CONSEQUENCES OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) ON HEPATIC STELLATE CELL ACTIVATION AND EXTRACELLULAR MATRIX REMODELING DURING CHRONIC LIVER INJURY

by

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A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in Biomolecular Sciences

Boise State University

May 2016

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the dissertation submitted by

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Dissertation Title: Consequences of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) on Hepatic Stellate Cell Activation and Extracellular Matrix Remodeling During Chronic Liver Injury

Date of Final Oral Examination: 22 April 2016

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ACKNOWLEDGEMENTS

I would like to thank my advisor, mentor, and friend, Dr. Kristen Mitchell, whose encouragement and enthusiasm convinced me to begin a graduate career. Her scientific expertise, dedication, and patience have made me the scientist I am today. I will forever be grateful to her. I would also like to thank members of my thesis committee, Dr. Ken Cornell, Dr. Julia Oxford, Dr. Allan Albig, and Dr. Daniel Fologea for their scientific input and support. I am also grateful to all of the current and past members of the Mitchell lab, who have contributed to my development as a scientist. Special thanks to Wendy Harvey, not only for her technical assistance and scientific input but also for her friendship, which kept me sane over the last 6 years. Finally, I would like to acknowledge my close friends and family members who tirelessly kept reminding me that I could do this. Last, but in no way least, my husband Joe and daughter Camas deserve my deepest appreciation for their love and encouragement, without which I could not have finished this dissertation.

This work was supported by the National Institutes of Health IDeA INBRE and COBRE Programs (P20GM103408 and P20GM109095), the National Science Foundation, Grants No. 0619793 and No. 0923535; the MJ Murdock Charitable Trust; and the Idaho State Board of Education.

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental pollutant and high-affinity ligand for the aryl hydrocarbon receptor (AhR). Exposure to TCDD elicits a spectrum of toxic effects, many involving aberrant cell proliferation, activation, and differentiation. The liver is a target organ for TCDD toxicity, and increasing evidence indicates that AhR signaling regulates genes that coordinate deposition and remodeling of the extracellular matrix (ECM) in the liver. Hepatic stellate cells (HSCs) are central to ECM remodeling in the liver. We recently reported that TCDD treatment increases the activation of human HSCs *in vitro*. The goal of this study was to determine if TCDD increases HSC activation *in vivo* using a mouse model of experimental liver fibrosis and to determine the consequences of TCDD treatment on ECM remodeling. To elicit fibrosis, C57BL6/ male mice were treated twice weekly for 8 weeks with 0.5 ml/kg carbon tetrachloride (CCl_4). TCDD (20 μg/kg) was administered once a week during weeks 7 and 8. Results indicate that TCDD increased liver damage in CCl_4-treated mice and increased activation of HSCs. However, TCDD treatment did not increase collagen deposition in the liver, nor did it exacerbate fibrosis. Instead, TCDD modulated expression and activity of ECM remodeling molecules associated with enhanced matrix turnover. These results support the hypothesis that TCDD increases HSC activation *in vivo*, and modulates ECM remodeling in response to chronic liver injury.

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LIST OF ABBREVIATIONS

AHH	Aryl Hydrocarbon Hydroxylase
AhR	Aryl Hydrocarbon Receptor
ALT	Alanine Amino Transferase
ARNT	AhR Nuclear Translocator
bHLH	Basic Helix-Loop-Helix
CCl_4	Carbon Tetrachloride
ECM	Extracellular Matrix
HAHS	Halogenated Aromatic Hydrocarbons
HSC	Hepatic Stellate Cell
MMP	Matrix Metalloproteinase
PAS	Per-ARNT-Sim
POPS	Persistent Organic Pollutants
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TEFS	Toxic Equivalency Factors
TEQ	Toxic Equivalency
XRE	Xenobiotic Response Element

CHAPTER ONE: INTRODUCTION

HALOGENATED AROMATIC HYDROCARBONS

Halogenated aromatic hydrocarbons (HAHs) are a family of toxic environmental pollutants that include dibenzofurans, naphthalenes, biphenyls, and chlorinated dibenzo-*p*-dioxin (Poland & Knutson, 1982). They have similar structures (Figure 1) and produce a similar pattern of toxic responses. Due to their ability to accumulate and persist in the environment, HAHs are also referred to as persistent organic pollutants (POPs) (Jones & de Voogt, 1999). The persistence of these chemicals is due to the fact that they tend to be lipophilic, which allows them to partition into the organic material in soils and into lipid compartments of organisms. Furthermore, these chemicals are typically resistant to biodegradation and metabolism (Jones & de Voogt, 1999), which allows them to bioaccumulate in the food chain (Poland & Knutson, 1982). These characteristics increase the likelihood of organisms becoming exposed to HAHs in the environment and through the food chain.

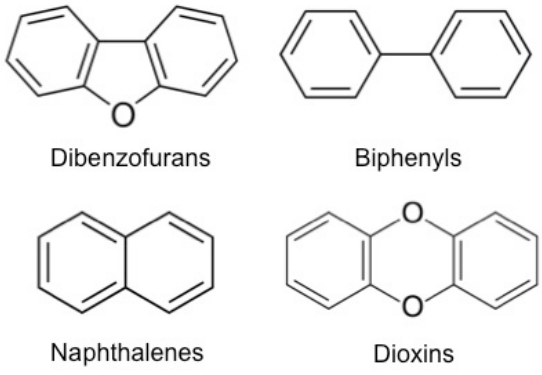

Figure 1. Structure of HAHs

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic and well-studied HAH and serves as the prototype for understanding how the HAH family of chemicals elicits toxicity (Poland & Knutson, 1982). The basic chemical structure of all dioxins consists of two benzene rings connected by two oxygen atoms and substituted with four to eight chlorine atoms. The various positions of the chlorine atoms give rise to up to 75 dioxin congeners (Schecter *et al.*, 2006). TCDD contains chlorine atoms at positions 2, 3, 7, and 8 on the benzene rings (Figure 2).

TCDD is often found in complex mixtures of HAHs, and it can persist in both environmental and biological samples that are exposed to such mixtures. In the environment, TCDD is primarily degraded by photolysis, which occurs when UV light (in the presence of a hydrogen donor) splits chlorine atoms off of TCDD (Skene *et al.*, 1989). However, the penetration of UV light into soil is typically shallow, which contributes to the long half-life of TCDD in soil, which can be 10 to 100 years (Seike *et al.*, 2007, Sinkkonen & Paasivirta, 2000). TCDD also persists in the human body because there is no process for metabolizing it. Adipose tissue and liver are major storage sites for TCDD (Schecter *et al.*, 1989). The half-life in the body is dependent on dose and body composition, and high amounts of body fat lead to increased persistence (Schecter *et al.*, 2006). Recent studies report that the half-life in humans is approximately 1 to 2 years (Sorg *et al.*, 2009).

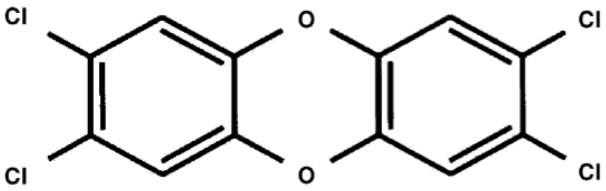

Figure 2. Chemical structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

TCDD Toxicity.

In animals, the toxic effects of TCDD include endocrine alterations, developmental and reproductive toxicity, hepatotoxicity, immunosuppression, carcinogenesis and death (Bock, 1994; Birnbaum, 1995). Studies of TCDD toxicity in humans have been primarily based on accidental exposures in which TCDD was found as part of a complex mixture. The most appropriate way to estimate the potential health risk of mixtures of dioxins is to express the toxicity as if a chemical mixture under study were pure TCDD (Van den Berg *et al.*, 1998). The total dioxin toxic equivalency (TEQ) value has been adopted internationally to express the toxicity of mixtures of dioxins. The TEQ equals the toxic equivalency factor, which expresses the toxic potencies of HAHs relative to that of TCDD, multiplied by the weight of the compound (Van den Berg *et al.*, 1998; Safe, 1993). In the general population, it is estimated that the mean TCDD TEQ level is 6 ng/kg in adipose tissue and 2 ng/kg in serum (Aylward & Hays, 2002). Current mean serum lipid TCDD levels are estimated to be between 0.5 and 1 ng/kg in the general population (Aylward & Hays, 2002). These levels are within the margins of exposure limits for some toxicological outcomes of TCDD, such as developmental reproductive toxicity, which is associated with TCDD body burden levels as low as 0.1 to 8 ng/kg

(White & Birnbaum, 2009). The observation of toxic effects of TCDD at these low exposure levels underscores the importance of understanding mechanisms of TCDD toxicity in order to adequately assess human health risks.

Sources of TCDD

TCDD is typically produced as an unintentional byproduct of industrial manufacturing and combustion processes. A classic historical example of unintentional TCDD production occurred during the manufacturing of the herbicide Agent Orange, which was used as a defoliant during the Vietnam War from the early 1960s to the early 1970s. Agent Orange was a 50/50 mixture of 2,4,5-trichlorophenooxyacetic acid (2,4,5-T) and dichlorophenoxyacetic acid (Hites, 2011). 2,4,5-Trichlorophenol serves as the raw material to produce 2,4,5-T, and TCDD was produced as a byproduct of this reaction. 2,4,5-T was further used to produce other herbicides. Although the quantity of TCDD impurity was small in these products, their widespread use sometimes resulted in the release of TCDD into the environment at levels that required clean up (Hites, 2011).

In addition to the use of TCDD-contaminated herbicides and other products, industrial accidents represent another source of human exposure to TCDD. For example, in 1976, in the town of Meda, Italy, an explosion at a chemical manufacturing plant produced a chemical cloud that was blown by the wind to the south. This cloud, which contained TCDD and a mix of other toxic chemicals, fell on the town of Seveso, where it contaminated soil, killed small animals, and caused dermal lesions in exposed humans. From the most contaminated areas, 730 people were eventually evacuated (Hites, 2011). Blood levels of TCDD in random samples of inhabitants in the most contaminated zones were between 9.8 and 89.9 ppt (Landi *et al.*, 1998). Another example of human exposure

to TCDD was in the early 1970s in the town of Times Beach, Missouri. A contractor used TCDD-contaminated oil from a chemical manufacturing plant to spray unpaved streets to control dust (Hites, 2011). By 1982, the town of Times Beach was evacuated and eventually disincorporated. It was not until 2001 that the Times Beach site was determined to no longer pose a significant threat to public health or the environment. Since the mid-1980s, when it became apparent that dioxins were a public health issue, lower amounts of dioxins have been entering the environment due to the abandonment of chlorinated phenol chemistry by large sectors of the chemical industry (Hites, 2011).

Currently, the combustion of waste products – particularly municipal waste and backyard burning of household waste – is the prominent source of TCDD in the environment (Dearfield *et al.*, 2013). These wastes often contain chlorine-based plastics, which form TCDD during combustion. Once released into the atmosphere, TCDD adsorbs onto dust particles and settles onto vegetation and in bodies of water, and accumulates in the soil. Livestock are exposed to TCDD through consumption of contaminated vegetation and feedstock, whereas humans are primarily exposed to dioxin through the consumption of TCDD-laden animal products (Dearfield *et al.*, 2013). Because it is lipophilic and poorly metabolized, TCDD partitions into the lipid compartments of organisms and bioaccumulates in the food chain. Therefore, the more animal fats consumed, the greater the risk of exposure to TCDD.

TCDD-Regulated Gene Battery

Dioxins induce a number of xenobiotic metabolizing enzymes, including two enzymes in the cytochrome P450 family, *Cyp1a1* and *Cyp1a2*, and four non-P450 enzymes: *Nqo1*, *Ald3a1*, *Ugta6*, and *Gstal* (Nebert *et al.*, 2000). This group of genes is

identified as a battery, which describes a set of functionally linked genes that are regulated in response to a particular endogenous or exogenous signal (Nebert *et al.*, 2000). The cytochrome P450 enzymes are almost exclusively Phase I metabolizing enzymes that metabolize substrates through oxidative degradation. The addition of oxygen into a substrate by Phase I enzymes can produce reactive intermediates, which can be carcinogenic, mutagenic, and/or toxic (Nebert *et al.*, 1990). The four non-P450 enzymes in the gene battery are Phase II metabolizing enzymes and act on the oxygenated intermediates produced during Phase I reactions. Phase II enzymes produce hydrophilic metabolites that are easily excreted (Nebert *et al.*, 1990). The coordination of Phase I and Phase II enzymatic reactions is critical for ensuring metabolic clearance of foreign substances from the body.

Of all of the dioxins, TCDD is the most potent inducer of cytochrome P450 1A1, which is also known as aryl hydrocarbon hydroxylase (AHH). AHH is an enzyme that oxygenates substrates, resulting in their metabolism (Whitlock, 1999). Although TCDD is a substrate for AHH, the positioning of the chlorine atoms on TCDD inhibits oxygenation. Therefore, TCDD is not metabolized through AHH activity. In fact, there are no known breakdown products of TCDD at all. The failure of TCDD to be metabolized in the body directly contributes to the long half-life of TCDD and its persistence in organisms (Whitlock, 1999).

It was found in the C57BL/6 and DBA/2 mouse strains that the extent of AHH induction by TCDD varied according to the strain of mice (Thomas *et al.*, 1972). Studies in strains that were unresponsive to TCDD identified a single genetic locus that conferred TCDD inducibility of AHH. This locus was named the Ah locus (Thomas *et al.*, 1972). In

these unresponsive mice, TCDD induced *Cyp1a1* to a lesser extent than in responsive mice, resulting in 10 times less induction of AHH activity (Nebert *et al.*, 1975). Subsequently, the protein product of the Ah locus was identified and characterized as a receptor called the aryl hydrocarbon receptor (AhR), which was found to bind to TCDD (Nebert *et al.*, 1975; Merchant *et al.*, 1992). These studies further demonstrated that the AhR controls the transcription of *Cyp1a1* in response to TCDD.

ARYL HYDROCARBON RECEPTOR (AhR)

The AhR is a soluble protein in the Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors. The PAS superfamily is named for the *Drosophila* circadian rhythm protein period (Per) protein, the mammalian AhR nuclear translocator (ARNT) protein, and the *Drosophila* neurogenic single-minded (Sim) protein (Burbach *et al.*, 1992; Schmidt *et al.*, 1993). Proteins in this family contain a conserved domain, referred to as a PAS domain, of approximately 250 to 300 amino acids. The primary amino acid sequence of the PAS domain is evolutionarily conserved across vertebrate species and functions in detection of and adaptation to environmental changes, such as circadian rhythm, hypoxia, and small metabolites (Nguyen & Bradfield; 2008, Gu *et al.*, 2000). As a PAS protein, the AhR facilitates adaptation to environmental changes through the mechanism of ligand binding and upregulation of xenobiotic metabolizing enzymes, such as those in the cytochrome P450 family (Gu *et al.*, 2000). The PAS domain contains two subdomains of approximately 50 amino acids: PAS-A and PAS-B. A schematic structure of the AhR protein is shown in Figure 3. The PAS domain is involved in hetero- and homodimerization with other PAS proteins and the docking of

molecular chaperone heat shock 90 (Hsp90) proteins (Abel & Haarmann-Stemmann, 2010). The AhR binds a ligand at the PAS-B domain, which induces AhR activation and subsequent gene transcription (Coumailleau *et al.*, 1995). The AhR is the only protein in this family that is ligand-activated, so it is the only receptor in the bHLH/PAS family.

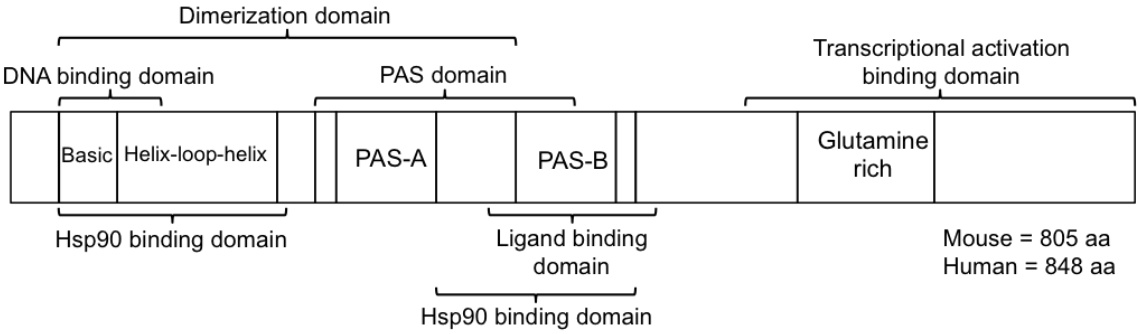

Figure 3. Schematic of functional domains within the AhR protein. This representation depicts domains that are common to the bHLH/PAS family of proteins and domains that are important in the classical pathway of AhR activation.

In the absence of TCDD, the AhR resides in the cytosol, where it is associated with cofactors such as tyrosine kinase c-src, two heat shock 90 proteins, the co-chaperone p23, and a protein called the immunophilin-like AhR interacting protein (AIP) (Ma & Whitlock, 1997; Denis *et al.*, 1988; Perdew, 1988; Abel & Haarmann-Stemmann, 2010). These cofactors maintain the AhR in a transcriptionally inactive state. Activation of the AhR occurs when TCDD binds the PAS-B domain of the receptor, which causes dissociation of the cofactors and reveals a nuclear localization sequence that permits the AhR to enter the nucleus (Lees & Whitelaw, 1999). Once in the nucleus, the AhR forms a heterodimer with another bHLH/PAS family member called the Ah receptor nuclear translocator (ARNT) and becomes transcriptionally active. Binding to ARNT is required to direct the AhR to enhancer elements of genomic targets (Gu *et al.*, 2000). The AhR/ARNT transcription factor binds DNA at consensus sequences known as xenobiotic

response elements (XREs), which contain the core bases 5'-GCGTG-3' in the promoter and enhancer regions of target genes (Lees & Whitelaw, 1999; Abel & Haarmann-Stemmann, 2010). The ability of the AhR/ARNT heterodimer to bind DNA at XREs is mediated primarily through the basic region of the helix-loop-helix domain (Ko *et al.*, 1997). Gene transactivation is accomplished through the transactivation domain of the AhR, which interacts with adjacent TATA and CCAATT boxes (Ko *et al.*, 1997). After the AhR/ARNT dimer binds DNA, several co-activators, such as SRC-1, p300, CBP, and Brg-1, are recruited to the complex. These co-activators not only relax the chromatin structure through histone modification, but also recruit components of the general transcription machinery to induce gene expression (Wang & Hankinson, 2002). Figure 4 illustrates the classical pathway of gene transcription by the AhR/ARNT transcription factor. In the absence of AhR nuclear translocation and DNA binding, TCDD loses many, if not all, of its toxic effects (Bunger *et al.*, 2003; Bunger *et al.*, 2008).

In mice, the Ah locus was found to have two alleles: Ahr^b and Ahr^d. The mouse strains C57BL/6 and DBA/2 are commonly used to contrast the responsiveness of the two alleles. C57BL/6 mice express the Ahr^b allele, whereas DBA/2 mice express the Ahr^d allele (Poland *et al.*, 1994). The Ahr^b allele is more responsive to TCDD than the Ahr^d allele, resulting in an approximate 10-fold difference in the dose of TCDD required to induce AHH activity (Nebert *et al.*, 1975). It has been shown that point mutations in the Ahr^d allele lower the ligand binding affinity, which accounts for the reduced sensitivity observed in mice that express this allele (Poland *et al.*, 1994). Another way to evaluate species responsiveness to TCDD is to compare the dose of chemical required to produce lethality in 50% of the test population. This dose is referred to as the LD_{50}. AhR point

mutations affecting ligand-binding affinity are shown to account for variations in the LD_{50} of TCDD between different strains of mice as well as between species. For example, the oral LD_{50} of TCDD in a guinea pig is estimated at 0.6 μg/kg, whereas the LD_{50} in a rabbit is 115 μg/kg (Schwetz *et al.*, 1973).

