disulfide isomerase A3, regucalcin, and agmatine ureohydrolase); (ii) strain-independent and only dependent on TCDD exposure (*e.g.* aldehyde dehydrogenase 3A1 and rat selenium-binding protein 2); (iii) dependent on both TCDD exposure and strain (*e.g.* oxidative stress-related proteins, apoptosis-inducing factor, and MAWD-binding protein). By integrating transcriptomic (microarray) data and genomic data (computational search of regulatory elements), we found that protein expression levels were mainly controlled at the level of transcription. These results reveal, for the first time, a subset of hepatic proteins that are differentially regulated in response to TCDD in a strain-specific manner. Some of these differential responses may play a role in establishing the major differences in TCDD response between these two strains of rats. As such, our work is expected to lead to new insights into the mechanism of TCDD toxicity and resistance.   *Molecular & Cellular Proteomics 5:882–894, 2006.*

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[1] is considered to be one of the most potent toxicants known and is the prototypical representative of the polyhalogenated aromatic hydrocarbon class of persistent environmental contaminants. Exposure of laboratory animals to TCDD results in a variety of tissue- and species-specific responses, ranging from the induction of xenobiotic-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1) to reproductive and developmental defects, teratogenicity, immunotoxicity and thymus atrophy, hepatotoxicity, wasting syndrome, and tumorigenesis (1, 2).

Evaluation of the risk posed by TCDD to humans is hampered by exceptionally large inter- and intraspecies variability both in wild animals and in laboratory species (for a review, see Ref. 3). Several studies have revealed that virtually all major toxic effects of dioxins are mediated by the specific binding of TCDD to a cytosolic protein, the aryl hydrocarbon receptor (AHR), which, upon ligand binding, translocates into the nucleus and heterodimerizes with the ARNT protein. This activated heterodimer binds to cognate *cis*-regulatory se-

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 2-DE, two-dimensional gel electrophoresis; AGMAT, agmatine ureohydrolase; AHR, aryl hydrocarbon receptor; AHRE, aryl hydrocarbon response element, also known as xenobiotic response element or dioxin response element; ALDH3A1, aldehyde dehydrogenase 3A1; ARE, antioxidant response element; ARNT, aryl hydrocarbon receptor nuclear translocator; ASS, arginosuccinate synthase; CA3, carbonic anhydrase 3; ER, endoplasmic reticulum; H/W, Han-Wistar (*Kuopio*); L-E, Long-Evans (*Turku/AB*); MAWBP, MAWD-binding protein; PDCD8, programmed cell death protein 8, also known as apoptosis-inducing factor; PDIA3, protein-disulfide isomerase 3; PON3, paraoxonase 3; RGN, regucalcin; SELENBP2, rat selenium-binding protein 2; SULT1A1, sulfotransferase 1A1; TF, transferrin; APOA-I, apolipoprotein A-I; CYP1A1, cytochrome P450 1A1.

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quences (the aryl hydrocarbon receptor response element, AHRE-I) and functions as a transcription factor to recruit coactivators and possibly to interact directly with the basal transcription machinery (4–6).

This relatively simple model for gene regulation by AHR has been so far only clearly demonstrated for the mouse CYP1A1 induction but may not universally explain AHR-mediated regulation. Recent studies suggest that the AHR may function not only as a traditional ligand-activated transcriptional factor but also as a novel ligand-activated coactivator (7). In addition to a series of studies showing interaction of the AHR-ARNT heterodimer with the estrogen receptor (8, 9) a novel response element (called the AHRE-II) has been characterized recently. The AHR-ARNT heterodimer can bind to the AHRE-II while associated with an unidentified factor (7). Binding of this complex appears to lead to the activation of a novel, functionally coherent gene battery (6).

In addition to its role in mediating response to xenobiotic ligands, the AHR status plays a crucial role in TCDD susceptibility, at least among laboratory animals, because a point mutation in the *Ahr* gene that leads to an abnormal C terminus transactivation domain has been associated with the exceptional resistance of Han/Wistar (*Kuopio*; H/W) rats to TCDD-mediated acute lethality (10, 11). A particularly useful tool for studying key mechanisms in dioxin toxicity is indeed the large interstrain difference between TCDD-sensitive Long-Evans (*Turku/AB*; L-E) and TCDD-resistant H/W rats (12–14). H/W rats are more than 1000 times more resistant to the acute lethality of TCDD than L-E rats, having oral LD_{50} values of >9600 and 10 μg of TCDD/kg of body weight, respectively.

Despite the abnormal AHR molecule, no substantial differences between the two strains could be detected in hepatic AHR levels, binding affinity of TCDD to the AHR, or specific binding of the activated AHR-ARNT heterodimer to DNA (11, 13). Furthermore H/W rats and L-E rats show similar sensitivity to induction of CYP1A1 activity, thymic atrophy, and embryotoxicity (3, 15, 16). The H/W strain displays some, but not all, of the characteristic toxic effects of TCDD exposure at doses similar to those needed for L-E rats.

The most striking divergence between the two strains appeared in feeding behavior and changes in body weight. In L-E rats TCDD induces an irreversible anorexia and body weight loss; H/W rats respond only marginally to TCDD in that respect. Further changes found exclusively in L-E rats are enhanced lipid peroxidation, elevations in free fatty acids, and severe hepatotoxicity (3).

Recently these biochemical effects have been classified into two categories: type I endpoints, such as CYP1A1 induction and thymus weight change, which are unaffected by strain differences, and type II endpoints, such as acute lethality, body weight change, and bilirubin levels, which are suppressed in H/W rats relative to L-E rats (3, 16). This classification suggests that there are at least two distinct AHR-mediated mechanisms that lead to different endpoints, namely those parallel to CYP1A1 induction and those parallel to lethality. Whether these mechanisms might be linked to distinct roles that the AHR plays in transcription is unclear, although this is a reasonable hypothesis given the structural divergence in the transactivation domain.

Overall these studies suggest that a large number of metabolic and/or signaling pathways might be involved in differential TCDD sensitivity between these rat strains. Therefore, there is a need to evaluate proteins from many signaling and metabolic pathways simultaneously. Proteomic analysis offers great opportunities for its ability to focus on simultaneous changes of a large number of proteins, which can reveal the complex interplay of different pathways at a single time point.

We adopted a global, proteome scale approach to investigate the extent to which TCDD exposure alters liver protein expression in H/W *versus* L-E rats to determine which, if any, protein markers are indicative of differences in dioxin susceptibility and thus are candidates for conferring resistance or sensitivity to TCDD. Although there have been several studies to investigate toxicological similarities and differences evoked by TCDD in these two strains, to our knowledge this is the first characterization of proteomic changes.

In this study, we also collected transcriptomic data (Affymetrix expression arrays) to reinforce and validate our proteomic results. To provide some insights into the regulatory networks controlling these combined (mRNA and protein) expression changes, we performed *in silico* searches for the canonical AHR response element (AHRE-I) and the antioxidant response element (ARE) in the promoters of genes identified in our proteomic study. We chose to study the presence of these motifs, which independently mediate the transcription of many different genes (17–20), because TCDD-mediating expression has been shown to be both direct (via the AHRE-I) or indirect (via the ARE) (21, 22).

In light of recent findings that the AHR may function as a ligand-activated coactivator (6, 7), we also searched for the presence of the AHRE-II sequence. The battery of genes regulated by the AHR through AHRE-I has been extensively characterized (23), but very few genes altered by AHR via the AHRE-II site have been discovered. So far, a total of 36 genes have been found that contain the AHRE-II motif conserved across human, mouse, and rat genomes, and over one-third of these genes respond to TCDD in rat liver (6).

