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Convergence of Hepcidin Deficiency, Systemic Iron Overloading, Heme Accumulation, and REV-ERB α/β Activation in Aryl Hydrocarbon Receptor-Elicited Hepatotoxicity

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Abstract

Persistent aryl hydrocarbon receptor (AhR) agonists elicit dose-dependent hepatic lipid accumulation, oxidative stress, inflammation, and fibrosis in mice. Iron (Fe) promotes AhR-mediated oxidative stress by catalyzing reactive oxygen species (ROS) production. To further characterize the role of Fe in AhR-mediated hepatotoxicity, male C57BL/6 mice were orally gavaged with sesame oil vehicle or 0.01–30 $\mu\text{g/kg}$ 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) every 4 days for 28 days. Duodenal epithelial and hepatic RNA-Seq data were integrated with hepatic AhR ChIP-Seq, capillary electrophoresis protein measurements, and clinical chemistry analyses. TCDD dose-dependently repressed hepatic expression of hepcidin (*Hamp* and *Hamp2*), the master regulator of systemic Fe homeostasis, resulting in a 2.6-fold increase in serum Fe with accumulating Fe spilling into urine. Total hepatic Fe levels were negligibly increased while transferrin saturation remained unchanged. Furthermore, TCDD elicited dose-dependent gene expression changes in heme biosynthesis including the induction of aminolevulinic acid synthase 1 (*Alas1*) and repression of uroporphyrinogen decarboxylase (*Urod*), leading to a 50% increase in hepatic heme and a 13.2-fold increase in total urinary porphyrins. Consistent with this heme accumulation, differential gene expression suggests that heme activated BACH1 and REV-ERB α/β , causing induction of heme oxygenase 1 (*Hmox1*) and repression of fatty acid biosynthesis, respectively. Collectively, these results suggest that *Hamp* repression, Fe accumulation, and increased heme levels converge to promote oxidative stress and the progression of TCDD-elicited hepatotoxicity.

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Conflict of Interest Statements

The authors declare that there are no conflicts of interest related to this work.

Graphical abstract

Keywords

TCDD; NAFLD; Iron; Hepcidin; Heme; REV-ERB α/β

INTRODUCTION

Metabolic syndrome (MetS) is a multifactorial disease characterized by obesity, increased triacylglycerol (TAG) levels, decreased high density lipoprotein (HDL) cholesterol levels, hypertension, and hyperglycemia. In the liver, MetS is manifested as non-alcoholic fatty liver disease (NAFLD), where benign and reversible hepatic fat accumulation (steatosis) progresses to chronic steatohepatitis (steatosis with inflammation) with fibrosis (collagen deposition) (Trauner *et al.*, 2010). The worldwide prevalence of MetS and NAFLD is continuously increasing, currently affecting ~30% of adults in the United States (Ford *et al.*, 2010). Although genetics, high caloric diet, and sedentary lifestyle are major contributing factors, emerging evidence suggests exposure to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds play an underappreciated role in the development of MetS and NAFLD (Casals-Casas and Desvergne, 2011).

TCDD is the prototypical ligand for a group of structurally diverse synthetic chemicals, natural products, and endogenous metabolites that activate the aryl hydrocarbon receptor (AhR) (Denison *et al.*, 2011). Upon ligand binding, the cytoplasmic AhR dissociates from chaperone proteins, followed by translocation to the nucleus and heterodimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT complex acts as a transcription factor, altering gene expression through binding to dioxin response elements (DREs) (Hankinson, 1995), although DRE-independent mechanisms of differential gene expression have also been reported (Huang and Elferink, 2012). In mice, a single bolus dose of TCDD induces hepatic steatosis with immune cell infiltration (Boverhof *et al.*, 2005; Kopec *et al.*, 2010b), which progresses to steatohepatitis with fibrosis following repeated dosing (Nault *et al.*, 2016a; Pierre *et al.*, 2014). TCDD-elicited hepatic inflammation and fibrosis are believed to be due, at least in part, to sustained oxidative stress mediated by AhR-dependent responses (reviewed in (Stohs, 1990)).

Oxidative stress involves disruption of the balance between pro-oxidants and anti-oxidants, in which accumulating reactive oxygen species (ROS) overwhelm neutralizing defenses. In B6C3F1 mice, hepatic superoxide anion radical ($O_2^{\bullet -}$) production increased following a

single acute dose of 10 µg/kg TCDD, while sub-chronic doses as low as 15 ng/kg/day TCDD also elevated ROS levels (Slezak *et al.*, 2000). TCDD-elicited hepatic ROS production is stimulated via several processes including the infiltration of activated macrophages and other phagocytes, the induction of cytochrome P450s and oxidoreductases, increased number of mitochondria and the partial uncoupling of oxidative phosphorylation, peroxisome proliferation, xanthine dehydrogenase/xanthine oxidase (XDH/XO) induction, and the inhibition of selenium-dependent glutathione (GSH) peroxidase. Increased production of hydrogen peroxide (H₂O₂), O₂^{•-}, and hydroxyl radical (•OH) results in lipid peroxidation, DNA damage, decreased membrane fluidity, and altered levels of GSH and NADPH (reviewed in (Stohs, 1990)).

Iron (Fe) is a micronutrient involved in several essential biological processes including oxygen transport, cellular respiration, and DNA synthesis. It is well established that Fe plays a fundamental role in TCDD-elicited hepatic oxidative stress. Cycling between the ferrous (Fe²⁺) and ferric (Fe³⁺) redox states allows Fe to catalyze the Haber-Weiss reaction, in which highly reactive •OH is generated from H₂O₂ and O₂^{•-}, leading to increased malondialdehyde (MDA) levels and other lipid peroxidation end products indicative of tissue damage and cell death. TCDD increased hepatic MDA levels in rats fed an Fe-supplemented diet, while Fe deficiency completely protected against MDA increases (al-Turk *et al.*, 1988). Similarly, *in vitro* lipid peroxidation in TCDD-treated rat hepatic microsomes was substantially reduced by desferrioxamine chelation (Al-Bayati and Stohs, 1987), further demonstrating a role for Fe in TCDD-elicited hepatic lipid peroxidation. Additionally, Fe-catalyzed oxidative damage is considered to be a causal factor in the development of hepatic fibrosis and hepatocellular carcinoma (George *et al.*, 1998; Kew, 2014).

Previous studies report that AhR activation causes accumulation of Fe and heme precursors (Goldstein *et al.*, 1973; Smith *et al.*, 2001), however the underlying mechanisms remain poorly understood. The objective of this study was to identify the altered biological processes and pathway interactions responsible for TCDD-elicited disruption of systemic Fe homeostasis and heme metabolism in mice. Using a systems biology approach, duodenal epithelial and hepatic RNA-Seq analyses were integrated with hepatic AhR ChIP-Seq data, capillary electrophoresis protein measurements, and clinical chemistry to investigate Fe absorption, transport, storage, and regulation. Our results demonstrate that TCDD repressed hepcidin, the master regulator of systemic iron homeostasis, leading to Fe accumulation in the serum and detection in urine. TCDD also altered hepatic heme biosynthesis and metabolism, leading to the activation of REV-ERBα/β, transcriptional repressors associated with the regulation of peripheral circadian rhythm and lipid metabolism. Taken together, these results suggest that disruption of Fe homeostasis plays a key role in TCDD-elicited oxidative stress and the progression of steatosis to steatohepatitis with fibrosis in mice.

