Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases human hepatic stellate cell activation

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a halogenated aromatic hydrocarbon that elicits toxicity through the aryl hydrocarbon receptor (AhR). In the liver, gross markers of TCDD toxicity are attributed to AhR activation in parenchymal hepatocytes. However, less is known regarding the consequences of TCDD treatment on non-parenchymal cells in the liver. Hepatic stellate cells (HSCs) are non-parenchymal cells that store vitamin A when quiescent. Upon liver injury, activated HSCs lose this storage ability and instead function in the development and maintenance of inflammation and fibrosis through the production of pro-inflammatory mediators and collagen type I. Reports that TCDD exposure disrupts hepatic retinoid homeostasis and dysregulates extracellular matrix remodeling in the liver led us to speculate that TCDD treatment may disrupt HSC activity. The human HSC line LX-2 was used to test the hypothesis that TCDD treatment directly activates HSCs. Results indicate that exposure to 10 nM TCDD almost completely inhibited lipid droplet storage in LX-2 cells cultured with retinol and palmitic acid. TCDD treatment also increased LX-2 cell proliferation, expression of α -smooth muscle actin, and production of monocyte chemoattractant protein-1 (MCP-1), all of which are characteristics of activated HSCs. However, TCDD treatment had no effect on *Colla1* mRNA levels in LX-2 cells stimulated with the potent profibrogenic mediator, transforming growth factor- β . The TCDD-mediated increase in LX-2 cell proliferation, but not MCP-1 production, was abolished when phosphoinositide 3-kinase was inhibited. These results indicate that HSCs are susceptible to direct modulation by TCDD and that TCDD likely increases HSC activation through a multifaceted mechanism.

1. Introduction

The liver is a target organ for toxicity resulting from exposure to the halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicity of TCDD and related chemicals is mediated through the aryl hydrocarbon receptor (AhR), which is a soluble protein in the basic helix-loop-helix/*Per-Arnt-Sim* superfamily (Massari and Murre 2000). Upon TCDD exposure, the activated AhR translocates to the nucleus, where it dimerizes with the Ah receptor nuclear translocator (ARNT) and binds to dioxin response elements (DREs) in the genome. In the liver, this sequence of events culminates in the induction of a battery of xenobiotic metabolizing enzymes and the onset of hepatotoxicity, as evidenced by hepatocellular damage, hepatomegaly, and hydropic degeneration (Hankinson 1995).

Studies of conditional AhR-deficient mice demonstrated that AhR signal transduction in parenchymal hepatocytes was necessary to produce classic endpoints of TCDD hepatotoxicity, namely hepatomegaly, increased serum alanine aminotransferase (ALT), and pathological changes (Walisser et al. 2005). Interestingly, upregulation of dioxin-inducible xenobiotic metabolizing enzymes was observed in both hepatocytes and non-parenchymal cells, albeit to different extents. Non-parenchymal cells contribute to liver homeostasis through diverse processes, such as wound healing, inflammation, immunity, and angiogenesis (Ishibashi et al. 2009). Therefore, AhR-mediated disruption of non-parenchymal cell activity could underlie the development of numerous hepatotoxic effects of TCDD, such as steatosis, inflammation, portal fibrosis, bile-duct hyperplasia and carcinogenesis (Yoshizawa et al. 2007). However, relatively little is known regarding the consequences of TCDD treatment on liver non-parenchymal cells.

Hepatic stellate cells (HSCs) are liver non-parenchymal cells involved in vitamin A storage and wound healing responses. In their quiescent form, HSCs store dietary vitamin A as retinyl esters inside cytoplasmic lipid droplets (Blomhoff et al. 1985). Upon liver injury, HSCs

become activated and lose this storage capacity. Activated HSCs transition to a myofibroblast-like phenotype, which is characterized by increased proliferation and contractility, production of proinflammatory and profibrogenic mediators, and synthesis of extracellular matrix components like collagen type I (Puche et al. 2013). Activated HSCs promote inflammation, innate immunity, and wound healing and are central to the development of liver fibrosis.

Several reports support the assertion that AhR signaling impacts HSC activation. One compelling line of evidence stems from studies in which TCDD was found to reduce vitamin A accumulation in the rodent liver (Hanberg et al. 1998; Håkansson and Ahlborg 1985; Håkansson and Hanberg 1989; Thunberg et al. 1980). Loss of vitamin A was attributed to increased mobilization and excretion of retinoids from the liver, which coincided with increased kidney and serum retinoid concentrations (Håkansson and Ahlborg 1985). When liver cells were separated, vitamin A levels in the non-parenchymal fraction from TCDD-treated rats were 30% lower than in control rats, whereas vitamin A content in parenchymal cells was not affected by TCDD (Håkansson and Hanberg 1989). TCDD also had no effect on the number of HSCs in the rat liver, which indicates that the loss of vitamin A storage most likely occurred as a result of HSC activation, rather than HSC deletion (Hanberg et al. 1996). However, there are conflicting reports as to whether or not TCDD activates HSCs in the rodent liver. For instance, Hanberg et al. reported that levels of the HSC activation marker α -smooth muscle actin (α SMA) were not increased in rats treated with TCDD (Hanberg et al. 1996). In contrast, Pierre et al. recently reported that treatment of mice with TCDD increased the expression of α SMA and collagen type I (Pierre et al. 2014). Hence, the consequences of TCDD exposure on HSC activation remain unclear.