Herein the combination of a genetic model of differential dioxin sensitivity combined with integrated genomic, proteomic, and transcriptomic data allowed us to identify a subset of hepatic proteins that might be involved in pathways that mediate the major differences in interstrain TCDD susceptibility. As such, these mechanistic findings may have a significant utility for improving human risk assessment and may provide pointers helping the search for new markers of TCDD human susceptibility.

EXPERIMENTAL PROCEDURES
Chemicals

2,3,7,8-TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was found to be over 99% pure by gas chromatography-mass spectrometry. It was dissolved in diethyl ether: adjusted volumes of the solution were mixed with corn oil after which the ether was allowed to evaporate. Dosing solutions were carefully mixed in a magnetic stirrer and sonicated for 20 min before dosing. Diethyl ether and corn oil were of analytical grade and purchased from Merck and from BDH Laboratory Supplies (Poole, England), respectively.

Animals and Treatment

Male L-E and H/W rats were obtained from the breeding colony of the National Public Health Institute (Kuopio, Finland). The rats were housed individually in stainless steel wire-bottomed cages, and they received commercial rat chow (R36; Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The ambient temperature in the animal room was 21 ± 1 °C, and the relative humidity was $55 \pm 10\%$. The rats were kept under a photoperiodic cycle of 12 h of light/12 h of dark in an air-conditioned animal room.

Rats (10 weeks old) were divided into experimental groups of five animals and given a single oral dose of 2,3,7,8-TCDD at 100 μg/kg of body weight in corn oil by oral gavage using a metal cannula with a ball tip. Control animals were dosed in the same manner with corn oil vehicle alone.

On day 5 postexposure rats were weighed and killed by decapitation. The liver was rapidly removed, divided in small aliquots, flash frozen in liquid nitrogen, and stored at -80 °C for subsequent analyses. All animal protocols were approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government, Finland.

Hepatic Protein Preparation for Two-dimensional Gel Electrophoresis

Frozen liver samples of ~300 mg in weight were ground into powder using a ceramic mortar and pestle chilled with liquid nitrogen. The frozen tissue was subsequently solubilized (at 1 ml/100 mg of frozen tissue weight) in a solution consisting of 5 M urea, 2 M thiourea, 2% CHAPS, 2% Zwittergent 3–10 detergent (Calbiochem), and a mixture of protease inhibitors (Complete, mini EDTA-free mixture; Roche Applied Science). DeStreak reagent (100 mM) (Amersham Biosciences) was added to protect cysteinyl groups and prevent nonspecific oxidation during the isoelectric focusing run.

The suspension was homogenized for ~1 min, sonicated for 3 min, and centrifuged at $100,000 \times g$ for 30 min at 10 °C. The pellet was discarded, and an aliquot of the supernatant was used to determine protein concentration using the PlusOne 2-D Quant kit (Amersham Biosciences).

Two-dimensional Gel Electrophoresis (2-DE)

For each rat liver sample, 150 μg of total protein were diluted to a final volume of 250 μl in the rehydration solution (5 M urea, 2 M thiourea, 2% CHAPS, 2% Zwittergent, 100 mM DeStreak, and 0.5% IPG buffer pH 3–10 linear (Amersham Biosciences) and then applied on immobilized pH 3–10 linear gradient strips (IPG strips; Amersham Biosciences). IPG strips were hydrated on an IPGphor apparatus (Amersham Biosciences) for 16 h at 30 V/h and then focused for 26 h until 50,000 V-h. After the first dimension was run, proteins were reduced by incubating individual strips for 15 min in a solution containing 50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 60 mM DTT (Amersham Biosciences). Proteins were then alkylated by incubating the strips for 15 min in a similar solution with DTT replaced by 100 mM iodoacetamide. The strips were then embedded in 0.7% (w/v) agarose on the top of 1-mm-thick acrylamide gels cast at 12%. Proteins were separated by molecular size by electrophoresis at 10 mA/gel. This was done overnight at 4 °C in a running buffer composed of 25 mM Tris, 250 mM glycine, 0.1% SDS. Gels were rinsed three times with deionized H_2O, fixed for 1 h in an aqueous solution with 50% methanol and 7% acetic acid, and then rinsed again with deionized H_2O. Finally gels were stained with colloidal Coomassie Blue (Pierce) for 4–5 h and then extensively washed with deionized H_2O.

Gel Image Analysis and Statistics

Stained gels were scanned at 16-bit resolution (Expression 1680 Pro, Epson), and the resulting TIFF images were analyzed with Progenesis work station software (version 2005; Nonlinear Dynamics, Newcastle upon Tyne, UK). Using Progenesis, the automatic analysis protocol for the images of the 20 gels included spot detection, warping, background subtraction, average gel creation, matching, and reference gel modification. Spot volumes were normalized against the total volume of all the spots in the gel.

Average gels were generated by the software for spot pattern comparison. They are a statistical combination of the gels in a group, showing mean spot values with associated error, which provide information about spot variation within the gel set. In this study an average gel was created for each experimental group by combining the individual gels for the five animals in a group. The criteria for including a spot in the average gel were that any spot must be present in at least four of the five individual gels. Spot editing (spot splitting corrections and match editing) was done sparingly and only on selected, complex areas of the gel.

Differential proteomic analysis between TCDD-treated and control groups used the statistical functions of the Progenesis software package. Briefly datasets were compared by unpaired two-tailed t tests (unadjusted $p < 0.05$). For each spot, the assumption of equal variance is tested with an F test, and the appropriate t test is applied. Additionally the assumption of normality inherent in a t test was verified with the Shapiro-Wilk test. Differences were considered significant when $p < 0.05$ was combined with thresholding for 2-fold changes in expression.

Protein Identification by Mass Spectrometry

In-gel Digestion—In-gel digestion was performed as described previously (24). Briefly the spots of interest were excised manually from the gel and digested with sequencing grade modified trypsin. Aliquots of the supernatant, containing tryptic peptides, were directly analyzed by mass spectrometry.

LC-MS/MS—Reverse-phase microbore LC was done using a Surveyor system (autosampler and MS pump) coupled to an ion trap mass spectrometer LCQ Deca XP^{Plus} (Thermo Finnigan) equipped with a standard electrospray source and operated in positive ion mode with an ion sprayer voltage of 4.6 kV and a capillary temperature of 220 °C.

Sample digest (20 μl) was first injected into a peptide microtrap (Michrom Bioresources Inc.) at a flow rate of 50 μl/min to concentrate and desalt it. The sample was then back-flushed with 0.1% HCOOH in H_2O, pH 3, from the microtrap to the analytical reverse-phase column at a flow rate of 12 μl/min. Peptide separation was performed using a packed capillary column (Aquasil C_{18} Kappa 100×0.5 mm, 3 μm; Thermo Electron Corp.). The mobile phases consisted of 1% HCOOH in water (A) and 100% CH_3CN (B). The linear solvent gradient was as follows: from 100% A to 34% B in A in 51 min.

Data were acquired sequentially in MS mode (scan range of 450–

2000 amu) and in data-dependent mode, recording the MS/MS spectra of the two most intense ions of each MS scan. The MS/MS spectra were acquired with an isolation width of 3.0 amu and normalized collision energy of 45%. Raw MS/MS data from each LC run were transformed into dta files using the instrument software (BioWorks version 3.1 SR1) with automatic selection of individual MS/MS spectra.