Although the mechanisms of TCDD toxicity remain poorly understood, most TCDD toxicity is attributed to changes in gene transcription (Walisser *et al.*, 2005; Bunger *et al.*, 2003; Bunger *et al.*, 2008). Studies within the last decade have shown that the AhR can alter gene transcription at sites distinct from consensus XREs. In fact, a new "non-consensus" XRE (NC-XRE) was recently identified that contains a 5'-GGGA-3' tetranucleotide motif instead of the 5'-GCGTG-3' core that was originally identified (Huang & Elferink, 2012). Furthermore, the AhR was found to bind this NC-XRE in an ARNT-independent manner (Huang & Elferink, 2012). A subsequent report from the same group demonstrated that the AhR binds to the NC-XRE after dimerizing with another protein called Krupple-like factor 6 (Wilson *et al.*, 2013). Another study found that the AhR interacts with nuclear factor-kappa beta (NF-κB) subunit RelA to activate c-myc gene transcription (Kim *et al.*, 2000; Tian *et al.*, 1999). Also, the AhR can associate with the NF-κB subunit RelB to mediate interleukin-8 gene transcription (Vogel *et al.*, 2007b). The identification of these AhR/non-ARNT heterodimers illustrates the complexity of understanding mechanisms of AhR activity and the ramifications of AhR activation on gene expression. This is further complicated by the finding that over 20 thousand mouse genes have been reported to have putative XREs (Dere *et al.*, 2011). However, in a hepatic global gene expression analysis, only 1,896 of these genes were

differentially expressed following TCDD treatment. Furthermore, 593 of these upregulated genes did not contain an XRE (Dere *et al.*, 2011).

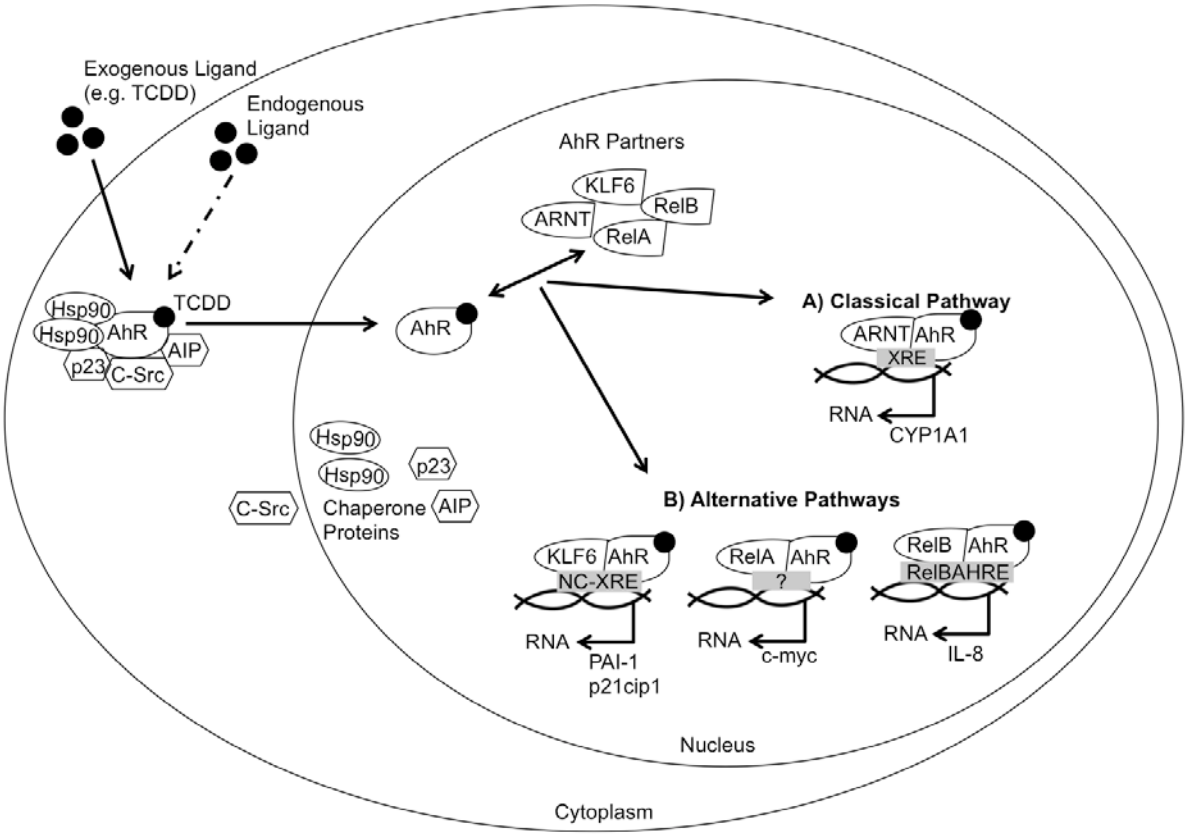

Figure 4. Pathways of AhR-regulated gene transcription. AhR activation by exogenous or endogenous ligand induces nuclear translocation. In the nucleus, the AhR binds to another transcriptional regulatory protein, and the heterodimer is recruited to DNA response elements of target genes. (A) In the classical pathway of AhR activation, the AhR modulates gene transcription by heterodimerizing with ARNT and binding to XREs. (B) According to recently identified alternative pathways of AhR activation, the AhR forms heterodimers with non-ARNT proteins, such as KLF6, RelA and RelB. Each AhR heterodimer targets a unique, non-XRE, sequence of DNA.

TCDD Hepatotoxicity

The AhR is highly expressed in the liver and contributes to the physiology of liver development, as the liver of AhR-null mice is about half the size of the liver in wild-type mice (Fernandez-Salguero *et al.*, 1996). Another prominent feature of AhR-null mice is a defect in the development of the liver vasculature, resulting in patent ductus venosus.

The liver in AhR-null mice also exhibits pronounced fibrosis in the portal tract (Fernandez-Salguero *et al.*, 1995). Finally, AhR-null mice display impaired retinoic acid catabolism, which results in elevated levels of retinoic acid, retinol, and retinyl palmitate (Gonzalez & Fernandez-Salguero, 1998). These findings support the notion that endogenous AhR signaling plays an important role in liver development and homeostasis.

The liver is a target organ for TCDD toxicity and has higher overall levels of TCDD than any other tissue except adipose tissue (Schecter *et al.*, 1989). The majority of research done on TCDD hepatotoxicity has focused on how TCDD treatment impacts hepatocytes, which comprise approximately 80% of the total liver volume (Kmieć, 2001). Hepatocytes are the major contributors to *Cyp1a1* induction in response to TCDD and mediate gross markers of TCDD hepatotoxicity, such as hepatomegaly, which refers to enlargement of the liver due to hypertrophy and hyperplasia of hepatocytes (Bock & Köhle, 2006). The direct action of TCDD on hepatocytes also produces increased serum alanine aminotransferase (ALT) levels and pathological changes in the liver (Walisser *et al.*, 2005). Nevertheless, non-parenchymal cells in the liver also express a functional AhR and are also putative targets for the toxic effects of TCDD. In addition to the aforementioned endpoints of hepatotoxicity, TCDD elicits the following toxic outcomes in the liver: inflammation, dysregulation of vitamin A homeostasis, steatosis, modulation of hepatocyte proliferation, and fibrosis. These endpoints of TCDD hepatotoxicity are discussed in further detail below.

Liver inflammation

In rodents, TCDD treatment has been shown to elicit moderate multifocal inflammatory foci in the liver (Kopec *et al.*, 2010). These foci consist mainly of

mononuclear cells and a smaller number of neutrophils (Boverhof *et al.*, 2006). Inflammatory cells are drawn to sites of injury by the action of chemokines. Increased levels of the chemokines monocyte chemoattractant protein-1 (MCP-1) and keratinocyte chemoattractant (KC) have been observed in mice exposed to a single dose of TCDD (Vogel *et al.*, 2007a). MCP-1 attracts macrophages, and KC, which is the mouse homolog for IL-8, attracts neutrophils to sites of injury. Both MCP-1 and IL-8 have been shown to be directly regulated by the AhR in response to TCDD (Sun *et al.*, 2004; Vogel *et al.*, 2007b).

Dysregulation of vitamin A homeostasis

The liver stores 80% of the body's vitamin A, which is essential for the maintenance of retinoid homeostasis in tissues and organs (Schreiber *et al.*, 2012). Vitamin A is involved in development and growth, vision, epithelial differentiation, immune function and reproduction (Ross *et al.*, 2000). Vitamin A is converted to retinol and stored in lipid droplets inside hepatic stellate cells (HSCs), which are non-parenchymal cells in the liver. TCDD has been shown to reduce the accumulation of vitamin A in the rodent liver (Thunberg *et al.*, 1980; Håkansson & Ahlborg, 1985). Loss of vitamin A has been attributed to increased mobilization and excretion of retinoids from the liver, which coincides with increased kidney and serum retinoid concentrations (Håkansson & Ahlborg, 1985). When liver cells were separated, vitamin A levels in the non-parenchymal fraction of the liver from TCDD-treated rats were 30% lower than in control rats, whereas vitamin A content in parenchymal cells was not affected by TCDD (Håkansson & Hanberg, 1989). Along these same lines, we recently found that TCDD inhibits lipid droplet storage in cultured human HSCs (Harvey *et al.*, 2016). Hence, the

consequences of TCDD on vitamin A homeostasis may be due to direct effects of TCDD on HSCs, rather than on hepatocytes.

Steatosis

TCDD-induced hepatic steatosis is characterized by an increase in hepatic triglycerides, vacuolization, and inflammatory cell infiltration in the liver (Angrish *et al.*, 2012). TCDD treatment induces hepatic steatosis in mice treated with a single dose of TCDD or with a single dose of TCDD in conjunction with a high fat diet (Lu *et al.*, 2011; Angrish *et al.*, 2012). The vacuolization that occurs during TCDD-induced hepatic steatosis results from the accumulation of triglycerides in hepatocytes and their subsequent packaging into lipid droplets (Boverhof *et al.*, 2006). Exposure to TCDD increases levels of hepatic triglycerides by enhancing their uptake into hepatocytes and also by inhibiting their secretion (Lu *et al.*, 2011; Lee *et al.*, 2010). Furthermore, microarray studies have revealed that TCDD increases expression of genes involved in lipid metabolism and immune responses (Boverhof *et al.*, 2006; Sun *et al.*, 2004)

Modulation of hepatocyte proliferation

Exposure to TCDD has been shown to suppress hepatocyte proliferation in vitro (Kolluri *et al.*, 1999) and in the mouse liver during regeneration (Bauman *et al.*, 1995; Mitchell *et al.*, 2006). Specifically, TCDD suppresses S-phase progression by eliciting a G1 cell cycle arrest (Kolluri *et al.*, 1999; Mitchell *et al.*, 2006). This cell cycle arrest appears to result from the TCDD-mediated induction of p27Kip1, which inhibits passage through the G1/S checkpoint of the cell cycle. In addition, TCDD can cause the AhR to associate with the E2F transcription factor to suppress the expression of genes needed for S-phase progression (Elferink, 2003; Puga *et al.*, 2000). Finally, a recent study found that

TCDD-induced suppression of *in vivo* hepatocyte proliferation also depends on induction of the p21Cip1 gene (Jackson *et al.*, 2014). Hence, there are multiple mechanisms by which TCDD may modulate hepatocyte proliferation, and this is an ongoing area of research.

It is interesting to note that the AhR can function as both a tumor suppressor and as a tumor promoter. Activation of the AhR by TCDD enhances tumor incidence in the liver of rodents (Pitot *et al.*, 1980). However, TCDD is not considered genotoxic, in that it does not bind to DNA and cause mutations. Instead, its mode of action has been determined to be one of tumor promotion (Pitot *et al.*, 1980). It is thought that TCDD promotes tumor development by modulating the rate of cell division, terminal differentiation, or apoptosis (Mandal, 2005). In contrast, it has been suggested that endogenous AhR activity (in the absence of TCDD) is important for suppressing tumors. This is supported by the finding that tumorigenesis increases when the AhR is absent altogether (Fan *et al.*, 2010). Thus, in the presence of TCDD, the AhR functions as a tumor promoter, whereas in the absence of exogenous ligand, it functions as a tumor suppressor.

Fibrogenesis

Fibrogenesis is a wound healing response characterized by the synthesis and deposition of extracellular matrix (ECM) material (Brenner *et al.*, 2000). Liver fibrosis refers to a pathological condition in which injury and inflammation drive the deposition of abnormal or excessive ECM (Wynn, 2008). TCDD treatment is shown to increase fibrogenic gene expression (Andreasen *et al.*, 2006). There is some evidence to suggest that the AhR regulates fibrogenic processes, as fibrotic lesions are observed in the liver of

AhR-null mice (Fernandez-Salguero *et al.*, 1996). Furthermore, several studies indicate that expression of fibrosis-related genes is modulated when the AhR is activated by TCDD. For example, chronic treatment of mice with TCDD was found to increase expression of the gene that encodes collagen type I (Pierre *et al.*, 2014), which is the primary collagen involved in liver fibrosis. Using a zebrafish model of fin regeneration, Andreasen *et al.* reported that TCDD treatment altered the expression of numerous genes involved in ECM synthesis and remodeling, including matrix metalloproteinases, tissue inhibitor of matrix metalloproteinases and collagen 1a1 (Andreasen *et al.*, 2006). Given the fibrotic phenotype of AhR-null mice, as well as the reported consequences of TCDD treatment on ECM remodeling activity, it is logical to speculate that AhR signaling could contribute to the regulation of fibrogenesis in the liver. However, no studies to date have determined how TCDD treatment impacts the pathogenesis of liver fibrosis, which is the focus of the research project described herein.

TCDD and Hepatic Stellate Cells

HSCs are non-parenchymal liver cells that lie at the interface of liver homeostasis and disease, as they have the ability to promote inflammation, innate immune responses, and wound healing (Ishibashi *et al.*, 2009). HSCs are uniquely positioned in the perisinusoidal space (Space of Disse), which facilitates their close interaction with parenchymal hepatocytes, endothelial cells, and Kupffer cells (Figure 5) (Hui & Friedman, 2003). When quiescent, HSCs function in retinoid homeostasis. In fact, quiescent HSCs store 80% of the body's vitamin A (Schreiber *et al.*, 2012). Chronic liver injury provokes HSCs to transition to a myofibroblast-like phenotype and acquire

fibrogenic properties. A range of liver insults, including viral hepatitis, steatohepatitis, toxicant exposure, and autoimmune disorders, can cause chronic liver injury (Bataller & Brenner, 2005). Activated HSCs are characterized by loss of vitamin A storage, enhanced proliferation, contractility, expression of αSMA, chemokine and growth factor production, and deposition of fibrillar collagens (Friedman, 2000).

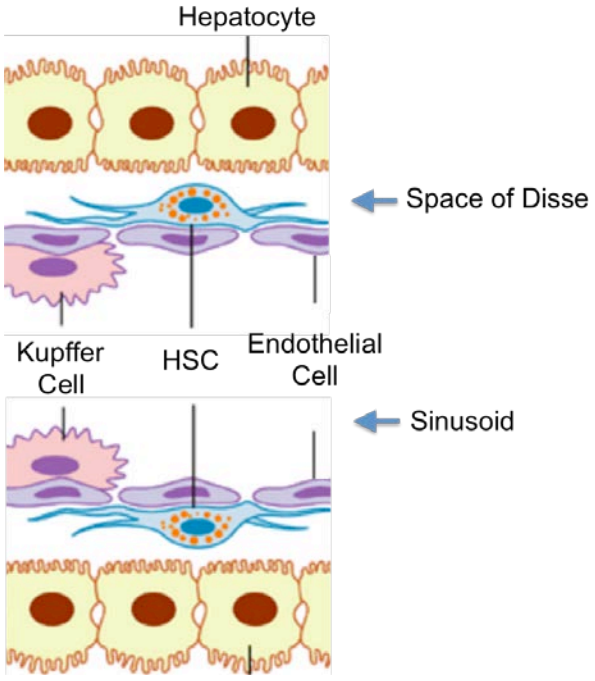

Figure 5. Cell distribution in the normal liver. Figure based on illustration in Hui & Friedman, 2003.

Liver fibrosis is a pathological condition characterized by excessive accumulation of ECM. Fibrosis results from a wound healing response to chronic injury and is characterized by an imbalance of ECM deposition and protease activity (Ghosh *et al.*, 2013). In response to chronic liver injury, which is typically accompanied by unresolved inflammation, activated HSCs are the central mediators of liver fibrosis (Lee & Friedman, 2011). These cells synthesize collagen type I, pro-fibrogenic mediators, as well as proteases and other molecules that contribute to ECM turnover and remodeling

(Duarte *et al.*, 2015). Liver fibrosis is a potentially reversible response, as removal of the insult or accompanying inflammation can cause HSCs do undergo apoptosis or revert to a quiescent state (Schuppan & Kim, 2013). However, the mechanisms by which HSCs become activated or revert to quiescence remain unclear.

There is some evidence that to support the notion that HSC activation may be an intriguing and overlooked target for TCDD toxicity. For example, TCDD is known to decrease vitamin A storage in the liver, which supports the notion that TCDD may modulate the ability of HSCs to regulate retinoid homeostasis (Hanberg *et al.*, 1998; Håkansson & Hanberg, 1989; Håkansson & Ahlborg, 1985; Thunberg *et al.*, 1980). In fact, given that one characteristic of activated HSCs is the loss of retinoid storage, it is conceivable that TCDD treatment increases HSC activation. However, there is a scarcity of data regarding the effects of TCDD on HSCs *in vivo*. One study demonstrated that the TCDD-induced loss of hepatic vitamin A did not correlate with changes in the number of HSCs or in expression of the HSC activation marker, αSMA (Hanberg *et al.*, 1996). In contrast, another study found that TCDD treatment increased both αSMA and collagen type I in the mouse liver, and this occurred through an AhR-dependent mechanism (Pierre *et al.*, 2014). Although this study did not directly examine the consequences of TCDD on HSCs, increased expression of these molecules is consistent with increased HSC activation.

Another reason to consider the possibility that HSCs are targeted by TCDD is based on the finding that HSCs retain TCDD for a relatively long period of time. In fact, the half-life of TCDD in hepatocytes is approximately 13 days, whereas the half-life of TCDD in HSCs is estimated at 52 days (Håkansson & Hanberg, 1989). It stands to reason

that the long half-life of TCDD in HSCs increases the likelihood of toxicity occurring in these cells. Furthermore, it leaves open the possibility that HSCs are direct cellular targets for TCDD toxicity. This is supported by recent studies in our lab, which show that TCDD treatment increases activation of the human HSC line, LX-2, leading to loss of vitamin A storage, increased proliferation, and increased αSMA expression, as well as increases in other endpoints of activation (Harvey *et al.,* 2016).

Given the diverse role of HSCs in liver homeostasis, as well as the contribution of activated HSCs to liver fibrosis, TCDD-mediated disruption of this population of cells could underlie the development of numerous hepatotoxic effects. Understanding how exposure to TCDD impacts HSC activation *in vivo* will be important for identifying a possible role for the AhR in regulation of fibrogenesis and for elucidating mechanisms of TCDD hepatotoxicity.

TCDD and ECM Remodeling

The ECM is composed of a network of proteins and sugars in the interstitial space and provides a physical scaffold and structural support for cells (Duarte *et al.*, 2015). The ECM can regulate various cellular functions through tissue stiffness, contact with cell receptors, and the sequestration and release of factors involved in cell proliferation and differentiation (Karsdal *et al.*, 2015). The composition of the ECM is important for regulating the activity and phenotypes of cells that are in contact with it. Indeed, dysregulation of ECM metabolism and deposition is now recognized to be associated with the development of chronic liver disease (Friedman, 2000; Duarte *et al.*, 2015).

In addition to impacting HSC activation and fibrogenesis, it is also possible that TCDD dysregulates ECM remodeling. Studies indicate that AhR activation can regulate genes involved in ECM deposition, such as collagens, as well as genes involved in ECM metabolism, such as matrix metalloproteinases (MMPs) (Andreasen *et al.*, 2007; Pierre *et al.*, 2014). In fact, TCDD treatment increases expression of ECM proteases in many cell lines, including bronchial epithelial cells, prostate cancer cells, and melanoma cells, as well as in zebrafish (Tsai *et al.*, 2014; Haque *et al.*, 2005; Villano *et al.*, 2006; Andreasen *et al.*, 2007). These reports support the idea that AhR signaling may contribute to chronic liver injury and fibrosis through the dysregulation of ECM deposition, composition, or breakdown. One of the goals of the research described in this dissertation was to determine how TCDD impacts molecules involved in ECM maintenance. For this reason, descriptions of some of these key molecules, including collagen and MMPs, are provided below.

