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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) increases necroinflammation and hepatic stellate cell activation but does not exacerbate experimental liver fibrosis in mice

Cheri L. Lamb^a, Giovan N. Cholico^a, Xinzhu Pu^b, Gerald D. Hagler^c, Kenneth A. Cornell^{a,b,d}, and Kristen A. Mitchell^{a,c,*}

^aBiomolecular Sciences Graduate Program, Boise State University, Boise, ID 83725

^bBiomolecular Research Center, Boise State University, Boise, ID 83725

^cDepartment of Biological Sciences, Boise State University, Boise, ID 83725

^dDepartment of Chemistry and Biochemistry, Boise State University, Boise, ID 83725

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 how TCDD impacts HSC activation *in vivo* using a mouse model of experimental liver fibrosis. To elicit fibrosis, C57BL/6 male mice were treated twice weekly for 8 weeks with 0.5 ml/kg carbon tetrachloride (CCl₄). TCDD (20 µg/kg) or peanut oil (vehicle) was administered once a week during the last 2 weeks. Results indicate that TCDD increased liver-body-weight ratios, serum alanine aminotransferase activity, and hepatic necroinflammation in CCl₄-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- β 1, 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 conclusion that TCDD increases CCl₄-induced liver damage and exacerbates HSC activation, yet collagen deposition and the development of fibrosis may be limited by TCDD-mediated changes in extracellular matrix remodeling.

*Corresponding Author: Kristen Mitchell, Ph.D., Associate Professor, Department of Biological Sciences, Boise State University, 1910 University Drive, Boise, ID 83725-1515, Ph: (208) 426-4620, kristenmitchell@boisestate.edu.

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Keywords

TCDD; hepatic stellate cell; liver; fibrosis; collagen

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) and 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). For example, exposure to TCDD was found to inhibit tissue re-growth in a zebrafish model of fin regeneration (Zodrow and Tanguay, 2003; Mathew *et al.*, 2006). TCDD treatment also inhibits rodent liver regeneration induced by partial hepatectomy (Bauman *et al.*, 1995; Mitchell *et al.*, 2006). Regeneration is regulated by numerous soluble mediators, including the potent pro-fibrogenic molecule transforming growth factor (TGF)- β 1 (Werner and Grose, 2003; Levesque *et al.*, 2007; Ho and Whitman, 2008; Thenappan *et al.*, 2010). Interactions between AhR and TGF- β 1 pathways have been documented, and AhR deficiency was found to increase secretion of TGF- β 1 (Zaher *et al.*, 1998; Guo *et al.*, 2004; Santiago-Josefat *et al.*, 2004). 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 has been shown to alter the expression of ECM proteins, including collagen and fibronectin (Riecke *et al.*, 2002; Andreasen *et al.*, 2006; Nottebrock *et al.*, 2006; Aragon *et al.*, 2008). 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 reportedly increases MMP expression in human keratinocytes, prostate cancer cells, and melanoma cells, and in zebrafish during fin regeneration (Murphy *et al.*, 2004; Haque *et al.*, 2005; Andreasen *et al.*, 2006; 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 protease inhibitors block 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 storage and mobilization of vitamin A. About 50 to 80% of the vitamin A in the body of mammals, including humans, is stored in HSCs in the form of lipid droplets (Blomhoff, *et al.*, 1990). 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). In fact, we recently found in a human HSC line, LX-2, that TCDD treatment increases activation of human HSCs and decreases their vitamin A lipid droplet storage (Harvey *et al.*, 2016). However, the consequences of TCDD on HSC activation *in vivo* are less clear. 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 (α SMA) (Hanberg *et al.*, 1996). However, another study recently found that chronic administration of TCDD in mice increased the expression of α SMA 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 1:10 in corn oil twice a week for 8 weeks (Figure 1). As a control for CCl_4 , mice were injected with corn oil alone. This treatment group is referred to as “Ctrl”. During weeks 7 and 8, mice were gavaged on the first day of each week (days 43 and 50) with TCDD (20 $\mu\text{g}/\text{kg}$; Cambridge Isotope Laboratories, Andover, MA) diluted in peanut oil. This dose of TCDD does not produce lethality and has been shown to reproducibly elicit endpoints of hepatotoxicity in other model systems (Pohjanvirta and Tuomisto, 1994; Whitlock, 1993). As a control for TCDD, mice were treated with an equivalent volume of peanut oil alone. This treatment group is referred to as “Veh”. At the end of the 8-week experiment, mice were euthanized by isoflurane overdosed followed by cervical dislocation. Liver was either flash-frozen in liquid nitrogen or fixed in Ultra Light Zinc Formalin Fixative (PSL Equipment, Vista, CA), and serum was collected and stored at -80°C until assayed. Six mice were used for each treatment group. Mice were housed in a 12:12 hour light/dark cycle, and food and water were available ad libitum. All animal experiments were approved by the Institutional Animal

