

Regulation of the *CYP1A1* Gene by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, but not β -Naphthoflavone or 3-Methylcholanthrene, is Altered in Hepatitis C Virus Replicon-Expressing Cells

Garret R. Anderson, Aliya Hasan, Hao Yin, Ishtiaq Qadri, and Linda C. Quattrochi

From the Department of Medicine (GRA, AH, LCQ), Department of Pediatrics (IQ), School of Medicine, and Department of Pharmaceutical Sciences, School of Pharmacy (HY), University of Colorado at Denver and Health Sciences Center, Denver, CO 80262

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Correspondence address:

Linda C. Quattrochi, Ph.D.

Department of Medicine, University of Colorado at Denver and Health Sciences Center

4200 E. 9th Avenue, Denver, CO 80262, U.S.A.

Tel.: (303) 315-3522, Fax: (303) 315-7180

E-mail: Linda.Quattrochi@UCHSC.edu

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Abbreviations: AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; β NF, β -naphthoflavone; CYP, cytochrome P450; HCV, hepatitis C virus; MC, 3-methylcholanthrene; NAC, N-acetylcysteine; NDGA, nordihydroguaiaretic acid; NS, nonstructural; PAHs, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UGT, UDP-glucuronosyltransferases.

ABSTRACT

Exposure to hepatitis C virus (HCV) can lead to the development of cirrhosis and hepatocellular carcinoma. To examine the effects of chronic HCV infection on hepatic cytochrome P4501A1 (CYP1A1) expression and function, we used a human hepatoma cell line expressing the HCV subgenomic replicon (Huh.8) to evaluate *CYP1A1* induction by the aryl hydrocarbon receptor (AhR) ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In this study, we demonstrate that the induction of *CYP1A1* expression in Huh.8 cells by TCDD, but not by β -naphthoflavone or 3-methylcholanthrene, was significantly diminished. TCDD exposure of Huh.8 cells resulted in greater than 55% suppression of *CYP1A1* transcription compared to the parent cell line Huh7, while protein levels and enzyme activities were further diminished. Suppression of *CYP1A1* mRNA expression in TCDD-treated Huh.8 cells was partially reversed following pretreatment with the antioxidants N-acetylcysteine and nordihydroguaiaretic acid, suggesting a role for oxidative stress. Induced *CYP1A1* message, protein, and enzyme activity were partially restored in a Huh7 cell line expressing the HCV replicon containing a deletion in the nonstructural protein, NS5A. Furthermore, adenoviral expression of NS5A in Huh7 partially suppressed TCDD-induced CYP1A1 protein and enzyme activity, implicating this protein in the mechanism of suppression. These findings demonstrate that TCDD-mediated AhR signaling is impaired in hepatocytes in which HCV is present, and that NS5A alone or in the presence of other nonstructural proteins of the subgenomic replicon is in part responsible.

The cytochrome P450s (CYP), a multigene family of heme-containing proteins, are responsible for the metabolism of numerous xenobiotics, including therapeutic drugs, environmental chemicals, and dietary constituents, as well as endogenous substrates, such as steroids and bile acids. Members of the CYP1 family include CYP1A1, 1A2, and 1B1. CYP1A1 is a highly inducible enzyme that plays a critical role in the bioactivation of certain chemicals, such as benzo[a]pyrene, to reactive intermediates associated with mutagenesis and carcinogenesis (Gonzalez and Gelboin, 1991). Induction is mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that plays a pivotal role in mediating the biological actions of a number of highly toxic chemicals including polychlorinated-dibenzo-*p*-dioxins (of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) is a prototype ligand), polychlorinated-dibenzofurans, coplanar biphenyls, and polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene and 3-methylcholanthrene (MC) (Burbach et al., 1992). Upon ligand binding, the cytosolic AhR migrates to the nucleus where it forms a dimer with Arnt (AhR nuclear translocator), which then binds to specific DNA recognition sites, referred to as Ah-response elements (AhRE, XRE, or DRE) located in the 5'-flanking region of target genes (reviewed in Denison and Whitlock, 1995). TCDD, a widespread environmental pollutant produced as the result of combustion and industrial processes, is one of the most potent agonists of the AhR, and thus a powerful inducer of CYP1A1 activity.