Collagen

As mentioned previously, chronic TCDD treatment was found to increase collagen type I in the mouse liver (Pierre *et al.*, 2014). However, less is known about the consequences of TCDD on other types of collagen. In addition to collagen type I, four other types of collagens are important for chronic liver disease: III, IV, V, and VI. All of these collagens, as well as collagen type I, have important roles in fibrogenesis, and all of them increase in response to chronic injury (Chen *et al.*, 2014; Yamamoto *et al.*, 1984; Murata *et al.*, 1984; Ala-Kokko *et al.*, 1987). Collagen types I and III are fibrillar-type collagens and are considered hallmarks of fibrotic disease (Chen *et al.*, 2014). Collagen

type I forms thick fibrils that predominately comprise scar tissue, while collagen type III forms finer fibrils that can be found in granulation tissue during the early stage of wound healing (Chen *et al.*, 2014). Collagen type IV is a nonfibril-forming collagen and a main component of basement membranes that form at the basal site of epithelia, endothelia, and around interstitial cells (Chen *et al.*, 2014; Karsdal *et al.*, 2015). In the liver, collagen type IV combines with lamina to form a basement membrane-like structure. Changes in these basement membranes have been linked to activation and deactivation of HSCs (Guyot *et al.*, 2006). Collagen type V forms fine fibrils, which are constituents of larger fibrils of collagens I and III (Chen *et al.*, 2014). Finally, collagen type VI is a nonfibril collagen that forms networks in basement membranes, dominating the subendothelial space (Chen *et al.*, 2014).

Fibrillar collagens can persist from months to years and, in the absence of disease, define the shape of tissues (Sottile & Hocking, 2002). Collagen molecules form chains that intertwine to form a trimeric left-handed helix (Canty & Kadler, 2005). These collagen chains are comprised of a repeating GLY-X-Y triplet responsible for the left-handed helix structure. At the N- and C- terminal domains are globular structures called propeptides that do not contain the repeating GLY-X-Y triplet motif. Proline and hydroxyproline usually occupy the X and Y positions. The propeptides play a role in collagen folding and processing. One left-handed helix can intertwine with two other left-handed helices to form a right-handed triple-helix (Canty & Kadler, 2005). Depending on the tissue type, collagen fibrils can reach diameters of ~500 nm and lengths of ~300 nm. The fibrils can be homotrimeric or heterotrimeric depending on the type of collagen. Table 1 identifies genes of the various chains that make up collagens in the liver.

Table 1: Collagens in the liver

Collagen Type	Genes	Structure / Function
I	COL1A1 COL1A2	Fibril / Scar formation
III	COL3A1	Fibril / Granulation tissue and forms dimers with type I
IV	COL4A1 COL4A2 COL4A5	Network / basement membrane
V	COL5A1 COL5A2 COL5A3	Fibril / Increases size of type I fibrils
VI	COL6A1 COL6A2 COL6A3	Network, basement membrane

Collagen Biosynthesis

Collagen molecules are assembled from procollagen molecules (Canty & Kadler, 2005). Biosynthesis of collagen begins in the endoplasmic reticulum (ER), where procollagen is cotranslationally translocated into the lumen. The chaperone protein HSP47 assists with folding and aggregation of procollagen (Kawasaki *et al.*, 2015). However, proper folding will not occur without conversion of proline residues to hydroxyproline residues, which requires prolyl 4-hydroxylase and its cofactor, vitamin C. In the absence of hydroxylation, unfolded procollagen remains within the ER, which results in ER stress (Kawasaki *et al.*, 2015). Procollagen is transported from the ER to the Golgi apparatus for the addition of N-linked oligosaccharides (Jürgensen *et al.*, 2011, Canty & Kadler, 2005). Then it is transported to the plasma membrane through the secretory pathway. To trigger fibril self-assembly, the N- and C- propeptides are removed. Collagen fibril growth is thought to occur by lateral and end-to-end fusion of

collagen fibrils in the ECM and also by the gradual accumulation of collagen molecules (Canty & Kadler, 2005).

Other molecules found in the ECM, such as decorin and integrin receptor α2 (ITGA2), can modulate collagen fibrillogenesis. Decorin can delay fibril assembly, which results in reduction of fibril diameter (Keene *et al.*, 2000), and ITGA2 can modulate fibril accretion (Girgert *et al.*, 2010). Additionally, both decorin and ITGA2 can affect the bioavailability and bioactivity of transforming growth factor (TGF)-β1, which is implicated in the induction and maintenance of excess matrix production through activation of HSCs (Huijun *et al.*, 2005; Kawelke *et al.*, 2011).

Another molecule important in fibrillogenesis is lysyl oxidase (LOX). This enzyme initiates the process of covalent intra- and intermolecular cross-linking of collagens (Perepelyuk *et al.*, 2013). Cross-linking increases the size of fibrils, which increases the stiffness of the collagenous matrix (Liu *et al.*, 2015). Considering the role that collagen deposition has in liver pathologies, perturbations at key collagen biosynthesis and fibrillogenesis steps could have diverse and even detrimental effects on liver homeostasis and disease.

Matrix Metalloproteinases (MMPs)

Following the build up of ECM molecules during a wound healing response, enzymes are needed to catabolize those molecules and return homeostasis to the ECM. MMPs are the largest class of proteinases that break down ECM molecules (Duarte *et al.*, 2015). TCDD treatment has been shown to increase MMPs in cell lines as well as in a zebrafish model of fin regeneration (Tsai *et al.*, 2014; Haque *et al.*, 2005; Villano *et al.*,

2006; Andreasen *et al.*, 2007). There are over 24 MMPs, which are divided into five main subgroups according to their substrate specificity: collagenases, stromelysins, gelatinases, matrilysins, and membrane-type MMPs (Rivera *et al.*, 2010; Duarte *et al.*, 2015). There are five constitutively expressed MMPs in the human liver, MMP-1, -2, -3, -11 and -14, and five constitutively expressed in the rodent liver, MMP-2, -3, -11, -13, and -14 (Table 2) (Duarte *et al.*, 2015; Calabro *et al.*, 2014). In the fibrotic human and rodent liver, MMP-8 and MMP-9 are also expressed (Arthur, 2000). Activated HSCs are the major producers of MMPs (Duarte *et al.*, 2015). However, Kupffer cells in both human and rodent livers also express MMP-9, and neutrophils express MMP-8 (Arthur, 2000; Duarte *et al.*, 2015). Furthermore, in the mouse liver, it has been found that hepatocytes can express MMP-2, -9, -13, and -14 (Calabro *et al.*, 2014). MMPs are secreted into the ECM in an inactive form (proMMP) that must be cleaved in order to function as a protease. MMP activity depends on zinc ion cofactors at the catalytic site (Duarte *et al.*, 2015). Mechanisms of MMP activation *in vivo* are still poorly understood.

Table 2. MMPs expressed by HSCs and heptocytes in the liver.

MMP:	Expressed in the liver by:	Type:
1	Human HSCs	Collagenase
2	Human and rodent HSCs and	Gelatinases
3	Human and rodent HSCs and	Stromelysin
11	Human and rodent HSCs and	Stromelysin
13	Rodent HSCs and Hepatocytes	Collagenase
14	Human and rodent HSCs and	Membrane-

MMP Activation

There are a number of potential mechanisms mediating activation of proMMPs *in vitro* (Ra & Parks, 2007). First, MMPs can be activated by cysteine switch, which involves an interaction between the thiol of a conserved cysteine residue in the prodomain and the zinc ion in the catalytic site (Figure 6) (Van Wart & Birkedal-Hansen, 1990). Disruption of this thiol-zinc interaction is a required step in the activation of all proMMPs. Second, MMPs can undergo allosteric activation, which is a component of the cysteine switch in which the thiol-zinc interaction is disrupted by chemicals such as sodium dodecyl sulfate. Third, MMP activation can occur through furin activation. Furin is a serine protease that cleaves the prodomain of MMPs that contain a furin cleavage site. Only about one-third of MMPs have furin cleavage sites. Fourth, some activated MMPs can cleave the prodomain of proMMPs *in vitro* and activate MMPs, although this mechanism is still poorly understood *in vivo* (Suzuki *et al.*, 1990; Nagase *et al.*, 1992). Finally, MMPs can be activated by plasmin and other serine proteinases. Plasmin is a serine protease from the precursor plasminogen. Plasmin is widely supported as an *in vivo* activator of proMMPs (Creemers *et al.*, 2000; Monea *et al.*, 2002; Ra & Parks, 2007). Evidence for this mechanism is derived from studies in plasmin knockout mice that demonstrate a correlation between absence of plasmin and decreased MMP activation (Ra & Parks, 2007; Creemers *et al.*, 2000).

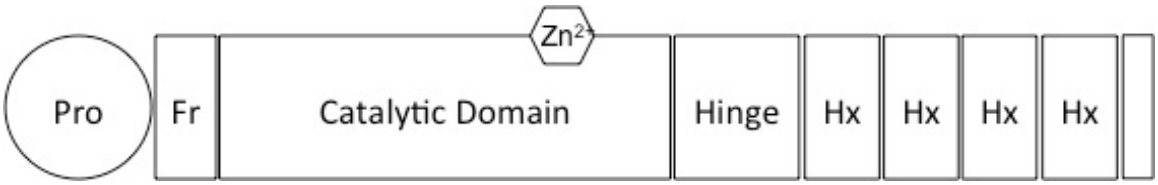

Figure 6. Typical conserved domains of the secreted MMP enzyme. Abbreviations: Pro, prodomain; Fr, furin cleavage site; Zn^{2+}, zinc binding site; Hx, hemopexin-like repeat.

MMP Inhibition

MMP activity can be controlled through direct inhibition of the catalytic site or through suppression of MMP activation mechanisms. One method of suppressing MMP activation is through the activity of plasminogen activator inhibitor-1 (PAI-1), which blocks the conversion of plasminogen to plasmin (Bergheim *et al.*, 2006). Tissue inhibitor of matrix metalloproteinases (TIMPs) are endogenous inhibitors of MMP activity and include TIMP-1, -2, -3, and -4 (Duarte *et al.*, 2015; Yoshiji *et al.*, 2000; Visse & Nagase, 2003; Piperi & Papavassiliou, 2012; Fowell *et al.*, 2011). TIMPs bind to MMPs in a 1:1 stoichiometric ratio and inactivate the catalytic site (Ra & Parks, 2007). During chronic liver injury, activated HSCs are the primary source of TIMPs, which supports the inclusion of TIMPs as markers of HSC activation (Fowell *et al.*, 2011; Boers *et al.*, 2006).

OVERVIEW OF RESEARCH PROJECT

In summary, there is substantial evidence to support the notion that activation of the AhR by TCDD may dysregulate wound healing processes in the liver. For example, exposure to TCDD reportedly increases activation of HSCs, although this has not been definitively proven *in vivo*. Activated HSCs produce collagen type I, which has been shown to be upregulated by TCDD. During chronic liver injury, deposition of collagen

type I increases ECM stiffness and produces fibrosis, a pathological condition for which there is no treatment. The ECM is degraded by matrix metabolizing enzymes, and TCDD treatment has been shown to modulate expression of molecules involved in ECM metabolism, such as MMPs and TIMPs. Based on these findings, it is logical to speculate that TCDD treatment could impact the development of fibrosis in response to chronic liver injury.

The goal of the research presented in this dissertation was to determine how exposure to TCDD modulates *in vivo* HSC activation and the development of liver fibrosis. To this end, we needed a model system of chronic liver injury in which HSCs are robustly activated, leading to the demonstrable development of fibrosis. One model that meets these requirements is chronic administration of carbon tetrachloride (CCl_4), which is a well-characterized model of experimental liver fibrosis (Scholten *et al.*, 2015). CCl_4 hepatotoxicity is dependent on metabolic activation by cytochrome P4502E1 to form the trichloromethyl free radical, CCl_3 (Wong *et al.*, 1998). The plasma membrane and membranes of organelles are targets of the CCl_3 radical in hepatocytes. The resulting lipid peroxidation causes necrosis in central lobular regions of the liver (Weber *et al.*, 2003). The resulting liver injury activates HSCs, resulting in measurable fibrosis endpoints, including collagen deposition (Scholten *et al.*, 2015). In brief, the experimental design we used was based on administering CCl_4 twice a week for 8 weeks to evoke HSC activation and fibrosis. TCDD was then added during the last two weeks of the experiment. This experimental approach was used for all of the studies described in this proposal. In Chapter Two, we tested the hypothesis that exposure to TCDD increases HSC activation, liver damage, and fibrosis in CCl_4-treated mice. HSC activation was

measured based on expression of αSMA and other activation markers. Liver damage was assessed based on gross markers of hepatotoxicity and histopathological analysis. Collagen deposition and fibrosis were measured using several biochemical techniques, as well as by histological analysis. Finally, we conducted a cursory analysis to identify the effects of TCDD on the activity and expression of several key molecules involved in ECM remodeling.

Results from these experiments led us to conduct a more extensive characterization of the consequences of TCDD treatment on ECM molecules. These data are presented in Chapter Three. Specifically, we tested the hypothesis that TCDD modulates the expression of molecules involved in ECM maintenance, including molecules that are important for collagen export and cross-linking. We also measured expression of enzymes that break down ECM components, as well as molecules that regulate enzyme activity in the ECM.

The results from Chapter Two and Chapter Three are summarized in the final chapter of this dissertation, which also includes future directions for this area of research.

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CHAPTER TWO: 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) INCREASES NECROINFLAMMATION AND HEPATIC STELLATE CELL ACTIVATION BUT DOES NOT EXACERBATE EXPERIMENTAL LIVER FIBROSIS IN MICE

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant and high-affinity ligand for the aryl hydrocarbon receptor (AhR). Increasing evidence indicates that AhR signaling contributes to wound healing, which involves the coordinated deposition and remodeling of the extracellular matrix. In the liver, wound healing is attributed to the activation of hepatic stellate cells (HSCs), which mediate fibrogenesis through the production of soluble mediators and collagen type I. We recently reported that TCDD treatment increases the activation of human HSCs *in vitro*. The goal of this study was to determine if TCDD increases HSC activation *in vivo* using a mouse model of experimental liver fibrosis. To elicit fibrosis, C57BL6/ male mice were treated twice weekly for 8 weeks with 0.5 ml/kg carbon tetrachloride (CCl_4). TCDD (20 μg/kg) was administered once a week during weeks 7 and 8. Results indicate that TCDD increased liver-body-weight ratios, serum alanine aminotransferase activity, and hepatic necroinflammation in CCl_4-treated mice. Likewise, TCDD treatment increased mRNA expression of HSC activation and fibrogenesis genes, namely α-smooth muscle actin, desmin, delta-like homologue-1, TGF-β1, and collagen type I. However, TCDD treatment did not exacerbate fibrosis, nor did it increase the collagen content of the liver.

Instead, TCDD increased hepatic collagenase activity and increased expression of matrix metalloproteinase (MMP)-13 and the matrix regulatory proteins, TIMP-1 and PAI-1. These results support the hypothesis that TCDD increases HSC activation *in vivo*. Furthermore, increased HSC activation and fibrogenesis in response to TCDD may be counteracted by concomitant alterations in extracellular matrix remodeling.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental contaminant and ligand for the aryl hydrocarbon receptor (AhR). The AhR belongs to the basic helix-loop-helix/PAS family of transcription factors and regulates gene expression through heterodimerization with the nuclear protein ARNT (Hankinson, 1995) as well as through interactions with other transcriptional regulatory proteins (Jackson *et al.*, 2015). Changes in gene expression are believed to mediate TCDD toxicity, although the mechanisms by which this occurs are incompletely understood. In addition to mediating TCDD toxicity, the AhR contributes to tissue homeostasis by regulating proliferation, differentiation and apoptosis (Barouki *et al.*, 2007).

Increasing evidence indicates that AhR signaling is important for tissue repair, which is a complex process that includes angiogenesis, inflammation, regeneration, and extracellular matrix (ECM) remodeling (Eming *et al.*, 2014). Exposure to TCDD was found to inhibit tissue re-growth in a zebrafish model of fin regeneration (Mathew *et al.*, 2006; Zodrow and Tanguay, 2003). TCDD treatment also inhibited rodent liver regeneration induced by partial hepatectomy (Bauman *et al.*, 1995; Mitchell *et al.*, 2006). Regeneration is regulated by numerous soluble mediators, including transforming growth

factor (TGF)-β1, which is also a potent pro-fibrogenic molecule (Ho and Whitman, 2008; Levesque *et al.*, 2007; Thenappan *et al.*, 2010; Werner and Grose, 2003). Interactions between AhR and TGF-β1 pathways have been documented, and AhR deficiency was found to increase secretion of TGF-β1 (Guo *et al.*, 2004; Santiago-Josefat *et al.*, 2004; Zaher *et al.*, 1998). It stands to reason that AhR signaling may also contribute to the regulation of fibrogenesis, and this notion is supported by the observation that AhR-null mice display fibrotic lesions in the liver (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996).

Fibrogenesis is initiated in response to injury and inflammation and results in ECM synthesis (Kisseleva and Brenner, 2008). Exposure to TCDD was found to alter the expression of ECM proteins, including collagen and fibronectin (Andreasen *et al.*, 2006; Aragon *et al.*, 2008; Nottebrock *et al.*, 2006; Riecke *et al.*, 2002). The AhR is also implicated in regulating the expression of matrix metalloproteinases (MMPs), which contribute to ECM remodeling through degradation of matrix proteins. For example, TCDD treatment was shown to increase MMP expression in human keratinocytes, prostate cancer cells, and melanoma cells, and in zebrafish during fin regeneration (Andreasen, *et al*., 2006; Haque *et al.*, 2005; Murphy *et al.*, 2004; Villano *et al.*, 2006). Additionally, TCDD was found to increase plasminogen activator inhibitors -1 and -2 (PAI-1, -2) (Gohl *et al.*, 1996; Son and Rozman, 2002). These serine proteases inhibit the activation of plasmin, which cleaves ECM molecules and activates pro-MMPs (Lu *et al.*, 2011). Hence, TCDD is implicated in the modulation of both ECM synthesis and degradation.

Liver fibrosis is characterized by the abnormal or excessive deposition of ECM in

response to injury and unresolved inflammation (Friedman, 2000). Liver fibrosis is mediated by hepatic stellate cells (HSCs), which are non-parenchymal cells that normally function in vitamin A storage. Upon injury, HSCs become activated, lose this storage capacity, and assume a myofibroblast-like phenotype characterized by proliferation, contractility, chemokine and growth factor production, and synthesis of fibrillar collagens (Puche *et al.*, 2013). TCDD is known to decrease vitamin A levels in the rodent liver, which supports the idea that TCDD might increase HSC activation (Hakansson and Hanberg, 1989). Indeed, we recently found that TCDD treatment increases activation of the human HSC line, LX-2 (Harvey *et al.*, 2016). However, the consequences of TCDD on HSC activation *in vivo* are unclear. One study reported that treatment of rats with a single dose of TCDD had no effect on expression of the HSC activation marker α-smooth muscle actin (αSMA) (Hanberg *et al.*, 1996). However, another study recently found that chronic administration of TCDD in mice increased the expression of αSMA and collagen type I (Pierre *et al.*, 2014).

The goal of this study was to determine how TCDD impacts *in vivo* HSC activation and fibrosis development during liver injury elicited by carbon tetrachloride (CCl_4). In the liver, CCl_4 is biotransformed by cytochrome P4502E1 into a trichloromethyl radical that causes lipid peroxidation resulting in membrane damage (Wong *et al.*, 1998). Chronic administration of CCl_4 causes widespread hepatocellular damage that promotes collagen deposition by activated HSCs (Mederacke *et al.*, 2013). We tested the hypothesis that TCDD treatment increases HSC activation and exacerbates liver fibrosis. We measured liver damage, expression of HSC activation markers, collagen synthesis and deposition, and the expression and activity of ECM remodeling

molecules.

MATERIALS AND METHODS

Animal Treatment. Male C57BL/6 mice (8-10 weeks old; Charles River, Wilmington, MA) were injected i.p. with 0.5 ml/kg CCl_4 (Sigma-Aldrich, St. Louis, MO) diluted in corn oil twice a week for 8 weeks. The ratio of CCl_4 to corn oil was 1:10. Control mice ("Ctrl") were injected with corn oil alone. During the last two weeks of the experiment, mice were treated weekly with TCDD (20 μg/kg by gavage; Cambridge Isotope Laboratories, Andover, MA) diluted in peanut oil or with peanut oil alone ("Veh"). Animals were euthanized at the end of the experiment, and liver was either flash-frozen in liquid nitrogen or fixed in Ultra Light Zinc Formalin Fixative (PSL Equipment, Vista, CA). Serum was collected and stored at -80° C until assayed. Six to seven mice were used per treatment group. All animal experiments were approved by the Institutional Animal Care and Use Committee at Boise State University.