Care and Use Committee at Boise State University and were conducted in compliance with the regulations and institutional policies that govern animal care and use.

Serum Alanine Aminotransferase (ALT) Activity

Serum samples were diluted 1:10 in phosphate buffered saline (PBS), and 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- μ m 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. Staining was quantified using ImageJ software (US National Institutes of Health), and the amount of staining was expressed as a percentage of total area stained. Sirius red-stained liver tissue was also scored for necroinflammation and fibrosis based on the Ishak Modified Histological Activity Index System (Ishak *et al.*, 1995) by a board-certified pathologist who was blinded to the treatment groups.

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). Four biological replicates were assayed per treatment group, and each sample was run in duplicate. Relative quantification was estimated using the C_q method normalized to GAPDH (Schmittgen and Livak, 2008). Gene expression data is presented as fold-change compared to Ctrl/Veh-treated mice.

Immunofluorescence Detection of α SMA

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 hours in immunofluorescence buffer (TBS with 1% bovine serum albumin and 2% fetal bovine serum). Cy3-conjugated anti- α SMA 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 α SMA 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 α SMA 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 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. Protein content was determined using a DC^{TM} Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA), and homogenates were diluted to 5 mg/ml. To reduce nonspecific binding of the anti-collagen type I antibody, liver homogenates were digested with pepsin prior to electrophoresis. To accomplish this, pepsin (Powder 1:3000; VWR, Radnor, PA) was diluted to 2 mg/ml in 2 N HCl, and 10 μL of this pepsin preparation was added to 100 μL of liver homogenate (500 μg protein). This sample was incubated for 2 hours at 20°C and 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 $\mu\text{L}/\text{lane}$) were resolved on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with an anti-collagen type I antibody (#AB765P, EMD Millipore, Hayward, CA). Undigested liver homogenates (25 μg protein/lane) were probed with an anti-actin antibody (Santa Cruz Biotech, Dallas, TX) and served as a quasi-loading control. Blots were subsequently incubated with species-specific, HRP-conjugated secondary antibodies, and bands were visualized with PierceTM 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 $1000 \times g$ 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 $\times$ 2.0 mm, 4 μm , Phenomenex, Torrance, CA) with a guard column and a flow rate of 150 $\mu\text{L}/\text{min}$. 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-buffered, formalin-fixed, paraffin-embedded liver tissue as described elsewhere (Hadler-Olsen *et al.*, 2010; Kumar *et al.*, 2014). Zinc-buffered formalin fixative has been shown to preserve the morphological and functional properties of tissues, including the enzymatic activity of matrix metalloproteinases (Beckstead, 1994), which are zinc-dependent endopeptidases that cleave extracellular matrix proteins. Briefly, liver tissue sections (8 μ m) were heated at 58°C for 12 hours, deparaffinized, and rehydrated. DQTM 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 CaCl₂ (pH 7.6). Tissue sections were incubated with the DQ-collagen solution for 12 hours at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and cover slips were mounted with Permount. Control slides were incubated with 20 mM EDTA for 1 hour prior to incubation with DQ-collagen substrate, which also contained 20 mM EDTA, in order to inhibit MMP activity. These slides were used to control for background autofluorescence. Fluorescent images were taken with an EVOS fluorescence microscope using a 20X objective.