In humans, infections from influenza, adenovirus, Herpes simplex, and HIV are associated with decreases in drug biotransformation and clearance (Renton, 2004). Only a few studies have reported the effects of hepatitis infection on drug metabolism,

including impairment of hepatic drug clearance in HCV patients, as measured by metabolism of the probe drug, antipyrine (Ali et al., 1995; Jorquera et al., 2001). Studies of CYP function have shown that CYP2A6 activity was depressed by hepatitis A (Pasanen et al., 1997) and induced by hepatitis B and C (Kirby et al., 1996); while Becquemont et al. (2002) found CYP3A4 and CYP2D6 activities to be significantly lower in HCV patients than healthy volunteers. Recently, it was shown that changes in mRNA expression of specific CYPs were linked to progression of HCV-associated hepatocellular carcinoma (Tsunedomi et al., 2005).

HCV is a positive-stranded RNA virus that belongs to the *Flaviviridae* family. Chronic infection can lead to cirrhosis and hepatocellular carcinoma (Alter, 1995). Many factors are associated with the development of HCV-related liver damage, including exposure to such environmental agents as cigarette smoke and alcohol. However, the molecular mechanisms leading to cell injury are unclear. Chronic infection leads to cellular oxidative stress, characterized by increases in cellular levels of reactive oxygen species (ROS), suggesting that ROS may be involved in producing the damage seen in chronic HCV infection (DeMaria et al., 1996). The development of subviral systems, consisting of stable high level expression of HCV subgenomic replicons, for the study of replication of the viral RNA in cultured cells (Lohmann et al., 1999; Blight et al., 2000) has facilitated studies on HCV replication and protein function. The subgenomic replicon is composed of six nonstructural proteins (NS) that perform various cellular functions. The NS5A protein plays a critical role in viral replication (Blight et al., 2000), and participates in numerous cellular functions, including activation of cellular transcription

factors via oxidative stress (Gong et al., 2001) and activation of the ER stress pathways (Waris et al., 2002).

To our knowledge, the relationship between HCV infection and the AhR signaling pathway has not been reported. However, recent studies have investigated the role of the AhR in viral replication. Exposure of cultured cells to AhR ligands increases the replication of human immunodeficiency virus type 1 (HIV) (Yao et al., 1995; Gollapudi et al., 1996; Ohata et al., 2003) and human cytomegalovirus (Murayama T et al., 2002). The proposed mechanisms for enhanced HIV replication include TCDD-dependent generation of thiol-sensitive reactive oxygen intermediates (Yao et al., 1995), activation of NF- κ B and production of TNF α (Gollapudi et al., 1996), and increased gene expression through AhR binding to a putative XRE (Yao et al., 1995; Ohata et al., 2003). Adult T-cell leukemia cell lines have elevated expression of AhR and *CYP1A1*, suggesting a link between increased AhR expression and ATL leukemogenesis (Hayashibara et al., 2003). Thus, the potential of infectious agents to alter AhR signal transduction pathways, including *CYP1A1* expression and function, could lead to increases in disease progression. Indeed, smoking, known to induce *CYP1A1* and hepatic *CYP1A2*, was recently shown to increase the severity of hepatic lesions in patients with chronic hepatitis C (Pessione et al., 2001; Hezode et al., 2003).

In this study we investigated the effect of HCV on the AhR signaling pathway by examining the induction of *CYP1A1* by TCDD and other AhR ligands. We demonstrate that transcriptional activation of the human *CYP1A1* gene by TCDD, but not β -naphthoflavone (β NF) or MC, is dramatically suppressed in Huh7 cells expressing the HCV subgenomic replicon. These findings will likely provide valuable insights into

mechanisms of dioxin toxicity and the interactions of non-inflammatory components of infectious agents on xenobiotic metabolism.

Materials and Methods

Chemicals and Reagents. Reagents were obtained as follows: TCDD from Chemsyn Science Laboratories (Lenexa, KS). β NF, MC, and N-acetylcysteine (NAC), nordihydroguaiaretic acid (NDGA) from Sigma Chemical Company (St. Louis, MO); [α - 32 P]dCTP, [γ - 32 P]ATP, and poly[d(I-C)] from GE Healthcare (Piscataway, NJ); 2' 7'-dichlorofluorescein diacetate from Alexis Biochemicals (San Diego, CA).