Serum Alanine Aminotransferase (ALT) Activity. Serum samples were diluted 1:10 in phosphate buffered saline (PBS). ALT activity was measured using the Infinity ALT (GPT) reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. This kinetic assay is based on the rate of decrease in absorbance due to the oxidation of NADH. The assay was read every 45 seconds for 3 minutes, and activity was expressed as U/L. Samples were run in duplicate.

Histopathology. Fixed liver tissue was paraffin-embedded and cut into 2-μm sections. Tissue sections were either stained with hematoxylin and eosin or with Sirius

Red as described elsewhere (Junqueira *et al.*, 1979). Images of stained tissues were taken with an Olympus BX53 compound microscope. Stained liver tissue was scored for necroinflammation and fibrosis by a board-certified pathologist based on the Ishak Modified Histological Activity Index System (Ishak *et al.*, 1995).

Quantitative Real-Time RT-PCR. Total RNA was extracted from 20 mg of frozen liver tissue using the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Norcross, GA). RNA concentration and purity were measured by ultraviolet (UV) absorbance, and quality was assessed on an agarose bleach gel (Aranda *et al.*, 2012). RNA was reverse-transcribed using the Applied Biosystems High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Gene-specific primers (Table 1) were used for quantitative real-time RT-PCR (qRT-PCR), which was performed using Roche FastStart Essential DNA Green Master reaction mix on a LightCycler® 96 thermocycler (Roche, Indianapolis, IN). Three biological replicates were assayed per treatment group, and all samples were run in duplicate. Relative quantification was estimated using the $\Delta\Delta C_q$ method normalized to GAPDH (Schmittgen and Livak, 2008).

Table 1. Mouse primer sequences used for qRT-PCR

Gene	Primer Sequence (5' to 3')	Temp (°C)
Col1a1	GTC CCT GAA GTC AGC TGC ATA TGG GAC AGT CCA GTT CTT CAT	60
Desmin	AGC GTG ACA ACC TGA TAG ACG TGA AGC TCA CGG ATC TCC TCT	60
DLK-1	GGA GAA AGG CCA GTA CGA ATG CTG TTG GTT GCG GCT ACT AT	58
GAPDH	CAA TGA CCC CTT CAT TGA CC GAT CTC GCT CCT GGA AGA TG	60
MMP-13	GCC CTG GGA AGG AGA GAC TCC AGG GGA TTC CCG CAA GAG TCG CAG G	55
PAI-1	TTC AGC CCT TGC TTG CCT C ACA CTT TAC TCC GAAGTC GGT	60
TIMP-1	CAC GGG CCG CCT AAG GAA CG GGT CAT CGG GCC CCA AGG GA	60
TGFβ1	TGC TAA TGG TGG ACC GCAA CAC TGC TTC CCG AAT GTC TGA	60
αSMA	TCC TCC CTG GAG AAG AGC TAC TAT AGG TGG TTT CGT GGA TGC	60

Immunofluorescence Detection of αSMA. Liver tissue sections were deparaffinized, rehydrated, and incubated at 95°C for 30 minutes in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.5% Tween-20, pH 9). Tissue sections were permeabilized in TBS (50 mM Tris, 150 mM NaCl, pH 7.6) with 0.025% Triton X-100 for 10 minutes at room temperature followed by blocking for 2 hr in immunofluorescence buffer (TBS with 1% bovine serum albumin and 2% fetal bovine serum). Cy3-conjugated anti-αSMA antibody (Sigma-Aldrich) was diluted 1:1000 and incubated on tissue sections overnight at 4°C. Nuclei were stained with DAPI, and cover slips were mounted with Permount. Expression of αSMA protein was visualized with a Zeiss LSM 510 confocal microscope using a 20X objective. Staining was quantified using ImageJ software (US National

Institutes of Health), and the amount of αSMA staining was expressed as a percentage of total area stained.

Western Blotting. Frozen liver tissue was homogenized in 50 mM HEPES, 150 mM NaCl, 10% Glycerol, 0.1% Tween 20, 7.5 mM EDTA, and 7.5 mM $MgCl_2{*}6H_2O$. Protein content was determined using a *DC*™ Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA), and homogenates were diluted to 5 mg/ml. Pepsin (2000 U/mg; VWR, Radnor, PA) was diluted to 2 mg/ml in 2 N HCl. For the digestion, 10 μl of this pepsin preparation was added to 100 μl of homogenate (500 μg protein), and the sample was incubated for 2 hours at 20ºC. Samples were then neutralized with 10 μl of 2 N NaOH and resuspended in SDS loading buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) containing 400 mM β-mercaptoethanol). Pepsin-digested samples (6 μl/lane) and undigested samples (25 μg protein/lane) were resolved on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with anti-collagen type I antibody (EMD Millipore, Hayward, CA) or anti-actin antibody (Santa Cruz Biotech, Dallas, TX). Blots were then incubated with HRP-conjugated secondary antibodies, and bands were visualized with Pierce™ ECL Western Blotting Substrate (Thermo Scientific).

***Hydroxyproline Quantification by LC/MS*.** Sample prep: Frozen liver samples (10 mg) were homogenized in 100 μl reagent-grade H_2O and hydrolyzed in 100 μl hydrochloric acid (12 M) at 95°C for 20 hours. Hydrolyzed samples were transferred to Phree® phospholipid removal columns (Phenomenex, Torrance, CA) and centrifuged at

1000 x *g* for 10 minutes. The resulting filtrates were transferred to autosampler vials for analysis. Linear calibration curves were constructed for quantification by spiking hydrolyzed homogenates with known concentrations of hydroxyproline standard. **LC-MS conditions:** Hydroxyproline levels were analyzed by LC-MS using a Dionex Ultimate 3000 LC system (Dionex, Sunnyvale, CA) attached to a Bruker MaXis Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer equipped with an electrospray ionization (ESI) source (Bruker Daltonics, Billerica, MA). Chromatographic separation was performed using a Synergi Hydro reverse phase column (150 x 2.0 mm, 4µm, Phenomenex, Torrance, CA) with a guard column and a flow rate of 150 µL/min. The column temperature was maintained at 40°C during the analysis. Samples were housed in an autosampler at 4°C. One µL of each sample was injected onto the column. The LC elution mobile phases consisted of A (5% methanol, 94.7% water, 0.2% formic acid) and B (94.5% acetonitrile, 0.2% formic acid). The gradient began at 0% B for 5 minutes, and increased linearly to 80% B over 5 min and maintained at this percentage for a further 10 min as a washing step. A post-column infusion of isopropanol (2mL/h) was used to enhance ionization. ***ESI-Q-TOF conditions:*** Analysis was performed in positive ion mode with a spray voltage of 3000V, endplate offset of -500V, nebulizer gas pressure of 1.5 bar, dry gas flow rate of 8 L/min, and dry gas temperature of 200°C. Peak area of the extracted ion chromatogram of hydroxyproline (132.067 [M+H]) were used for quantification.

In Situ Zymography. Collagenase activity was measured using *in situ* zymography of zinc-formalin fixed liver tissue as described elsewhere (Hadler-Olsen *et*

al., 2010; Kumar *et al.*, 2014). Briefly, liver tissue sections (8 μm) were heated at 58°C for 12 hr, deparaffinized, and rehydrated. DQ™ collagen (Thermo Fisher Scientific) was dissolved in reagent grade water and diluted 1:50 in a reaction buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.6). Tissue sections were incubated with the DQ-collagen solution for 12 hr at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and cover slips were mounted with Permount. Fluorescent images were taken with an EVOS fluorescence microscope using a 20X objective.

Immunohistochemistry for MMP-13. Paraffin-embedded liver tissue sections (2 μm) were incubated with an anti-MMP-13 antibody (#ab75606, Abcam, Cambridge, MA) overnight at 4°C. Tissues were then stained with 3,3-diaminobenzidine using a commercially available kit (R&D Systems, Minneapolis, MN). Tissues were counterstained with hematoxylin. Images were captured with an Olympus BX53 compound microscope, and staining was quantified using ImageJ software.

Statistical Analyses. Statistical analyses were performed using Prism version 6.0 (GraphPad Software, La Jolla, CA). Data were evaluated by two-way analysis of variance followed by a Bonferroni's multiple comparisons test to evaluate differences between treatment groups. Data were considered significantly different at $p < 0.05$.

RESULTS

Hepatotoxic effects of TCDD in CCl_4-treated mice.

To investigate how TCDD treatment impacts liver damage and fibrogenesis during chronic CCl_4 administration, gross markers of hepatotoxicity were evaluated. TCDD treatment induced marked hepatomegaly in control-treated and CCl_4-treated mice (Figure 1), which corresponded with increased liver weights and liver-to-body-weight ratios (Table 2). CCl_4 treatment alone had no effect on liver weight or liver-to-body-weight ratios. Serum activity levels of alanine aminotransferase (ALT) were measured as an indication of hepatocellular necrosis. Treatment with either TCDD or CCl_4 alone caused an elevation in serum ALT activity levels, and the combination of the two appeared to have an additive effect (Table 2). These results are consistent with other endpoints of toxicity reported for mice treated with either TCDD or CCl_4. It is worth noting that 40% mortality was observed two days after the last injection of TCDD in CCl_4-treated mice (data not shown), whereas no mortality occurred in the other treatment groups.

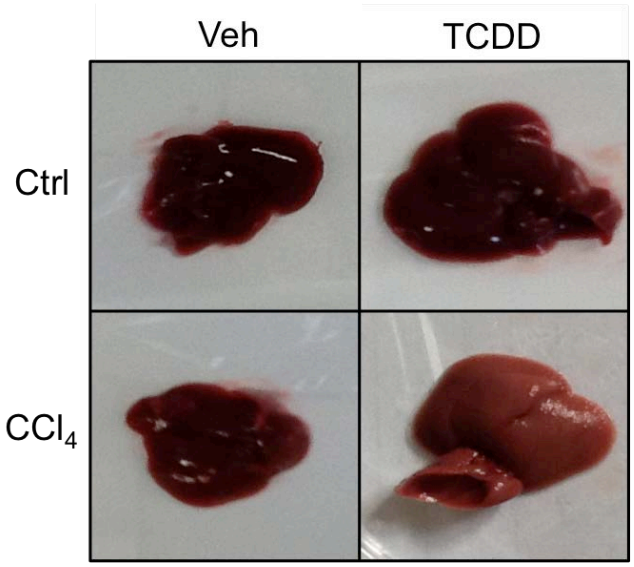

Figure 1. TCDD treatment, but not CCl_4 administration, elicits hepatomegaly. C57BL/6 mice were treated with CCl_4 for 8 weeks, and TCDD (20 μg/kg) was administered during the last two weeks. Photographs reveal representative differences in liver size among mice in each treatment group.

Table 2. Consequences of TCDD on liver weight, body weight, and serum ALT activity in CCl_4-treated mice

	Ctrl/Veh	Ctrl/TCDD	CCl_4/Veh	CCl_4/TCDD
Liver weight	1.50 ± 0.07	1.74 ± 0.58[a]	1.38 ± 0.06	1.72 ± 0.07[a]
Body weight	28.93 ± 0.66	27.35 ± 0.77	26.76 ± 0.92	26.25 ± 1.11
Liver weight/body weight	0.052 ± 0.002	0.064 ± 0.002[a,b]	0.052 ± 0.002	0.066 ± 0.001[a,b]
ALT (U/L)	27.22 ± 12.26	198.70 ± 44.27[b]	130.74 ± 10.38[b]	282.22 ± 46.67[a,b]

Values represent mean ± SEM. [a]$p < 0.05$ when compared to CCl_4/Veh-treated mice. [b]$p < 0.05$ when compared to Ctrl/Veh-treated mice.

TCDD increases necroinflammation in the liver of CCl_4-treated mice.

During chronic CCl_4 administration, ongoing liver injury and inflammation are the driving factors that elicit fibrogenesis (Weber *et al.*, 2003). Histological analysis revealed that TCDD treatment alone induced mild inflammation, based on the presence of hepatic inflammatory foci that were not present in vehicle-treated mice (Figure 2, A-D). Analysis of livers from mice treated with CCl_4 revealed ballooning degeneration of hepatocytes, coagulation necrosis and necrotic bridge formation (Figure 2, E-F). Administration of TCDD to CCl_4-treated mice evoked widespread coagulation necrosis and inflammation (Figure 2, G-H). To further assess liver damage and inflammation, a more detailed histopathological analysis was conducted using the Ishak Modified Histological Activity Index system (Ishak, *et al*., 1995). Administration of either TCDD or CCl_4 alone was determined to increase all four endpoints of necroinflammation measured in this system: 1) periportal or periseptal interface ("piecemeal") necrosis; 2) confluent necrosis; 3) focal lytic necrosis, apoptosis, and focal inflammation; and 4) portal inflammation (Table 3). Based on the combined score for these endpoints and the staging categories of the Ishak system, mice treated with either TCDD or CCl_4 were determined to display "mild" necroinflammation. In contrast, administration of TCDD to CCl_4-treated mice resulted in a combined necroinflammation score that was more than twice as high. This was due to a marked increase in confluent necrosis, as well as portal inflammation and periportal or periseptal interface hepatitis.

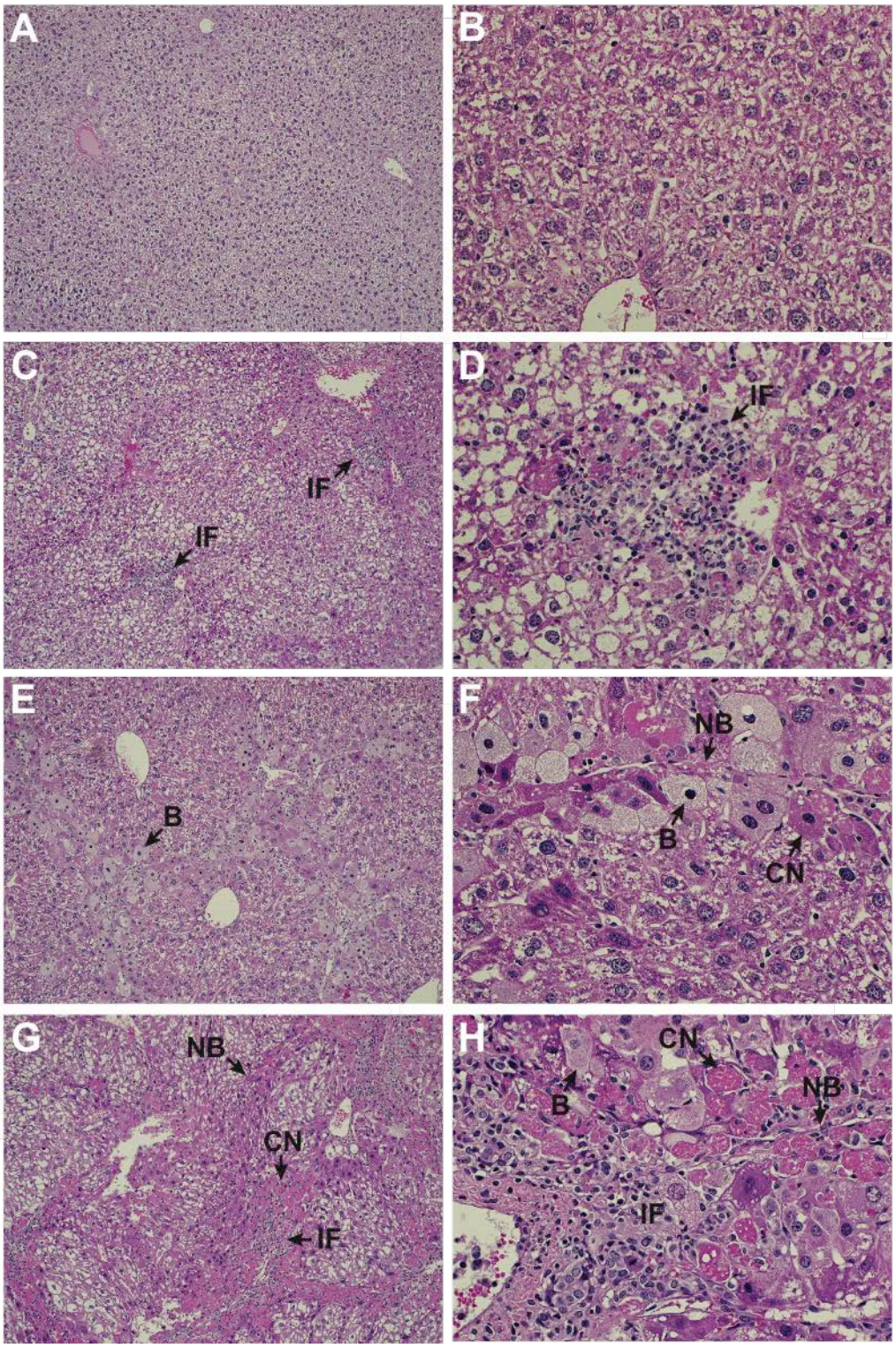
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Figure 2. TCDD increases necroinflammation in the liver of CCl_4-treated mice. Representative photomicrographs showing pathological changes in the liver based on hematoxylin and eosin staining at 100x *(A,C,E,G)* and 400x *(B,D,F,H)* magnifications. *(A, B)* Liver from a Ctrl/Veh-treated mouse with normal liver architecture. *(C, D)* Representative liver from a Ctrl/TCDD-treated mouse reveals presence of inflammatory foci ("IF"). *(E, F)* Liver from a CCl_4/Veh-treated mouse contains balloon ("B") cells, coagulation necrosis ("CN") and necrotic bridge ("NB") formation. *(G, H)* Liver from a CCl_4/TCDD-treated mouse reveals widespread coagulation necrosis, infiltration of inflammatory cells, ballooning hepatocytes, and necrotic bridges.

Table 3. Effects of TCDD treatment on necroinflammation during CCl_4-induced liver fibrosis

	Ctrl/Veh	Ctrl/TCDD	CCl_4/Veh	CCl_4/TCDD
Periportal or periseptal interface hepatitis (0-4)	0	1.86 ± 0.34^{a}	1.50 ± 0.22^{a}	$4 \pm 0^{a,b}$
Confluent necrosis (0-6)	0	1.14 ± 0.26^{a}	1.33 ± 0.21^{a}	$5 \pm 0^{a,b}$
Focal lytic necrosis, apoptosis, and focal inflammation (0-4)	0	1.71 ± 0.29^{a}	1 ± 0	1.33 ± 0.33^{a}
Portal inflammation (0-4)	0	1.86 ± 0.34^{a}	1.83 ± 0.17^{a}	$3.33 \pm 0.33^{a,b}$
Combined necroinflammation score:	0	6.57 ± 0.81^{a} (mild)	5.67 ± 0.33^{a} (mild)	$13.67 \pm 0.33^{a,b}$ (severe)

Necroinflammation was assessed using the Ishak Modified Histological Activity Index System (Ishak, Baptista, Bianchi, Callea, De Groote, Gudat, Denk, Desmet, Korb and MacSween, 1995). Numbers in parentheses indicate the scoring range for each feature. Values represent mean ± SEM. $^{a}p < 0.05$ when compared to Ctrl/Veh-treated mice. $^{b}p < 0.05$ when compared to CCl_4/Veh-treated mice.

TCDD increases expression of HSC activation markers.

Although other myofibroblast precursors exist in the liver, HSCs are the primary source of activated myofibroblasts in response to chronic administration of CCl_4 (Iwaisako *et al.*, 2014). To determine how TCDD exposure impacted the activation of these cells in response to CCl_4, expression of the HSC activation marker αSMA was

measured. Based on immunofluorescence staining, αSMA expression increased in response to CCl_4 treatment (Figure 3A). Quantification of staining revealed that administration of TCDD to CCl_4-treated mice evoked a 2-fold increase in αSMA expression compared to mice treated with CCl_4 alone (Figure 3B). While this effect was not statistically significant, similar results were produced when mRNA levels were measured. In fact, αSMA mRNA expression was about 40 times higher in mice treated with both TCDD and CCl_4 as compared to all other treatment groups (Figure 3C). A similar effect of TCDD was observed in the mRNA levels of desmin (Figure 3D) and delta-like homolog 1 (DLK-1; Figure 3E), which are two other markers selectively expressed on activated HSCs (Zhu *et al.*, 2012).