MATERIALS & METHODS

Animal Handling and Treatment

Postnatal day 25 (PND25) male C57BL/6 mice weighing within 10% of each other were obtained from Charles River Laboratories (Kingston, NY) and housed in Innovive Innocages (San Diego, CA) containing ALPHA-dri bedding (Shepherd Specialty Papers, Chicago, IL)

in a 23°C environment with 30–40% humidity and a 12-hour light/dark cycle (7am–7pm). Aquavive water (Innovive) and Harlan Teklad 22/5 Rodent Diet 8940 (Madison, WI) were provided *ad libitum*. Mice were acclimated for 4 days (d) prior to treatment. 4-week old mice were used because juveniles are more sensitive to TCDD compared to adults (Vos and Penninks, 1987). Animals (n=8) were orally gavaged with sesame oil vehicle (Sigma-Aldrich, St. Louis, MO), 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 30 µg/kg TCDD (AccuStandard, New Haven, CT) every 4d for a total of 28d (7 exposures; Supplementary Figure S1). The doses used compensate for the relatively short duration of the study compared to lifelong cumulative human exposure from diverse AhR ligands, the bioaccumulative nature of halogenated AhR ligands, and differences in the metabolism and half-life of TCDD between humans (1–11 years (Sorg *et al.*, 2009; Wolfe *et al.*, 1994)) and mice (8–12d (Gasiewicz *et al.*, 1983)). In addition, these doses result in hepatic tissue levels that span background serum concentrations reported in the United States, Germany, Spain, and the United Kingdom to serum levels reported in Viktor Yushchenko 4–39 months following poisoning (Nault *et al.*, 2016a). Hepatic samples from female C57BL/6 mice treated with a single bolus dose of 3,3',4,4',5-pentachlorobiphenyl (PCB126; 3000 µg/kg), 2,3,7,8-tetrachlorodibenzofuran (TCDF; 3000 µg/kg), and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153; 300 mg/kg) were collected as previously described (Kopec *et al.*, 2010a; Kopec *et al.*, 2010b). All animal procedures were approved by the Michigan State University (MSU) All-University Committee on Animal Use and Care.

Sample Collection

At 7, 15, and 23d after the first dose, mice (vehicle and 30 µg/kg TCDD groups) were fasted for 6 hours (h) (access to water but not food) prior to tail blood glucose measurements using a FreeStyle Lite meter (Abbott Laboratories, IL). At 22d after the first dose, oral glucose tolerance tests (OGTT) were performed (vehicle and 30 µg/kg TCDD groups; fasted for 6h). Briefly, at time 0 minutes (min) animals were orally gavaged with 2 g/kg glucose in a 25% solution and tail blood glucose was measured at 0, 5, 15, 25, 30, 60, and 120 min. At 26d after the first dose, mice (vehicle and 30 µg/kg TCDD groups) were temporarily transferred to Innocages lacking bedding for 2h, with access to water but not food. Any urine expelled during this period was immediately collected, frozen in liquid nitrogen, and stored at –80°C. At 28d after the first dose, mice (all dose groups; fasted for 6h) were weighed and blood was collected from the submandibular vein prior to cervical dislocation. The duodenum (entire length from the stomach to the duodenojejunal flexure) was removed, flushed with Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS; Sigma) and opened longitudinally. The epithelial lining was scraped into either an empty vial for protein analysis or a vial containing 1.3 mL TRIzol (Invitrogen, Carlsbad, CA) for RNA extraction, frozen in liquid nitrogen, and stored at –80°C. Liver, gonadal white adipose tissue (gWAT), and brown adipose tissue were removed, weighed, frozen in liquid nitrogen, and stored at –80°C.

Clinical Chemistry

Total Fe levels (Fe²⁺ and Fe³⁺, heme and non-heme) were measured in serum, urine, and liver (vehicle, 3–30 µg/kg TCDD groups) using inductively coupled plasma mass spectrometry (ICP-MS) at the MSU Diagnostic Center for Population and Animal Health (DCPAH). Free heme (oxidized heme) was quantified in liver homogenates (vehicle and 30

µg/kg TCDD groups) using a commercial colorimetric assay (MAK036; Sigma). Total and direct (conjugated) bilirubin levels were measured in serum and urine (vehicle and 30 µg/kg TCDD groups) using commercial colorimetric assays (B7576 and B7538, respectively; Pointe Scientific, Canton, MI). All colorimetric end products were measured using a Tecan (Mannedorf, Switzerland) Infinite M200 plate reader. Indirect (unconjugated) bilirubin levels were calculated using indirect bilirubin = total bilirubin - direct bilirubin. Total iron binding capacity (TIBC) of serum (vehicle and 30 µg/kg TCDD groups) was measured through a direct ferric chloride colorimetric assay performed by MLabs (University of Michigan, Ann Arbor, MI). TIBC values were used to calculate transferrin saturation using transferrin saturation = serum Fe/TIBC × 100%. Total porphyrins in the urine (vehicle and 30 µg/kg TCDD groups) were quantified using a modified spectrophotometric method (Wolff *et al.*, 2013). Uroporphyrin (8-carboxyporphyrin) accounts for 90% of mouse hepatic porphyrins in TCDD-elicited porphyria (Goldstein *et al.*, 1973) and therefore the molar absorptivity of uroporphyrin (541,000 M⁻¹ cm⁻¹) was used to calculate the concentration of total urinary porphyrins.

RNA Extraction and RNA-Sequencing

Frozen duodenal epithelium scrapings and liver samples (all doses) were homogenized in TRIzol (Ambion, Waltham, MA) using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated as previously described (Boverhof *et al.*, 2005) and quantified using a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE) at 260 nm. Purity was assessed by calculating the A₂₆₀/A₂₈₀ ratio and RNA quality was analyzed using the Caliper LabChip GX (Perkin Elmer, Waltham, MA).

Duodenal and hepatic dose-dependent gene expression (all dose groups) was examined using RNA-Seq performed at the Research Technology Support Facility Genomics Core (rtsf.natsci.msu.edu/genomics). Duodenal libraries from three independent biological replicates (n=3) were prepared using the Ovation Mouse RNA-Seq System 1–16 sample preparation kit (NuGen, San Carlos, CA), with an additional DNase step. Hepatic libraries from three independent biological replicates (n=3) were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). Libraries were quantified and sequenced using a read depth of ~30M per sample, as previously described (Nault *et al.*, 2015). Quality was determined using FASTQC v0.11.2/v0.11.3 and adaptor sequences were removed using Trimmomatic v0.32/v0.33. Reads were mapped to the mouse reference genome (GRCm38 release 81) using Bowtie2 v2.2.3/v2.2.6 and TopHat2 v2.0.12/v2.1.0.

Following semi-parametric normalization, data were analyzed through an empirical Bayes method in which model-based *t* values were used to calculate posterior probability P1 (*t*) values on a per gene and dose basis (Eckel *et al.*, 2004; Eckel *et al.*, 2005). Bayesian models take into account adjacent data points while considering trends within a dose-response dataset. In addition, P1(*t*) values can be used to rank and prioritize genes based on the probability of differential expression. For TCDD-mediated differential gene expression, fold changes were calculated relative to vehicle controls. Genes were considered differentially expressed if fold change ≥ 1.5 and statistical P1(*t*) value ≥ 0.8 at one or more doses. RNA-Seq datasets for the duodenal epithelium and liver were deposited in the Gene Expression

Omnibus (GEO; accession number GSE87542 and GSE87519, respectively) and are available as Supplementary Tables S1 and S2, respectively.

Putative DRE Identification and Hepatic AhR ChIP-Seq

Putative dioxin response elements (pDREs) with matrix similarity scores (MSS) 0.856 were previously identified (Nault *et al.*, 2016b). ChIP-Seq was performed on liver samples from male C57BL/6 mice 2h following a single oral dose of 30 µg/kg TCDD as previously described (Nault *et al.*, 2016b). Reads were mapped to the mouse reference genome (GRCm38 release 81) using Bowtie 2.0.0 and alignments were converted to SAM format using SAMTools v0.1.2.

Enrichment Analyses and KEGG Pathway Integration

Differentially expressed genes (DEGs) in the duodenal epithelium were analyzed for enriched functions using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7. Only Gene Ontology (GO) Biological Processes and Molecular Functions were considered. Enrichment scores (ES) 1.3 were considered significantly enriched, representing the -log scale geometric mean *p*-value of 0.05.

CytoKEGG 0.0.5 within Cytoscape 3.2.0 was used to generate metabolism pathways with additional manual curation. Metabolite levels were obtained from clinical chemistry analyses or a published hepatic metabolomics dataset (Nault *et al.*, 2016b). The largest fold change for differential gene expression and metabolite levels are represented regardless of dose. The fold change for AhR enrichment (upper left corner) and the number of pDREs (upper right corner) are also provided.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed for select genes as previously described (Boverhof *et al.*, 2005). Forward and reverse primer sequences and amplicon sizes are provided in Supplementary Table S3. PCR amplification was conducted on a Bio-Rad CFX Connect Real-Time PCR Detection System. Gene expression relative to vehicle control was calculated using the 2^{-CT} method and the geometric mean of 3 housekeeping genes *ActB*, *Gapdh*, and *Hprt*. Gene expression data are plotted relative to vehicle control.