Immunohistochemistry for MMP-13

Paraffin-embedded liver tissue sections (2 μ m) 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) and counterstained with hematoxylin. Images were captured with an Olympus BX53 compound microscope.

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 CCl₄-treated mice

To investigate how TCDD treatment impacts liver damage and fibrogenesis during chronic CCl₄ administration, we began by evaluating gross markers of hepatotoxicity. TCDD treatment induced marked hepatomegaly in control-treated and CCl₄-treated mice (Figure 2), which corresponded with increased liver weights and liver-to-body-weight ratios (Table 2). CCl₄ treatment alone had no effect on liver weight or liver-to-body-weight ratios. The livers from CCl₄/TCDD-treated mice were also visually distinguishable from the other treatment groups based on the slightly pale appearance (Figure 2). Serum activity levels of alanine aminotransferase (ALT) were measured as an indication of hepatocellular necrosis. Treatment with either TCDD or CCl₄ 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 CCl₄ (Mejia *et al.*, 2013; Scholten *et al.*, 2015). It is worth noting that 40% mortality was observed two days after the last injection of TCDD in CCl₄-treated mice (data not shown), whereas no mortality occurred in the other treatment groups.

TCDD increases necroinflammation in the liver of CCl₄-treated mice

During chronic CCl₄ 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 (Figure 3, A-D). Analysis of livers from mice treated with CCl₄ revealed ballooning degeneration of hepatocytes, coagulation necrosis and necrotic bridge formation (Figure 3, E-F). Administration of TCDD to CCl₄-treated mice evoked widespread coagulation necrosis and inflammation (Figure 3, 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 CCl₄ 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 CCl₄ were determined to display “mild” necroinflammation. In contrast, administration of TCDD to CCl₄-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.

TCDD increases expression of HSC activation markers

HSCs are the primary source of activated myofibroblasts during chronic administration of CCl₄ (Iwaisako *et al.*, 2014). To determine how TCDD exposure impacted HSC activation in response to CCl₄, expression of the HSC activation marker α SMA was measured. Based on immunofluorescence staining, α SMA expression increased in response to CCl₄ treatment (Figure 4A). Quantification of staining revealed that administration of TCDD to CCl₄-treated mice evoked a 2-fold increase in α SMA expression compared to mice treated with CCl₄ alone (Figure 4B). While this effect was not statistically significant, similar results were produced when mRNA levels were measured. In fact, α SMA mRNA expression was about 40 times higher in mice treated with the combination of TCDD and CCl₄ as compared to all other treatment groups (Figure 4C). A similar effect of TCDD was observed in the mRNA levels of desmin (Figure 4D) and delta-like homolog 1 (DLK-1; Figure 4E), which are two other markers selectively expressed by activated HSCs (Zhu *et al.*, 2012).

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

Based on increased HSC activation, we hypothesized that TCDD treatment would increase fibrogenesis in CCl₄-treated mice. Increased production and activation of the profibrogenic mediator TGF- β 1, as well as synthesis and deposition of collagen, are important for the pathogenesis of fibrosis (Kisseleva and Brenner, 2008). Results indicate that exposure to TCDD increased TGF- β 1 mRNA levels regardless of CCl₄ treatment (Figure 5A). CCl₄

treatment increased mRNA levels of Col1a1, which encodes the alpha-1 chain of collagen type I, whereas TCDD treatment alone had no effect (Figure 5B). In mice that received both TCDD and CCl₄, 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, CCl₄ administration increased the deposition of collagen in the liver (Figure 5C). However, exposure to TCDD did not appear to increase collagen content, and this finding was confirmed when staining was quantified (Figure 5D). 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 5E). Moderate fibrosis was observed in CCl₄-treated mice, and TCDD treatment did not increase the fibrosis score in these mice.