Tandem mass spectra were analyzed using Phenyx version 1.9 (GenBio, Geneva, Switzerland), the MS/MS search engine developed by Geneva Bioinformatics, against the National Center for Biotechnology Information (NCBI) non-redundant (nr) database (version August 31, 2005, 2,524,882 sequences) (25). The search was enzymatically constrained for trypsin and allowed for one missed cleavage site. Further search parameters were as follows: no restriction on molecular weight and isoelectric point; taxonomy, *Rattus norvegicus*; fixed modification, carbamidomethylation of cysteine; variable modification, oxidation of methionine.

A summary table is available (Supplemental Table 1) that contains a concise restatement of the main submission parameters including algorithm, scoring models, thresholds, and rounds of calculations that are specific to Phenyx. The basic principle of two rounds is that the first round processes all the proteins in the designated search space, and the second round only processes the proteins that passed the first round. The first round parameters need to be stringent enough to sufficiently validate protein identification (*i.e.* parent error tolerance of 0.8 Da). The second round parameters make it possible to open the search criteria (*i.e.* parent error tolerance of 2 Da), to increase the sequence coverage, by searching for combinatorial modifications or other special features. A two-round search therefore identifies proteins according to a first set of parameters and then performs a more exhaustive search on the proteins while saving computation time and reducing the random match rate. The mass tolerance for the fragment ions is included in the scoring scheme determined by the algorithm chosen (LCQ) to resolve the data. An Excel spreadsheet is available (Supplemental Table 2) derived from the Phenyx Database/AC/Peptide view results page and contains all information concerning peptide identifications.

RNA Isolation and Microarray

The full microarray studies will be reported in forthcoming publications.[2,3] Briefly total hepatic RNA was isolated from each animal using standard techniques. The mRNA expression study used Affymetrix RAE230-2 arrays; hybridizations were performed according to the manufacturer's standard protocol. Data were preprocessed with the GCRMA algorithm (26) as implemented in the affy package (version 1.6.7) (27) for R (version 2.1.1). Statistical significance was determined by fitting linear models followed by a false discovery rate adjustment for multiple testing and an empirical Bayes moderation of standard error as implemented in the limma package (version 2.0.2) (28) for R (version 2.1.1). ProbeSets were deemed significantly differentially expressed at the $p < 1 \times 10^{-3}$ level.

In Silico Response Element Search

Conserved AHRE-I, AHRE-II, and ARE motifs in the upstream regulatory region of genes encoding proteins of interest were identified as described previously (6). Briefly genomic sequence was downloaded from the University of California Santa Cruz genome browser database (29) for the most recent mouse (mm5) and rat (rn3) assem-

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TABLE I
Response element sites assessed in the study

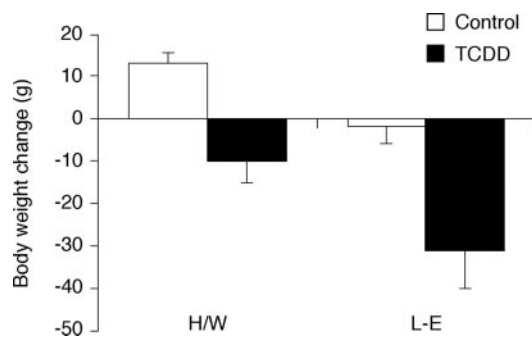| Regulatory element name	Sequence
AHRE-I (core)	GCGTG
AHRE-I (extended)	TNGCGTG
AHRE-I (full)	(T/G)NGCGTG(A/C)(G/C)A
AHRE-II	CATGN$_6$C(T/A)TG
ARE	TGACNNNGC

Fig. 1. **Body weight changes of H/W and L-E rats after a single oral dose of 100 μg/kg TCDD.** *Bars* represent means $\pm$ S.D. of five individual rats.

blies. Using custom Bioperl-based scripts (30) the regions between -5000 and $+1000$ relative to each RefSeq transcriptional start site were extracted from the genomic assembly and searched for three variations of the AHRE-I sequence as well as the ARE and AHRE-II sequences. Sequence patterns used in the searches are given in Table I.

RESULTS

Animal General Health

As expected, the body weight of TCDD-treated L-E rats was decreased by 11% during the postdose period of 5 days (Fig. 1). Body weight of H/W rats was only marginally affected (decrease of 3%). The rats did not show any other signs of toxicity.

Proteome Analysis

We compared global hepatic protein expression patterns of sensitive Long-Evans with resistant Han/Wistar rats after a single oral dose of TCDD to determine which, if any, protein markers are indicative of differences in dioxin susceptibility. Fig. 2 shows the 2-DE average gel representative of each treatment group. Image analysis detected a comparable number of spots in the four average gels (spot number, 785 $\pm$ 6.5, mean $\pm$ S.D.).

Overall 21 protein species showed a statistically significant change in abundance of at least 2-fold as a result of the genetic background of the rat and/or of the TCDD treatment. All these protein species were positively identified by peptide sequencing (LC-MS/MS). Results of identifications are summarized in Table II. Detailed information on protein/peptide identification is available in Supplemental Table 2.
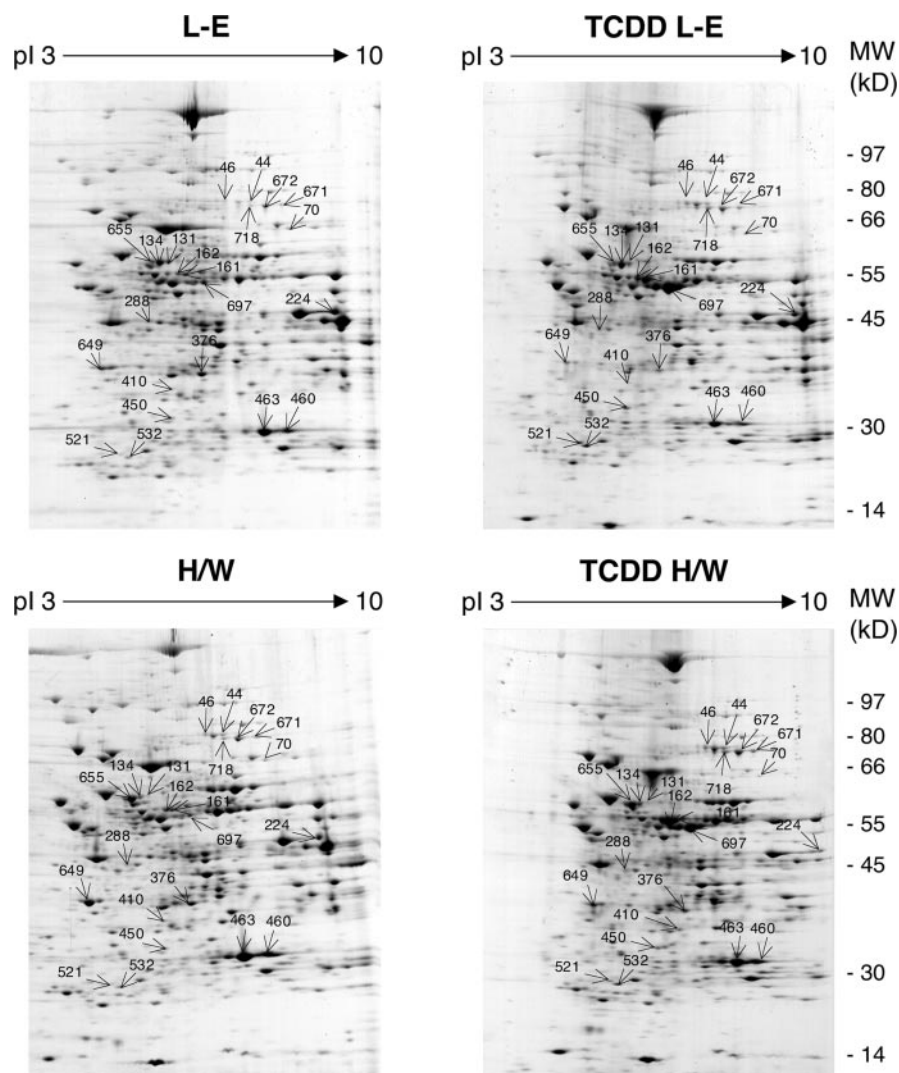
FIG. 2. **Colloidal Coomassie Brilliant Blue-stained 2-DE gel (Progenesis average gel image) of protein liver extracts (150 μg) from untreated L-E, untreated H/W, TCDD-treated L-E, and TCDD-treated H/W rats.** Spots showing differences in abundance are indicated by *arrows*. *Numbers* refer to spot number from the Progenesis image analysis results.