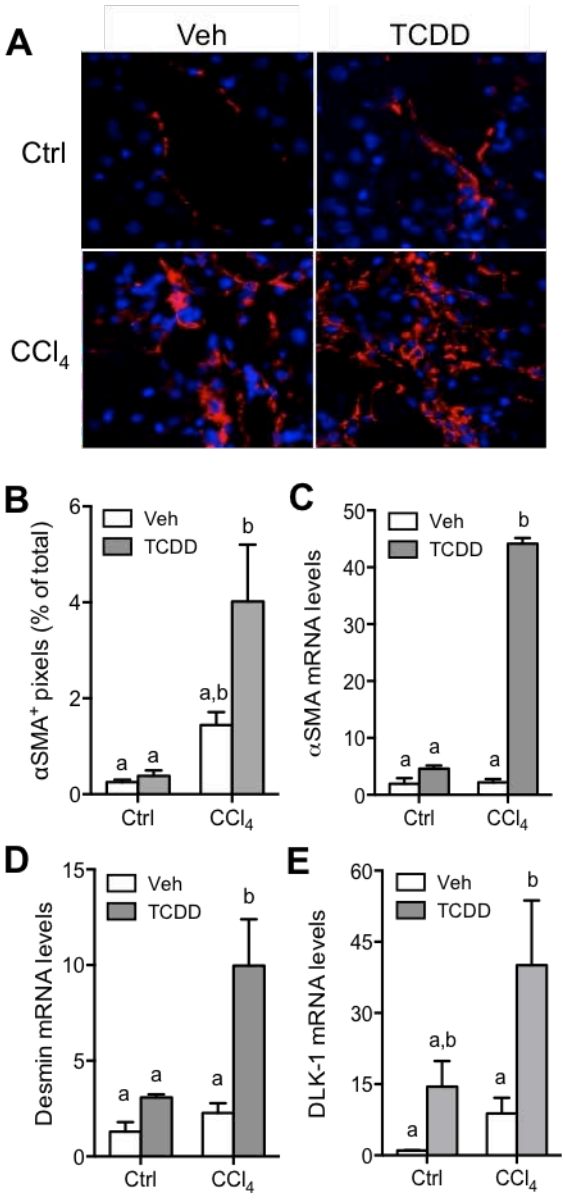

Figure 3. TCDD increases markers of HSC activation in CCl_4-treated mice. *(A)* Immunofluorescence was used to detect expression of alpha-smooth muscle actin (αSMA; red) in paraffin-embedded liver tissue; nuclei were stained with DAPI (blue). *(B)* αSMA immunofluorescence staining was quantified and expressed as a percentage of the total area stained. *(C-E)* Hepatic mRNA levels (mean ± SEM) of αSMA, desmin, and DLK-1 are represented relative to Ctrl/Veh-treated mice (n=3). Means that do not share a letter are significantly different from each other ($p < 0.05$).

Consequences of TCDD on expression of pro-fibrogenic molecules and fibrosis development.

Based on increased HSC activation, it was logical to hypothesize that TCDD treatment would increase fibrogenesis in CCl_4-treated mice. Increased production and activation of the profibrogenic mediator TGF-β1, as well as synthesis and deposition of collagen, are important for the pathogenesis of fibrosis (Kisseleva and Brenner, 2008). We found that exposure to TCDD increased TGF-β1 mRNA levels regardless of CCl_4 treatment (Figure 4A). Analysis of mRNA levels of Col1a1, which encodes the alpha-1 chain of collagen type I, revealed no overt changes in mice treated with TCDD or CCl_4 alone (Figure 4B). However, in mice that received both TCDD and CCl_4, Col1a1 mRNA levels increased more than 100-fold.

Fibrosis is characterized by the deposition of fibrillar collagens, which can be visualized in tissues stained with Sirius Red. As expected, CCl_4 administration increased the deposition of collagen in the liver (Figure 4C). However, exposure to TCDD did not appear to increase collagen content, and this finding was confirmed when staining was quantified (Figure 4D). Liver tissue stained with Sirius Red was also used to stage fibrosis on a 0-6 scale using the Ishak Modified Histological Activity Index system. The administration of TCDD alone did not initiate fibrosis (Figure 4E). Moderate fibrosis was observed in CCl_4-treated mice, and TCDD treatment did not increase the fibrosis score in these mice.

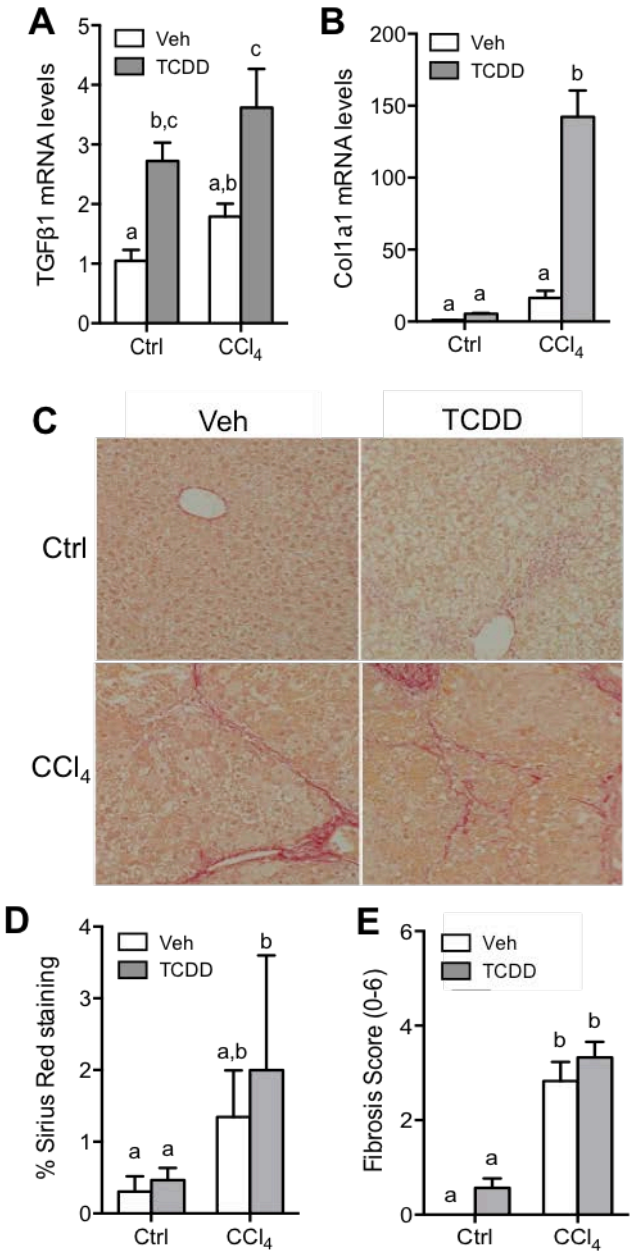

Figure 4. Consequences of TCDD on the development of fibrosis in CCl_4-treated mice. *(A, B)* Hepatic mRNA levels (mean ± SEM) of TGFβ1 and Col1a1 expressed relative to Ctrl/Veh-treated mice (n=3). Means that do not share a letter are significantly different from each other ($p < 0.05$). *(C)* Representative photomicrographs of liver tissue stained with Sirius Red to visualize collagen deposition (10X magnification). *(D)* Sirius Red staining was quantified and expressed as a percentage of total area (mean ± SEM). *(E)* Sirius Red-stained liver tissues were evaluated and scored for fibrosis based on the Ishak Modified Histological Activity Index System (Ishak *et al.*, 1995). This system uses numerical scoring (0-6) to evaluate fibrosis-related architectural changes in the liver, such as fibrous expansion in portal areas and the development of fibrous septa and bridging. Means that do not share a letter are significantly different from each other ($p < 0.05$).

TCDD does not increase the collagen content in the liver of CCl_4-treated mice

Because TCDD treatment did not appear to increase collagen content or fibrosis in CCl_4-treated mice despite increased mRNA levels of TGF-β1 and Col1a1, we performed additional analyses to determine how TCDD impacted the collagen content of the liver. Western blot analysis revealed that CCl_4 administration increased expression of collagen type I protein, and TCDD had no additive effect on protein levels (Figure 5A). Further analysis was performed using mass spectrometry to quantify hepatic levels of hydroxyproline, which is a major component of collagen (Figure 5B). Results indicate that CCl_4 administration increased the amount of hydroxyproline in the liver, and no overt increases were detected when TCDD was administered to CCl_4-treated mice (Figure 5C). Collectively, these results reinforce the notion that TCDD treatment does not increase the collagen content in the liver of CCl_4-treated mice.

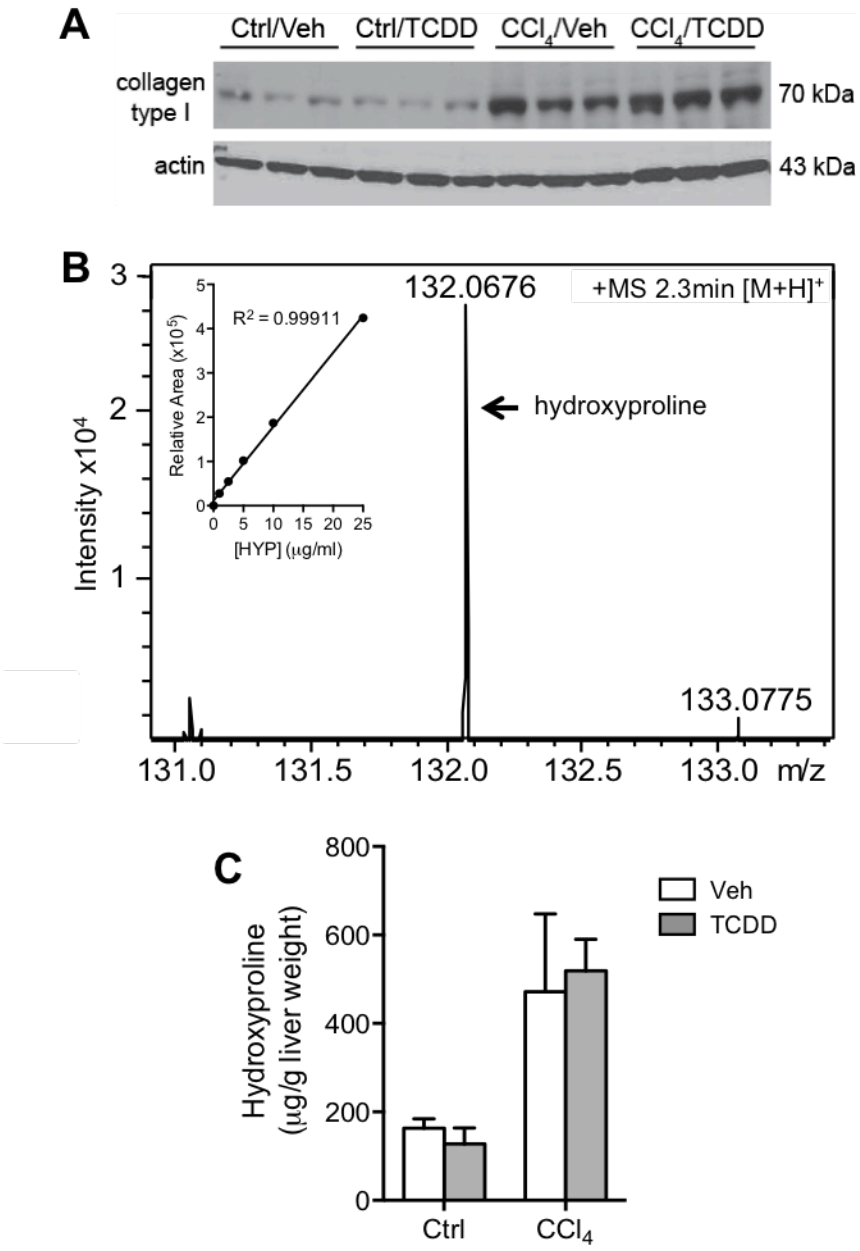

Figure 5. TCDD treatment does not increase collagen protein levels in the liver of CCl_4-treated mice. *(A)* Western blot analysis of collagen type I protein levels in pepsin-digested liver homogenates. Actin levels were evaluated in undigested liver homogenates (25 μg protein/lane). *(B)* Mass spectrum of hydroxyproline, which was identified based on its mass and retention time. *Inset:* Hydroxyproline standard curve, based on injecting different concentrations of hydroxyproline standard and measuring ratio of area to concentration. *(C)* Data represent average hydroxyproline content (mean ± SEM) in liver of mice from all four treatment groups (n=3). Means were not significantly different at $p < 0.05$.

Collagenase expression and activity is increased in TCDD-treated mice.

The finding that TCDD increased liver injury, inflammation, and HSC activation in CCl_4-treated mice but did not exacerbate fibrosis development led us to speculate that TCDD may increase collagen breakdown in the ECM. Such a finding would presumably explain why TCDD treatment had no overt impact on the collagen content in the liver. Using *in situ* zymography, we found that TCDD markedly increased collagenase activity, and this effect was especially pronounced in mice that were treated with CCl_4 (Figure 6A).

MMP-13 is a prominent murine collagenase that is important for cleavage of ECM components, and its expression decreases after induction of liver fibrosis (Giannandrea and Parks, 2014). Analysis of MMP-13 protein and mRNA expression revealed a trend towards increased expression in response to TCDD, although differences between treatment groups were not statistically significant (Figure 6 B-C). MMP activity is regulated through interactions with tissue inhibitor of metalloproteinases (TIMP) proteins, which reversibly bind to MMPs and inhibit their proteolytic activities. Analysis of TIMP expression revealed that TCDD treatment resulted in a 2-fold increase in TIMP-1 mRNA levels in CCl_4-treated mice (Figure 6D). Another mechanism by which MMP activity is regulated is by plasmin, which converts pro-MMPs into their active form (Giannandrea and Parks, 2014). The conversion of plasminogen to plasmin is inhibited by plasminogen activator inhibitors (PAI). Measurement of PAI-1 mRNA levels revealed a marked and robust increase in TCDD-treated mice, regardless of CCl4 treatment (Figure 6E). Taken together, these results indicate that TCDD treatment may dysregulate the expression and activity of molecules involved in ECM remodeling.

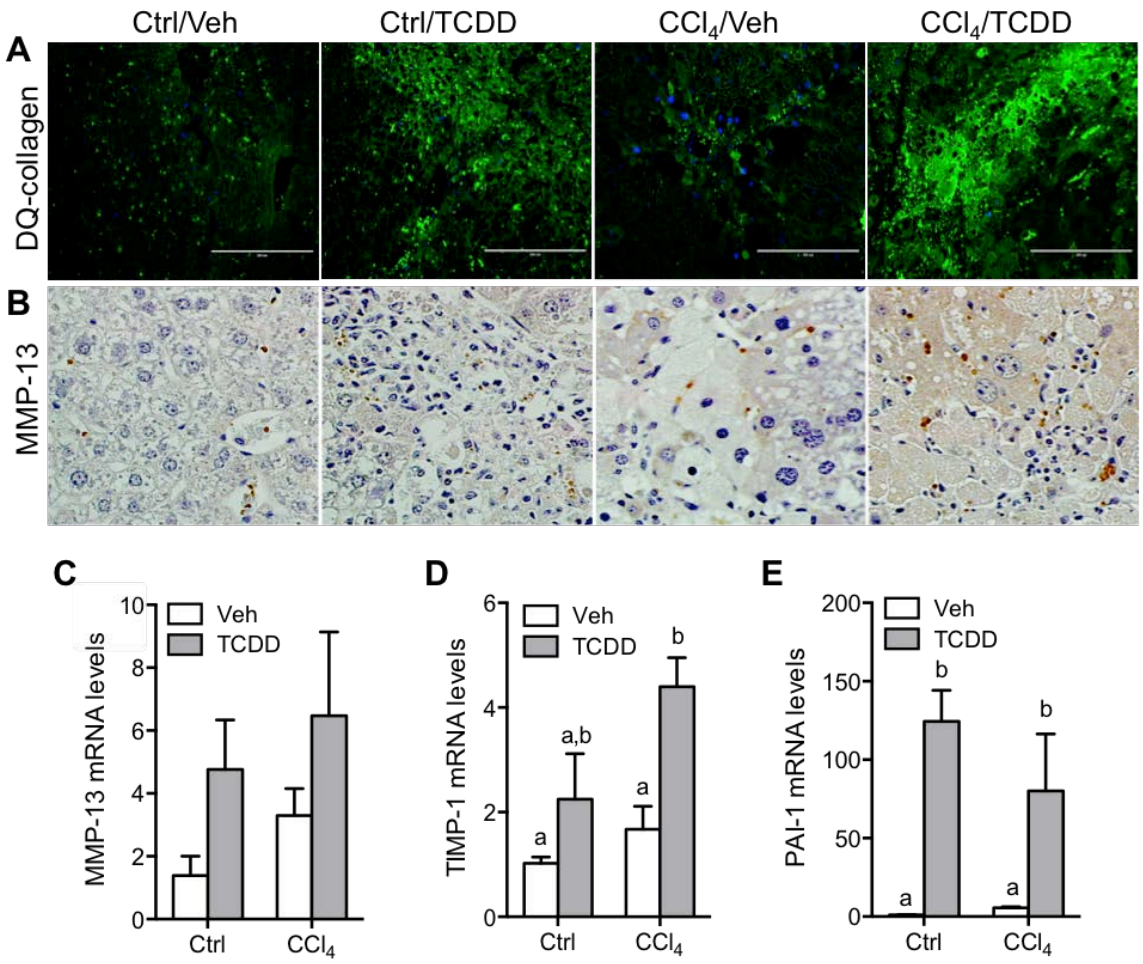

Figure 6. TCDD treatment increases collagenase activity and alters the expression of matrix remodeling molecules. *(A)* In situ zymography of zinc-buffered, formalin-fixed mouse liver tissue using DQ-collagen substrate. Green fluorescence (FITC) reveals collagenase activity; nuclei were stained with DAPI (blue). Images are representative of liver sections from three mice per treatment group. Scale bar depicts 200 μm. *(B)* Representative MMP-13 protein expression detected in paraffin-embedded liver tissue using immunohistochemistry (40X magnification). *(C-E)* Hepatic mRNA levels (mean ± SEM) of MMP-13, TIMP-1 and PAI-1 expressed relative to Ctrl/Veh-treated mice (n=3). Means that do not share a letter are significantly different from each other ($p < 0.05$).

DISCUSSION

The goal of this study was to determine how TCDD treatment impacts the *in vivo* activation of HSCs and the subsequent development of liver fibrosis. The CCl_4 model of experimental liver fibrosis was selected because it elicits robust HSC activation in response to liver injury. One of our major findings was that TCDD treatment increased liver damage in CCl_4-treated mice. This corroborates a recent report that pretreatment of mice with TCDD two days prior to a single injection of CCl_4 increased liver injury compared to mice treated with CCl_4 alone (Mejia-Garcia *et al.*, 2013). This effect was shown to occur through an AhR-dependent mechanism, as TCDD failed to increase liver injury in AhR-null mice treated with CCl_4. These same authors also found that TCDD treatment increased expression of CYP2E1, which would presumably enhance the conversion of CCl_4 into the reactive trichloromethyl radical and increase liver damage in TCDD-pretreated mice. In light of this report, we also measured CYP2E1 protein levels and found that TCDD had no effect on CYP2E1 expression (data not shown). The discrepancy between these studies could stem from the fact that Mejia-Garcia *et al.* measured CYP2E1 expression 72 hours after a single dose of TCDD (80 μg/kg), whereas we used two doses of TCDD (20 μg/kg), over a period of two weeks. Indeed, dose and time effects have been shown for other P450 enzymes (Santostefano *et al.*, 1998).

It is also possible that TCDD increases liver damage in CCl_4-treated mice by enhancing liver inflammation. Our observation that TCDD increased the prevalence of inflammatory foci in the liver of CCl_4-treated mice is consistent with other reports that TCDD enhances hepatic inflammation (Pierre *et al.*, 2014). The inflammatory response associated with hepatocellular necrosis has been shown to heavily recruit neutrophils,

which can induce lipid peroxidation through production of reactive oxygen species (Huebener *et al.,* 2015). Hence, recruitment of activated neutrophils to the liver can potentially exacerbate hepatocellular necrosis. However, reports vary as to the consequences of TCDD treatment on neutrophil recruitment. For example, exposure to TCDD was found to increase neutrophil recruitment to the lung during virus infection (Teske *et al.,* 2005). But, in a model of liver injury induced by concanavalin A, TCDD did not increase the influx of neutrophils to the liver despite increased production of neutrophil chemoattractants (Fullerton *et al.*, 2013). At this point, the mechanism by which TCDD increases liver damage in our model system is unclear.