TCDD does not increase the collagen content in the liver of CCl₄-treated mice

Because TCDD treatment did not appear to increase collagen content or fibrosis in CCl₄-treated mice despite increased mRNA levels of TGF- β 1 and Col1a1, additional analyses were performed to determine how TCDD impacted collagen content of the liver. Western blot analysis revealed that CCl₄ administration increased expression of collagen type I protein, and TCDD had no additive effect on protein levels (Figure 6A). Further analysis was performed using mass spectrometry to quantify hepatic levels of hydroxyproline, which is a major component of collagen (Figure 6B). Results indicate that CCl₄ administration increased the amount of hydroxyproline in the liver, and no overt increases were detected when TCDD was administered to CCl₄-treated mice (Figure 6C). Collectively, these results strengthen the notion that TCDD treatment does not increase the hepatic collagen content in CCl₄-treated mice.

Collagenase expression and activity is increased in TCDD-treated mice

The finding that TCDD increased liver injury, inflammation, and HSC activation in CCl₄-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 CCl₄ (Figure 7A).

MMP-13 is a prominent murine collagenase that is important for cleavage of ECM components (Giannandrea and Parks, 2014). A preliminary analysis of MMP-13 protein and mRNA levels revealed a trend towards increased expression in response to TCDD, although differences between treatment groups were not statistically significant (Figure 7 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 CCl₄-treated mice (Figure 7D). 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 7E). Taken together, these results indicate that TCDD treatment may dysregulate the expression and activity of molecules involved in ECM remodeling.

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 CCl₄ 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 CCl₄-treated mice. This corroborates a recent report that pretreatment of mice with TCDD two days prior to a single injection of CCl₄ increased liver injury compared to mice treated with CCl₄ 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 CCl₄ (Mejia-Garcia *et al.*, 2013). Our finding that TCDD treatment increases HSC activation in CCl₄-treated mice could result from a direct effect of TCDD on HSCs or it could reflect a compensatory response to the increased liver damage observed in CCl₄/TCDD-treated mice.

It is possible that TCDD increases liver damage in CCl₄-treated mice by enhancing liver inflammation. The finding that TCDD increased the prevalence of inflammatory foci in the liver of CCl₄-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). Yet 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). An alternative explanation for how TCDD exacerbates liver damage in CCl₄-treated mice stems from other reports that TCDD inhibits hepatocyte proliferation (Bauman *et al.*, 1995; Kolluri *et al.*, 1999; Mitchell *et al.*, 2006). After toxicant exposure, hepatocyte proliferation is important for maintaining liver function and homeostasis. Hepatocytes that do not enter the cell cycle are susceptible to collateral damage due to the release of soluble mediators from dying cells (Limaye *et al.*, 2003). Such increased liver damage in cell cycle-delayed hepatocytes could contribute to increased liver damage in TCDD-treated mice. Future studies are needed to determine the mechanism by which TCDD exacerbates CCl₄-induced liver damage. The finding that TCDD treatment increased HSC activation in CCl₄-treated mice could also reflect a direct effect of TCDD on HSCs. This notion is supported by our previous finding that TCDD treatment increases activation of the human HSC line, LX-2 (Harvey *et al.*, 2016). 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 it is possible that TCDD-induced alterations in this population of cells have been inadvertently overlooked. Reports in the literature do indicate that a single dose of TCDD suppresses vitamin A

storage in the rat liver, which is consistent with HSC activation (Thunberg *et al.*, 1980; Hakansson and Hanberg, 1989). However, TCDD was found to have no effect on expression of the HSC activation marker, α SMA. It is conceivable that acute exposure to TCDD alone (i.e. in the absence of a hepatotoxicant such as CCl₄) does not provide a sufficient stimulus to evoke the activation of quiescent HSCs 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 CCl₄-treated mice when HSC activation is initiated in response to liver damage (Figure 4). Along these same lines, it was recently reported that chronic exposure to TCDD increased expression of α SMA and collagen type I (Pierre *et al.*, 2014), although HSC activation was not formally assessed. The authors of the study, in which mice were treated with TCDD at 25 μ g/kg/week for six weeks, reported that chronic TCDD administration increased liver damage, which could have provided the stimulus necessary to initiate HSC activation. Our study used a similar dose of TCDD (20 μ g/kg), but it was administered only twice and towards the end of the experiment, when HSCs presumably would have been activated in response to CCl₄-induced liver damage. It is interesting to note that the fibrosis associated with chronic exposure to TCDD appears to occur in the portal region of the liver (NTP, 2004; Ovando *et al.*, 2010). In contrast, collagen deposition in CCl₄-treated mice is initiated in centrilobular regions, as fibrous septa form connections between central veins (Hatori *et al.*, 2015). We did not find any evidence of periportal fibrosis in CCl₄ mice regardless of TCDD treatment (data not shown). Given the association between TCDD and periportal fibrosis, it is intriguing to consider the possibility that chronic TCDD treatment targets the activation of portal fibroblasts, which represent another source of myofibroblasts precursors in the liver distinct from HSCs. An analysis of collagen distribution in the liver of mice chronically treated with TCDD and CCl₄, as well as the isolation of distinct populations of myofibroblasts from the liver, could prove useful in identifying mechanisms by which TCDD impacts fibrogenesis.