Proteins Differentially Expressed in a Strain-specific Manner in Untreated Rats—Fig. 3 provides an overview of the expression patterns and the relative abundance of the proteins whose constitutive expression in liver was different between untreated H/W *versus* L-E strains.

Protein-disulfide isomerase A3 (PDIA3) was positively identified in multiple forms (see Table II) with similar molecular weight but shifted pI, suggesting post-translational modifications. The expression of the more basic forms of PDIA3 (PDIA3b and PDIA3m) was 4.4- and 3.3-fold lower in the resistant H/W strain than in the sensitive L-E strain ($p = 0.004$ and $p < 0.0001$, respectively). In contrast, the abundance of the more acidic form of PDIA3 (PDIA3a) was significantly higher (3.3-fold; $p = 0.001$) in the resistant strain compared with the sensitive one. Furthermore the expression of regucalcin (RGN) and agmatine ureohydrolase (AGMAT) was more than 2-fold higher (2.8- and 2.14-fold, $p = 0.02$ and $p < 0.0001$, respectively) in the liver of H/W rats compared with the liver of L-E rats.

Proteins Differentially Expressed after TCDD Exposure in Both Strains—Fig. 4 illustrates the expression patterns and the relative abundance of proteins whose expression is significantly modulated by TCDD in both strains, suggesting that these changes are not related to the genetic background of the rats.

TCDD strongly induced the expression of two proteins, aldehyde dehydrogenase 3A1 (ALDH3A1) and selenium-binding protein 2 (SELENBP2). Overall the effect of TCDD was more pronounced in the sensitive L-E strain than in the H/W strain with the -fold increase in expression of ALDH3A1 twice as great in L-E rats (5.9-fold, $p < 0.0001$) than in H/W rats (2.7-fold, $p = 0.003$).

Interestingly the rat SELENBP2 was present in two forms with a similar molecular weight but a different pI: a basic form (SELENBP2b) and a more acidic form (SELENBP2a) (Fig. 5). TCDD exposure enhanced the expression of the two SELENBP2 isoforms similarly in both strains, although SELENBP2a and SELENBP2b appeared to be induced about

TABLE II

Rat liver proteins identified by LC-MS/MS showing variant expression levels

Detailed information on peptide/protein identification are reported in Supplemental Table 2. AC, accession number.

Spot no.	Identified protein	Symbol	NCBInr AC	Theor./Exp.[a]pI	Theor./Exp. molecular mass	No. pept.[b]	Cov[c]	Score[d]
					kDa		*%*	
44	Transferrin	TFa1[e]	gi|33187764	7.4/7.4	76/75	14	24.6	102
46	Transferrin	TFa[e]	gi|33187764	7.4/7.2	76/75	22	26.4	157.3
70	Programmed cell death protein 8	PDCD8	gi|14279176	9.1/8	66.1/67	2	3.1	14.2
131	Protein-disulfide isomerase A3	PDIA3b[e]	gi|38382858	6/6.1	56.6/57	14	28.7	112.5
134	Protein-disulfide isomerase A3	PDIA3m[e]	gi|38382858	6/5.9	56.6/57	37	574.9	228.8
161	Selenium-binding protein 2	SELENBP2b[e]	gi|18266692	6.2/6.2	52.5/53	70	79.9	392
162	Selenium-binding protein 2	SELENBP2a[e]	gi|18266692	6.2/6.1	52.5/53	55	69.1	282.2
224	Argininosuccinate synthetase	ASS	gi|25453414	8.1/9	46.5/46	18	30.8	116.5
288	Paraoxonase 3	PON3	gi|51854237	5.6/5.3	39.4/40	14	32.8	81.2
376	Sulfotransferase family 1A, phenol-preferring, member 1	SULT1A1	gi|1091600	6.8/6.8	34/35	18	33	99.9
410	Agmatine ureohydrolase	AGMAT	gi|60688189	7.2/6.2	38/33	5	10.8	38.3
450	MAWD-binding protein	MAWBP	gi|51491893	6.1/6.1	32/31	18	36.5	86.4
460	Carbonic anhydrase 3	CA3b[e]	gi|31377484	7.2/7.7	29.4/30	33	47.7	140.4
463	Carbonic anhydrase 3	CA3a[e]	gi|31377484	7.2/7.4	29.4/30	14	36.5	76.5
521	Apolipoprotein A-I	APOA-Ia[e]	gi|55747	5.6/5.1	30/27	10	28.2	62.6
532	Apolipoprotein A-I	APOA-Ib[e]	gi|55747	5.6/5.2	30/26	15	37.5	95.3
649	Regucalcin	RGN	gi|13928740	5.4/5	33.4/34	45	62.9	188.3
655	Protein-disulfide isomerase A3	PDIA3a[e]	gi|38382858	6/5.6	56.6/53	42	61	230
671	Transferrin	TFb1[e]	gi|33187764	7.4/7.9	76/78	22	30.9	160.2
672	Transferrin	TFb[e]	gi|33187764	7.4/7.6	76/78	20	25.4	126.5
697	Aldehyde dehydrogenase family 3, member A1	ALDH3A1	gi|2392057	6.5/6.8	50.3/52	38	44.2	202.5
718	Transferrin	TFa2[e]	gi|33187764	7.4/7.4	76/78	16	21.6	105.3

[a] Theor., theoretical, data-based annotations; Exp., experimental, from two-dimensional gels.

[b] Number of valid peptide matches found for the given protein.

[c] Cov, the percent ratio of all amino acids from valid peptide matches to the total number of amino acids in the protein.

[d] The protein score is a function calculated from the individual normalized z-scores of validated peptides. Peptide z-score refers to the distribution of calculated scores compared with that of random peptide sequences to find the mean and variance (www. phenyx-ms.com). Database redundancy is handled and solved by the Phenyx software. If a proteins shares all of its validated peptides with another protein, then it is considered to be a subset and will not appear in the best scoring protein list. It appears in the protein Details panel under Subset for the principal and better scoring parameters. Therefore, the entries reported (NCBInr AC) refer exclusively to the best scoring protein found by the search engine.

[e] Arbitrary label assigned to the different isoforms.