In the present study, use of the CCl_4 model system provided the means to elicit HSC activation, and then to determine how administration of TCDD impacted these cells. Exposure to TCDD was found to increase HSC activation in CCl_4-treated mice. This finding could be due to a direct effect of TCDD on HSCs, which is supported by our previous finding that TCDD treatment directly increases activation of the human HSC line, LX-2 (Harvey *et al.,* 2016) However, increased HSC activation could also occur as a result of the increased severity of liver damage observed in CCl_4/TCDD-treated mice. Generally speaking, HSCs have not been extensively investigated as a target for TCDD toxicity. Furthermore, few studies of TCDD hepatotoxicity have utilized a model system in which robust HSC activation would be expected to occur, so is possible that TCDD-induced alterations in this population of cells may have been inadvertently overlooked. Reports in the literature do indicate that a single dose of TCDD can suppress vitamin A storage in the rat liver, which is consistent with HSC activation (Hakansson and Hanberg, 1989; Thunberg *et al.*, 1980). However, TCDD was found to have no effect on

expression of the HSC activation marker, αSMA. It is conceivable that acute exposure to TCDD alone does not provide the stimulus needed to evoke the complete activation of quiescent HSCs into myofibroblasts in the rodent liver. Nevertheless, it appears to be sufficient to impact this transition in LX-2 cells, which already exist in a quasi-activated state (Xu *et al.*, 2005), and in the liver of CCl_4-treated mice, in which HSC activation was already elicited due to liver damage (Figure 3). Along these same lines, it was recently reported that chronic exposure to TCDD increased expression of αSMA and collagen type I (Pierre *et al.*, 2014), although HSC activation was not formally assessed. The authors of the study reported that chronic TCDD administration increased liver damage, and this could have provided the stimulus necessary to initiate HSC activation. Hence, it is logical to surmise that investigating how TCDD impacts HSC activity will require the use of model systems in which liver injury is substantial enough to evoke robust HSC activation.

Given that TCDD treatment increased the activation of HSCs, which are the central mediators of liver fibrosis, it was not surprising that TCDD also increased TGF-β1 and Col1a1 mRNA levels in CCl_4-treated mice. TGF-β1 is activated in response to reactive oxygen species generated from chronic liver injury and inflammation (Schon and Weiskirchen, 2014), and the active form of TGF-β1 induces Col1a1 expression (Fan *et al.*, 2013). Col1a1 expression can also be stimulated by platelet derived growth factor (PDGF), a potent mitogen expressed that drives HSC proliferation (Kisseleva and Brenner, 2008). There is evidence to suggest that either of these collagen-stimulating pathways could be impacted by TCDD treatment (Chang *et al.*, 2007; Jaguin *et al.*, 2015).

Despite increased expression of TGF-β1 and Col1a1 mRNA levels, we found no evidence that TCDD treatment exacerbated fibrosis in CCl_4-treated mice, based on histopathological analysis as well as measurements of collagen and hydroxyproline content in the mouse liver. Instead, we found that TCDD may activate pathways leading to collagen degradation in CCl_4-treated mice, based on increased collagenase activity and possibly increased MMP-13 activity in the liver of CCl_4/TCDD-treated mice. These findings corroborate other studies in which TCDD was reported to increased expression of the collagenase MMP-13 in zebrafish and cell culture (Andreasen *et al.*, 2007; Andreasen, *et al*., 2006). It is possible that TCDD directly activates MMP-13 gene expression, based on the identification of a consensus XRE in the promoter region of the zebrafish MMP-13 gene (Andreasen, *et al*., 2006) as well as in the mouse MMP-13 gene (data not shown). Increased collagenase activity and MMP-13 expression would support the notion that increased collagen synthesis in CCl_4/TCDD-treated mice is essentially balanced by increased collagen breakdown, leading to no net increase in fibrosis when compared to mice treated with CCl_4 alone.

Nevertheless, the notion that TCDD increases collagenase activity in CCl_4-treated mice must be reconciled with the observation that TCDD also increased expression of TIMP-1 and PAI-1, which inhibit MMP activity. TIMP-1 directly inhibits MMPs in a stoichiometric 1:1 ratio. It is conceivable that TIMP-1 expression increased in CCl_4/TCDD -treated mice in response to increased MMP-1 expression. However, TIMP-1 levels in CCl_4/TCDD -treated mice could still be insufficient for suppressing MMP activity. The other inhibitor, PAI-1, inhibits the conversion of plasminogen to plasmin, which activates MMPs. Increased PAI-1 expression could occur in response to elevated

TGF-β1 levels in CCl_4/TCDD -treated mice (Liu *et al.*, 2010). On the other hand, TCDD has been shown to increase PAI-1 directly through binding of the AhR to a non-consensus XRE in the promoter region of the PAI-1 gene (Wilson *et al.*, 2013). Interestingly, binding of the AhR to this non-consensus XRE involves the interaction of AhR with KLF6, a transcription factor that is known to represses fibrogenic gene expression in quiescent HSCs (Ghiassi-Nejad *et al.*, 2013). It is formally possible that, when activated by TCDD, the AhR usurps KLF6 and prevents it from suppressing fibrogenic gene expression. Finally, there is evidence to suggest that MMPs can be activated through plasmin-independent pathways (Hahn-Dantona *et al.*, 1999; Suzuki *et al.*, 1990), which would allow these proteolytic enzymes to break down collagen despite increased PAI-1 expression.

In summary, results from this study demonstrate that *in vivo* HSC activation is increased by TCDD. Whether this occurs due to a direct effect of TCDD on HSCs or through the exacerbation of hepatocellular damage remains to be determined. Furthermore, data presented herein support the hypothesis that TCDD treatment can modulate ECM remodeling *in vivo*. Collectively, these findings implicate a role for TCDD-induced AhR activation in regulating myofibroblast activation and the pathogenesis of fibrosis.

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CHAPTER THREE: ARYL HYDROCARBON RECEPTOR ACTIVATION BY TCDD MODULATES EXPRESSION OF EXTRACELLULAR MATRIX REMODELING GENES DURING CHRONIC LIVER INJURY

ABSTRACT

The aryl hydrocarbon receptor (AhR) is a soluble, ligand-activated transcription factor that mediates the toxicity of the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Increasing evidence implicates a role for AhR activation in regulating the expression of genes involved in extracellular matrix (ECM) deposition and metabolism. We recently reported that TCDD treatment increased collagen type I expression, as well as collagen breakdown, during chronic liver injury elicited by carbon tetrachloride (CCl_4) administration. The goal of the present study was to determine how TCDD treatment impacts the expression of genes known to regulate collagen synthesis and breakdown in the injured liver. C57Bl/6 male mice were treated twice weekly for 8 weeks with 0.5 ml/kg CCl_4, and TCDD (20 μg/kg) was administered once a week during weeks 7 and 8. TCDD treatment was found to increase expression of procollagen genes for collagen types I, III, IV and VI and molecules involved in collagen processing and maturation (HSP47, decorin, and lysyl oxidase). Despite these increases, there were no overt effects of TCDD on collagen distribution or organization in the injured liver. TCDD also enhanced gelatinase activity and mRNA levels of matrix metalloproteinases (MMP)-3, MMP-8, MMP-9, and MMP13, as well as the MMP inhibitor, TIMP-1. Finally, TCDD increased expression of enzymes in the plasminogen

activator/plasmin system that regulates MMP activation. Specifically, TCDD decreased plasminogen mRNA levels, but increased mRNA levels of urokinase and tissue plasminogen activator (uPA/tPA) and plasminogen activator inhibitor (PAI)-1. These findings support the conclusion that TCDD modulates gene expression related to ECM remodeling but does not significantly impact collagen deposition during chronic liver injury.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a soluble protein in the basic helix-loop-helix Per/ARNT/Sim family of transcriptional regulators that contribute to developmental processes, adaptation to environmental stress, and xenobiotic metabolism (Beischlag *et al.* 2008; Gu *et al.* 2000; Hankinson 1995). The AhR mediates the toxicity associated with exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is an environmental contaminant and high-affinity ligand for this receptor (Fernandez-Salguero *et al.* 1996). After ligand binding, the AhR translocates from the cytoplasm to the nucleus, where it forms a heterodimer with the AhR nuclear translocator protein (ARNT). The AhR/ARNT complex binds to DNA at xenobiotic response elements (XREs) and modulates gene transcription. A growing body of evidence indicates that the AhR also interacts with other co-regulatory proteins in addition to ARNT and can modulate the expression of genes that do not contain XREs (Jackson *et al.* 2015), which underscores the increasing complexity of AhR-mediated gene regulation. Such AhR-dependent changes in gene expression are believed to underlie most of the toxic responses to TCDD. In the absence of TCDD, endogenous AhR activation is implicated in regulating the expression of genes

important for a number of normal developmental and physiological processes (Barouki *et al.* 2007; Mitchell and Elferink 2009).

Emerging evidence implicates a role for AhR signaling in the deposition and metabolism of extracellular matrix (ECM) components. The ECM is comprised of a network of proteins, such as collagens, glycoproteins, and proteoglycans, which are deposited in interstitial spaces and provide mechanical and structural support to cells (Karsdal *et al.* 2015). The ECM also regulates various cellular processes, such as survival, migration, proliferation and differentiation, by modulating tissue stiffness, communicating with the intracellular cytoskeleton, and sequestering and releasing growth factors (Kim *et al.* 2011). AhR activation by TCDD has been found to modulate the expression of ECM proteins, such as collagen and fibronectin (Andreasen *et al.* 2006; Aragon *et al.* 2008; Nottebrock *et al.* 2006; Pierre *et al.* 2014; Riecke *et al.* 2002). Expression of matrix metalloproteinases (MMPs), which are responsible for the degradation of ECM components, also appears to be targeted by TCDD. For example, *in vitro* TCDD treatment has been shown to increase MMP expression in human keratinocytes, prostate cancer cells, and melanoma cells (Haque *et al.* 2005; Murphy *et al.* 2004; Villano *et al.* 2006). Insight into the effect of TCDD on ECM maintenance and remodeling also stems from studies in a zebrafish regeneration model, in which amputation of the caudal (tail) fin initiates epimorphic regeneration accompanied by a wound healing response. Using this model, Andreasen et. al. reported that TCDD treatment increased the expression of MMP-9 and MMP-13 (Andreasen *et al.* 2007). In addition, exposure to TCDD induced a localized fibrosis in the regenerating fin, where collagen accumulated as an unorganized fibrotic deposit at the basement membrane. In a

separate study, gene expression analysis revealed that the largest number of genes impacted by TCDD during fin regeneration were those involved in ECM remodeling and structure (Andreasen *et al.* 2006). Collectively, these reports support the notion that TCDD dysregulates ECM homeostasis, and this most likely occurs through a mechanism that includes AhR-mediated changes in gene expression.

Disruptions of ECM metabolism and deposition are known to impact the development of liver disease (Duarte *et al.* 2015; Friedman 2000). Liver fibrosis is a pathological condition characterized by the deposition of excessive or abnormal ECM components, including collagen type I (Friedman 2000). In the liver, collagen is synthesized by myofibroblast precursors, namely hepatic stellate cells (HSCs). Upon liver injury, HSCs transition from quiescent, vitamin A-rich cells into activated myofibroblasts, characterized by increased proliferation, contractility, and synthesis of collagen type I (Friedman 2008). One well-established model system to investigate HSC activation and ECM modulation is experimental liver fibrosis induced by chronic carbon tetrachloride (CCl_4) administration. In the liver, CCl_4 is metabolized by cytochrome P4502E1 to a trichloromethyl radical that causes membrane damage through lipid peroxidation (Wong *et al.* 1998). Chronic treatment of mice with CCl_4 causes widespread centrilobular necrosis and inflammation, which drive HSC activation and the development of fibrosis (Mederacke *et al.* 2013).

We recently found that exposure to TCDD increased liver damage and HSC activation in mice treated with CCl_4 for 8 weeks (Lamb *et al.* in review). However, TCDD did not increase the deposition of collagen or the severity of liver fibrosis in CCl_4-treated mice, despite increased expression of genes encoding collagen type I and the

potent profibrogenic mediator, transforming growth factor (TGF)-β1. Results further indicated that TCDD increased collagenase activity in the liver of CCl_4-treated mice. Increased breakdown of ECM in CCl_4/TCDD-treated mice could explain why collagen deposition and fibrosis development were not exacerbated, despite increases in other endpoints of fibrogenesis.

Collagen biosynthesis begins with the transcription of procollagen genes and is facilitated by various intercellular and extracellular molecules (Frantz *et al.* 2010). For example, heat shock protein-47 (HSP47), within the endoplasmic reticulum, is required for proper triple helical folding and trafficking of procollagen to the Golgi apparatus (Widmer *et al.* 2012). Another molecule, decorin, facilitates collagen fibril assembly in the extracellular matrix during fibrillogenesis (Baghy *et al.* 2012). Skin fibroblasts, lacking decorin, produce significantly thinner collagen fibrils then the same fibroblasts with exogenously added decorin and mice lacking the decorin gene have enhanced expression of TGF-β1, resulting in increased hepatic fibrosis (Ruhland *et al*. 2007; Baghy *et al*. 2011). Lysyl oxidase (LOX) catalyzes cross-linking of collagen fibers, which marks the last step in collagen biosynthesis (Liu *et al.* 2015).

Collagen breakdown is achieved through the activity of numerous MMPs. MMP expression is regulated at the transcriptional level, and these proteins are synthesized as inactive zymogens called proMMPs (Caley *et al.* 2015). MMP activity is regulated by enzymatic inhibition and activation. Endogenous proteins called tissue inhibitors of metalloproteinase (TIMPs) inhibit MMP activity. Numerous mechanisms activate MMPs, including the plasminogen activator/plasmin system (Duarte *et al.* 2015). Plasmin is produced through the cleavage of plasminogen by tissue plasminogen activator (tPA) and

urokinase plasminogen activator (uPA), and this pathway is suppressed by plasminogen activator inhibitor (PAI)-1. Plasmin can directly convert proMMPs into enzymatically active MMPs, and some of these active MMPs can further activate other proMMPs.

The goal of the present study was to determine how TCDD treatment impacts the expression of genes related to ECM synthesis, deposition, and breakdown during chronic liver injury induced by CCl_4 administration. We measured gene expression related to collagen synthesis, processing, and cross-linking and assessed the impact of TCDD on the organization and dispersion of fibrillar collagens in the injured liver. Expression of MMPs and the molecules that activate or inhibit them were also measured to determine how TCDD modulates ECM breakdown.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (8-10 weeks old; Charles River, Wilmington, MA) were injected i.p. with 0.5 ml/kg CCl_4 (Sigma-Aldrich, St. Louis, MO) diluted in corn oil or with corn oil alone ("Ctrl") twice a week for 8 weeks. During the last two weeks of the experiment, mice were gavaged once weekly with 20 μg/kg TCDD (Cambridge Isotope Laboratories, Andover, MA) diluted in peanut oil or with peanut oil alone ("Vehicle"). At the end of the experiment, animals were euthanized, and liver was either flash-frozen in liquid nitrogen or fixed in Ultra Light Zinc Formalin Fixative (PSL Equipment, Vista, CA). All animal experiments were approved by the Institutional Animal Care and Use Committee at Boise State University and conducted according to the established policies and guidelines of this committee.

Quantitative Real-Time RT-PCR. Total RNA was extracted using the Omega Bio-Tek E.Z.N.A.® Total RNA Kit, and genomic DNA was eliminated using the Omega RNAse Free DNAse Set (Norcross, GA) from 20 mg of frozen liver tissue. RNA concentrations and purity were measured by ultraviolet (UV) absorbance. Quality and elimination of genomic DNA were assessed using an agarose bleach gel (Aranda *et al.* 2012). RNA was reverse-transcribed using the Applied Biosystems High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA). Gene-specific primers (Table 1) were used for quantitative real-time RT-PCR (qRT-PCR), which was performed using a Light Cycler® 96 Thermocycler and FastStart™ Essential DNA Green Master reaction mix (Roche, Indianapolis, IN). All samples were analyzed in duplicate from three mice per treatment group. Relative quantification was estimated using the $\Delta\Delta C_q$ method normalized to GAPDH (Schmittgen and Livak 2008).

Table 1. qRT-PCR primers and annealing temperatures used in this study.

Gene	**Primer Sequence**	**Annealing Temp (°C)**
Col1a1	FWD: GTCCCTGAAGTCAGCTGCATA REV: TGGGACAGTCCAGTTCTTCAT	60
Col3a1	FWD: CCTGGTGGAAAGGGTGAAAT REV: CGTGTTCCGGGTATACCATTAG	62
Col4a3	FWD: TCCTGGGGAAATGGGAAAGC REV: CTGCCTACGGATGGTTCTCC	64
Col4a5	FWD: TGCTCCTGAGAGATCGGCTT REV: GTTATGCTGGTGCACTTGGG	58
Col6a1	FWD: TCCCACCCACACAGAACAAC REV: CACTGAGAGGTGTCGTGTCC	58
Col6a2	FWD: TGACGCTGTTCTCTGACCTG REV: TTGTGGAAGTTCTGCTCGCC	58

Col6a3	FWD: CTGATGGCACCTCTCAGGAC REV: GTCACTTCCAACATCGAGGC	58
Dcn	FWD: AAGGGGGCCGATAAAGTTTC REV: CTGGGTTGAAAACCTCCTGC	58
Lox	FWD: CTGCACACACACAGGGATTG REV: AGCTGGGGTTTACACTGACC	56
Mmp2	FWD: ACCCAGATGTGGCCAACTAC REV: TACTTTTAAGGCCCGAGCAA	63
Mmp3	FWD: GTCCTCCACAGACTTGTCCC REV: GGGAGTTCCATAGAGGGACTGA	65
Mmp8	FWD: TACAGGGAACCCAGCACCTA REV: GGGGTTGTCTGAAGGTCCATAG	64
Mmp9	FWD: AAGGCAGCGTTAGCCAGAAG REV: GCGGTACAAGTATGCCTCTGC	63
Mmp13	FWD: GCCCTGGGAAGGAGAGACTCCAGG REV: GGATTCCCGCAAGAGTCGCAGG	55
Mmp14	FWD: GCCCTCTGTCCCAGATAAGC REV: ACCATCGCTCCTTGAAGACA	58
Plat	FWD: CAGAGATGAGCCAACGCAGA REV: TTCGCTGCAACTTCGGACAG	58
Plau	FWD: CATCCAGTCCTTGCGTGTCT REV: CCAAGTACACTGCCACCTTCA	62
Plg	FWD: ACTCAAGGGACTTTCGGTGC REV: TCAGATACTCGACGCGGTTG	58
Serpine1	FWD: TTCAGCCCTTGCTTGCCTC REV: ACACTTTACTCCGAAGTCGGT	60
Serpinh1	FWD: GGGAACGGATCGCTCCAAA REV: GGACCTGTGAGGGTTTACCAG	67

Timp1	FWD: CACGGGCCGCCTAAGGAACG REV: GGTCATCGGGCCCCAAGGGA	60
Timp2	FWD: GCCAAAGCAGTGAGCGAGAAG REV: CACACTGCTGAAGAGGGGGC	56
Timp3	FWD: AAGAAAAGAGCGGCAGTCCC REV: TTTGGCCCGGATCACGATG	60
Timp4	FWD: TATGGTAGGTGGGCTGACTGT REV: AGTTGAGACAGTGGGAGTAGGA	64

Measurement of Collagen Fibril Organization. Fixed liver tissue was paraffin-embedded, cut into 2-μm sections, and stained with Sirius Red as described elsewhere (Junqueira *et al.* 1979). Birefringence of stained liver tissues was visualized using an Olympus BX53F polarizing microscope. Photographs were taken at 600x magnification to focus on septa formation in the damaged liver of CCl_4-treated mice. Images were then converted to 8-bit grayscale and analyzed with FiberFit software to calculate fiber dispersion (κ) (Morrill *et al.* 2016). Ten images were analyzed from each mouse liver; three mice were evaluated per treatment group. Septa formation was not detected in the livers of mice that did not receive CCl_4, and these samples were excluded from the FiberFit analysis.

In Situ Zymography. Gelatinase activity was examined using *in situ* zymography of zinc-formalin fixed liver tissue as described elsewhere (Hadler-Olsen *et al.* 2010; Kumar *et al.* 2014). Briefly, tissue sections (8 μm) were heated at 58°C for 12 hours then deparaffinized and rehydrated. DQ™-gelatin (Thermo Fisher Scientific) was dissolved in reagent-grade water and diluted 1:50 in a 50 mM Tris-HCl buffer containing 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.6). Tissue sections were incubated with the DQ™-gelatin

solution for 12 hours at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and cover slips were mounted with Permount (Thermo Fisher Scientific). Fluorescent images were taken with an EVOS™ fluorescence microscope (Thermo Fisher Scientific) with a 20X objective. Fluorescence was quantified using ImageJ software (US National Institutes of Health) and expressed as a percentage of the area in the microscope field of view.