Protein Measurements

Duodenal epithelial scrapings, liver (ground by mortar and pestle), and serum samples (vehicle and 30 µg/kg TCDD groups) were sonicated on ice in RIPA buffer supplemented with protease inhibitor cocktail (Sigma). Samples were centrifuged and total protein in the supernatant was measured using the bicinchoninic acid (BCA) assay (Sigma). The WES capillary electrophoresis system (ProteinSimple, San Jose, CA) was used with the following antibodies: ACO1 (1:65; ab126595; Abcam, Cambridge, MA), FTH1 (1:65; ab65080; Abcam), FTL1 (1:65; ab69090; Abcam), HAMP/HAMP2 (1:10; ab190775; Abcam), HAO1 (1:30; ab93137; Abcam), HP (1:650; ab131236; Abcam), HPX (1:10; ab90947; Abcam), IREB2 (1:65; ab181153; Abcam), SLC11A2 (DMT1; 1:10; ab123085; Abcam), SLC40A1 (1:30; NBP1-21502; Novus Biologicals, Littleton, CO), TFRC (1:30; ab84036; Abcam), and TRF (1:30; ab82411; Abcam). Primary antibodies were detected using a goat anti-rabbit

secondary antibody conjugated to horseradish peroxidase. Chemiluminescence signals were analyzed with Compass software (ProteinSimple). Protein levels in the duodenum and liver were normalized to SDHA levels, while protein levels in the serum were normalized to sample volume.

RESULTS & DISCUSSION

Gross Morphology & Histopathology

Hepatic TCDD levels in female C57BL/6 mice gavaged every 4d for 28d approach steady state and are comparable to levels reported in human serum following accidental or intentional exposures (Nault *et al.*, 2016a). In this study, 30 µg/kg TCDD decreased terminal body weight by 27% (Supplementary Figure S2A), despite no significant change in daily food intake (data not shown). Absolute liver weights increased 18–30% at 1–10 µg/kg TCDD, while relative liver weight displayed an 18–57% dose-dependent increase at 0.3–30 µg/kg TCDD (Supplementary Figure S2B). There was a 46% and 28% decrease in absolute and relative gonadal white adipose tissue weight, respectively, at 30 µg/kg TCDD (Supplementary Figure S2C). No change in terminal brown adipose tissue was observed (not shown).

TCDD and related AhR agonists are reported to elicit hepatic lipid accumulation, immune cell infiltration, periportal fibrosis, and bile duct proliferation in mice (Birnbaum *et al.*, 1990; Jones and Greig, 1975; McConnell *et al.*, 1978; Pierre *et al.*, 2014; Vos *et al.*, 1974). Similarly, we observe these same histopathological features in the livers of C57BL/6 mice orally gavaged with TCDD every 4d for 28d, where the incidence and severity are consistently greater in males compared to females (Fader *et al.*, 2015; Nault *et al.*, 2016a) (Fader *et al.*, 2017). Specifically, male mice exhibit hepatic lipid accumulation at 0.3 µg/kg determined through Oil Red O (ORO) staining and inflammatory cell infiltration (primarily macrophages) at 3 µg/kg determined through F4/80 labeling. Picrosirius red (PSR) and hematoxylin & eosin (H&E) staining reveal periportal fibrosis and bile duct proliferation, respectively, at 30 µg/kg (Fader *et al.*, 2017). Hepatic vacuolization indicative of lipid accumulation is first observed in mice 18h after a single oral dose of 30 µg/kg TCDD, while immune cell infiltration and individual cell apoptosis are evident at 168h (Boverhof *et al.*, 2005).

Resident hepatic macrophages (Kupffer cells) and other innate immune cells play an important role in the development of these TCDD-elicited hepatic lesions. Following activation, Kupffer cells release pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6, which trigger immune cell (circulating monocytes, neutrophils) recruitment. Additionally, macrophage-derived TGFβ is an important activator of hepatic stellate cells, leading to excess extracellular matrix deposition and fibrosis (Nault *et al.*, 2016a). However, this manuscript primarily focuses on the role of hepatocytes in TCDD-elicited hepatotoxicity.

Fe Levels

Total Fe (Fe²⁺ and Fe³⁺, heme and non-heme) levels were measured in serum, urine, and liver extracts. ICP-MS detected a dose-dependent increase in Fe from 314 pg/dL in controls

to 816 µg/dL in serum at 30 µg/kg TCDD (Figure 1A). Fe was not detected in control urine but was present in the urine of all mice at 30 µg/kg TCDD, with an average level of 0.37 µg/mL (Figure 1B). A modest dose-dependent increase in total hepatic Fe levels was observed, although it was not statistically significant ($p=0.086$) due to inter-animal variability (Figure 1C). Previous studies report a 60% increase in hepatic Fe levels in mice receiving 4 weekly doses of 25 µg/kg TCDD (Goldstein *et al.*, 1973). Interestingly, the rate of Fe-catalyzed lipid peroxidation is dependent on the amount of free catalytically active Fe^{2+} rather than total Fe (Gruger and Tappel, 1970). Intracellular Fe is normally sequestered in the ferric state by the storage protein ferritin. However, TCDD facilitates the mobilization of catalytically active Fe^{2+} through induction of cytochrome P450s (Rowley and Sweeney, 1984) and *Xdh/Xo* which generate hepatic ROS (Nault *et al.*, 2016b). Ascorbate, which also releases Fe^{2+} from ferritin (Mazur *et al.*, 1955), increased 1.3-fold in hepatic extracts and 124.4-fold in urine of TCDD-treated mice (Nault *et al.*, 2016a). Consequently, TCDD can facilitate catalytically active Fe^{2+} mobilization through diverse mechanisms.

In blood, Fe is bound to transferrin (TRF), a plasma glycoprotein containing two high-affinity Fe^{3+} -binding sites. Serum TIBC increased from 236 µg/dL in controls to 534 µg/dL in TCDD-treated mice, indicating an increase in blood TRF levels, consistent with a 2.2-fold increase in TRF protein levels in the liver, the primary site of TRF synthesis (Figure 1D–E). This TIBC increase was proportional to increases in serum Fe, and therefore transferrin saturation (serum iron/TIBC $\times$ 100%) was unaffected by TCDD (Figure 1F).

Duodenal Differential Gene Expression

The duodenum is the intestinal segment predominantly responsible for the absorption of dietary minerals including Fe. Analysis of duodenal epithelial differential gene expression detected 22,541 expressed genes, of which 969 were differentially expressed ($|\text{fold change}| \geq 1.5$, $P(t) \leq 0.8$) at one or more doses. The majority of differentially expressed genes (DEGs) exhibited dose-dependent induction. AhR battery members including *Cyp1a1* (284.0-fold), *Cyp1a2* (117.1-fold), and *Cyp1b1* (51.0-fold) were among the most induced genes, while cubilin (*Cubn*; 4.2-fold), apolipoprotein L10A (*Apol10a*; 4.0-fold), and glutathione peroxidase 2 (*Gpx2*; 3.8-fold) were among the most repressed genes.