In this study, we investigated the direct consequences of TCDD treatment on the human HSC line, LX-2. This cell line is derived from healthy liver tissue and grown in reduced serum, and gene expression in these cells is reportedly >98% similar to primary human HSCs (Friedman et al. 1992; Xu et al. 2005). LX-2 cells display a quasi-activated phenotype, characterized by the expression of surface markers and growth factor receptors that are also detected in activated HSCs in vivo, but not in quiescent cells (Xu et al. 2005). Furthermore, LX-2 cell activation is responsive to matrix stiffness, and activation increases over time when LX-2 cells are grown on uncoated plastic (Xu et al. 2005). In the present study, experiments were performed to test the hypothesis that TCDD treatment directly increases LX-2 cell activation. The endpoints of activation included loss of vitamin A storage, increased proliferation, elevated production of pro-inflammatory and pro-fibrogenic mediators, and production of collagen type I.

2. Materials and Methods

2.1. LX-2 Cell Culture

The human hepatic stellate cell line, LX-2, was kindly provided by Dr. Scott Friedman (Mt. Sinai School of Medicine, New York, NY). LX-2 cells were cultured in DMEM with 5% fetal bovine serum (FBS) and penicillin/streptomycin at 37° C in 5% CO₂, and plated in six-well tissue culture plates (1-2x10⁵ cells/well) or on glass cover slips. At 70% confluency, cells were treated with either 10 nM TCDD (Cambridge Isotope Labs, Andover, MA) diluted in dimethyl sulfoxide (DMSO) or with an equivalent volume of DMSO alone (0.2% vol/vol). This concentration of TCDD has been shown to reproducibly elicit differential gene expression in human hepatoma cells, human liver adult stem cells, and primary hepatocytes from human, mouse, and rat (Dere et al. 2011; Forgacs et al. 2013; Gohl et al. 1996; Kim et al. 2009). The 10 nM concentration of TCDD is not overtly toxic to primary hepatocytes (Black et al. 2012) and is

about 10-fold less than the hepatic TCDD concentration in rats 7 days after a single subcutaneous dose of 3 µg/kg TCDD (Abraham et al. 1988). After 4 to 96 hours, cells were collected with 0.25% trypsin + EDTA, and viable cells were counted by trypan blue exclusion. Cell lysates and supernatant were stored at -80°C until used. For the PI3 kinase inhibition experiments (Fig. 7), cells were treated with 25 nM wortmannin (Enzo, New York, NY) 30 minutes prior to administration of TCDD or DMSO.

2.2. RT-PCR to Measure LX-2 Cell Gene Expression

RNA was collected from LX-2 cell lysates using a commercially available RNA isolation kit (Qiagen, Valencia, CA). For each sample, 1 µg of RNA was transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA), and 50 ng of cDNA was then amplified by PCR, using the primer sequences and annealing temperatures listed in Table 1 (Chen et al. 2011; Guo et al. 2009). The PCR protocol consisted of a 5-min denaturation step at 95° C, followed by 30 cycles (30 s at 95° C, 30 s at the annealing temperature, and 30 s at 72° C), and a final extension for 5 min at 72° C. PCR products were resolved on 2% agarose gels, and bands were visualized with ethidium bromide. GAPDH was used as a loading control. Band intensities of the PCR products were compared to the band intensity of GAPDH in the same sample. Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD) to quantitate expression levels of MCP-1.

2.3. Detection of Lipid Storage in LX-2 Cells

To induce the uptake and storage of vitamin A, LX-2 cells (2×10^5 cells) were plated on 25 x 25 mm glass cover slips and cultured for 48 hr with 5 µM all-trans retinol (Thermo Fisher

Scientific, Waltham, MA) and 100 μ M palmitic acid (MP Biomedicals, Santa Ana, CA). TCDD (10 nM) or DMSO (0.1% vol/vol) was either added at the same time as the retinol and palmitic acid (Figure 2A) or else 48 hr after retinol and palmitic acid (Figure 2B). At the end of the culture period, cells were fixed in 1% formalin overnight, and lipid droplets were stained with Oil Red O (Alfa Aesar, Ward Hill, MA). Cells were counterstained with hematoxylin (Bi-Biomics, Nampa, ID). Red-stained intracellular lipid droplets were visualized by light microscopy.

2.4. Measurement of MCP-1 by ELISA

MCP-1 concentrations were measured in LX-2 cell culture supernatants using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Samples were run in duplicate. The limit of detection for this assay was 5 pg/ml.

2.5. Immunofluorescent Detection of α SMA

LX-2 cells were plated on 25 x 25 mm glass cover slips and treated for 24 hr with either 10 nM TCDD or DMSO. Cells were then fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.5% Triton X-100 in 10 mM Tris-EDTA (pH 9) for 10 minutes at RT. Cover slips containing cells were then incubated overnight with an anti- α SMA antibody (Thermo Fisher Scientific) at a 1:100 dilution followed by incubation with DyLight 549-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent

images were collected using a Zeiss LSM 510 Meta system with Axiovert Observer Z1 inverted microscope and ZEN 2009 imaging software (Carl Zeiss, Thornwood, NY).