Given that TCDD treatment increased the activation of HSCs, which are the central mediators of CCl₄-induced liver fibrosis, it was not surprising that TCDD also increased TGF- β 1 and Col1a1 mRNA levels in CCl₄-treated mice. TGF- β 1 is activated in response to reactive oxygen species generated from chronic liver injury and inflammation (Schon & Weiskirchen, 2014), and the active form of TGF- β 1 induces Col1a1 gene 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- β 1 and Col1a1 mRNA levels, we found no evidence that TCDD treatment exacerbated fibrosis in CCl₄-treated mice, based on histopathological analysis as well as measurements of collagen and hydroxyproline content in the mouse liver. Instead, it appeared that TCDD may activate pathways leading to collagen degradation in CCl₄-treated mice, based on increased collagenase activity and possibly increased MMP-13 activity in the liver of CCl₄/TCDD-treated mice. These findings corroborate other studies in which TCDD was reported to increase expression of the collagenase MMP-13 in zebrafish and cell culture (Andreasen *et al.*, 2006; Andreasen *et al.*, 2007). 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 CCl₄/TCDD-treated mice is essentially counteracted by increased collagen breakdown, leading to no net increase in fibrosis when compared to mice treated with CCl₄ alone. Future studies will be necessary to identify the cellular source of MMP-13, which could include macrophages that infiltrate the fibrotic scar.

The notion that TCDD increases collagenase activity in CCl₄-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, and it is conceivable that TIMP-1 expression increased in CCl₄/TCDD-treated mice in response to increased MMP-1 expression. However, it is possible that TIMP-1 levels in CCl₄/TCDD-treated mice still remained insufficient for suppressing MMP activity. Another inhibitor, PAI-1, inhibits the conversion of plasminogen to plasmin, the latter of which activates MMPs. Increased PAI-1 expression could occur in response to elevated TGF- β 1 levels in CCl₄/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 repress fibrogenic gene expression in quiescent HSCs (Ghiassi-Nejad *et al.*, 2013). It is conceivable 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 (Suzuki *et al.*, 1990; Hahn-Dantona *et al.*, 1999), 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 that occurs in response to liver injury.

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Highlights

- TCDD increased liver damage and inflammation in mice treated with CCl₄.
- TCDD treatment enhanced markers of hepatic stellate cell activation and fibrogenesis.
- TCDD did not increase the deposition of collagen type I or the severity of liver fibrosis.
- TCDD increased hepatic collagenase activity and expression of matrix metalloproteinase-13.

Figure 1. Experimental Design

Mice were injected i.p. with 0.5 ml/kg CCl_4 or corn oil alone twice a week for 8 weeks.

During weeks 7 and 8, mice were gavaged on the first day of each week (days 43 and 50) with 20 μ g/kg TCDD or vehicle (peanut oil).

Figure 2. TCDD treatment, but not CCl₄ administration, elicits hepatomegaly
C57BL/6 mice were treated with CCl₄ 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.