DAVID identified 25 enriched functional clusters ($ES \geq 1.3$) within the 969 duodenal DEGs, including an Fe- and heme-binding cluster consisting of 17 cytochrome P450s and several Fe homeostasis genes. For example, ferritin heavy chain 1 (*Fth1*), lactotransferrin (*Ltf*), and solute carrier 40A1 (*Slc40a1*; aka ferroportin) were repressed 1.7-, 1.6-, and 2.1-fold, respectively. Functional analysis also identified clusters related to immune response and inflammation. Several MHC class II genes (*Cd74*, *H2-Oa*, *H2-Ob*, *H2-Aa*, *H2-Ab1*, *H2-Eb1*, *H2-DMa*, *H2-DMb1*) involved in antigen processing and presentation were repressed, consistent with TCDD-elicited depletion of antigen presentation cells such as intestinal macrophages and dendritic cells (Fader *et al.*, 2015). Genes involved in T and B lymphocyte differentiation were also differentially expressed, including thymus cell antigen 1 (*Thy1* repressed 1.9-fold), interleukin-15 (*Il15* induced 1.5-fold), and interleukin-7 receptor (*Il7r* induced 1.5-fold), providing further evidence of duodenal lymphocyte population changes or disruption of lymphocyte activation. Clusters associated with lipid homeostasis and

metabolism, including intestinal digestion and absorption, lipid transport, steroid biosynthesis, and vitamin A metabolism (e.g., *Adh1* induced 1.7-fold, *Rdh10* induced 1.7-fold, *Aldh1a1* repressed 1.9-fold, *Rdh16* repressed 1.9-fold) were also enriched. Various cholesterol transporters such as ATP-binding cassette A1 (*Abca1*), ATP-binding cassette G8 (*Abcg8*), scavenger receptor B1 (*Scarb1*), and NPC1-like 1 (*Npc1l1*) were repressed, while long chain fatty acid transporter Cd36 antigen (*Cd36*) was induced. Overall, these changes are consistent with reports that TCDD alters dietary fatty acid/cholesterol absorption and vitamin A metabolism in the jejunum (Fader *et al.*, 2015).

Hepatic Differential Gene Expression

Hepatic RNA-Seq analysis detected 19,935 expressed genes, of which 9,313 were differentially expressed (|fold change| ≥ 1.5, $P1(t) \leq 0.8$) at one or more doses with the majority exhibiting dose-dependent induction. Highly induced AhR battery members included *Cyp1a1* (1,771-fold), flavin containing monooxygenase 3 (*Fmo3*; 1,491-fold), and *Cyp1b1* (1,436-fold). Carboxylesterase 3A (*Ces3a*), carbonic anhydrase 3 (*Car3*), and major urinary protein 7 (*Mup7*) were repressed at 3,062-, 2,198-, and 1,800-fold, respectively. Overall, the number of male liver DEGs (9,313) exceeded the previously reported number of female hepatic DEGs (3,677) (Nault *et al.*, 2015). In general, genes exhibited greater differential expression in males compared to that in females.

A previously published functional analysis of the hepatic transcriptome after repeated TCDD dosing identified dysregulation of lipid biosynthesis and metabolism, central carbon metabolism, and oxidative stress defense pathways (Nault *et al.*, 2016b). The current study focuses specifically on hepatic genes related to iron and heme homeostasis. A list of 119 mouse genes involved in Fe homeostasis was compiled by searching the literature and the Gene Ontology (GO) database for: (i) Fe ion homeostasis GO:0055072, (ii) Fe ion transport GO:0006826, (iii) regulation of Fe ion transport GO:0034756, (iv) heme metabolic process GO:0042168, (v) heme biosynthetic process GO:0006783, and (vi) heme transport GO:0015886. Approximately 114 of these 119 Fe-related genes were expressed in the liver, of which 73 exhibited differential expression following TCDD treatment (Figure 2). The observed gene expression changes were consistent with Fe accumulation and disrupted Fe homeostasis, which are discussed below.

Systemic Fe Regulation

Hepcidin is the master regulator of systemic Fe homeostasis. It limits serum levels by inhibiting duodenal absorption of dietary Fe, release of recycled heme Fe from macrophages, and efflux of Fe stores from hepatocytes (Zhao *et al.*, 2013). While the human and rat genomes contain a single hepcidin (*Hamp*) gene, the mouse locus has undergone a duplication event, leading to two closely related isoforms, *Hamp* and *Hamp2* (Ilyin *et al.*, 2003). Homozygous *Hamp* knockout mice exhibit Fe accumulation in several tissues, and increased serum Fe and ferritin levels, similar to hereditary hemochromatosis in humans (Lesbordes-Brion *et al.*, 2006). Transgenic mice overexpressing hepatic *Hamp* develop severe Fe-deficiency anemia (Nicolas *et al.*, 2002), while *Hamp2* overexpression in the liver has no effect on Fe regulation, suggesting HAMP and HAMP2 are not redundant (Lou *et al.*, 2004).

RNA-Seq analysis identified dose-dependent repression of *Hamp* (max 5.9-fold) and *Hamp2* (max 37.0-fold) at 10–30 µg/kg TCDD, which was confirmed by qRT-PCR (Figure 3A–B). Total HAMP (HAMP and HAMP2) protein was reduced 4.8-fold in the liver, consistent with the transcriptional repression (Figure 3C). No AhR binding occurred within ±10 kb of the *Hamp* or *Hamp2* transcription start sites. Other AhR agonists including PCB126 and TCDF also repressed *Hamp* expression 1.7-(not significant) and 2.8-fold, respectively, while non-dioxin-like PCB153 had no effect, suggesting *Hamp* repression involves the AhR (Figure 3D). *Hamp2* was repressed by PCB126 (6.3-fold), TCDF (19.7-fold), and PCB153 (2.2-fold), suggesting it may be regulated by both AhR and non-AhR mechanisms (Figure 3E).

Hepcidin reduces serum Fe levels via the internalization and degradation of SLC40A1 (aka ferroportin), a transmembrane Fe exporter expressed on macrophages, hepatocytes, and the basal membrane of duodenal enterocytes. Consistent with reductions in HAMP, SLC40A1 protein was increased 1.6-fold in the liver (Figure 3F). This would facilitate Fe efflux from hepatocytes and Kupffer cells into the blood, consistent with Fe accumulation in the serum and negligible hepatic increases. However, TCDD-elicited macrophage infiltration may also contribute to hepatic increases in SLC40A1. In the duodenal epithelium, SLC40A1 protein levels remained unchanged by TCDD (Figure 3G), despite 2.1-fold repression of *Slc40a1* mRNA. Thus, under conditions of TCDD-elicited HAMP deficiency, duodenal SLC40A1 protein levels were maintained, enabling continuous dietary Fe uptake across the basal duodenal membrane despite increasing serum Fe levels. Hepcidin is also reported to regulate dietary Fe absorption through proteasomal degradation of the apical solute carrier 11A2 (SLC11A2, aka DMT1) (Brasse-Lagnel *et al.*, 2011), however duodenal SLC11A2 protein levels were unaffected by treatment (Figure 3H). Studies using rat duodenal loops have shown that TCDD increases dietary Fe absorption, where enterocyte-to-blood transfer mediated by SLC40A1 was substantially more affected than SLC11A2-mediated lumen-to-enterocyte transfer (Manis and Kim, 1979).

Intracellular Fe Regulation

Intracellular Fe homeostasis is also regulated by two iron response element (IRE)-binding proteins, aconitase 1 (ACO1, aka IREB1) and IRE-binding protein 2 (IREB2). During Fe deficiency, ACO1 and IREB2 bind to IREs within the 5' UTR or 3' UTR of target genes, leading to translational repression or mRNA stabilization, respectively. In contrast, abundant Fe lowers ACO1 and IREB2 binding affinity for IREs. TCDD repressed *Aco1* and *Ireb2* 2.4- and 1.5-fold, respectively, while ACO1 and IREB2 hepatic protein levels were reduced 3.2- and 1.6-fold, respectively (Figure 4A–B), suggesting transcriptional and/or translational repression due to Fe accumulation. Lower ACO1 and IREB2 levels may increase translation of 5' UTR IRE-containing transcripts and reduce mRNA stabilization of transcripts containing 3' UTR IREs. For example, hydroxyacid oxidase 1 (*Hao1*) mRNA, which contains a 3' UTR IRE, was repressed 856.2-fold possibly due to reduced ACO1 and IREB2 levels, although AhR enrichment was also observed. The corresponding protein was undetectable in liver extracts from TCDD-treated animals (Figure 4C). In contrast, TCDD had no effect on hepatic protein levels of ferritin heavy (FTH1) and light (FTL1) chains, despite 5' UTR IREs within both genes (Figure 4D–E).