1.5-fold more by TCDD treatment in L-E rats (3.2- and 5-fold, $p = 0.022$ and $p < 0.0001$, respectively) than in H/W rats 2-fold ($p = 0.024$) and 3.4-fold ($p = 0.004$), respectively.

Proteins Whose Levels Were Altered by TCDD Exposure in the Sensitive L-E Strain Only—Fig. 6 provides an overview of the expression patterns and relative abundances of eight proteins whose levels were altered in the sensitive L-E strain, but not in the resistant H/W strain, following TCDD exposure.

The abundances of two putative isoforms of carbonic anhydrase 3 (CA3a and CA3b), of the programmed cell death protein 8 (PDCD8), of sulfotransferase 1A1 (SULT1A1), and of argininosuccinate synthetase (ASS) were approximately halved by TCDD treatment. On the contrary, TCDD exposure induced the expression of paraoxonase 3 (PON3; 2.4-fold, $p = 0.002$), MAWD-binding protein (MAWBP; 3.2-fold, $p < 0.0001$), and two forms of apolipoprotein A-I (APOA-Ia and

APOA-Ib, 2.9- and 5.8-fold, $p < 0.0001$ and $p = 0.0002$, respectively). We also observed statistically significant increases in the expression of multiple isoforms of transferrin (isoforms arbitrary labeled as TFa, -a1, -a2, -m, -b, and -b1) in the range of 2.0–3.4-fold relative to untreated control animals. Interestingly in the dioxin-resistant H/W rats no liver proteins (apart from ALDH3A1 and SELENBP2) showed levels significantly ($p < 0.05$) different from untreated animals with a -fold change ≥ 2 after TCDD treatment (data not shown).

Integration of Transcriptomic and Proteomic Data

To supplement our proteomic results, we focused on transcripts corresponding to the 13 proteins, identified by LC-MS/MS, differentially expressed between untreated rat strains and after the TCDD treatment. Table III shows the changes in mRNA
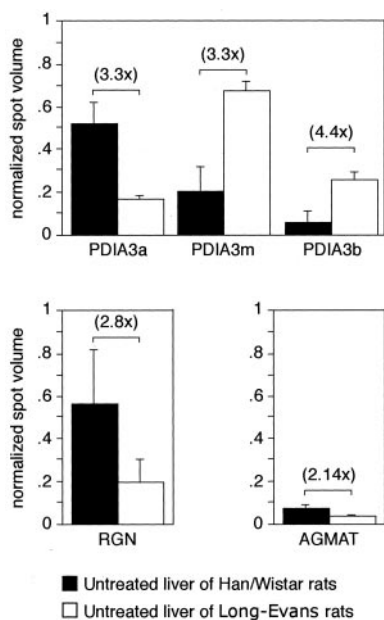
FIG. 3. **Expression patterns for the hepatic proteins whose abundance differed significantly (*t* test, *p* < 0.05) between liver extracts of untreated H/W and L-E rats.** Each *bar* represents the average spot abundance expressed as normalized volume ± S.D. The *vertical axis* shows spot normalized volume. *Numbers* in *parentheses* indicate protein expression -fold difference.
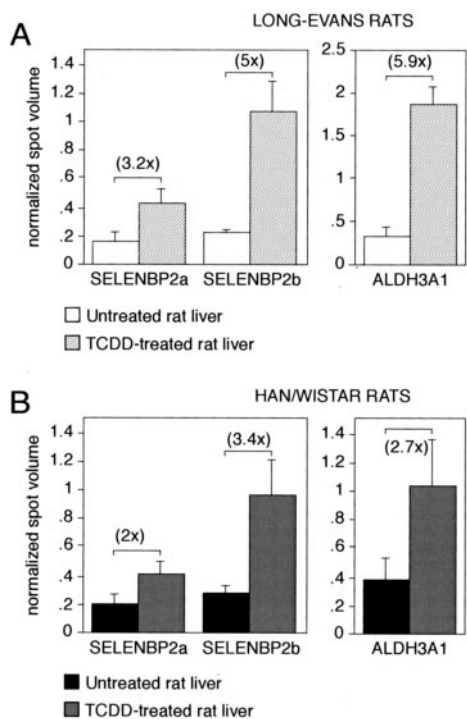

FIG. 4. **Expression patterns for the hepatic proteins whose abundance changed significantly (*t* test, *p* < 0.05)) in liver extracts after TCDD treatment in both H/W and L-E strains.** Each *bar* represents the average spot abundance expressed as normalized volume ± S.D. The *vertical axis* shows spot normalized volume. *Numbers* in *parentheses* indicate protein expression -fold change.
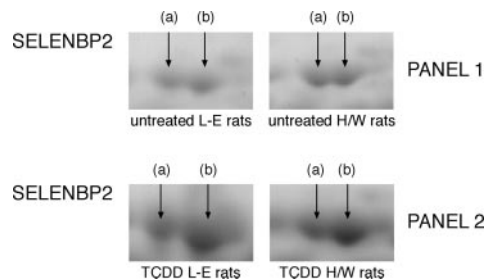

FIG. 5. **Enlarged regions of the 2-DE average gel image showing the potential additional isoforms of the SELENBP2 in untreated rats (L-E and H/W) (*PANEL 1*) and in TCDD-treated rats (L-E and H/W) (*PANEL 2*).** *Lowercase letters* in *parentheses* indicate an arbitrary label assigned to the different isoforms.
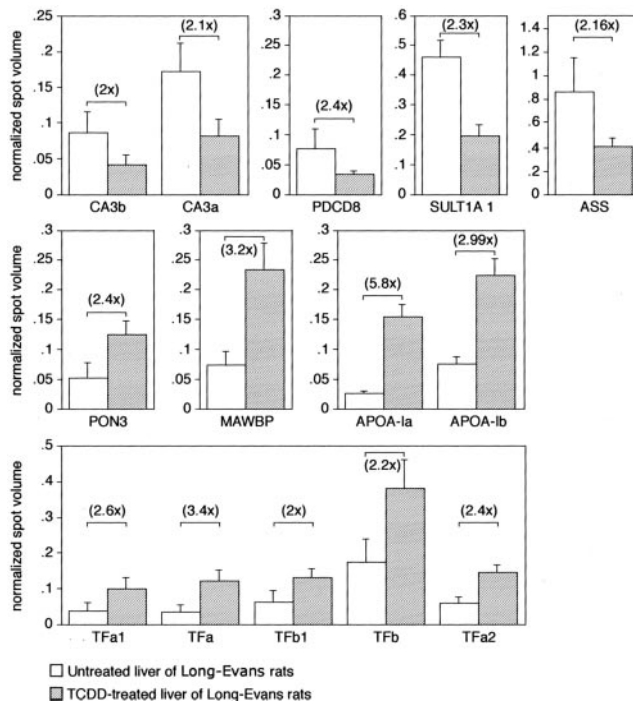

FIG. 6. **Expression patterns for the hepatic proteins whose abundance changed significantly (*t* test, *p* < 0.05) in liver extract of sensitive L-E rats after TCDD treatment.** Each *bar* represents the average spot abundance expressed as normalized volume ± S.D. The *vertical axis* shows spot normalized volume. *Numbers* in *parentheses* indicate protein expression -fold change.

expression for 12 genes from microarray analyses on L-E and H/W animals after TCDD exposure. One gene, *Agmat*, was not present on the Affymetrix arrays used for this analysis.