Cell Culture. The cell lines used in this study, Huh7, Huh.8 and Ava.1, were provided by Dr. Charles Rice (Rockefeller University, New York) and are described in (Blight et al., 2000). In brief, the Huh.8 cell line contains an HCV derived expression vector stably integrated into a Huh7 background. The expression vector includes the HCV proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B, linked to the antibiotic selection marker G-418. The Ava.1 cell line is similar to Huh.8, but with a 47 amino acid deletion within the NS5A region, rendering this nonstructural protein nonfunctional. All cell lines were maintained as a monolayer using Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) and heat-inactivated 10% fetal bovine serum from Hyclone (Logan, UT). G-418 (geneticin)(Invitrogen) was added to a final concentration of 800 μ g/ml to medium used for culturing Huh.8 and Ava.1 cell lines. For treatments, cells were exposed to TCDD, β NF, or MC at concentrations indicated in figure legends or vehicle (0.1% DMSO). NDGA was dissolved in DMSO, and NAC was dissolved in DMEM plus 25 mM HEPES and adjusted to pH 7.1 immediately before treatments.

RNA Isolation, Northern Blot Analyses, and Quantitative Real-time RT-PCR.

Total RNA was extracted from near confluent cells using the RNeasy Kit (Qiagen, Valencia, CA). Northern blot analysis was performed by electrophoresis of total RNA (10 µg) through a 1% agarose-2.2 M formaldehyde gel, followed by blotting onto a Hybond nylon membrane (GE Healthcare). CYP1A1 and β-actin cDNA probes were labelled with [α -³²P]dCTP using the Random Prime Kit (Invitrogen), and hybridized to the blots as previously described (Quattrochi et al., 1985). Images were quantified by phosphorimaging in the STORM 840 Phosphorimager (GE Healthcare) and using Image Quant software from Molecular Dynamics. For quantitative real-time RT-PCR, total RNA was treated with DNase I prior to analysis. Real-time RT-PCR was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) with the following primers: CYP1A1 forward primer, 5'GAATTCAGCGTGCCACTGG-3', reverse primer, 5'-GGCATGCTTCATGGTTAGCC-3', and TaqMan probe, 5'-CGTGAAGGTGGACATGACCCCCAT-3', giving a product of 69 bp; AhR forward primer, 5'-CCTCCTTCTTGCCCTTCACC-3', reverse primer, 5'-GGATTTGACTTGATTCCTTCAGCT-3', and TaqMan probe, 5'-CCGGTGCAGAAAACAGTAAAGCCAATCC-3', giving a product of 75 bp; ARNT forward primer 5'-TAGTGCCCTGGCTCGAAAA-3', reverse primer, 5'-CCGCAAGGACTTCATGTGAGA-3', and TaqMan probe, 5'-CAGACAAGCTAACCATCTTACGCATGGCA-3', giving a product of 73 bp. Each real-time RT-PCR reaction was performed in duplicate, and normalized to the ribosomal RNA levels in the same sample.

Transient Transfections and Luciferase Activity Assays. Cells were plated at a density of 125,000 cells/well in 12-well plates. Transfections were performed using Fugene 6 Transfection Reagent following manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Five hours after transfection, DMEM containing 10% FBS was added to each well and cultures incubated overnight. The culture media was removed after incubation for 24 h with the transfection reagent-DNA complexes, and the cells were then treated for 24 h with xenobiotics dissolved in DMSO. Control cells received media containing 0.1% DMSO. Following treatment, cells were rinsed with phosphate buffered saline (PBS) and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity of cellular lysates was quantified with a Packard LumiCount luminometer. *Firefly* luciferase activity was determined from three independent transfections, and normalized against *Renilla* luciferase activities of the pRL-null control vector obtained from the same culture.

Western Blot Analyses. Whole cell lysates were prepared by sonication of cell pellets in 250 mM sucrose/10 mM Tris (pH 7.5) buffer. Protein concentrations were determined using the Bradford microassay from BioRad (Hercules, CA). Western blots were prepared by electrophoresis of whole cell lysates or nuclear extracts through 4-20% PAGE gels, and transferred to PVDF membranes (MSI, Westboro, MA) overnight at 4°C. Blots were probed with antibody raised against human CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA) or AhR and Arnt antibodies (obtained from Dr. Christopher A. Bradfield, University of Wisconsin) and subsequently with anti-actin

antibody (Oncogene Research Products, San Diego, CA) to normalize the amount of protein loaded in each lane. As a positive control, each blot was run with one lane containing recombinant CYP1A1 (BD Gentest, Woburn, MA). Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL), and visualized by UVP BioImaging Systems camera and LabWorks acquisition software (Upland, CA). Quantifications were performed using NIH Image v1.63 software.

Isolation of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA).