The transferrin receptor (TFRC), which is regulated by intracellular Fe levels, mediates cellular uptake of TRF-bound Fe via receptor-mediated endocytosis. Under low Fe conditions, *Tfrc* mRNA is stabilized by the presence of 5 IREs within the 3' UTR. Despite negligible increases in hepatic Fe levels, TCDD induced hepatic *Tfrc* mRNA and protein 2.3- and 3.0-fold, respectively, in the absence of AhR enrichment (Figure 4F). Hemochromatosis (HFE) is a membrane protein which competes with Fe³⁺-TRF for overlapping binding sites on TFRC. With serum Fe accumulation, TRF saturation increases and TRF dislodges HFE from its overlapping binding sites on TFRC (D'Alessio *et al.*, 2012). HFE then forms an iron-sensing complex with juvenile hemochromatosis type 2 (HFE2), transferrin receptor 2 (TFR2), and bone morphogenetic protein 6 (BMP6), using the cell surface receptor neogenin (NEO1) as a scaffold protein (D'Alessio *et al.*, 2012; Zhao *et al.*, 2016). Complex assembly initiates the canonical BMP signaling pathway, in which SMAD transcription factors induce *Hamp* transcription. The importance of HFE2, TFR2, and NEO1 in *Hamp* expression has been confirmed in human genetic disorders, human liver transplant studies, null mouse models, and mutation analysis studies. Hepatic *Hfe2*, *Tfr2*, and *Neo1* were repressed 2.6-, 6.1, and 1.6-fold, respectively, by TCDD, which may compromise HFE-HFE2-TFR2-BMP6 complex assembly thereby limiting *Hamp* and *Hamp2* transcription (Figure 5). AhR enrichment within *Hfe2* and *Neo1* was increased 6.2- and 3.2-fold, respectively, suggesting AhR involvement in *Hamp* and *Hamp2* repression. Alternatively, Qian *et al* propose that PCB126 and PCB153 repress hepatic *Hamp* in female mice in an estrogen response element (ERE)-dependent manner by mimicking estrogen activity (Qian *et al.*, 2015).

Heme Biosynthesis and Metabolism

Heme, a cyclic tetrapyrrole cofactor containing Fe²⁺, is essential for multiple biological functions including the capacity to bind gases and facilitate electron transfer in redox reactions. However, due to its ability to oxidize biological macromolecules and activate apoptotic enzymes, free heme must be incorporated into hemoproteins to minimize its toxicity. In our study, hepatic levels of free hemin (oxidized heme) increased from 510 pmole/mg in controls to 782 pmole/mg in TCDD-treated samples (Figure 6A).

Mammals are equipped with soluble scavengers to prevent heme and hemoprotein toxicity. Free hemoglobin in blood is captured by haptoglobin (HP) and removed by interaction with scavenger receptor CD163. TCDD repressed hepatic expression of *Hp* 7.5-fold and reduced serum protein levels 1.6-fold (Figure 6B), possibly through AhR-dependent transcriptional repression given the 2.1-fold increase in AhR enrichment. This would reduce HP buffering capacity while increasing hemoglobin oxidation to methemoglobin and the release of free heme. Free heme is bound by hemopexin (HPX), a high affinity plasma glycoprotein that is primarily expressed by the liver. HPX delivers heme to hepatocytes where it is endocytosed through LDL receptor-related protein 1 (LRP1). Hepatic *Hpx* was repressed 3.1-fold by TCDD, consistent with a 1.4-fold decrease in serum protein levels (Figure 6C), while AhR enrichment was increased 6.6-fold. Similarly, TCDD repressed *Lrp1* 1.8-fold, with a 7.0-fold increase in AhR genomic binding. In addition, a truncated form of α_1 -microglobulin (A1M, encoded by *Ambp*) binds and degrades extracellular heme but was dose-dependently repressed 9.0-fold, thereby further compromising antioxidant defenses against free heme.

Both HP and HAMP are acute phase proteins, synthesized during initiation of the acute phase response (APR). The APR is an innate immune response triggered by inflammation, infection, or stress, which aims to re-establish homeostasis and promote healing. In addition to *Hp* and *Hamp*, TCDD repressed several acute phase proteins including serum amyloid A proteins (*Saall*; 1.6-fold, *Saa4*; 96.7-fold), serum amyloid P-component (*Apcs*; 50.8-fold), and orosomucoids (*Orm1*; 13.3-fold, *Orm3*; 9.8-fold). Moreover, TCDD repressed metallothionein 1 and 2 (*Mt1* and *Mt2*) 10.6- and 17.5-fold, respectively, which are also considered APR members given their role as free radical scavengers. Most acute phase proteins lack both a pDRE and AhR genomic enrichment, suggesting negative-feedback regulation.

Hepatic heme oxygenase 1 (*Hmox1*), an inducible enzyme which catabolizes heme to Fe²⁺, biliverdin, and CO, was induced 6.5-fold (Figure 7). Heme is the most potent physiological inducer of *Hmox1* following binding to BACH1, a cap'n'collar basic region leucine zipper transcription factor, although oxidative stress and cytokines are also known inducers (Sun *et al.*, 2002). BACH1 forms heterodimers with small Maf proteins including MafF, MafG, and MafK, which bind Maf recognition elements (MAREs) within the promoter region of target genes to repress transcription. As heme accumulates, it binds to the heme regulatory motif (HRM) within BACH1, reducing DNA-binding activity and inducing nuclear export (Sun *et al.*, 2002). Maf proteins then form heterodimers with transcriptional activators such as NRF1 (*Nfe2l1*, repressed 1.6-fold) and NRF2 (*Nfe2l2*, induced 5.1-fold), to induce MARE-driven expression of target genes including *Hmox1*. Hepatic expression of *Bach1* was induced 1.9-fold by TCDD, while *Maf*, *Maff*, and *Mafk* were induced 1.6-, 34.7-, and 3.0-fold, respectively. AhR enrichment was identified at several Maf-related gene loci, including 8.3-, 2.5-, 3.3, and 1.9-fold enrichment within *Maf*, *Mafb*, *Maff*, and *Mafk*, respectively. *Hmox1* induction is likely in response to TCDD-elicited heme accumulation; however, the 3.3-fold increase in AhR genomic enrichment within the *Hmox1* promoter may also be a contributing factor.

In addition, TCDD elicited a 3.3-fold induction of biliverdin reductase B (*Bilvrb*) which metabolizes biliverdin to bilirubin, the latter being an effective peroxy radical scavenger that inhibits heme-induced lipid peroxidation. Several UDP-glucuronosyltransferases involved in bilirubin conjugation were also induced including *Ugt1a1*, *Ugt1a7c*, *Ugt1a8*, and *Ugt1a9* (2.2-, 51.2-, 4.8-, and 4.8-fold, respectively) (Figure 7). In the serum, total bilirubin was increased 2.4-fold at 30 µg/kg TCDD, while conjugated (direct) and unconjugated (indirect) bilirubin levels exhibited no clear treatment effect (Figure 6D–F). Similarly, TCDD increased total, direct, and indirect bilirubin levels in the urine 2.6-, 1.8-, and 4.2-fold, respectively (Figure 6G–I). Conjugated-bilirubin in the blood is transported into hepatocytes by solute carrier organic anion transporter 1A1 (*Slco1a1*) and 1B2 (*Slco1b2*), and then exported to the biliary tract by ATP-binding cassette C2 (*Abcc2*) and ATP-binding cassette G2 (*Abcg2*). Hepatic *Slco1a1*, *Slco1b2*, and *Abcg2* were repressed 1,186-, 7.9-, and 3.0-fold, respectively, by TCDD, consistent with increased serum and urine bilirubin levels. Unconjugated bilirubin in the blood binds to albumin (ALB) to limit tissue uptake and toxicity, however TCDD repressed hepatic *Alb* 3.7-fold. In humans, reducing the bilirubin-binding capacity of ALB increases the risk of bilirubin toxicity in the brain (kernicterus) and skin (jaundice). Furthermore, bilirubin can be oxidized back to biliverdin by ROS, creating a

redox cycle that draws NADPH away from other reactions including ROS defense responses (Nault *et al.*, 2016b). Interestingly, bilirubin activates the AhR and induces *Cyp1a1* mRNA levels, likely to enhance bilirubin elimination (Sinal and Bend, 1997).