2.6 Induction and measurement of procollagen $\alpha 1(I)$ in LX-2 cells. LX-2 cells were cultured for 48 hr in DMEM with 0.2% FBS. Cells were then treated for 24 hr with 2.5 ng/ml human recombinant transforming growth factor (TGF)- $\beta 1$ (R&D Systems, Minneapolis, MN) and either TCDD (10 nM) or DMSO. Total RNA was extracted using the Omega Bio-Tek E.Z.N.A.[®] Total RNA Kit (Norcross, GA) and was reverse-transcribed using the Applied Biosystems High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Procollagen $\alpha 1(I)$ -specific primers (Table 1) were used for quantitative real-time RT-PCR using FastStart Essential DNA Green Master reaction mix on a Roche Light Cycler 96 (Indianapolis, IN). Samples were analyzed in duplicate from 5 biological replicates per treatment group. Relative quantification was estimated using the $\Delta\Delta C_t$ method normalized to β -actin.

2.7. Statistics

Data were analyzed using a two-way analysis of variance followed by a Bonferroni's post-hoc test for multiple comparisons or by a Student's *t*-test for comparison between two groups. Statistical significance was determined using Prism 4 software (GraphPad Software, San Diego, CA). Data were considered statistically significant at $p < 0.05$.

3. Results

3.1. LX-2 Cells Express a Functional AhR

AhR and AhR-regulated gene expression was investigated in LX-2 cells treated with either DMSO or 10 nM TCDD for 24 hours. Gene expression was measured by RT-PCR. AhR mRNA was detected in LX-2 cells and expression was not markedly different between treatment groups (Fig. 1). TCDD treatment increased expression of the AhR-regulated gene, *Cyp1a1*, which indicates that the AhR is responsive to TCDD and participates in DRE-mediated signaling in LX-2 cells.

3.2. TCDD Treatment Decreases Vitamin A Storage in LX-2 Cells

TCDD treatment has been reported to decrease vitamin A stores in the rat liver (Hanberg et al. 1996; Hanberg et al. 1998; Håkansson and Hanberg 1989). Given that HSCs store the majority of vitamin A in the liver (Senoo et al. 2010), and that these cells lose this storage capacity upon activation (Friedman 2008), we hypothesized that TCDD treatment would reduce vitamin A storage in LX-2 cells. To test this, LX-2 cells were cultured with retinol and palmitic acid in the presence of 10 nM TCDD or DMSO, and lipid droplets were identified with oil red O stain. After 48 hr, abundant lipid droplets were detectable in DMSO-treated cells (Fig. 2A). However, little to no lipid droplets were detected in TCDD-treated cells. In a separate experiment, LX-2 cells were pretreated with retinol and palmitic acid to induce lipid storage and promote cellular quiescence, and then TCDD or DMSO was added for an additional 48 hr. Under these conditions, TCDD treatment had no effect on the lipid content (Fig. 2B). Hence, TCDD

treatment suppressed lipid storage in quasi-activated LX-2 cells but not in quiescent cells that were preloaded with lipid.

3.3. TCDD Treatment Increases Proliferation of LX-2 Cells

To test the hypothesis that TCDD treatment enhances HSC activation, LX-2 cells were treated with 10 nM TCDD or DMSO, and viable cells were counted 24, 48, 72 and 96 hr later. No statistically significant changes in proliferation were observed before 72 hr of culture. However, TCDD treatment significantly increased the number of viable cells at 72 and 96 hr compared to DMSO-treated cells (Fig. 3), supporting the idea that TCDD increases HSC activation.

3.4. TCDD Treatment Increases MCP-1 Production by LX-2 Cells

In addition to proliferation, another characteristic of activated HSCs is the production of cytokines, chemokines and growth factors. One soluble mediator known to be produced by HSCs is monocyte chemoattractant protein-1 (MCP-1), which regulates monocyte and lymphocyte trafficking (Marra et al. 1999). LX-2 cells were treated with 10 nM TCDD or DMSO for 24 hr, and MCP-1 levels were measured by ELISA. MCP-1 levels were elevated in DMSO-treated cells 24 hr after culture, which presumably reflects culture-induced activation of LX-2 cells (Xu et al. 2005). TCDD treatment further increased MCP-1 production by about two-fold (Fig. 4A). Likewise, MCP-1 mRNA levels were significantly increased in TCDD-treated cells at 24 hr compared to vehicle-treated cells (Fig. 4B).

3.5. TCDD Treatment Increases α SMA Expression in LX-2 Cells

To further test the consequences of TCDD treatment on LX-2 cell activation, we measured expression of α SMA, which is a myofibroblast marker expressed on activated HSCs. LX-2 cells were treated with 10 nM TCDD or DMSO for 24 hr, and α SMA expression was examined by immunofluorescent staining and confocal microscopy. Minimal α SMA expression was detected in untreated LX-2 cells (Fig. 5A), and α SMA expression was largely unchanged in cells treated with DMSO for 24 hr (Fig. 5B). However, a marked increase in α SMA expression was detected in LX-2 cells cultured for 24 hr in the presence of TCDD (Fig. 5C). This finding supports the notion that TCDD treatment promotes the transition of LX-2 cells to a myofibroblast-like phenotype characteristic of activated HSCs.

3.6. TCDD Treatment Does Not Increase Collagen Type I Production in LX-2 Cells

Activated HSCs are an abundant source of collagen type I, which is important for wound healing and excessively produced during liver fibrosis. In the liver, HSCs produce collagen in response to profibrogenic mediators, namely transforming growth factor (TGF)- β 1. To determine if TCDD treatment enhances collagen type I production in HSCs, LX-2 cells were cultured for 24 hr with TGF- β 1 and either DMSO or TCDD, and *Colla1* mRNA levels were measured. TGF- β 1 stimulated *Colla1* gene expression, but expression did not change between DMSO- and TCDD-treated cells (Figure 6). Likewise, TCDD had no effect on *Colla1* mRNA levels in unstimulated LX-2 cells.