Nuclear protein fractions were isolated from near confluent cells as described by Denison et al. (1988). Cells were treated with TCDD or β NF for 24 h prior to extraction of nuclear proteins. Oligonucleotides were supplied by Operon Biotechnologies (Huntsville, AL). For the preparation of the CYP1A1-XRE DNA probe, the oligonucleotides 5'-CCGGCTCGCGTGAGAAGCG-3' and 5'-CGCTTCTCACGCGAGCCGG-3' were annealed together overnight at 37°C. The probe was labeled with 32 P at the 5' ends using T4 polynucleotide kinase (Invitrogen) and [γ - 32 P]ATP. Labeled probes were purified through TE-10 columns (BD Biosciences Clontech, Palo Alto, CA). For EMSA, 4 μ g of nuclear extract was incubated in a DNA binding buffer containing radiolabelled probe, 10 mM Tris (pH 8.0), 75 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM DTT, 2 μ g poly[d(I-C)], and 10% glycerol. Protein-DNA complexes were separated on 6% PAGE 1X TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA) gels. Images were quantified by phosphorimaging in the STORM 840 Phosphorimager.

P450-Glo Assay. Detection of CYP1A1 activity was performed using a P450-Glo CYP1A1 Assay Kit (Promega). The assay was performed using cultured cells according to kit specifications. Cells were plated on 96-well plates at 30,000 cells/well. The following day, cells were exposed to TCDD for 24 h. The next day, cells were rinsed one time with PBS and medium containing 50 μ M of the CYP1A1 specific substrate, luciferin 6' chloroethyl ether (luciferin-CEE), was added, and cells incubated at 37 °C for 3 h. Following the incubation, the reaction was terminated by adding 50 μ L of luciferase detection reagent, and luciferase activity quantified with a Packard LumiCount luminometer. For each treatment group, one set of wells was assayed in the absence of substrate and values obtained subtracted from substrate wells.

Determination of ROS Production. ROS production in Huh7 and Huh.8 cells was measured spectrofluorometrically using the cell permeable 2',7'-dichlorofluorescein-diacetate (DCF-DA) probe. DCF-DA is converted to its fluorescent product dichlorofluorescein by ROS. Cells were plated on 96-well black plates (Nunc, Roskilde, Denmark) at 25,000 cells/well (approximately 80% confluency). The following day, media was changed to serum-free, phenol red-free DMEM, DCF-DA added to a final concentration of 5 μ M, and cells incubated for 30 min. At the end of the incubation period, cells were rinsed one time with PBS and media containing xenobiotics was added. Fluorescence readings were taken immediately and after various times of treatment from 15 min to 24 h using a SpectraMax Gemini EM fluorometer and SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Recombinant Adenovirus Expression of NS5A. The construction of the recombinant adenovirus expressing NS5A is described in (Qadri et al., 2004). Huh7 cells were infected with a recombinant adenovirus expressing NS5A (Ad-NS5A) or expressing green fluorescent protein (Ad-GFP), as a control, at a concentration of approximately 2×10^3 adenovirus particles per cell for 24 h prior to the addition of TCDD. The following day, media was changed and 10 nM TCDD was added to culture plates for an additional 24 h. Cells were subsequently harvested for RNA and whole cell protein extracts. Expression of Ad-GFP in Huh7 cells was detected using conventional fluorescence microscopy as an index of efficiency of infection.

Statistics. Statistics were performed using InStat Instant Statistics (Prism 4 GraphPad Software, San Diego, CA). Statistical differences between values were determined by a one-way ANOVA, followed by either Bonferroni or Dunnett multiple comparisons *post hoc* tests or Student *t* test. A $p < 0.05$ is considered statistically significant.

Results

Differential Effects of AhR Ligands on *CYP1A1* Gene Transcription in HCV Replicon-expressing Cells. To examine whether the AhR pathway is affected by the presence of the HCV genomic replicon, we treated the parent cell line (Huh7) and the cells containing the HCV subgenomic replicon (Huh.8) with TCDD, β NF, or MC, and assayed for the expression of *CYP1A1* message. We found, as expected, that exposure of Huh7 cells to all three AhR ligands resulted in substantial induction of *CYP1A1* mRNA (Fig. 1A). In contrast, exposure of Huh.8 cells to TCDD resulted in a significant reduction in *CYP1A1* mRNA as compared to Huh7 cells. Data from Northern blots were confirmed by quantitative real-time RT-PCR data (Fig. 1B). The expression in Huh.8 cells of TCDD-induced *CYP1A1* was diminished by 55% when compared to Huh7 cells. Time course and dose response experiments indicated that the effects on *CYP1A1* mRNA expression occur early (6 h) and at all doses tested (Fig. 2). In stark contrast, treatment of Huh.8 cells with β NF or MC resulted in an approximately 2 to 3-fold enhanced expression of *CYP1A1* compared to Huh7 cells (Fig. 1).