Intracellular heme levels are also regulated by (i) ABCG2, an ATP-binding cassette transporter that exports urate, protoporphyrin IX, hemin, and heme into the blood, and (ii) SLC48A1 (aka HRG1), which facilitates endosome to cytosol heme transport. TCDD repressed hepatic *Abcg2* 3.0-fold and induced hepatic *Slc48a1* 7.6-fold. Reduced export capacity with increased endosomal output likely increases the cytosolic heme pool, contributing to oxidative stress, induction of heme degradation, and diminished BACH1-Maf-mediated gene repression. Putative heme importers *Mfsd7c* (aka *Flvcr2*) and *Slc46a1* (aka *Hcp1*) were repressed 5.4- and 2.0-fold, respectively, suggesting extracellular sources do not contribute to intracellular heme accumulation.

Cyp1a1, *1a2*, and *1b1* induction was accompanied by an equally dramatic repression of several highly expressed cytochrome P450 subfamilies such as *2c* (*2c29*, *2c44*, *2c50*, *2c70*, and *2c54* repressed 8.7-, 62.3-, 41.6-, 68.6-, and 550.5-fold, respectively), *2d* (*2d9*, *2d26*, *2d34*, and *2d40* repressed 5.8-, 32.4-, 14.8-, and 75.4-fold, respectively), *2e1* (repressed 7.6-fold), *2j* (*2j5*, and *2j9* repressed 32.1, and 17.5-fold, respectively), and *3a* (*3a13*, *3a16*, and *3a25* repressed 17.0-, 5.2-, and 6.0-fold, respectively) (Figure 8). Cytochrome P450 protein degradation involves lysosomal, ubiquitin-independent/dependent, and 20S/26S proteasomal mechanisms. Cytochrome P450 isoforms also differ in their turnover rate, with half-lives ranging from 7 to 37h (Yang *et al.*, 2008). Other hemoproteins were also repressed, including indoleamine 2,3-dioxygenase 2 (*Ido2*; 11.9-fold) and sulfite oxidase (*Suox*; 7.5-fold) (Figure 8). Heme released from degradation is not recycled, but catabolized by *Hmox1*. Hemoprotein degradation likely also contributes to transient increases in free heme, biliverdin, bilirubin, CO, and Fe²⁺.

After erythroid progenitors, hepatocytes have the highest rate of heme biosynthesis, with 50% of hepatic heme committed to cytochrome P450 synthesis (Chiabrando *et al.*, 2014). The first and rate limiting step of heme biosynthesis, which involves the condensation of glycine with succinyl-CoA to produce delta-aminolevulinic acid (ALA), is catalyzed by mitochondrial delta-aminolevulinic acid synthase 1 (*Alas1*). TCDD induced hepatic *Alas1* 4.1-fold in the absence of AhR enrichment, consistent with the TCDD-elicited hepatic hemin increases. However, 6 of the other 7 heme biosynthesis enzymes were repressed, including aminolevulinic acid dehydratase (*Alad*), hydroxymethylbilane synthase (*Hmbs*), uroporphyrinogen III synthase (*Uros*), uroporphyrinogen decarboxylase (*Urod*), coproporphyrinogen oxidase (*Cpox*), and protoporphyrinogen oxidase (*Ppox*) (2.9-, 1.3-, 2.4-, 2.0-, 1.9-, and 1.6-fold, respectively) (Figure 7). Furthermore, hepatic expression of *Alas2*, which is typically specific to erythroid-derived cells, was repressed 65.6-fold.

Porphyria cutanea tarda (PCT), the most common human porphyria, is a photosensitive skin disease caused by over-production of hepatic porphyrins, resulting in high urinary concentrations. Biochemically, PCT is characterized by a UROD deficiency that causes uroporphyrinogen III accumulation, although nutritional and/or environmental interactions, such as exposure to AhR agonists, are typically required for disease expression (Smith *et al.*,

2001). In the presence of Fe and CYP1A2, uroporphyrinogen III is oxidized to uroporphomethene, a UROD inhibitor, and finally, uroporphyrin III, a urinary metabolite identified in rodent porphyria models and PCT patients. Uroporphyrin III is not a substrate for heme synthesis and thus it accumulates in the liver, skin, and urine (Smith *et al.*, 2001). While TCDD induces hepatic uroporphyria in rodent models, CYP1A2 null mice are completely protected against uroporphyrin accumulation (Smith *et al.*, 2001).

At 30 µg/kg TCDD, total urinary porphyrin levels increased 13.2-fold (Figure 6J), consistent with the dark tea color of the collected urine (Figure 6K). This is in agreement with the 2.0-fold repression of *Urod*, mirroring the genetic deficiency in human PCT, and the 4.1-fold induction of *Alas1* which initiates heme biosynthesis and increases precursor levels, as previously reported (Goldstein *et al.*, 1973). Transport of glycine, ALA, and coproporphyrinogen III between the cytosol and mitochondria may also be impaired by the 1.6-, 2.9- and 1.7-fold repression of *Slc25a38*, *Abcb10*, and *Abcb6*, respectively. Furthermore, TCDD induced *Cyp1a2* 20.0-fold, leading to uroporphyrinogen III oxidation and porphyrin accumulation. Lastly, TCDD-elicited mobilization of hepatic Fe²⁺ likely contributes to the development of porphyria as Fe exacerbates TCDD-elicited porphyria in mice (Smith *et al.*, 1998) while Fe deficiency is completely protective (Sweeny *et al.*, 1979).

REV-ERBα/β-Regulated Hepatic Metabolism

In addition to serving as a prosthetic group for BACH1, heme also activates the transcriptional repressors REV-ERBα (*Nr1d1*) and REV-ERBβ (*Nr1d2*). TCDD induced hepatic *Nr1d1* and *Nr1d2* 4.0- and 3.1-fold, respectively, favoring REV-ERBα/β repressive activity as heme accumulates. Heme-activated REV-ERBα/β compete with retinoic acid receptor-related orphan receptors α and γ (*Rora* and *Rorc* were repressed 1.3- and 5.2-fold, respectively) for binding to ROR response elements (RORE). Once bound, REV-ERBα/β recruit the nuclear receptor corepressor-histone deacetylase 3 (NCoR-HDAC3), resulting in the transcriptional repression of RORE-regulated genes. AhR genomic enrichment was increased 3.2-, 2.2-, and 2.9-fold within *Nr1d1*, *Nr1d2*, and *Rorc*.

Comparing hepatic DEGs from TCDD treated mice to RORα/RORγ double knockout mice (Kang *et al.*, 2007) revealed that 64 out of 111 genes exhibit the same profile, suggesting TCDD treatment mimics RORα/RORγ knockout due to heme-mediated REV-ERBα/β activation (Figure 9). Furthermore, a REV-ERBα and HDAC3 co-localization study identified ~100 hepatic genes associated with lipid biosynthesis and metabolism (Feng *et al.*, 2011), of which 66 were repressed by TCDD (Figure 10). REV-ERBα/β-mediated coordinated repression is consistent with the inhibition of *de novo* fatty acid synthesis and β-oxidation, and in line with TCDD-elicited disruption of lipid metabolism and hepatic steatosis (Boverhof *et al.*, 2005; Nault *et al.*, 2016b).

REV-ERBα/β transcriptional repressors serve as links between the positive and negative loops that regulate peripheral circadian expression. The positive loop consists of aryl hydrocarbon receptor nuclear translocator-like (ARNTL) and circadian locomotor output cycles kaput (CLOCK), which form a heterodimer and induce circadian output mediators. Accordingly, several circadian-regulated genes were repressed by TCDD, including *Arntl* (aka *Bmal1*; 23.9-fold), *Clock* (2.6-fold), neuronal PAS domain protein 2 (*Npas2*, 36.3-fold),

elongation of very long chain fatty acids-like 3 (*Elovl3*, 736.3-fold), apolipoprotein A-I (*Apoa1*, 43.7-fold), and apolipoprotein C-III (*Apoc3*, 9.3-fold). There is also a connection between disruption of the hepatic peripheral clock and MetS/NAFLD development (reviewed in (Li *et al.*, 2012)). In mice, loss-of-function of *Clock* causes hyperlipidemia, hepatic steatosis, and obesity (Turek *et al.*, 2005), while liver-specific *Arntl*/knockout leads to exaggerated glucose clearance and hypoglycemia in the fasted state (Lamia *et al.*, 2008). In our study, fasting blood glucose decreased 1.7- and 2.1-fold at 15 and 23 days, respectively, after the initial TCDD dose (Figure 11A). TCDD also increased glucose tolerance 22 days after the initial dose (Figure 11B–C), comparable to results in TCDD-treated female mice (Nault *et al.*, 2016a). Although this suggests that TCDD disrupted the peripheral clock regulating hepatic metabolism, further studies which specifically consider time of day of dosing and tissue harvesting are required.