Statistical Analysis. Statistical analyses were performed using Prism (version 6; GraphPad Software, La Jolla, Ca.). Data were evaluated by two-way analysis of variance followed by a Bonferroni's multiple comparisons test to evaluate differences between treatment groups. Data were considered significantly different at $p < 0.05$.

RESULTS

Consequences of TCDD treatment on procollagen mRNA levels in CCl_4-treated mice.

To determine how TCDD treatment impacts procollagen synthesis during chronic liver injury, we measured the mRNA levels of genes that encode procollagen type I and III (fibrillar collagens) and types IV and VI (non-fibrillar collagens). Chronic CCl_4 treatment significantly increased *Col1a1* and *Col4a5* in the mouse liver (Figure 1). Administration of TCDD to CCl_4-treated mice further increased expression of *Col1a1* but not *Col4a5*. Trends indicated that CCl_4-treatment also increased transcript levels of *Col3a1* and *Col6a3*, but these elevations were not statistically significant. Nevertheless, the combination of TCDD and CCl_4 significantly increased transcript levels of both of

these procollagen genes compared to mice that did not receive CCl_4. Finally, TCDD treatment elevated *Col4a3* mRNA levels in mice that were not treated with CCl_4, but this increase was not observed in mice that received both TCDD and CCl_4. Collectively, these findings support a general trend in which exposure to TCDD increases procollagen gene expression in the liver of CCl_4-treated mice. Moreover, TCDD impacts the expression of procollagen isoforms that encode both fibrillar and non-fibrillar collagens.

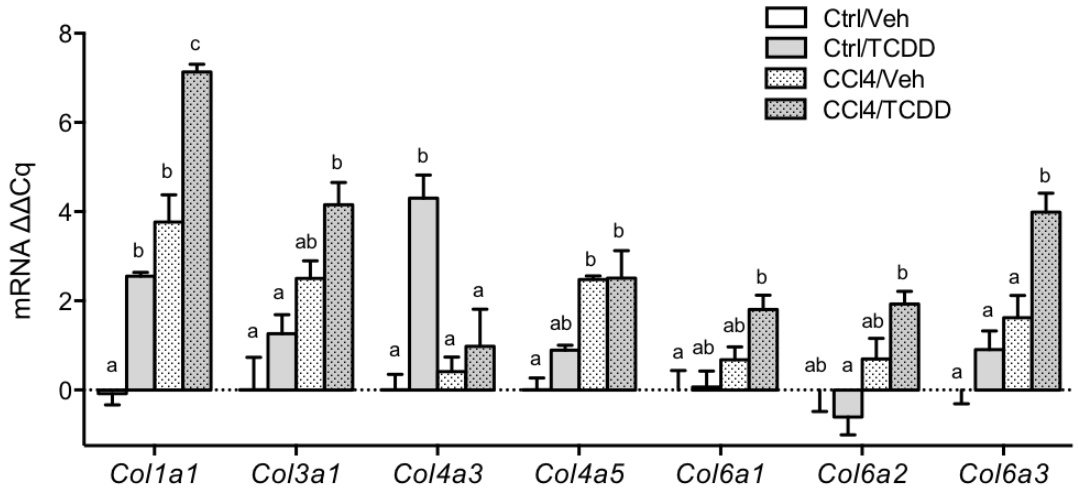

Figure 1. Consequences of TCDD treatment on collagen mRNA levels in the liver of CCl_4-treated mice. Collagen mRNA expression was measured by qRT-PCR and normalized to GAPDH. Data represent mean (± SEM) of three mice per treatment group. Within the data set for each gene, means that do not share a letter are significantly different from each other ($p < 0.05$).

TCDD increases mRNA levels of collagen processing molecules in CCl_4-treated mice.

Collagen synthesis requires not only expression of procollagen genes, but also processing of procollagen, assembly of fibrils, and cross-linking of fibers. To identify how TCDD impacts these events during CCl_4-induced liver injury, we measured

transcript levels of *Serpinh1* (HSP47), *Lox* (LOX), and *Dcn* (decorin). HSP47 is required for proper folding and trafficking of procollagen, whereas decorin, and lysyl oxidase contribute to fibril assembly and fiber cross-linking in the ECM (Canty and Kadler 2005; Widmer *et al.* 2012). Results indicate that when TCDD was administered to CCl_4-treated mice, *Serpinh1* and *Lox* mRNA levels increased 4 to 6 fold compared to mice treated with CCl_4 alone (Figure 2). In contrast, *Dcn* mRNA levels were significantly decreased in CCl_4/TCDD-treated mice. These results support the notion that TCDD significantly modulates the expression of genes encoding collagen-processing molecules during chronic liver injury.

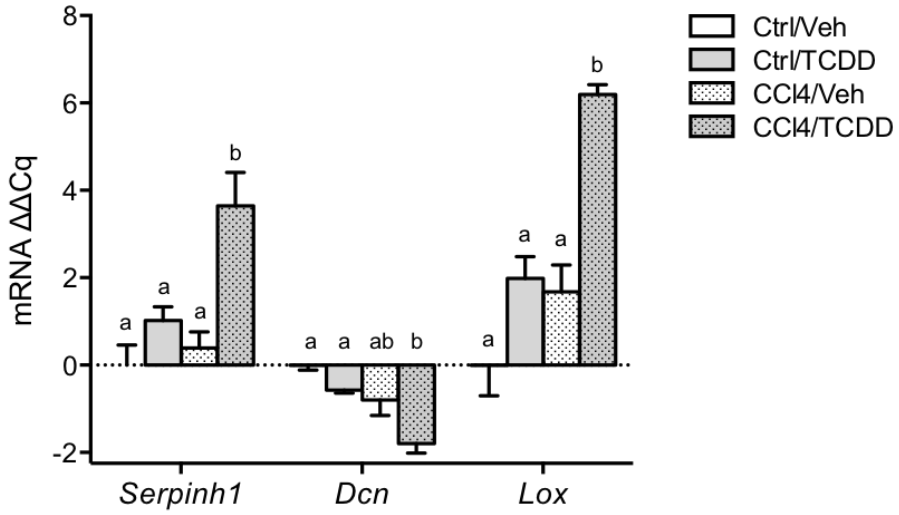

Figure 2. TCDD treatment alters expression of collagen processing molecules in the liver of CCl_4-treated mice. Transcript levels of *Serpinh1* (HSP47), *Dcn* (decorin) and *Lox* (lysyl oxidase) were measured by qRT-PCR and normalized to GAPDH. Data represent mean (± SEM) of three mice per treatment group. Within the data set for each gene, means that do not share a letter are significantly different from each other ($p < 0.05$).

TCDD treatment does not affect collagen fiber organization in the liver of CCl_4-treated mice.

The observation that TCDD altered the expression of ECM processing molecules in CCl_4-treated mice led us to speculate that it would subsequently impact the fibrillar collagen network. To test this, collagen fibers were visualized in liver tissue stained with Sirius Red, which aligns with fibrillar collagens and enhances their birefringence under cross-polarized light (Junqueira *et al.* 1979; Rich and Whittaker 2005). Polarized microscopy of stained tissue revealed the presence of thick, strongly birefringent yellow fibers in the septa of livers from CCl_4-treated mice (Figure 3A). However, based on visual inspection, TCDD treatment had no overt impact on the appearance of collagen. The effects of TCDD on collagen fiber organization were further evaluated using the free software application, FiberFit, which provides a rapid, two-dimensional analysis of fiber networks (Morrill *et al.* 2016). Results indicate that TCDD had no effect on fiber dispersion, which is a measure of disorder (Figure 3B). Hence, despite the TCDD-mediated increase in expression of procollagen genes and genes encoding collagen-processing molecules, TCDD did not appear to dysregulate collagen fiber organization in the ECM.

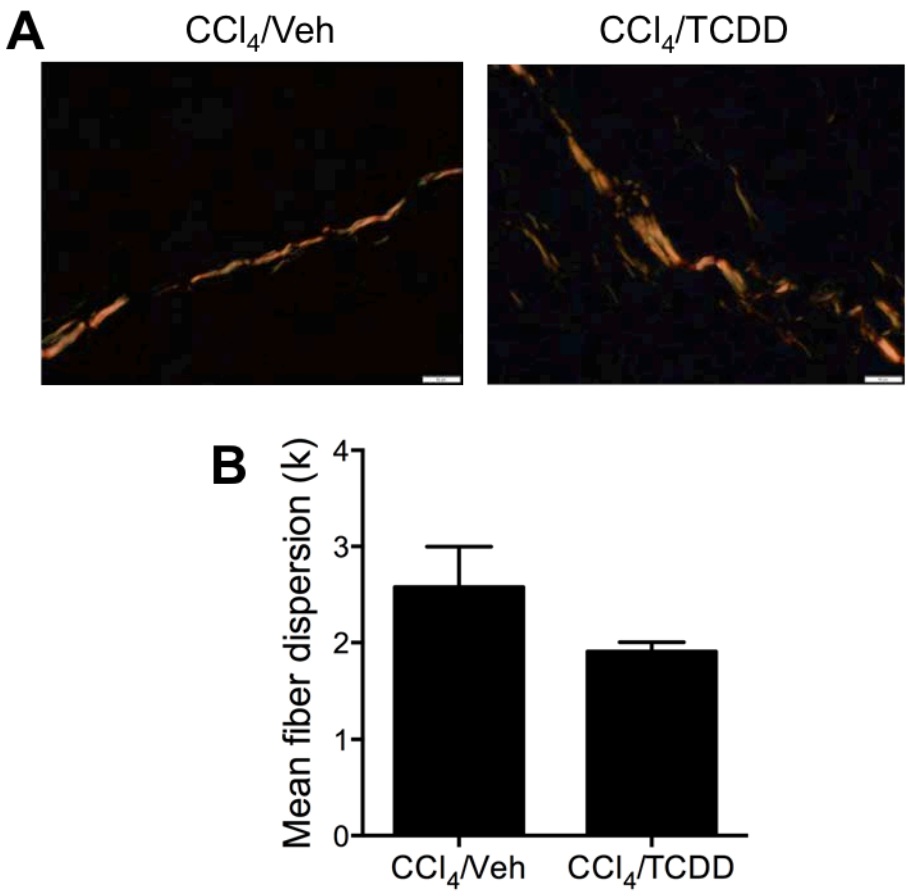

Figure 3. Exposure to TCDD does not impact collagen fiber organization in the liver of CCl_4-treated mice. *(A)* Polarized microscopy facilitates the visualization of collagen fiber birefringence in liver tissue stained with Sirius Red (600x magnification). Photomicrographs depict representative fibers in septa of liver from a mouse treated with CCl_4 and peanut oil vehicle (left) or with CCl_4 and TCDD (right). Scale bars represent 10 μm. Collagen network organization was evaluated by analyzing Sirius Red-stained liver tissues with the FiberFit software application (Morrill *et al.* 2016). Ten photomicrographs were evaluated per mouse; three mice were analyzed in each treatment group. Data represent mean (± SEM) fiber dispersion *(B)*. No statistically significant changes were found between treatment groups ($p < 0.05$).

Expression of ECM remodeling enzymes is elevated in the presence of TCDD.

ECM maintenance requires not only the synthesis and deposition of matrix molecules, but also their degradation and turnover, which is regulated by the proteolytic activity of MMPs. MMP expression is largely regulated at the transcriptional level

(Rodriguez *et al.* 2010). To determine how TCDD treatment impacts MMP gene expression in the liver of CCl_4-treated mice, we measured transcript levels of mouse MMPs known to be important in chronic liver injury. *Mmp-8* and *Mmp-13* encode enzymes that function primarily as collagenases, and expression of these genes was markedly increased by TCDD, regardless of CCl_4 treatment (Figure 4). *Mmp-2* and *Mmp-9* are referred to as gelatinases, and they degrade not only gelatin, but also collagen type IV, laminin, elastin and fibronectin (Rodriguez *et al.* 2010). While TCDD had no effect on *Mmp-2* transcript levels, it increased *Mmp-9* expression in CCl_4-treated mice. Likewise, the combination of TCDD and CCl_4 increased *Mmp-14* (membrane-type MMP) expression compared to mice treated with TCDD alone, although this increase was modest. *Mmp-3* (stromelysin) mRNA levels were about 3 to 5 times higher in TCDD-treated mice, regardless of CCl_4 treatment. Generally speaking, these results support the conclusion that TCDD treatment increases MMP gene expression during CCl_4-induced liver injury.

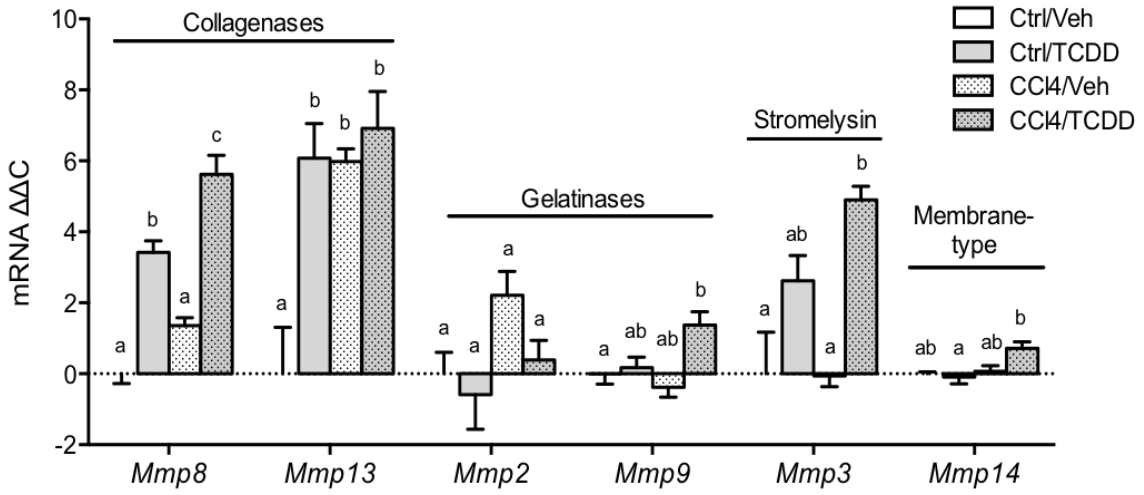

Figure 4. Effects of TCDD treatment on mRNA levels of MMPs in the liver of CCl_4-treated mice. MMP mRNA expression was measured by qRT-PCR and normalized to GAPDH. Data represent mean (± SEM) of three mice per treatment group. Within the data set for each gene, means that do not share a letter are significantly different from each other ($p < 0.05$).

TCDD increases gelatinase activity in the liver of CCl_4-treated mice.

MMP activity is central to ECM remodeling and has been implicated in both the promotion and attenuation of liver injury (Duarte *et al.* 2015). We recently found that TCDD treatment increased collagenase activity in the liver of CCl_4-treated mice (Lamb *et al.* in review). During fibrotic liver injury, collagenases cleave the native helix of fibrillar collagens to produce gelatin, which can be degraded by MMPs, namely MMP-2 and MMP-9 (Iredale *et al.* 2013). We used *in situ* zymography to measure gelatinase activity in the liver. Whereas gelatinase activity was barely detectable in mice treated with CCl_4/Veh (Figure 5A), it was significantly induced when TCDD was administered to CCl_4-treated mice (Figure 5B). When administered alone, TCDD did not increase gelatinase activity.

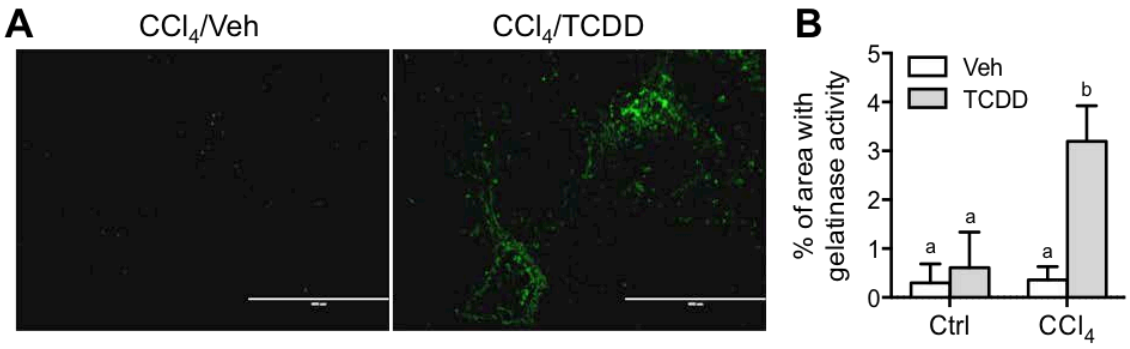

Figure 5. TCDD treatment increases gelatinase activity in the liver of CCl_4-treated mice. *(A)* In situ zymography of zinc-buffered, formalin-fixed liver tissue using DQ™-gelatin. Green fluorescence indicates gelatinase activity. Photomicrographs (100x magnification) are representative of three mice per treatment group. Scale bars represent 400 μm. *(B)* Quantification of gelantinase activity based on the percentage of green fluorescence coverage per field of liver tissue. Ten fields were analyzed per mouse; three mice were evaluated per treatment group. Data represent mean (± SEM) of three mice per treatment group. Means that do not share a letter are significantly different from each other ($p < 0.05$).

Consequences of TCDD treatment on TIMP mRNA expression in CCl_4-treated mice

MMP activity is not only regulated by gene expression, but also by controlled enzymatic activation and inhibition (Caley *et al.* 2015). All known MMPs can be inhibited by four homologous members of the TIMP family. TIMP1 is a strong inhibitor of many MMPs, but the gelatinases MMP-2 and MMP-9 are also inhibited by other TIMPs. For example, TIMP2, TIMP3 and TIMP4 can inhibit MMP-2 activity, and TIMP3 inhibits MMP-9 (Caley *et al.* 2015). Analysis of TIMP gene expression confirmed our previous finding that TCDD treatment increased *Timp1* expression (Figure 6). It further revealed that TCDD had no impact on the mRNA level of *Timp2*, *Timp3*, or *Timp4* regardless of CCl_4 treatment (Figure 6). Hence, modulation of TIMP gene expression by TCDD appears to be limited to *Timp1*.

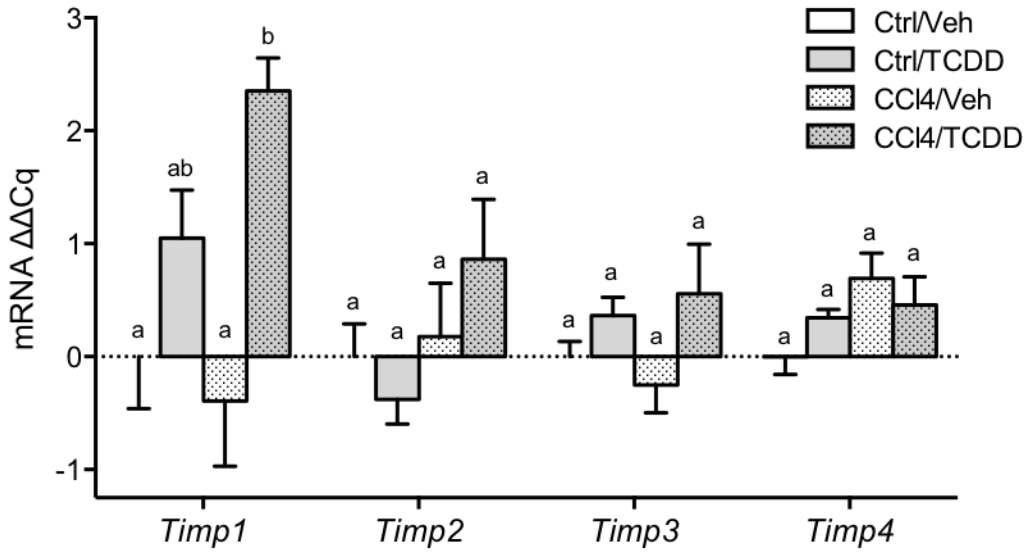

Figure 6. Consequences of TCDD treatment on TIMP mRNA levels in the liver of CCl_4-treated mice. TIMP mRNA expression was measured by qRT-PCR and normalized to GAPDH. Data represent mean (± SEM) of three mice per treatment group. Within the data set for each gene, means that do not share a letter are significantly different from each other ($p < 0.05$).

TCDD treatment modulates expression of molecules in the plasminogen activator/plasmin system.