A change in the steady-state levels of TCDD-induced *CYP1A1* message suggested that the transcription of the *CYP1A1* gene was impaired in TCDD-treated Huh.8 cells. To test this, we transiently transfected a *CYP1A1*-luciferase plasmid (1A1Luc), containing the *CYP1A1* promoter sequences from +292 to - 1612 (Postlind et al., 1993), into both cell lines and treated with TCDD or MC. We found that luciferase activity in transfected Huh.8 cells treated with TCDD was significantly reduced compared to Huh7 cells (Fig.

3A). The decrease in reporter gene activity is consistent with the decrease in *CYP1A1* mRNA levels (approximately 60%). Treatment of Huh.8 cells with MC resulted in a significant increase in luciferase activity, consistent with increased *CYP1A1* mRNA from MC-treated cells. These findings indicate that TCDD affects AhR signaling differently than non-dioxin AhR ligands in HCV replicon-expressing cells. Since β NF and MC were capable of inducing the expression of *CYP1A1* mRNA, we reasoned that Huh.8 cells possessed a functional AhR. To directly test this, we performed EMSA experiments to examine AhR binding to XREs in both cell lines. We found that nuclear AhR binding was reduced in extracts from TCDD-treated Huh.8 cells compared to Huh7, but the decrease was less than the magnitude of the transcription response (approximately 30% vs. 55% suppression, respectively)(Fig. 3B). We believe these findings are not the result of unequal loading (i.e., non-specific complex is less in Huh.8) since we obtained the same results using nuclear extracts from three different experiments. Furthermore, increased AhR binding was observed in β NF-treated Huh.8 vs. Huh7, consistent with mRNA data. Quantitative real-time RT-PCR of RNA from TCDD-treated cells indicated no difference in AhR mRNA expression between cell lines (Huh7, 964 ± 60 pg/ng rRNA vs. Huh.8, 1032 ± 347), while Arnt expression was slightly elevated in Huh.8 cells (Huh7, 735 ± 30 pg/ng rRNA vs. Huh.8, 1428 ± 420). AhR and Arnt protein levels were also similar between cell lines, as were changes in the subcellular distribution of the AhR with TCDD exposure (Fig. 4).

CYP1A1 Protein Expression and Enzyme Activity are Suppressed in Huh.8.

Next, we confirmed the transcriptional data by examining the expression of CYP1A1

protein and enzyme activity in Huh7 and Huh.8 cells. Whole cell lysates were prepared from cells exposed for 24 h to either TCDD, β NF, or MC. Increases in the level of CYP1A1 protein were observed in Huh7 cells treated with all three agents (Fig. 5A), consistent with induced message (Fig. 1). We observed a significant decrease in TCDD-induced CYP1A1 protein expression in Huh.8 cells (mean \pm SE, $13.8 \pm 4.2\%$ of Huh7), and slight increases in β NF- and MC-induced CYP1A1 protein ($125.4 \pm 3.1\%$, $211 \pm 8.0\%$, respectively). To further characterize the regulation of the *CYP1A1* gene in Huh.8 cells, we utilized the P450-Glo assay to measure CYP1A1-mediated enzyme activity. CYP1A1 enzyme activity was measured directly in cultured cells following treatment with 10 nM TCDD for 24 h (Fig. 5B). The data shown in Figure 5B revealed that CYP1A1 enzyme activity in Huh.8 cells was only 7% of Huh7 and not statistically significant from untreated cells.

Effects of ROS on TCDD-induced CYP1A1 Expression in Huh.8 Cells. The preceding experiments demonstrated the significant effect of the HCV subgenomic replicon on AhR signaling and *CYP1A1* induction by TCDD. A potential mechanism for virus-induced down-regulation of induced *CYP1A1* expression is a change in the redox state of the cells resulting in changes in gene transcription. One explanation for our findings is that increases in cellular oxidative stress from replication of the HCV genomic replicon (Gong et al., 2001) and from TCDD-mediated production of ROS in Huh.8 cells results in a decrease in the induced transcription of the *CYP1A1* gene. To test this, we examined the role of ROS generation on *CYP1A1* transcription by preincubating Huh.8 cells with N-acetylcysteine (NAC), a thiol antioxidant and cysteine precursor that