SUMMARY

Dysregulation of Fe homeostasis plays an important role in NAFLD and MetS development (reviewed in (Aigner *et al.*, 2015)). Although there is no evidence that Fe significantly affects hepatic lipid accumulation, increased levels are associated with increased risk of steatohepatitis, fibrosis, and HCC, suggesting that Fe promotes the progression of steatosis to more severe liver pathologies (Fargion *et al.*, 2001; George *et al.*, 1998; Kew, 2014). NAFLD patients often exhibit ‘dysmetabolic iron overload syndrome’ (DIOS), characterized by moderate hepatic Fe accumulation, normal transferrin saturation, and elevated hepatic and serum HAMP levels (Aigner *et al.*, 2015). In DIOS, elevated inflammatory cytokines (IL-6, TNF- α), LPS, and excess Fe trigger HAMP synthesis, thereby reducing Fe export from hepatocytes and macrophages.

In contrast to NAFLD and DIOS in humans, TCDD repressed *Hamp* expression, mimicking the underlying genetic cause of human hereditary hemochromatosis (HH), in which excessive dietary absorption leads to systemic Fe overloading. HAMP deficiency is the unifying mechanism responsible for both classic HH caused by a *Hfe* mutation, as well as juvenile HH caused by mutations in *Hfe2* or *Hamp* (Roetto and Camaschella, 2005). Our results suggest that TCDD decreased *Hamp* transcription by repressing expression of components of the HFE-HFE2-TFR2-BMP6 ‘iron sensing’ complex, which is required for SMAD-mediated expression of *Hamp* and *Hamp2*. Consequently, HAMP is unavailable to initiate the endocytosis and eventual degradation of SLC40A1, leading to continuous absorption of dietary Fe and accumulation in the serum and urine.

On average, HH patients exhibit hepatic Fe concentrations ~12-fold higher compared to patients with other chronic liver diseases, along with transferrin saturation values >80% (normal range = 15–55%) and serum Fe levels reaching ~160–200 $\mu\text{g/dL}$ (normal range = 50–170 $\mu\text{g/dL}$) (Kowdley *et al.*, 1997; Parkash and Akram, 2015). In 2-month-old *Hamp* knockout mice, hepatic Fe levels increased 5-fold, which worsened to 15-fold at 8 months of age. Serum Fe levels also increased ~1.25-fold at 2 months, but remained constant across time (Lesbordes-Brion *et al.*, 2006). Unlike HH patients and *Hamp* null mice, TCDD caused greater Fe accumulation in the serum compared to the liver, while transferrin saturation was unchanged due to corresponding increases in serum transferrin. However, our mice were

only ~2 months old and hepatic Fe accumulation may have increased with age if the study had been prolonged. Additionally, substantial strain differences are reported in *Hfe* knockout models of HH. *Hfe* null mice on a C57BL/6 background exhibit more modest hepatic Fe accumulation and smaller increases in transferrin saturation compared to C3H or AKR nulls (Fleming *et al.*, 2001), suggesting other genetic factors influence Fe dysregulation and that C57BL/6 mice are comparatively resistant to hepatic Fe accumulation. Nevertheless, even moderate hepatic Fe accumulation has been associated with the progression of liver disease in combination with other underlying liver injuries (i.e., chronic HCV infection, oxidative stress) (Isom *et al.*, 2009).

Although total Fe levels are often used as a biomarker for HH, oxidative stress is dependent on the redox state of Fe present (Gruger and Tappel, 1970). TCD accelerates the release of catalytically active Fe^{2+} from ferritin thereby increasing the pool of free Fe and ROS production through the Haber-Weiss reaction (Rowley and Sweeney, 1984). The induction of cytochrome P450s and *Xdh/Xo*, as well as accumulating heme levels from *de novo* synthesis and net repression of hepatic hemoproteins, further contribute to oxidative stress fueled by hepatic fat accumulation and lipid peroxidation. Free heme is not only a source of redox-active Fe, but it also promotes inflammation through activation of endothelial cells and neutrophils, leading to chemotaxis, leukocyte recruitment, and pro-inflammatory cytokine expression (Kumar and Bandyopadhyay, 2005). In addition, TCDD repressed several scavenging proteins (i.e., HP and HPX) and defense responses (i.e., APR), thus compromising anti-oxidant cellular defense. As defense systems become overwhelmed, accumulating tissue damage leads to cell death and hepatotoxicity. Increased porphyrin biosynthesis through induction of *Alas1* draws succinyl-CoA away from the TCA cycle, reducing ATP production and putting further pressure on strained cellular bioenergetics. However, only a fraction of synthesized porphyrins is converted to heme due to repression of *Urod* and induction of *Cyp1a2*, leading to porphyrin accumulation.

Heme also serves as the biological ligand for several transcription factors including BACH1 and REV-ERB α/β , which regulate heme catabolism and peripheral circadian clocks, respectively (Raghuram *et al.*, 2007; Sun *et al.*, 2002). In the liver, circadian oscillations are controlled by environmental cues (i.e., zeitgebers) that entrain or synchronize the biological rhythm to a 24h light/dark cycle. For example, feeding-fasting cycles regulate the core clock, as well as output regulators such as the REVERBs and RORs that subsequently coordinate the oscillatory expression patterns of downstream genes. Accumulating evidence suggests that AhR activation regulates peripheral circadian clocks (Wang *et al.*, 2011). TCDD-elicited differential gene expression and AhR enrichment suggest that interactions with circadian regulators may modulate the phase, period and/or amplitude of oscillating gene expression associated with fatty acid biosynthesis/metabolism, contributing to the progression of TCDD-elicited hepatotoxicity. Peripheral clock disruption has been associated with hepatic lipid accumulation, dysregulated glucose homeostasis, and the progression of NAFLD pathologies (Li *et al.*, 2012).

In summary, TCDD-elicited hepatotoxicity involves the convergence of primary AhR-mediated effects (e.g., *Cyp1a1/1a2* induction, hepatic fat accumulation) and elicited tertiary responses (e.g., *Hamp* repression, Fe^{2+} overloading, heme accumulation, oxidative stress)

across the liver-intestine axis (Figure 12). This suggests that AhR-mediated toxicity is a cumulative response to the overall burden of multiple disrupted pathways, as opposed to a single adverse effect. It is consistent with the multiple “hit” paradigm proposed in numerous liver injury models where multiple factors contribute to disease progression and severity. Consequently, each response and the resulting interactions contribute to the overall pathological burden of toxicity. Additional comparative studies are required to identify which perturbations exert the greatest influence and are most relevant to the population of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

TCDD represses hepatic hepcidin expression, leading to systemic iron overloading

Dysregulation of heme biosynthesis is consistent with heme and porphyrin accumulation

Heme-activated REV-ERB α/β repress circadian-regulated hepatic lipid metabolism

Disruption of iron homeostasis promotes TCDD-elicited steatohepatitis with fibrosis

Figure 1.

Total iron (Fe) levels in (A) serum, (B) urine, and (C) liver of male C57BL/6 mice orally gavaged with sesame oil vehicle or 3–30 µg/kg TCDD every 4 days for 28 days, as determined through inductively coupled plasma mass spectrometry (ICP-MS) analysis. For (B), the number of animals in which Fe was detected in the urine is indicated. (D) Serum total iron binding capacity (TIBC), (E) hepatic transferrin (TRF) protein levels, and (F) serum transferrin saturation of male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 µg/kg TCDD every 4 days for 28 days. Bars represent the average $\pm$ standard error of the mean for at least 4 biological replicates. Statistical significance (* $p < 0.05$) was determined using one-way ANOVA analysis followed by Dunnett's post-hoc test (A,C) or a Student's t-test (D–F) performed in SAS 9.3, or Chi-Square analysis based on the presence or absence of Fe performed in R 3.3.1 (B).