In Table III, the M values represent $\log_2$ expression ratios relative to vehicle-treated controls (*i.e.* $\log_2|TCDD|$ − $\log_2|Vehicle|$). *p* values were generated by model-based *t* tests and have been adjusted for false discovery rate control to avoid multiple testing concerns. Statistical significance was set at the $p < 10^{-3}$ level.

In the L-E strain, nine of the 12 genes whose protein levels were affected by TCDD also showed a significant change in their mRNA expression after TCDD treatment. Interestingly

TABLE III

mRNA expression of the study gene subset from array analyses after 4 days in H/W and L-E rats following TCDD exposure

Gene subset refers to all the identified proteins in this study (differently expressed in untreated rat strains and after TCDD treatment).

Gene/protein name	Abbreviation	Gene ID[a]	H/W rats		L-E rats	
			M[b]	p[c]	M	p
Agmatine ureohydrolase	*Agmat*	298607	NA[d]	NA	NA	NA
Aldehyde dehydrogenase family 3, member A1	*Aldh3a1*	25375	10.5	1.08E−08	11.3	4.97E−10
Apolipoprotein A-I	*ApoA-I*	25081	0.4	0.270	1.1	5.21E−05
Programmed cell death protein 8	*Pdcd8*	83533	0.1	0.628	−0.1	0.562
Argininosuccinate synthetase	*Ass*	25698	−0.2	0.629	−0.9	1.11E−04
Carbonic anhydrase 3	*Ca3*	54232	0.0	1.000	−6.9	4.58E−06
MAWD-binding protein	*Mawbp*	171564	1.3	6.84E−07	1.3	3.77E−08
Paraoxonase 3	*Pon3*	312086	1.2	6.84E−07	1.4	7.52E−09
Protein-disulfide isomerase A3	*Pdia3*	29468	−0.1	0.894	0.2	0.376
Regucalcin	*Rgn*	25106	0.3	0.320	−0.6	6.01E−03
Selenium-binding protein 2	*Selenbp2*	140927	1.7	1.01E−05	2.1	6.19E−08
Sulfotransferase family 1A, phenol-preferring, member 1	*Sult1a1*	83783	−0.6	0.441	−2.1	2.57E−05
Transferrin	*Tf*	24825	−0.2	0.308	0.6	8.40E−06

[a] NCBInr EntrezGene.
[b] M, $\log_2$ units of differential expression (*e.g.* +1.0 indicates 2-fold induction).
[c] p, ProbeSets were deemed significant at the $p < 1 \times 10^{-3}$ level (see "Experimental Procedures" for statistical analysis).
[d] NA, not available in the Affymetrix arrays used.

the genes displaying the highest mRNA induction by TCDD were *Selenbp2* and *Aldh3a1* in both L-E and H/W rats. In the resistant H/W strain, two further transcripts were significantly up-regulated by TCDD (*Mawbp* and *Pon3*), although their corresponding protein levels did not change. TCDD did not alter the abundance of either *Rgn* or *Pdia3* transcripts or proteins.

A plot comparing mRNA expression with protein expression for all the proteins characterized is shown in Fig. 7. Overall the mRNA differential expression analysis displays concordant changes (direction of change) with the protein abundance changes. Some data points fall on the axes indicating that these loci deviate from a positive correlation between transcript and protein expression. However, each of these points corresponds to cases where the changes did not reach statistical significance.

Analysis of Response Element Search

Table IV provides the number of matches to five different transcription factor binding site motifs for the chosen subset of rat genes and their murine orthologs. The numbers of AHRE-I, AHRE-II, and ARE motifs found in the region −5000 to +1000, relative to the transcriptional start site, are given for both rat and mouse orthologs. This search was not performed for the *Agmat* and *ApoA-I* genes due to the lack of definite position for their transcriptional start site in the current annotation of the rat genome.

The extended AHRE-I element was present in all rat genes except for *Tf* and *Sult1a1*. The full AHRE-I binding sequence, which occurs by chance very rarely in the genome, was found twice in the promoter of the *Ass* rat gene. Five genes (*Aldh3a1*, *Ass*, *Ca3*, *Selenbp2*, and *Sult1a1*) showed the AHRE-II motif in both rat and mouse. Interestingly the core ARE sequence was not found in the *Pdcd8* and *Rgn* rat genes, although it was present in their mouse orthologs.
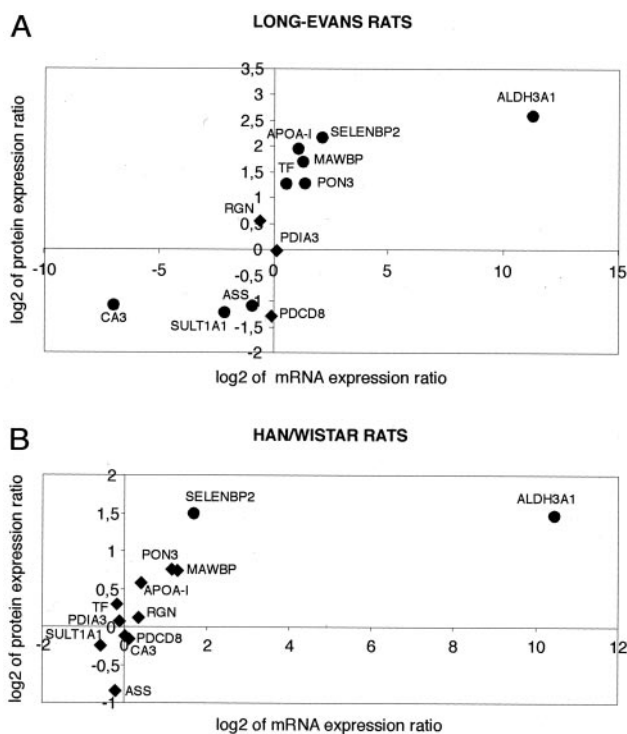
FIG. 7. **Plot of the correlation between mRNA and protein expression changes following TCDD treatment.** The $\log_2$ value of the mRNA and protein ratio of expression (treated/untreated) was calculated and plotted for all proteins identified by LC-MS/MS. *A*, mRNA-protein correlation in TCDD-treated L-E rats. *B*, mRNA-protein correlation in TCDD-treated H/W rats. The names of the proteins as abbreviations are shown. *Circles* and *diamonds* indicate statistically significant and nonsignificant changes, respectively, both in mRNA and protein expression.

DISCUSSION

The fact that the molecular mechanisms of dioxin toxicity are still poorly understood complicates dioxin risk assess-

TABLE IV
Genes containing AHRE and ARE elements in rat and their mouse orthologs

Gene subset refers to all the identified proteins in this study (differently expressed in untreated rat strains and after TCDD treatment).