eliminates oxygen free radicals, and nordihydroguaiaretic acid (NDGA), an antioxidant and broad spectrum inhibitor of lipoxygenase. Cells were exposed to TCDD and various concentrations of NAC or NDGA, and *CYP1A1* mRNA quantified by real-time RT-PCR (Fig. 6). The suppression of TCDD-induced *CYP1A1* mRNA was partially reversed by NAC or NDGA pretreatments. *CYP1A1* mRNA expression from Huh.8 cells exposed to TCDD and 20 mM NAC was $72.7 \pm 9.5\%$ of TCDD-treated Huh7 cells (Fig. 6A); while *CYP1A1* mRNA levels from cells treated with TCDD and 15 μ M NDGA was $86.6 \pm 17.5\%$ (Fig. 6B). Treatment of both cell lines with NDGA alone increased constitutive *CYP1A1* message by approximately 3-fold (data not shown). Since constitutive *CYP1A1* mRNA levels are elevated 10-fold in the Huh.8 cells (see Fig. 9B), a further increase with NDGA treatment may have accounted for some of the changes seen with TCDD and NDGA cotreatments (Fig. 6B). Nonetheless, these results suggest a partial contribution by ROS to the suppression of TCDD-induced regulation of *CYP1A1* gene expression.

To assess the ability of TCDD itself to initiate ROS production in Huh.8 cells, we used the ROS-sensitive fluorescent probe, DCF-DA, to directly measure ROS in treated cells. Huh7 and Huh.8 cells were exposed to TCDD, MC, or β NF, and DCF fluorescence measured between 15 min and 24 h following treatments. Increased fluorescence over control cells from inducer exposure was observed only after 6 h (data not shown). A 24 h treatment with TCDD had no effect in Huh7, but increased ROS production in Huh.8 by approximately 2-fold over control (Fig. 7). MC and β NF treatments increased ROS production in both cell lines (approximately 2-fold in Huh7 and 3-fold in Huh.8). ROS production from inducer exposed cells was blocked by co-treatment with 20 mM NAC (data not shown).

The Role of NS5A on Suppression of TCDD-induced CYP1A1 Expression in Huh.8 Cells. The HCV protein NS5A functions, alone or in the context of other HCV NS proteins, to increase ROS production through induction of oxidant stress pathways (Qadri et al., 2004; Gong et al., 2001). To examine its role in modulating induced *CYP1A1* expression, we took two approaches. First, we utilized a cell line, Ava.1, which expresses the HCV subgenomic replicon identical to that of Huh.8 but containing a deletion of 47 amino acids in the C-terminus of NS5A, rendering this protein non-functional. We found treatment of Ava.1 cells with TCDD partially alleviated the down-regulation of induced *CYP1A1* expression (Fig. 8A). The HCV replicon-mediated decrease in *CYP1A1* mRNA levels was reversed by approximately 16% in NS5A-defective cells (left panel); while protein levels and enzyme activity were more dramatically increased; approximately 35% for protein (right panel), and 30% for enzyme activity (data not shown). Although TCDD-induced *CYP1A1* expression was not fully restored in the Ava.1 cell line, these findings suggested that NS5A plays a role in the suppression. To test the direct involvement of this HCV protein, Huh7 cells were infected with recombinant adenovirus expressing NS5A. We found that expression of NS5A suppressed TCDD-induced *CYP1A1* protein expression by approximately 32% (Fig. 8B), in good agreement with results from the Ava.1 cell line

TCDD-induced UGT1A Expression in Huh.8 Cells. The preceding studies demonstrate an effect of HCV on the TCDD-activated AhR pathway. To determine whether this effect is specific for the *CYP1A1* gene or whether it is associated with

changes in other AhR target genes, we used semi-quantitative RT-PCR to analyze the expression of two UDP-glucuronosyltransferases, UGT1A1 and 1A6, known to be regulated via AhR (Sugatani et al., 2004; Auyeung et al., 2003). We found that TCDD-induced UGT1A1 mRNA is slightly suppressed, while 1A6 is considerably suppressed in Huh.8 cells, indicating that our observations are not specific for *CYP1A1* induction (UGT1A1: $86.7 \pm 10.4\%$ and UGT1A6: $61.0 \pm 1\%$ of Huh7) (Fig. 9). Interestingly, we found that UGT1A basal levels are substantially suppressed in Huh.8 relative to Huh7 (UGT1A1: $38.0 \pm 12.2\%$; UGT1A6: $44.6 \pm 7.2\%$ of Huh7), results that are opposite to the constitutive expression of *CYP1A1* in Huh.8 cells (Fig. 9B). Indeed, we found constitutive *CYP1A1* mRNA expression in Huh.8 cells to be increased approximately 10-fold over untreated Huh7 cells, consistent with increased reporter gene activity (Fig. 3A).