Figure 3.

qRT-PCR analysis of hepatic dose-dependent differential gene expression of (A) hepcidin (*Hamp*) and (B) hepcidin 2 (*Hamp2*) in male C57BL/6 mice orally gavaged with sesame oil vehicle or 0.01–30 µg/kg TCDD every 4 days for 28 days. (C) Total hepatic HAMP (HAMP and HAMP2) protein levels in male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 µg/kg TCDD every 4 days for 28 days. Ligand-dependent repression of (D) *Hamp* and (E) *Hamp2* 24h after a single oral gavage with AhR agonists (3000 µg/kg PCB126 and 3000 µg/kg TCDF) and a non-AhR agonist (300 mg/kg PCB153) (Kopec *et al.*, 2010a; Kopec *et al.*, 2010b). Protein levels of (F) hepatic SLC40A1 (ferroportin), (G) duodenal epithelial SLC40A1, and (H) duodenal epithelial SLC11A2 in male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 µg/kg TCDD every 4 days for 28 days. Bars represent the average $\pm$ standard error of the mean for at least 3 biological replicates. Red dashed line indicates a fold change of 1. Statistical significance (* $p < 0.05$) was determined using one-way ANOVA analysis followed by Dunnett's test (A–B) or a Student's t-test (C–H) performed in SAS 9.3.

Figure 4.

Hepatic (A) aconitase 1 (ACO1), (B) iron response element binding protein 2 (IREB2), (C) hydroxyacid oxidase 1 (HAO1), (D) ferritin heavy chain 1 (FTH1), (E) ferritin light chain 1 (FTL1), and (F) transferrin receptor (TFRC) protein levels in male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 µg/kg TCDD every 4 days for 28 days. Bars represent the average $\pm$ standard error of the mean (SEM) for at least 3 biological replicates. ND indicates 'not detected'. Statistical significance (* $p < 0.05$) was determined using a Student's t-test performed in SAS 9.3.

Figure 5.

Proposed mechanism of *Hamp* and *Hamp2* repression by TCDD. (A) When Fe levels are sufficient, saturated Fe^{3+} -TRF binds to TFRC, dislodging HFE from its overlapping binding site on TFRC. HFE is then available to form a complex containing TFR2, NEO1, HFE2, BMP6, and BMPR, leading to SMAD-induced transcription of *Hamp* and *Hamp2*. Hepcidin controls systemic Fe levels by initiating internalization and eventual degradation of SLC40A1. (B) Under low Fe conditions, TRF does not interfere with TFRC-HFE binding, the complex required for SMAD signaling is not assembled, and *Hamp/Hamp2* are not transcribed (modified from (Zhao *et al.*, 2013)). Consequently, SLC40A1 continues to transport dietary Fe into the systemic circulation. (C) Despite sufficient Fe levels and the binding of Fe^{3+} -TRF to TFRC, TCDD compromises HFE-HFE2-TFR2-BMP6 complex assembly through repression of *Tfr2*, *Neo1*, and/or *Hfe2*, limiting *Hamp* and *Hamp2* transcription. Low hepcidin levels allow duodenal SLC40A1 to continue exporting dietary Fe into portal circulation, while hepatic SLC40A1 effluxes intracellular Fe into the blood thereby limiting hepatic Fe accumulation. Unregulated intestinal Fe absorption and hepatic efflux increases serum Fe levels, resulting in ROS production, oxidative stress, and hepatotoxicity. Red and blue indicate increased and decreased levels, respectively.

Figure 6.

(A) Hepatic heme levels in the liver, and (B) serum haptoglobin (HP) and (C) serum hemopexin (HPX) levels in male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 $\mu\text{g/kg}$ TCDD every 4 days for 28 days. Total, direct, and indirect levels of bilirubin in the serum (D–F) and urine (G–I) of male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 $\mu\text{g/kg}$ TCDD every 4 days for 28 days. (J) Total porphyrins in urine samples collected on day 26 from male C57BL/6 mice orally gavaged with sesame oil control or 30 $\mu\text{g/kg}$ TCDD every 4 days. (K) Urine samples collected from 3 mice treated with 30 $\mu\text{g/kg}$ TCDD

exhibit a dark tea color, compared to urine samples from 3 sesame oil controls. Bars represent the average $\pm$ standard error of the mean (SEM) for at least 4 biological replicates. Statistical significance ($*p < 0.05$) was determined using a Student's t-test performed in SAS 9.3.

Figure 7.

TCDD-elicited differential expression of heme biosynthesis and metabolism. Genes are identified as rectangles and metabolites as circles. Color scale represents the $\log_2(\text{fold change})$ for differential gene expression and altered metabolite levels. Grey symbols indicate that the metabolite was not measured. The upper left corner of genes indicates maximum AhR enrichment fold change where '-' indicates no AhR enrichment, and the upper right corner indicates the number of pDREs (MSS = 0.856).

Figure 8.

TCDD-elicited hepatic differential expression of genes encoding (A) cytochrome P450 enzymes and (B) other hemoproteins. Male C57BL/6 mice were orally gavaged with sesame oil vehicle or TCDD (0.01–30 $\mu\text{g/kg}$) every 4 days for 28 days. Color scale represents the $\log_2$ (fold change) for differential gene expression, as determined through RNA-Seq analysis ($n=3$). The presence of pDREs (MSS ≥ 0.856) and AhR enrichment peaks (FDR ≤ 0.05) at 2h are shown as green boxes. Relative gene expression level indicating the number of reads aligned to each transcript is also shown, where yellow represent lower expression (< 500 reads) and pink represents higher expression (> 10,000).

Figure 9.

Heat map comparing hepatic TCDD-elicited differential gene expression to ROR α /RORY double knockout (KO) mice (Kang *et al.*, 2007). Color scale represents the log₂(fold change) for differential gene expression, where fold change (FC) was determined relative to sesame oil vehicle for TCDD-treated mice and relative to wild type for ROR α /RORY KO mice. For each gene, the maximal TCDD-elicited response ($|FC| \geq 1.5$) was classified as consistent (yes vs. no) with the ROR α /RORY double KO based on the direction of differential expression. The presence of pDREs (MSS ≥ 0.856) and AhR enrichment peaks (FDR 0.05) at 2h are shown as green boxes. Relative gene expression level indicating the number of reads aligned to each transcript is also shown, where yellow represents lower expression (< 500 reads) and pink represents higher expression ($> 10,000$).

Figure 10.

TCDD-elicited hepatic differential expression of REV-ERBa target genes associated with lipid biosynthesis and metabolism identified by Feng et al (Feng *et al.*, 2011). Male C57BL/6 mice were orally gavaged with sesame oil vehicle or TCDD (0.01 – 30 µg/kg) every 4 days for 28 days. Color scale represents the log₂(fold change) for differential gene expression, as determined through RNA-Seq analysis (n=3). The presence of pDREs (MSS 0.856) and AhR enrichment peaks (FDR = 0.05) at 2h are shown as green boxes. Relative gene expression level indicating the number of reads aligned to each transcript is also shown, where yellow represents lower expression (< 500 reads) and pink represents higher expression (> 10,000).

Figure 11.

Effects of TCDD on blood glucose levels. (A) Fasting blood glucose levels on days 7, 15, and 23 in male C57BL/6 mice orally gavaged with sesame oil vehicle or 30 μ g/kg TCDD every 4 days. (B) Oral glucose tolerance test at 22d in male C57BL/6 mice receiving an oral gavage of sesame oil vehicle or 30 μ g/kg TCDD every 4 days, and (C) the corresponding area under the curve. Data points represent the average $\pm$ standard error of the mean (SEM) for at least 3 biological replicates. Statistical significance (* $p < 0.05$) was determined using two-way ANOVA analysis followed by Tukey's test (A) or a Student's t-test (C) performed in SAS 9.3.

Figure 12.

Convergence of AhR-mediated disruption of systemic Fe homeostasis and heme metabolism involving interactions between the intestine, serum, liver, and urine. Red, blue, or grey indicate increased, decreased, or unchanged levels, respectively.