Identified gene	Rat		Mouse		AHRE-I counts[a]						AHRE-II counts		ARE counts	
					Core		Extended		Full					
	Gene ID	Location	Gene ID	Location	Rat	Mm[b]	Rat	Mm	Rat	Mm	Rat	Mm	Rat	Mm
Agmatine ureohydrolase	298607	5q36	75986	4 E1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Aldehyde dehydrogenase family 3, member A1	25375	10q22	11670	11 34.25 cM	8	11	2	2	0	0	2	1	3	3
Apolipoprotein A-I	25081	8q23-q24	11806	9 27.0 cM	NA	11	NA	3	NA	0	NA	0	NA	1
Programmed cell death protein 8	83533	Xq35	26926	X 17.0 cM	10	7	4	1	0	0	0	1	0	2
Argininosuccinate synthetase	25698	3p12	11898	2 20.0 cM	18	13	3	1	2	0	2	1	4	3
Carbonic anhydrase 3	54232	2q23	12350	3 11.7 cM	5	1	1	1	0	0	1	2	4	4
MAWD-binding protein	171564	20p11	68371	10 B4	12	7	2	1	0	0	1	0	3	3
Paraoxonase 3	312086	4q13	269823	6 0.5 cM	3	14	1	2	0	0	0	2	4	1
Protein-disulfide isomerase A3	29468	3q35	14827	2 69.0 cM	12	8	4	2	0	0	2	0	4	0
Regucalcin	25106	Xq12	19733	X A1.3	4	2	1	0	0	0	1	1	0	2
Selenium-binding protein 2	140927	2q34	20341	3 43.25 cM	13	6	3	3	0	0	0	1	1	2
Sulfotransferase family 1A, phenol-preferring, member 1	83783	1q36	20887	7 4.0 cM	8	1	0	0	0	0	2	2	1	3
Transferrin	24825	8q32	22041	9 56.0 cM	3	9	0	3	0	0	0	0	3	5

[a] Counts, number of occurrences, number of times each motif appears in the region between -5000 and $+1000$ relative to the transcriptional start site in the gene.

[b] Mm, mouse.

ment. Only one mechanism of dioxin action, the direct induction of CYP1A1, has been elucidated in detail (5). However, despite its direct regulation by the AHR, CYP1A1 induction is not predictive of dioxin toxicity.

A useful tool for studying the mechanisms of dioxin toxicity is the large sensitivity difference in TCDD-induced lethality between H/W and L-E rat strains. Although there is a large sensitivity difference in certain endpoints of TCDD toxicity (*e.g.* acute lethality, wasting, and hepatotoxicity), other responses, such as induction of CYP1A1, remain similar between these strains (3). A wide range of clinical chemistry variables and biochemical parameters have been investigated in an effort to rationalize the differences between these two strains, but no clear molecular factors responsible for TCDD resistance in the H/W rat strain have been identified.

The aim of this study was to investigate the extent to which hepatic levels of specific proteins differ between H/W and L-E rat strains both under physiologic conditions and after TCDD exposure. The TCDD dose utilized in this study is a discriminating dose at which maximal CYP1A1 induction is present in both strains, but type II endpoints, such as increased serum levels of aspartate aminotransferase, free fatty acid, and bilirubin, show dramatic changes only in the sensitive L-E rats (31). Thus, evaluation of differential protein expression patterns following TCDD treatment may yield important clues about the molecular factors responsible for TCDD resistance/lethality in this animal model.

For the first time, we provide evidence that differences in the liver proteome of the L-E and H/W rats exist even before any treatment is applied. A substantial change in the expression of different isoforms of PDIA3 was observed between the strains. PDIA3 (also known as Erp57, Er-60, and GRP58) introduces disulfides into proteins and catalyzes the rearrangement of incorrect disulfides during oxidative protein folding in the endoplasmic reticulum (ER). It is a chaperone that inhibits aggregation of denatured proteins (32).

Up to now, post-translational modifications of PDIA3 have not been well characterized. Recently Sakai *et al.* (33) suggested that PDIA3 undergoes dephosphorylation during ischemia and reperfusion in a rat heart model, although the physiological implication of these modifications was not addressed. The observed pI differences between adjacent spots of PDIA3 and the overexpression of the more acidic form of PDIA3 in the H/W strain might suggest a peculiar post-translational modification profile of this protein in the TCDD-resistant strain.

It is relevant that PDIA3 can interact with calreticulin, one of the major Ca^{2+}-binding proteins of the ER membrane, and that Ca^{2+} modulates the interaction between these two proteins (34, 35). It has been reported that PDIA3 modulates the redox state of the ER, providing dynamic control of ER Ca^{2+} homeostasis. Optimal $[Ca^{2+}]$ in the ER is necessary for protein folding, and calcium depletion inhibits protein folding and maturation and facilitates protein degradation. This indirect link of PDIA3 with the Ca^{2+} signaling pathway suggests that Ca^{2+} homeostasis and its control would be of importance in explaining the resistance of H/W rat. This speculation is strengthened by our finding that basal expression of proteins

directly and/or indirectly involved in the Ca^{2+} signaling is elevated in HW rats.

In particular, we observed that RGN abundance was 3 times higher in untreated H/W than in untreated L-E rats. RGN is an intracellular Ca^{2+} regulator. It blunts cell death caused by intracellular Ca^{2+} accumulation by enhancing plasma membrane Ca^{2+} pumping activity (36). It has been reported that TCDD treatment of mouse hepatoma cells causes a rapid increase in Ca^{2+} influx rates from extracellular sources (37). We speculate that elevated basal levels of this protein may confer to the H/W strain a protection against disruption of calcium homeostasis as might be mediated by TCDD.

The putative importance of Ca^{2+} signaling pathway in the puzzling TCDD resistance of H/W rats may also be supported by the abundance of AGMAT protein in H/W livers relative to TCDD-sensitive L-E livers. This enzyme represents a potentially important mechanism for regulating the biological effect of agmatine, which is formed by decarboxylation of arginine (38). Agmatine has neuronal and vascular properties through a myriad of effects on calcium channels and undergoes a complex interaction with the nitric oxide system (39, 40). Moreover agmatine by itself has an important role in polyamine homeostasis, inhibiting the activity of the ornithine decarboxylase, a highly regulated enzyme that catalyzes decarboxylation of ornithine to form putrescine (41). Indeed it has been reported that TCDD might also inhibit ornithine decarboxylase (42). Thus the increased expression of AGMAT might provide a supply of putrescine for polyamine biosynthesis when the primary pathway is somehow altered.

Polyamines are involved in the synthesis of nucleic acids and proteins. A polyamine deficiency may result in growth arrest or apoptosis (43). Interestingly polyamines are thought to act as intracellular second messengers by modulating Ca^{2+} flux and mobilizing intracellular calcium stores (44, 45). A decrease in polyamine concentrations in critical organs may play an important role in the toxic effects of TCDD, and this might be linked to the disrupted calcium homeostasis observed following TCDD exposure (37, 46). In such a framework, a strengthened expression of AGMAT in H/W rats might provide an alternative route for polyamine biosynthesis and might counteract the detrimental effect of TCDD on polyamine biosynthesis.

The picture that emerges is one in which a peculiar control of the Ca^{2+} signaling pathway and homeostasis might have a pivotal role in conferring resistance to TCDD in the H/W strain. Interestingly there are clear differences in bone geometry and mineral density between untreated H/W and L-E rats (47). Long bones and lumbar vertebra of H/W rats are shorter and thinner than those of L-E rats, and the cortical bone mineral density is higher in the long bones of H/W rats.[4] The bones of

H/W rats are also generally more resistant to TCDD-induced alterations in bone geometry, mineral density, and mechanical strength. However, whether the observed differences between H/W and L-E rats in Ca^{2+} signaling and homeostasis might contribute to the strain differences in bone structure and TCDD sensitivity needs to be investigated further.

Remarkably dioxin did not have any effect on expression of these proteins or their mRNA transcripts in either strain, suggesting that the regulation of these genes is not driven mainly by TCDD. It is unclear how to rationalize this observation with the known AHR dependence of dioxin toxicity. It is possible that the expression of these genes is mediated by the AHR in a dioxin-independent fashion, and indeed many such genes have been identified recently (48).