3.7. Role of PI3K Signaling in TCDD-Mediated LX-2 Cell Activation

To begin to understand how TCDD treatment increases certain endpoints of HSC activation, we examined phosphatidylinositol 3-kinase (PI3K) activity, which is known to

control numerous cellular functions including proliferation, survival, and migration (Porta et al. 2014). PI3K signaling has been reported to facilitate HSC proliferation and collagen type I gene transcription, whereas inhibitors of PI3K signaling suppress extracellular matrix deposition and expression of profibrogenic factors (Gentilini et al. 2000; Reif et al. 2003; Son et al. 2009). To investigate the role of this signaling pathway in the TCDD-mediated activation of HSCs, LX-2 cells were treated with wortmannin to inhibit PI3K during treatment with either 10 nM TCDD or DMSO. As shown in Fig. 7A, TCDD treatment failed to enhance LX-2 cell proliferation when cells were pretreated with wortmannin. However, wortmannin had no impact on the TCDD-induced increase in MCP-1 production (Fig. 7B).

4. Discussion

The present study investigated how TCDD treatment impacts the activation of HSCs, which are liver non-parenchymal cells that function in vitamin A storage, inflammation, and wound healing. Of the studies that have investigated TCDD toxicity in HSCs, most analyzed this population of cells indirectly in the intact rodent liver or in primary cultured liver cells. The availability of the human LX-2 cell line makes it possible to investigate the consequences of TCDD exposure in a pure population of HSCs in vitro. Because gene expression in LX-2 cells is remarkably similar to gene expression in isolated human HSCs, these cells are a physiologically relevant resource (Xu et al. 2005). The observation that human LX-2 cells express a functional AhR (Fig. 1) is consistent with a recent report that mouse HSCs express a ligand-responsive AhR (Zhang et al. 2012) and establishes the suitability of LX-2 cells for investigating AhR activity. It is generally accepted that the AhR mediates most, if not all, of the toxic responses to TCDD (Hankinson 1995). Hence, it stands to reason that the consequences of TCDD treatment on LX-2

cells are likewise mediated through the AhR, although this was not formally tested in the present study.

There is substantial evidence that TCDD treatment reduces vitamin A storage in the rodent liver (Håkansson and Ahlborg 1985; Hanberg et al. 1996; Hanberg et al. 1998; Håkansson and Hanberg 1989; Thunberg et al. 1980). Reports that TCDD treatment suppressed vitamin A accumulation in HSCs by 30% without affecting the vitamin A content of parenchymal hepatocytes (Håkansson & Hanberg 1989) and inhibited the storage of newly ingested vitamin A in the liver (Håkansson and Ahlborg 1985) led us to speculate that TCDD would similarly suppress vitamin A storage in LX-2 cells. Indeed TCDD treatment was found to inhibit the formation of cytoplasmic lipid droplets in LX-2 cells that were simultaneously treated with retinol. In the intact liver, suppression of vitamin A storage has been shown to correlate with TCDD levels, as vitamin A storage was only inhibited before 60-90% of the TCDD was eliminated from the liver (Håkansson and Hanberg 1989). While not formally proven, inhibition of vitamin A storage by TCDD may reflect a direct effect of TCDD, which was shown to preferentially accumulate in HSCs (Håkansson & Hanberg 1989). In fact, the half-life of TCDD was reportedly 52 days in HSCs (compared to 13 days in hepatocytes), which raises the possibility that cytoplasmic lipid droplets in HSCs are a storage depot for TCDD in the liver. It stands to reason that increased duration of exposure to TCDD would increase the likelihood of TCDD toxicity in HSCs. Hence, LX-2 cells may prove useful for understanding the fate and persistence of TCDD in HSCs and for correlating intracellular TCDD concentrations with endpoints of cellular toxicity.

In contrast to the suppressive effects on lipid storage, TCDD treatment had no apparent effect on the mobilization of pre-stored lipids in LX-2 cells. This was surprising because TCDD

was shown elsewhere to increase the mobilization of endogenous retinoids from the liver (Hakansson et al. 1988), so it seemed likely that TCDD would likewise diminish the lipid content in LX-2 cells. The rationale for preloading LX-2 cells with retinol was to drive these cells to a quiescent, retinol-storing phenotype that would better approximate HSCs in a healthy, intact liver. It is possible that, when driven to this quiescent state, LX-2 cells became less sensitive to the direct effects of TCDD. In fact, it could be argued that quiescent HSCs in the intact liver may also be relatively insensitive to the direct effects of TCDD, as the increased mobilization of endogenous retinoids from HSCs in TCDD-treated animals could be attributed to an indirect consequence of TCDD treatment. Perhaps the TCDD-induced increase in retinoid mobilization requires another cue, such as a molecule produced by injured hepatocytes or a metabolite created through retinoic acid metabolism. The absence of such signals in the LX-2 model system could explain why TCDD treatment failed to increase the mobilization of stored retinol from quiescent cells. Loss of vitamin A stores is a characteristic feature of activated HSCs, but it remains unclear whether loss of lipid droplets is actually required for HSC activation or merely a coincidence (Beaven et al. 2011; Blaner et al. 2009; Friedman 2008).