Discussion

The present study was designed to examine whether chronic hepatitis C virus infection alters the molecular mechanisms regulating induced cytochrome P450 gene expression in the absence of classic inflammatory mediators (*e.g.* cytokines produced by Kupffer cells). Using novel human hepatoma cell lines expressing the HCV subgenomic replicon, we found that TCDD-induced *CYP1A1* transcription and enzyme activity were dramatically suppressed. In contrast, AhR-dependent increases in *CYP1A1* expression by other AhR ligands were not suppressed, but were in fact enhanced in the Huh.8 cell line that contains the HCV replicon. The AhR signaling pathway leading to induced *CYP1A1* gene expression is well-described. In addition to *CYP1A1*, other AhR responsive genes have been identified and characterized by various methods, including more recently, microarray analyses (Puga et al., 2000; Fletcher et al., 2005). These studies found that in addition to numerous induced genes, dioxin also inhibited the expression of a number of genes. However, the molecular mechanism by which TCDD-activated AhR signal transduction leads to gene repression is still largely unknown. Most AhR agonists have been shown to function in an identical manner with respect to activation of gene transcription; thus, our findings that β NF and MC treatment of Huh.8 cells does not suppress *CYP1A1* gene expression are unique and may provide insights into the mechanism of dioxin-induced toxicity.

The endoplasmic reticulum (ER) is the site of viral replication, and all of the HCV nonstructural proteins remain associated with this membrane. This association leads to

ER stress which involves release of calcium from the ER, changes in the mitochondrial permeability transition pore and increases in intracellular ROS. As a result of increases in the level of ROS, including hydrogen peroxide, superoxide radicals, and hydroxyl radicals, the transcription factors, STAT-3 and NFκB, are activated and migrate to the nucleus where they regulate target genes (Waris et al., 2002). Evidence suggests similar increases in oxidative stress occur in Huh7 cells expressing the HCV replicon (Qadri et al., 2004; Gong et al., 2001). It has been demonstrated that increased oxidative stress down-regulates *CYP1A1* transcription (reviewed in Barouki and Morel, 2001), and that *CYP1A1* activity can lead to induced and sustained oxidative stress in the presence of ligands that are poorly metabolized (*e.g.* dioxin, polychlorinated biphenyls) (Shertzer et al., 1998; Schlezinger et al., 1999). Therefore, we reasoned that HCV replicon-induced oxidative stress in addition to TCDD-mediated increases in ROS could contribute to the decrease in induced *CYP1A1* expression reported here. In experiments to test this, we found that the antioxidants, NAC and NDGA, partially reversed the suppression (Fig. 6), indicating that suppression is mediated, in part, by an increase of ROS elicited from the HCV replicon or due to a decreased capacity of the replicon-expressing cells to scavenge ROS. However, direct measurements of ROS in Huh.8 cells exposed to various AhR ligands indicated that all tested agents increase ROS production (Fig. 7). Taken together, these findings suggest that ROS may play a partial role in the down-regulation of TCDD-induced *CYP1A1* transcription in Huh.8 cells, but do not explain differences in the ligand-dependent effects.

Evidence demonstrating decreased TCDD-induced *CYP1A1* transcription in Huh.8 cells suggests that the AhR signaling pathway is impaired in the presence of the HCV

replicon in a ligand-dependent fashion. Additional insights into mechanisms underlying the suppression were provided by DNA binding studies. Results of EMSA (Fig. 3B) and Western blotting (Fig. 4) indicate that there is sufficient AhR present in the nucleus of TCDD-treated Huh.8 cells to drive a high level of gene transcription. These results suggest that the XRE-bound receptor may not be fully functional. Furthermore, the observation that the amount of bound AhR in TCDD nuclear extracts from Huh.8 cells is over twice the amount from β NF extracts, suggests that the decrease in AhR binding activity contributes to, but does not completely explain, the changes in *CYP1A1* transcription. This finding again emphasizes the striking differences between AhR ligands in inducing *CYP1A1* gene transcription in Huh.8 cells. The lack of complete transactivational capacity of the TCDD-activated AhR could be the result of events occurring upstream or downstream of XRE binding. HCV NS proteins are localized to the endoplasmic reticulum and therefore participate in signal transduction pathways that are initiated in the cytoplasm, including generation of ROS and activation of transcription factors (*e.g.* AP-1, NF κ B, STAT-3). Therefore, HCV-induced changes in the cellular redox state could alter the number of available (*i.e.*, functional) AhR in the cytosol. Support for this idea is provided by the recent findings that different residues in the ligand binding domain of AhR affect function of low-affinity ligands, but not TCDD (Backlund and Ingelman-Sundberg, 2004), and that phosphorylations in HSP90 modulate the formation of a functional AhR complex (Ogiso et al., 2004). These conclusions suggest the possibility that HCV-induced signaling pathways may modify residues of the cytosolic AhR, resulting in changes in ligand binding. Although we do not presently know how β NF and MC treatment of Huh.8 cells results in enhanced, rather than

repressed, *CYP1A1* gene expression, the evidence presented here supports a ligand-dependent interaction of the AhR with the HCV replicon. Future studies of such interactions should provide important insights into AhR signaling.