In both sensitive and resistant rats, TCDD strongly induced only two proteins, ALDH3A1 and SELENBP2, and in each case their mRNA transcripts showed correlated and concordant increases in our microarray analyses. Although the increased level of ALDH3A1 is a well known mechanism of detoxification of damaging electrophilic aldehydes (49), the expression regulation of SELENBP2 by dioxin is a novel observation, although its hepatic induction by a dioxin-like pentachlorobiphenyl (PCB126) has been reported (50).

SELENBP2 has been recently suggested to participate in the late stage of intra-Golgi protein transport (51). However, neither its physiological role nor its transcriptional regulation have been determined.

Our *in silico* transcription factor binding site search showed, for the first time, that the *Selenbp2* gene possesses both AHREs and conserved AREs similar to the *Aldh3a1* gene. Thereby a similar mechanism of transactivation might be hypothesized for these two genes where the induction is concomitantly mediated by dioxin and by oxidative stress (52).

Because SELENBP2 and ALDH3A1 transcript and protein levels respond to dioxin in a similar fashion in both sensitive and resistant rat strains, it is likely that they are involved in TCDD toxicities that are similar between the two strains, *i.e.* type I responses. However, they have to be excluded as major determinants of the differential sensitivity to acute toxicity between the two rat strains.

As expected, TCDD treatment did not cause any further changes in protein expression response in resistant rats, whereas it did affect the abundance of many additional hepatic proteins in sensitive rats. The majority of these dioxin-responsive proteins lie in the pathways leading to well known TCDD toxic effects. For example, the induction of TF, the iron-binding protein that carries ferric ion between the site of its absorption to its sites of storage and utilization, is in line with the widely reported alterations in heme synthesis and catabolism and the disruption of iron homeostasis, both well characterized aspects of TCDD-related hepatic toxicity (53–55).

It has to be underlined that, in the injured liver, TF is one of several genes that are expressed immediately after injury to

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[4] N. Stern, S. Larsson, M. Viluksela, J. T. Tuomisto, J. Tuomisto, J. Tuukkanen, T. Jämsä, P. M. Lind, and H. Håkansson, manuscript in preparation.

mesenchymal cells known as stellate cells (56). Therefore the higher expression of TF might be viewed also as a putative marker of a progression of liver damage in the sensitive strain.

TCDD exerts many of its effects by binding to the AHR and inducing cytochrome P450 gene expression. One by-product of enhanced P450 activity is an increased incidence of electron transfer to molecular oxygen leading to reactive oxygen species formation and lipid peroxidation (57).

In this oxidative stress scenario, the increased expression of PON3 and APOA-I, both associated with high density lipoprotein, might be viewed as a protective response to the oxidative degradation of lipoproteins evoked by TCDD. Moreover this is consistent with the findings that hepatic lipid peroxidation, measured as the amount of thiobarbituric acid-reactive substances, was induced by TCDD dose dependently in L-E but not in H/W rats (58).

Again the decreased abundance, following TCDD exposure, of CA3, a cytosolic enzyme that has an important role in defending the cell against oxidative damage and reactive oxygen species-induced cell death, might account for the suppression of the defense system for oxidative stress (59). It cannot be excluded, however, that the down-regulation of its hepatic expression might be due to a direct effect of TCDD because it has been suggested recently that AHR ligands could elicit CA3 suppression (60).

Following TCDD exposure, L-E rats showed a decreased abundance of ASS, one of the key enzymes of the urea cycle, and of SULT1A1, which is involved in the sulfonation of xenobiotics. Such alterations might be viewed as a secondary effect of TCDD because primary TCDD-dependent effects such as the modulation of the concentration of glucocorticoids, glucagons, and insulin are all known to play roles in the liver-specific transcriptional regulation of these proteins (61, 62).

Interestingly in the TCDD-treated L-E strain we observed down-regulation of PDCD8, whose modulation has not been associated previously with dioxin exposure. The decreased expression of PDCD8, also known as apoptosis-inducing factor, might suggest an alteration of apoptosis in the liver cells because, in response to some death stimuli, PDCD8 is released by mitochondria and translocates into the nucleus. In the nucleus it binds DNA and triggers caspase-independent cell death (63). The decrease in PDCD8 levels might be consistent with the suggested reduction of apoptosis by TCDD as one possible epigenetic mechanism of hepatocarcinogenesis observed in rat studies on liver tumor promoting activity of TCDD (31, 64).

The MAWBP is another novel target for TCDD. The up-regulation of this protein was found exclusively in the L-E strain after TCDD exposure. This is in accordance with a very recent observation that the abundance of MAWBP increases in the liver of Sprague-Dawley rats treated with either a single high dose of TCDD or with a daily low dose of TCDD (65). Unfortunately the biochemical and physiological role of the MAWBP has not been yet clarified.

The majority of abundance changes in the TCDD-responsive proteins are concordant with those reported at the message levels. We analyzed RNA samples collected 4 days after TCDD exposure, thus preceding the 5-day time point used for the proteomic analysis in a different group of animals. However, the good correlation between our proteomic and transcriptomic results suggests that most regulation takes place at the transcriptional level for these genes.

We tried to decipher patterns of transcriptional regulation through the computational characterization of the upstream regulatory regions of these genes. In particular, we identified the presence and location of the major TCDD-associated response elements: AHRE-I, AHRE-II, and ARE.

The composite structure of AHRE and/or ARE elements (one or more motifs) necessary to mediate induction strength remains unclear. Recently microarray analysis using hepatic tissue from mice treated with TCDD for 24 h identified 739 genes that exhibited a significant change in expression with 192 of these genes possessing at least one AHRE (23).

Collectively our results from the *in silico* search provide evidence that the novel TCDD-responsive genes identified in this study are likely regulated through one of the three characterized motifs: AHRE-I, AHRE-II, or the ARE. In addition, we found that the AHRE-II induction mechanism might be utilized by four rat genes (*Ass*, *Ca3*, *Rgn*, and *Sult1a1*) not reported previously. The presence of AHRE-II element in the promoter region of the *Aldh3a1* gene has been observed already (6).

Although the presence of such novel motifs in genes encoding these proteins does not account for strain-specific toxicities, it might indicate that part of the divergent sensitivity could be explained through the coactivation aspect of AHR function. More importantly, the linkage of motif searching, mRNA expression profiling, and proteomic analysis allows us, for the first time, to begin to link changes in protein levels with specific mechanisms of transcriptional regulation.

In conclusion, we successfully identified several proteins that may contribute to strain-specific sensitivity differences in TCDD toxicity. We have highlighted (i) the potential importance of differential basal proteome profile between the rat strains as a potential contributor to divergent sensitivity, (ii) the identification of novel and plausible mediators integral to major TCDD toxicity pathways conferring sensitivity, and (iii) the identification of several novel dioxin-responsive proteins (*e.g.* PDCD8, MAWBP, and SELENBP2) whose deregulation may lead to new insights into the molecular mechanism of dioxin toxicity.

Finally and critically, we have provided evidence that protein expression in a model of dioxin toxicity is primarily regulated at the level of transcription. This is reasonable given that the AHR is essential to major forms of dioxin toxicity and that the function of the AHR is as a transcriptional regulator. Furthermore the fact that several novel dioxin-responsive proteins possess AHRE-II regulatory elements within their promoters suggests that some of the species differences in di-

oxin sensitivity might be mediated via the novel coactivator function of AHR. If confirmed, this would have a significant impact on human risk assessment studies.

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