Another characteristic of HSCs transitioning to a myofibroblast-like phenotype is proliferation, and we found that TCDD treatment increased the proliferation of cultured LX-2 cells. Interestingly, there was a lag period before increased proliferation was detected in LX-2 cells, irrespective of DMSO or TCDD treatment. During this time, LX-2 cells begin to undergo culture-induced activation and transition towards a more activated phenotype. One molecule that is upregulated in activated HSCs is the β subunit of platelet-derived growth factor receptor (PDGFR) (Wong et al. 1994). PDGF is the most potent mitogen for HSCs (Friedman and Arthur 1989) and also the primary mitogen found in FBS (Ross et al. 1986), in which the cells are

cultured. Hence, the lag in LX-2 cell proliferation could reflect the time needed to increase PDGFR expression and enhance responsiveness to PDGF. From such reasoning, it follows that increased proliferation in TCDD-treated LX-2 cells could result from a TCDD-mediated increase in PDGFR expression or responsiveness. As a receptor tyrosine kinase, PDGFR activates downstream signal transduction pathways, including the PI3K/AKT pathway that promotes HSC proliferation (Niu et al. 2007; Reif et al. 2003; Son et al. 2009). Our finding that TCDD does not increase proliferation when PI3K is inhibited supports the notion that TCDD may enhance proliferation through a mechanism that involves PDGFR/PI3K/Akt signaling.

Yet another indication of HSC activation is α SMA expression, which was also increased in LX-2 cells treated with TCDD. This finding conflicts with another report in which exposure of rats to a single dose of TCDD did not change the expression of α SMA in HSCs (Hanberg et al. 1996). One explanation for this discrepancy between in vitro and in vivo effects of TCDD on α SMA expression could stem from differences in HSC activation levels. For example, HSCs in a healthy rodent liver would presumably be found in a quiescent state, whereas cultured LX-2 cells exist in a quasi-activated state characterized by moderate proliferation and low levels of α SMA expression. It stands to reason that a single dose of TCDD may not provide the stimulus needed to initiate proliferation or α SMA expression in quiescent HSCs in the rodent liver, whereas it appears to be sufficient to enhance these endpoints in quasi-activated cells in culture. This notion is strengthened by a recent report demonstrating that chronic TCDD exposure increases α SMA expression in the rodent liver (Pierre et al. 2014). Furthermore, we recently found that TCDD treatment enhances α SMA expression in the livers of carbon tetrachloride-treated mice, a commonly used model system of HSC activation and experimental liver fibrosis (manuscript in preparation).

TCDD treatment also increased MCP-1 production by LX-2 cells. MCP-1 is produced by activated HSCs and can function in an autocrine loop to further increase HSC activation (Friedman 2008; Marra 1999; Marra et al. 1993; Reeves and Friedman 2002). By binding to the chemokine receptor CCR2, MCP-1 regulates the infiltration of monocyte subsets into the liver, which can further perpetuate inflammation and liver injury (Karlmark et al. 2009). TCDD treatment was also found to increase MCP-1 expression in the mouse liver as early as one day after treatment, although increased expression was not attributed to a specific cell type (Vogel et al. 2007). In the present study, the TCDD-induced increase in MCP-1 production by LX-2 cells was not affected when PI3K signaling was inhibited. At first glance, this was somewhat surprising because PI3K has been shown elsewhere to mediate the production of MCP-1 by HSCs during hepatic steatosis (Wobser et al. 2009). However, it is logical to speculate that TCDD may directly induce MCP-1 expression via AhR binding to a DRE in the MCP-1 gene, thereby bypassing HSC activation pathways altogether. This notion is backed by a report that TCDD induces MCP-1 expression in the mouse liver through a mechanism that requires AhR nuclear localization (Vogel et al. 2007), which is a prerequisite for AhR transcriptional activity.

While TCDD treatment enhanced some endpoints of LX-2 cell activation, it did not increase *Colla1* expression, which is a hallmark of fully activated HSCs. This was somewhat unexpected given that TCDD increased other endpoints of LX-2 cell activation. It is formally possible that LX-2 cells reached an upper threshold of stimulation in response to the potent profibrogenic mediator TGF- β 1, and this could have prevented additional upregulation of *Colla1* in response to TCDD. An alternative explanation could be that increased *Colla1* expression by TCDD requires additional signals that are absent in cultured LX-2 cells. This is supported by a recent report in which chronic TCDD treatment induced hepatic *Colla1*

expression in mice (Pierre et al. 2014). During liver fibrosis, the production of pro-fibrogenic mediators and deposition of collagen are driven by ongoing liver injury and inflammation (Czaja 2014). Hence, it is not surprising that the increased *Colla1* expression in TCDD-treated mice was reported to coincide with increased expression of inflammatory cytokines (Pierre et al. 2014). Although liver damage was not directly assessed in this study, other reports indicate that TCDD treatment increases serum levels of alanine aminotransferase and other endpoints of hepatocyte damage (Walisser et al. 2005). Hence, it is possible that TCDD treatment indirectly increases *Colla1* expression in HSCs by increasing inflammation and/or liver injury. This theory could be further explored using animal models of liver injury or a co-culture system of hepatocytes and HSCs.

In conclusion, results from this study indicate that HSCs are susceptible to direct modulation by TCDD treatment in vitro. Furthermore, exposure to TCDD likely increases HSC activation through a multi-faceted mechanism that includes PI3K signaling. The use of LX-2 cells could be useful for investigating mechanisms by which TCDD treatment impacts the fate of vitamin A in HSCs and for exploring the significance of AhR signaling to myofibroblast activation in the liver. Given the multifunctional role of HSCs, understanding how AhR activation impacts this unique population of cells may shed light on mechanisms of TCDD hepatotoxicity and on the physiological significance of AhR signaling during liver homeostasis and injury.