MMP activation is regulated through numerous mechanisms, including the plasminogen activator/plasmin system, in which tPA and uPA mediate the conversion of plasminogen to plasmin, which directly activates numerous proMMPs (Duarte *et al.* 2015). PAI-1 suppresses MMP proteolytic activity by inhibiting tPA/uPA, and the PAI-1 gene is known to be regulated by AhR activity (Huang and Elferink 2012; Son and Rozman 2002; Wilson *et al.* 2013). To determine how TCDD impacted this pathway of MMP activation, we measured expression of *Plg* (plasminogen), *Plat* (tPA), *Plau* (uPA), and *Serpine1* (PAI-1). Results indicate that TCDD induced a modest, yet statistically

significant, decrease in *Plg* mRNA levels in CCl_4-treated mice (Figure 7). Levels of *Plat* and *Plau* expression were markedly elevated in CCl_4/TCDD-treated mice, with *Plat* levels being about 12-times higher than levels in control mice. Finally, exposure to TCDD increased PAI-1 (*Serpine1*) gene expression regardless of CCl_4-treatment. Hence, based on gene expression data, TCDD treatment modulated the expression of the plasminogen activator/plasmin system.

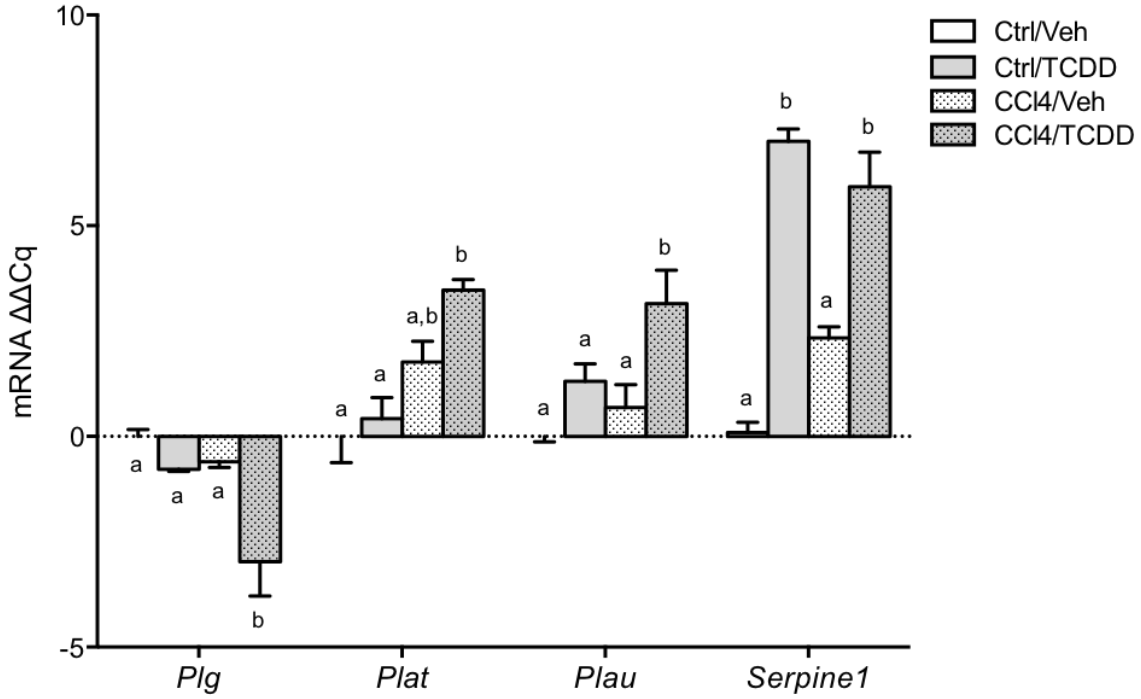

Figure 7. Exposure to TCDD modulates expression of genes in the plasminogen activator/plasmin system. Transcript levels of *Plg* (plasminogen), *Plat* (tPA), *Plau* (uPA) and *Serpine1* (PAI-1) were measured by qRT-PCR and normalized to GAPDH. Data represent mean (± SEM) of three mice per treatment group. Within the data set for each gene, means that do not share a letter are significantly different from each other ($p < 0.05$).

DISCUSSION

The present study investigated the consequences of TCDD treatment on expression of molecules involved in collagen biosynthesis and ECM metabolism during chronic liver injury. We recently reported that exposure to TCDD increased HSC activation and mRNA levels of TGF-β1 and collagen type I in the injured liver without increasing hepatic collagen content or exacerbating fibrosis (Lamb *et al.* in review). This led us to speculate that TCDD treatment may dysregulate ECM remodeling activities, including collagen synthesis.

During fibrosis, the collagen content in the liver can increase up to ten-fold (Rojkind *et al.* 1979). Our results indicate that TCDD treatment alone increased mRNA levels of *Col1a1* and *Col4a3*. This observation corroborates other reports in which exposure to TCDD increased collagen type I and IV (Aragon *et al.* 2008; Nottebrock *et al.* 2006; Pierre *et al.* 2014; Riecke *et al.* 2002; Thackaberry *et al.* 2005). In CCl_4/TCDD-treated mice, there was a marked increase in expression of *Col3a1*, *Col4a5*, *Col6a1*, *Col6a2*, and *Col6a3* mRNA compared to Ctrl/Veh-treated mice. Collagen type III is structurally similar to collagen type I and is the first collagen to increase during chronic liver disease (Wells 2008). Collagen type IV is the primary component of basement membranes, and its expression increases during fibrosis (Ala-Kokko *et al.* 1987). Collagen type VI is also upregulated in liver fibrosis and has been shown to stimulate DNA synthesis and inhibit apoptotic cell death in HSCs *in vitro* (Mak *et al.* 2014). This is intriguing because we previously reported that exposure to TCDD increases HSC proliferation *in vitro* (Harvey *et al.* 2016) and increases HSC activation markers in the liver of CCl_4-treated mice (Lamb *et al.* in review). It is possible that increased expression

of collagen type VI, as well as other types of collagen, contributes to the effects of TCDD in the CCl_4 model system.

The finding that certain collagen genes were upregulated by TCDD treatment only, while others were increased by the combination of TCDD and CCl_4, implies that the AhR may differentially regulate gene expression in the healthy and injured liver. Increasing evidence supports a role for AhR signaling in regulating collagen deposition, including the discovery that AhR knockout mice develop liver fibrosis and have elevated TGF-β1 and collagen expression (Carvajal-Gonzalez *et al.* 2009; Fernandez-Salguero *et al.* 1995; Zaher *et al.* 1998). In addition, it was recently reported that AhR knockdown increased *Col1a1* and *Col4a4* mRNA levels in retinal pigment epithelial cells and choroidal endothelial cells (Choudhary *et al.* 2015). Collectively, these findings implicate a role for AhR activity in regulating collagen gene expression. Future studies that investigate how AhR knockdown impacts gene expression during chronic liver injury will expand our understanding of how the AhR regulates ECM remodeling during states of health and disease.

Not only did TCDD increase the expression of collagen genes, but it also modulated gene expression for several key proteins involved in collagen synthesis. For example, administration of TCDD to CCl_4-treated mice increased gene expression of HSP47, which resides in the endoplasmic reticulum and is involved in the folding and shuttling of collagen molecules to the Golgi (Kawasaki *et al.* 2015). Increased HSP47 levels reportedly contribute to fibrosis by facilitating the excessive assembly and intracellular processing of procollagen molecules, leading to fibrotic lesions (Taguchi and Razzaque 2007). Furthermore, suppression of HSP47 expression was found to reduce

collagen accumulation and delay fibrotic progression (Sunamoto *et al.* 1998). Both procollagen and HSP47 gene expression are regulated by TGF-β1 (Pan and Halper 2003). We previously found that TGF-β1 gene expression was increased in CCl_4/TCDD-treated mice, and speculate that this could drive HSP47 and *Col1a1* expression in our model system. However, TCDD treatment was shown to suppress both *Col1a1* and HSP47 gene expression during fin regeneration in zebrafish, despite increased TGF-β1 expression (Andreasen *et al.* 2007; Andreasen *et al.* 2006).

Decorin is a secreted proteoglycan that has a dual role in liver fibrosis. First, it functions as a naturally occurring TGF-β1 antagonist, and its genetic ablation has been shown to increase ECM deposition, impair matrix degradation, and increase HSC activation (Baghy *et al.* 2012). Second, decorin facilitates the development of normal collagen morphology by binding to the collagen triple helix and preventing the lateral fusion of fibrils (Weber *et al.* 1996). We found that TCDD suppressed decorin gene expression in CCl_4-treated mice. Other studies demonstrate a possible role for AhR signaling in decorin expression. For instance, decorin expression was increased in fibroblasts and vascular smooth muscle cells from AhR-knockout mice (Chang *et al.* 2007; Guo *et al.* 2004).

LOX initiates the cross-linking of collagen fibers, which is important for collagen organization and perhaps also for conferring resistance to proteolytic degradation by MMPs (Kagan and Li 2003). Consistent with this role of LOX, administration of the irreversible LOX inhibitor β-aminopropionitrile (BAPN) to CCl_4-treated mice was reported to reduce collagen cross-linking and produced fibrotic septa with less organized collagen fibers (Liu *et al.* 2015). Our finding that TCDD increased LOX expression in

CCl_4-treated mice could possibly be explained as a compensatory response to increased collagen synthesis, as could the TCDD-induced increase in HSP47. It is worth noting that Andreasen *et al.* found that TCDD treatment suppressed not only LOX2 and HSP47 expression during zebrafish fin regeneration, but also prolyl-4-hydroxylase α1 and 2, which help stabilize collagen crosslinks (Andreasen *et al.* 2006). Based on the role of these molecules in collagen processing and organization, their reduced expression may underlie the accumulation of disorganized collagen observed in the regenerating fin tissue (Andreasen *et al.* 2007). In contrast, we found no evidence that TCDD impacted collagen fiber organization in the liver of CCl_4-treated mice. Increased expression of LOX and HSP47, as well as decreased expression of decorin, could be one possible explanation for this observation.

One of the most consistently reported consequences of TCDD treatment on ECM remodeling is increased MMP expression (Hillegass *et al.* 2006). TCDD treatment increases the expression and activity of MMPs in numerous and diverse cell types, including keratinocytes, macrophages, and endometrial cells (Igarashi *et al.* 2005; Murphy *et al.* 2004; Vogel *et al.* 2004). In the zebrafish model of fin regeneration, TCDD upregulated MMP-13 (Andreasen *et al.* 2006). Similarly, TCDD increased expression of MMP-13, as well as other MMPs, in the fetal mouse heart (Thackaberry *et al.* 2005). These reports support our observation that TCDD increased *Mmp-3*, *Mmp-8*, *Mmp-9*, *Mmp-13* and *Mmp-14* in the mouse liver. MMP-8 and MMP-13 function primarily as collagenases, and these were robustly increased by TCDD regardless of CCl_4 treatment. This is intriguing because it supports our previous finding that TCDD increases collagenase activity in the liver of CCl_4-treated mice (Lamb *et al.* in review). During

ECM breakdown, MMPs with collagenase activity will partially denature collagen, resulting in the production of gelatin, which is metabolized primarily by the gelatinases MMP-2 and MMP-9 (Duarte *et al.* 2015). Decreased gelatinase activity, particularly MMP-2, is associated with increased liver fibrosis development (Preaux *et al.* 1999). The increase in gelatinase activity we observed in CCl_4/TCDD-treated mice could reflect a compensatory response to increased collagenase activity. Furthermore, TCDD also increased expression of MMP-3 (stromelysin) and MMP-14 (membrane-type), both of which have been found to exhibit some collagenase and gelatinase activity.

MMP activity is inhibited through interactions with TIMP proteins, as well as other endogenous inhibitors (Arpino *et al.* 2015). TIMP1, in particular, is associated with ECM proteolysis during fibrosis, and *Timp1*$^{-/-}$ mice display increased liver injury, inflammation, and fibrosis following CCl_4-treatment (Wang *et al.* 2011). TIMP1 is a strong inhibitor of most MMPs except some of the membrane-type MMPs. However, the gelatinase MMPs are inhibited by other TIMPs as well. Specifically, TIMP1 and TIMP3 inhibit MMP-9, and TIMPs 2, 3 and 4 inhibit MMP-2 (Baker *et al.* 2002). In the CCl_4 model system, TCDD treatment increased TIMP1 but had no effect on expression of TIMPs 2, 3 or 4. Thus, it is possible that the expression of TIMPs in CCl_4/TCDD-treated mice was not sufficient to counteract MMP activity. Other studies have reported that TIMP expression is modulated by *in vitro* and *in vivo* TCDD exposure as well (Andreasen *et al.* 2006; Hanlon *et al.* 2005; Martinez *et al.* 2002; Mizutani *et al.* 2004).

Our results demonstrate that TCDD treatment produced changes in the plasminogen activator/plasmin system that modulates MMP activation. TCDD was found to modestly but significantly decrease plasminogen expression in CCl_4-treated mice.

Because MMPs are activated by plasmin, which is produced from plasminogen, this would presumably lead to decreased MMP activation. Given that TCDD increased both collagenase and gelatinase activity in the CCl_4 model system, it is possible that the observed decrease in plasminogen gene expression was not physiologically relevant. It is also possible that increased expression of tPA and uPA compensated for any decrease in plasminogen expression. The TCDD-mediated increase in uPA gene expression corroborates another report showing that TCDD upregulated uPA protein in a human keratinocyte cell line (Gaido and Maness 1995). It is interesting to note that this TCDD-induced increase in uPA appeared to occur through a post-transcriptional mechanism that included changes in mRNA stability (Gaido and Maness 1995; Shimba *et al.* 2000). This same study reported that TCDD increased PAI-2 at the transcriptional level. PAI-1 expression is also recognized as an AhR-regulated target gene and is transcriptionally induced by TCDD through a mechanism that involves heterodimerization of the AhR with KLF-6 and the recruitment of this complex to a non-consensus XRE (Huang and Elferink 2012; Son and Rozman 2002; Wilson *et al.* 2013). We found that TCDD treatment increased PAI-1 gene expression regardless of CCl_4 treatment and presume that this reflects a direct effect of TCDD through AhR-regulated gene expression. Based on our finding that TCDD did not suppress collagenase or gelatinase activity in CCl_4-treated mice, it is possible that increased PAI-1 expression in CCl_4/TCDD-treated mice failed to offset increased tPA/uPA activity. However, MMPs can also be activated through non-plasmin pathways, which leaves open the possibility that MMP activation is increased in CCl_4/TCDD-treated mice despite inhibition of the plasminogen activator/plasmin system by PAI-1.

In conclusion, results from this study demonstrate that AhR activation by TCDD modulates ECM remodeling during chronic liver injury. TCDD treatment was found to increase expression of procollagen genes and molecules involved in collagen processing and maturation. Despite these increases, there were no overt effects of TCDD on collagen distribution or organization in the injured liver. Furthermore, TCDD enhanced gelatinase activity and mRNA levels of several MMPs and TIMP-1. Finally, TCDD increased expression of enzymes in the plasminogen activator/plasmin system that negatively regulates MMP activation. These findings support the conclusion that TCDD modulates gene expression related to ECM remodeling but does not significantly impact collagen deposition during chronic liver injury.

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CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The overall goal for the studies presented in this dissertation was to determine the consequences of TCDD treatment on *in vivo* HSC activation and ECM remodeling. Specifically, we focused our efforts on elucidating these effects during the development of fibrosis in response to chronic liver injury. To do this, we used a mouse model of repeated administration of CCl_4, which elicits liver damage and stimulates HSC activation, leading to the development of fibrosis. Our major findings were that exposure to TCDD increased liver damage, increased HSC activation, and dysregulated ECM gene expression, but did not exacerbate liver fibrosis.

Exposure to TCDD was shown to increase liver damage in CCl_4-treated mice. This is supported by the observation that TCDD treatment elevated serum ALT activity and doubled the combined necroinflammation score in CCl_4-treated mice. Increased inflammation is important because it can perpetuate liver injury and facilitates the development of fibrosis.

HSCs are the central mediators of fibrosis, and we found that TCDD treatment enhances the activation of these cells in the liver of mice treated with CCl_4. This observation corroborates our previous finding that TCDD treatment increases the activation of a human HSC line (Harvey *et al.*, 2016). It also supports the notion that TCDD may directly affect HSCs. This idea is interesting because HSCs have not been particularly well studied as a cellular target for TCDD toxicity. These cells may have

been overlooked due to the fact that TCDD does not appear to elicit robust changes in these cells when they are quiescent, which would occur in the healthy liver. Instead, it is possible that TCDD may only affect these cells as they are becoming activated or already in a state of activation. For this reason, the use of the CCl_4 model system, in which HSCs are known to be activated, is well suited studying how TCDD impacts HSCs.

It is unclear if the TCDD-induced increase in HSC activation *in vivo* is due to a direct or indirect effect of TCDD. It is possible that TCDD could directly target HSCs and activate them through changes in AhR-regulated gene expression. Many of the genes that we see upregulated by TCDD in the CCl_4 model system are attributed to HSCs, which supports the idea that HSCs could be directly affected by TCDD. However, it is also possible that TCDD activates HSCs indirectly by increasing hepatocyte damage, which could exacerbate inflammation and oxidative stress and further activate HSCs. Considering the role that HSCs have in liver homeostasis and pathology, understanding how TCDD disrupts this cell population may reveal novel mechanisms of TCDD hepatotoxicity.

Exposure to TCDD dysregulated the expression of genes involved in ECM maintenance and remodeling. TCDD treatment was found to modulate genes involved in synthesis, processing, and cross-linking of collagen, as well as enzymes and inhibitors involved in ECM remodeling. One of the characteristics of activated HSCs is increased deposition of collagen type I into the ECM. However, we found that TCDD treatment failed to increase the collagen content in the liver despite the TCDD-induced increase in *Col1a1* mRNA. The incongruences between collagen mRNA levels and hepatic collagen protein content could not be explained by TCDD-induced expression of genes involved in

collagen synthesis and fibril formation. However, the finding that TCDD-induced expression and activity of MMPs could indicate that any increase in collagen synthesis by TCDD may be counteracted by increased collagen degradation. Changes in ECM remodeling activities could explain why TCDD treatment failed to exacerbate liver fibrosis in CCl_4-treated mice, despite increased liver injury and HSC activation. Understanding how TCDD impacts ECM remodeling, as well as HSC activation, could lead to identifying a new role for the AhR in limiting the development of fibrosis. This is significant because there are currently no FDA-approved, anti-fibrotic therapies.

FUTURE DIRECTIONS

Based on the finding that TCDD increases activation of HSCs *in vivo*, it is logical to next address whether HSCs are the direct cellular target for TCDD or if increased HSC activation occurs due to the effect of TCDD on hepatocytes. One approach to address this question would be to determine the effect of TCDD treatment in the CCl_4 model system using mice in which either HSCs or hepatocytes do not express an AhR. To this end, the Cre-Lox recombination technology would be particularly suitable for selectively deleting the AhR in either HSCs or in hepatocytes in the mouse liver. This system is based on the activity of an enzyme called Cre recombinase, which targets DNA sequences called Lox elements, which are inserted onto either side of a target gene (Orban *et al*., 1992). Activity of the Cre recombinase can be controlled through the use of promoter sequences that are only expressed in specific cell types. When the Cre recombinase is expressed in a particular cell type, it will then target the Lox elements and delete the target gene. Currently, our laboratory is breeding mice that have the AhR gene flanked by Lox, with

Cre recombinase expression being regulated either by the albumin promoter for AhR deletion in hepatocytes, or by the GFAP promoter to delete the AhR in HSCs.

Using these Cre-Lox mice in the CCl_4 model system, it should be relatively straightforward to determine if TCDD-induced activation of HSCs is dependent on expression of the AhR in hepatocytes or HSCs. Endpoints for testing HSC activation would include those described in Chapter Two. If we found that TCDD induced HSC activation in mice that did not express the AhR in hepatocytes, then we would conclude that HSCs are either directly targeted by TCDD, or that TCDD induces HSC activation indirectly through another mechanism that does not involve hepatocytes. However, if exposure to TCDD failed to activate HSCs in these mice, then we would conclude that HSC activation requires expression of the AhR in hepatocytes. The next step would be to determine if HSC activation required expression of the AhR in HSCs. This would be addressed by performing the same experiments using Cre/GFAP mice, in which the AhR is deleted in the HSC population. Understanding how exposure to TCDD impacts HSC activation *in vivo* will be important for identifying a possible role for the AhR in regulation of fibrogenesis and for elucidating mechanisms of TCDD hepatotoxicity.

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