Figure 3. TCDD increases necroinflammation in the liver of CCl₄-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 CCl₄/Veh-treated mouse contains balloon (“B”) cells, coagulation necrosis (“CN”) and necrotic bridge (“NB”) formation. (*G, H*) Liver from a CCl₄/TCDD-treated mouse reveals widespread coagulation necrosis, infiltration of inflammatory cells, ballooning hepatocytes, and necrotic bridges.

Figure 4. TCDD increases markers of HSC activation in CCl₄-treated mice

(A) Immunofluorescence was used to detect expression of alpha-smooth muscle actin (α SMA; red) in paraffin-embedded liver tissue; nuclei were stained with DAPI (blue). (B) α SMA immunofluorescence staining was quantified and expressed as a percentage of the total area stained. (C-E) Hepatic mRNA levels (mean $\pm$ SEM) of α SMA, desmin, and DLK-1 are represented relative to Ctrl/Veh-treated mice (n=4). Means that do not share a letter are significantly different from each other ($p < 0.05$).

Figure 5. Consequences of TCDD on the development of fibrosis in CCl₄-treated mice (A, B) Hepatic mRNA levels (mean $\pm$ SEM) of TGFβ1 and Col1a1 expressed relative to Ctrl/Veh-treated mice (n=4). 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. (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. Six mice were evaluated per treatment group.

Figure 6. TCDD treatment does not increase collagen protein levels in the liver of CCl₄-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. Hydroxyproline 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 $\pm$ SEM) in liver of mice from all four treatment groups ($n=4$). Means were not significantly different at $p < 0.05$.

Figure 7. 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 (20X). Green fluorescence reveals collagenase activity; nuclei were stained with DAPI (blue). Scale bar depicts 200 μ m. (B) Representative MMP-13 protein expression detected in paraffin-embedded liver tissue using immunohistochemistry (40X). Scale bar depicts 100 μ m. (C-E) Hepatic mRNA levels (mean $\pm$ SEM) of MMP-13, TIMP-1 and PAI-1 expressed relative to Ctrl/Veh-treated mice (n=4). Means that do not share a letter are significantly different from each other ($p < 0.05$).

Table 1

Mouse primer sequences used for qRT-PCR

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

Table 2Consequences of TCDD on liver weight, body weight, and serum ALT activity in CCl₄-treated mice

	Ctrl/Veh	Ctrl/TCDD	CCl ₄ /Veh	CCl ₄ /TCDD
Liver weight (g)	1.50 ± 0.07	1.74 ± 0.58 [*]	1.38 ± 0.06	1.72 ± 0.07 [*]
Body weight (g)	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 ^{*†}	0.052 ± 0.002	0.066 ± 0.001 ^{*†}
ALT (U/L)	27.22 ± 12.26	198.70 ± 44.27 [†]	130.74 ± 10.38 [†]	282.22 ± 46.67 ^{*†}

Values represent mean ± SEM.

^{*}
p < 0.05 when compared to CCl₄/Veh-treated mice.[†]
p < 0.05 when compared to Ctrl/Veh-treated mice. Six individual mice were evaluated per treatment group.

Table 3Effects of TCDD treatment on necroinflammation during CCl₄-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 *	1.50 ± 0.22 *	4 ± 0 * [†]
Confluent necrosis (0-6)	0	1.14 ± 0.26 *	1.33 ± 0.21 *	5 ± 0 * [†]
Focal lytic necrosis, apoptosis, and focal inflammation (0-4)	0	1.71 ± 0.29 *	1 ± 0	1.33 ± 0.33 *
Portal inflammation (0-4)	0	1.86 ± 0.34 *	1.83 ± 0.17 *	3.33 ± 0.33 * [†]
Combined necroinflammation score:	0	6.57 ± 0.81 * (mild)	5.67 ± 0.33 * (mild)	13.67 ± 0.33 * (severe)

Necroinflammation was assessed using the Ishak Modified Histological Activity Index System (Ishak *et al.*, 1995). Numbers in parentheses indicate the scoring range for each feature. Values represent mean ± SEM.

* $p < 0.05$ when compared to Ctrl/Veh-treated mice.

[†] $p < 0.05$ when compared to CCl₄/Veh-treated mice. Six individual mice were evaluated per treatment group.