Figure Legends

Fig. 1. LX-2 cells express a functional AhR. LX-2 cells were treated with DMSO or 10 nM TCDD for 24 hr. Levels of *AhR* and *Cyp1a1* mRNA were measured by RT-PCR. *GAPDH* was used as a loading control. Results are representative of four samples from each treatment group.

Fig. 2. TCDD treatment modulates lipid storage in LX-2 cells. (A) LX-2 cells were treated for 48 hours with 5 μ M retinol (Ret) and 100 μ M palmitic acid (PA) in the presence of DMSO or 10 nM TCDD. Cells were then stained with oil red O to visualize lipid droplets (arrows). (B) LX-2 cells were cultured with retinol and palmitic acid for 48 hr to induce lipid storage, and then TCDD (10 nM) or DMSO was added for an additional 48 hr. Magnification 200x. Results are representative of three samples from each treatment group.

Fig. 3. TCDD treatment increases LX-2 cell proliferation. Cells were treated with 10 nM TCDD or DMSO. Viable cells were enumerated based on trypan blue exclusion at 24, 48, 72 and 96 hr after treatment. Data represent the mean $\pm$ SEM (n=3). Results are representative of four different experiments. * p <0.05 when compared to DMSO-treated cells at same time point.

Fig. 4. TCDD treatment increases the production of MCP-1 by LX-2 cells. Cells were treated with 10 nM TCDD or DMSO for 24 hr. (A) MCP-1 protein levels in supernatants were measured by ELISA. Data represent the mean $\pm$ SEM (n=3). (B) MCP-1 mRNA levels were measured in cell lysates after 24-hr treatment by semi-quantitative RT-PCR. * p <0.05 when compared to DMSO-treated control (n=3).

Fig. 5. TCDD treatment increases α SMA expression in LX-2 cells. LX-2 cells were grown on glass-cover slips, treated with 10 nM TCDD or DMSO for 24 hr, and α SMA expression was detected by fluorescence microscopy (anti- α SMA, red; DAPI, blue). (A) Untreated LX-2 cells at 0 hr. (B) LX-2 cells treated for 24 hr with DMSO. (C) LX-2 cells treated for 24 hr with TCDD. Magnification 630x.

Fig. 6. TCDD treatment does not increase collagen type I production by LX-2 cells. LX-2 cells were cultured for 24 hr with 2.5 ng/ml TGF- β 1 and either TCDD (10 nM) or DMSO. *Colla1* mRNA levels were measured by qRT-PCR. Samples were analyzed in duplicate from 5 biological replicates per treatment group. Relative quantification was estimated using the $\Delta\Delta C_t$ method normalized to β -actin. Error bars represent 95% confidence intervals, * $p < 0.05$.

Fig. 7. PI3K inhibition suppresses the effect of TCDD on LX-2 cell proliferation but not on MCP-1 production. Cells were pretreated for 30 min with 25 nM wortmannin (WM) to inhibit PI3K signaling prior to being cultured for 72 hr in TCDD (10 nM) or DMSO. (A) Average (mean $\pm$ SEM) number of viable cells in WM-treated cultures. (B) Average (mean $\pm$ SEM) levels of MCP-1 in the supernatant of WM-treated cells. Results are representative of three independent experiments, * $p < 0.05$.

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REFERENCES

- Abraham, K., Krowke, R. and Neubert, D. 1988. Pharmacokinetics And Biological-Activity Of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin .1. Dose-Dependent Tissue Distribution And Induction Of Hepatic Ethoxyresorufin O-Deethylase In Rats Following A Single Injection. *Archives of Toxicology* 62, 359-368.
- Beaven, S.W., Wroblewski, K., Wang, J.H., Hong, C., Bensinger, S., Tsukamoto, H. and Tontonoz, P. 2011. Liver X Receptor Signaling Is a Determinant of Stellate Cell Activation and Susceptibility to Fibrotic Liver Disease. *Gastroenterology* 140, 1052-1062.
- Black, M.B., Budinsky, R.A., Dombkowski, A., Cukovic, D., LeCluyse, E.L., Ferguson, S.S., Thomas, R.S. and Rowlands, J.C. 2012. Cross-species Comparisons of Transcriptomic Alterations in Human and Rat Primary Hepatocytes Exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin. *Toxicological Sciences* 127, 199-215.
- Blaner, W.S., O'Byrne, S.M., Wongsiriroj, N., Kluwe, J., D'Ambrosio, D.M., Jiang, H.F., Schwabe, R.F., Hillman, E.M.C., Piantadosi, R. and Libien, J. 2009. Hepatic stellate cell lipid droplets: A specialized lipid droplet for retinoid storage. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1791, 467-473.
- Blomhoff, R., Rasmussen, M., Nilsson, A., Norum, K.R., Berg, T., Blaner, W.S., Kato, M., Mertz, J.R., Goodman, D.S., Eriksson, U. and Peterson, P.A. 1985. Hepatic Retinol Metabolism - Distribution Of Retinoids, Enzymes, And Binding-Proteins In Isolated Rat-Liver Cells. *Journal of Biological Chemistry* 260, 3560-3565.
- Chen, L., Charrier, A.L., Leask, A., French, S.W. and Brigstock, D.R. 2011. Ethanol-stimulated differentiated functions of human or mouse hepatic stellate cells are mediated by connective tissue growth factor. *J Hepatol* 55, 399-406.
- Czaja, A.J. 2014. Hepatic inflammation and progressive liver fibrosis in chronic liver disease. *World Journal of Gastroenterology* 20, 2515-2532.
- Dere, E., Lee, A.W., Burgoon, L.D. and Zacharewski, T.R. 2011. Differences in TCDD-elicited gene expression profiles in human HepG2, mouse Hepa1c1c7 and rat H4IIE hepatoma cells. *Bmc Genomics* 12, 14.
- Forgacs, A.L., Dere, E., Angrish, M.M. and Zacharewski, T.R. 2013. Comparative Analysis of Temporal and Dose-Dependent TCDD-Elicited Gene Expression in Human, Mouse, and Rat Primary Hepatocytes. *Toxicological Sciences* 133, 54-66.
- Friedman, S.L. 2008. Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiological Reviews* 88, 125-172.
- Friedman, S.L. and Arthur, M.J.P. 1989. Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell

proliferation via induction of platelet-derived growth factor receptors. *Journal of Clinical Investigation* 84, 1780-1785.

Friedman, S.L., Rockey, D.C., McGuire, R.F., Maher, J.J., Boyles, J.K. and Yamasaki, G. 1992. Isolated hepatic lipocytes and Kupffer cells from normal human liver: morphological and functional characteristics in primary culture. *Hepatology* 15, 234-243.

Gentilini, A., Marra, F., Gentilini, P. and Pinzani, M. 2000. Phosphatidylinositol-3 kinase and extracellular signal-regulated kinase mediate the chemotactic and mitogenic effects of insulin-like growth factor-I in human hepatic stellate cells. *J Hepatol* 32, 227-234.

Gohl, G., Lehmkoetter, T., Munzel, P.A., Schrenk, D., Viebahn, R. and Bock, K.W. 1996. TCDD-inducible plasminogen activator inhibitor type 2 (PAI-2) in human hepatocytes, HepG2 and monocytic U937 cells. *Carcinogenesis* 17, 443-449.

Guo, J., Loke, J., Zheng, F., Hong, F., Yea, S., Fukata, M., Tarocchi, M., Abar, O.T., Huang, H., Sninsky, J.J. and Friedman, S.L. 2009. Functional linkage of cirrhosis-predictive single nucleotide polymorphisms of Toll-like receptor 4 to hepatic stellate cell responses. *Hepatology* 49, 960-968.

Hakansson, H. and Ahlborg, U.G. 1985. The Effect Of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (Tcdd) On The Uptake, Distribution And Excretion Of A Single Oral Dose Of 11,12-H-3 Retinyl Acetate And On The Vitamin-A Status In The Rat. *Journal of Nutrition* 115, 759-771.

Hakansson, H., Johansson, L. and Ahlborg, U.G. 1988. Effects Of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (Tcdd) On Tissue-Levels Of Vitamin-A And On The Distribution And Excretion Of The Endogenous Pool Of Vitamin-A In The Marginally Vitamin A Sufficient Rat. *Chemosphere* 17, 1781-1793.

Hanberg, A., Kling, L. and Hakansson, H. 1996. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the hepatic stellate cell population in the rat. *Chemosphere* 32, 1225-1233.

Hanberg, A., Nilsson, C.B., Trossvik, C. and Håkansson, H. 1998. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the lymphatic absorption of a single oral dose of [3H]retinol and on the intestinal retinol esterification in the rat. *J Toxicol Environ Health A* 55, 331-344.

Hankinson, O. 1995. The Aryl-Hydrocarbon Receptor Complex. *Annual Review of Pharmacology and Toxicology* 35, 307-340.

Håkansson, H. and Hanberg, A. 1989. The distribution of [14C]-2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its effect on the vitamin A content in parenchymal and stellate cells of rat liver. *J Nutr* 119, 573-580.

Ishibashi, H., Nakamura, M., Komori, A., Migita, K. and Shimoda, S. 2009. Liver architecture, cell function, and disease. *Seminars in Immunopathology* 31, 399-409.

Karlmark, K.R., Weiskirchen, R., Zimmermann, H.W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C. and Tacke, F. 2009. Hepatic Recruitment of the Inflammatory Gr1(+) Monocyte Subset Upon Liver Injury Promotes Hepatic Fibrosis. *Hepatology* 50, 261-274.

Kim, S., Dere, E., Burgoon, L.D., Chang, C.-C. and Zacharewski, T.R. 2009. Comparative Analysis of AhR-Mediated TCDD-Elicited Gene Expression in Human Liver Adult Stem Cells. *Toxicological Sciences* 112, 229-244.

Marra, F. 1999. Hepatic stellate cells and the regulation of liver inflammation. *J Hepatol* 31, 1120-1130.

Marra, F., Romanelli, R., Giannini, C., Failli, P., Pastacaldi, S., Arrighi, M., Pinzani, M., Laffi, G., Montalto, P. and Gentilini, P. 1999. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. *Hepatology* 29, 140-148.

Marra, F., Valente, A., Pinzani, M. and Abboud, H. 1993. Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. *J Clin Invest* 92, 1674-1680.

Massari, M.E. and Murre, C. 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* 20, 429-440.

Niu, L.W., Wang, X.H., Li, J., Huang, Y., Yang, Z., Chen, F.H., Ni, H.C., Jin, Y., Lu, X.W. and Cao, Q. 2007. Leptin stimulates alpha 1(I) collagen expression in human hepatic stellate cells via the phosphatidylinositol 3-kinase/Akt signalling pathway. *Liver International* 27, 1265-1272.

Pierre, S., Chevallier, A., Teixeira-Clerc, F., Ambolet-Camoit, A., Bui, L.C., Bats, A.S., Fournet, J.C., Fernandez-Salguero, P., Aggerbeck, M., Lotersztajn, S., Barouki, R. and Coumoul, X. 2014. Aryl hydrocarbon receptor-dependent induction of liver fibrosis by dioxin. *Toxicol Sci* 137, 114-124.

Porta, C., Paglino, C. and Mosca, A. 2014. Targeting PI3K/Akt/mTOR Signaling in Cancer. *Front Oncol* 4, 64.

Puche, J.E., Saiman, Y. and Friedman, S.L. 2013. Hepatic Stellate Cells and Liver Fibrosis. *Comprehensive Physiology* 3, 1473-1492.

Reeves, H.L. and Friedman, S.L. 2002. Activation of hepatic stellate cells--a key issue in liver fibrosis. *Front Biosci* 7, d808-826.

Reif, S., Lang, A., Lindquist, J.N., Yata, Y., Gabele, E., Scanga, A., Brenner, D.A. and Rippe, R.A. 2003. The role of focal adhesion kinase-phosphatidylinositol 3-kinase-akt signaling in hepatic stellate cell proliferation and type I collagen expression. *J Biol Chem* 278, 8083-8090.

Ross, R., Raines, E.W. and Bowenpope, D.F. 1986. The Biology Of Platelet-Derived Growth-Factor. *Cell* 46, 155-169.

Senoo, H., Yoshikawa, K., Morii, M., Miura, M., Imai, K. and Mezaki, Y. 2010. Hepatic stellate cell (vitamin A-storing cell) and its relative - past, present and future. *Cell Biol Int* 34, 1247-1272.

Son, G., Hines, I.N., Lindquist, J., Schrum, L.W. and Rippe, R.A. 2009. Inhibition of phosphatidylinositol 3-kinase signaling in hepatic stellate cells blocks the progression of hepatic fibrosis. *Hepatology* 50, 1512-1523.

Thunberg, T., Ahlborg, U.G., Hakansson, H., Krantz, C. and Monier, M. 1980. Effect Of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin On The Hepatic Storage Of Retinol In Rats With Different Dietary Supplies Of Vitamin-A (Retinol). *Archives of Toxicology* 45, 273-285.

Vogel, C.F., Nishimura, N., Sciallo, E., Wong, P., Li, W. and Matsumura, F. 2007. Modulation of the chemokines KC and MCP-1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice. *Arch Biochem Biophys* 461, 169-175.

Walisser, J.A., Glover, E., Pande, K., Liss, A.L. and Bradfield, C.A. 2005. Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types. *Proceedings of the National Academy of Sciences of the United States of America* 102, 17858-17863.

Wobser, H., Dorn, C., Weiss, T.S., Amann, T., Bollheimer, C., Büttner, R., Schölmerich, J. and Hellerbrand, C. 2009. Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells. *Cell Res* 19, 996-1005.

Wong, L., Yamasaki, G., Johnson, R.J. and Friedman, S.L. 1994. Induction Of Beta-Platelet-Derived Growth-Factor Receptor In Rat Hepatic Lipocytes During Cellular Activation In-Vivo And In Culture. *Journal of Clinical Investigation* 94, 1563-1569.

Xu, L., Hui, A., Albanis, E., Arthur, M., O'Byrne, S., Blaner, W., Mukherjee, P., Friedman, S. and Eng, F. 2005. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 54, 142-151.

Yoshizawa, K., Heatherly, A., Malarkey, D.E., Walker, N.J. and Nyska, A. 2007. A critical comparison of murine pathology and epidemiological data of TCDD, PCB126, and PeCDF. *Toxicol Pathol* 35, 865-879.

Zhang, H.F., Lin, X.H., Yang, H., Zhou, L.C., Guo, Y.L., Barnett, J.V. and Guo, Z.M. 2012. Regulation of the activity and expression of aryl hydrocarbon receptor by ethanol in mouse hepatic stellate cells. *Alcohol Clin Exp Res* 36, 1873-1881.

TABLE 1. PCR primers used in this study.

Gene	Primer Sequences	Annealing temp (°C)
<i>Ahr</i>	5'-CCACTTCAGCCACCATCCAT-3' 5'-AAGCAGGCGTGCATTAGACT-3'	54
<i>Cyp1a1</i>	5'-GGCCACATCCGGGACATCACAGA-3' 5'-GGGGGATGGTGAAGGGGACGAA-3'	58
<i>MCP-1</i>	5'-CGCGAGCTATAGAAGAATCAC-3' 5'-TTGGGTTGTGGAGTGAGTGT-3'	51
<i>GAPDH</i>	5'-GTCAACGGATTTGGCGTATT-3' 5'-AAAGTTGTCATGGATGACCTTGGC-3'	51
<i>Colla1</i>	5'-AACATGACCAAAAACCAAAAGTG-3' 5'-CATTGTTTCCTGTGTCTTCTGG-3'	56
<i>β-actin</i>	5'-GATGAGATTGGCATGGCTTT-3' 5'-GAGAAGTGGGGTGGCTT-3'	56

FIGURE 1

FIGURE 2

Figure 3

FIGURE 3

Figure 4

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7