In addition to changes in induced expression, we found that the presence of the HCV replicon increased the constitutive level of *CYP1A1* transcription. These findings are similar to those reported for adult T-cell leukemia where *CYP1A1* mRNA expression was increased in the absence of exogenous ligand and shown to be partially due to the actions of the viral transactivator protein, Tax (Hayashibara et al., 2003). The mechanism for increased *CYP1A1* mRNA in Huh.8 cells may involve an endogenous AhR ligand present in HCV-replicating cells. Alternatively, other cellular factors altered by the presence of the HCV replicon could increase the rate of *CYP1A1* gene transcription. Although Western blotting was not sufficient to detect CYP1A1 protein in untreated Huh.8 cells, mRNA levels were increased at least 10-fold over untreated Huh7 cells. Constitutive expression of *CYP1A1*, not normally found in liver, may have biological implications. PAHs, known substrates for CYP1A1 metabolism, are converted to intermediate metabolites that form DNA adducts and cause toxicity. Historically, activation of procarcinogens (*e.g.* PAHs) by CYP1A1 was considered a critical event leading to mutagenesis and carcinogenesis. Recent advances in genetic engineering have been able to address the *in vivo* significance of metabolic activation of PAHs through the use of CYP1A1 null mice (Uno et al., 2004; Nebert et al., 2004). These intriguing studies provide evidence that detoxification of procarcinogens by CYP1A1 may afford protection from toxicity depending on several factors, including target organ, route of administration, and subcellular content and localization. Thus, increased levels of

constitutive CYP1A1 in HCV infected hepatocytes might be viewed as providing a detoxification function rather than metabolic activation. On the other hand, it appears that a large portion of CYP1A1 protein may be catalytically non-functional in Huh.8 cells (Fig. 5B). Therefore, the possibility exists that in HCV-infected patients, cigarette smoking or exposure to other environmental agents that are substrates for CYP1A1, could be harmful, potentially leading to a more rapid progression of liver disease associated with viral infection.

Finally, TCDD suppression of gene transcription in Huh.8 cells is not restricted to *CYP1A1* because we found similar results with UGT1A (Fig. 9). Interestingly, the constitutive UGT1A levels were also significantly reduced in Huh.8 cells, a finding opposite to that of *CYP1A1*. Constitutive and induced UGT1A expression are also regulated through the electrophile response element; thus, it is possible that this signal transduction pathway, which responds to oxidant stress by activating antioxidant genes, is impaired in Huh.8 cells. Indeed, inhibiting this signal transduction pathway would result in a decreased ability of the cellular defense mechanisms to detoxify ROS. Our studies demonstrate that expression and function of drug metabolizing enzymes are substantially modified in this *in vitro* model of HCV. These findings may have major implications for the progression of HCV-mediated liver disease as well as in patient treatment, especially if CYP1A and other cytochrome P450s or phase II enzymes are similarly modified *in vivo*.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Differential effects of AhR ligands on expression of *CYP1A1* mRNA in the parent Huh7 cell line and the HCV replicon-expressing cell line, Huh.8. Cells were treated with vehicle control (C), 10 μ M β NF (B), 2 μ M MC, or 10 nM TCDD (T) for 24 h. (A) Representative Northern blot. (B) Quantitative real-time RT-PCR. The histogram represents the ratio of *CYP1A1* mRNA to ribosomal RNA (rRNA). The values denote the mean of four independent experiments, with error bars representing standard error of the mean (SEM). Statistical differences between group mean values (Huh7, open bars vs. Huh.8, closed bars) were determined using the unpaired t test. * $p < 0.05$, ** $p < 0.005$.

Figure 2. Suppression of TCDD-induced *CYP1A1* mRNA expression is time- and dose-dependent. (A) Time course. Cells were treated with 10 nM TCDD for 6, 12, and 24 h. (B) Dose response. Cells were treated with 0.1, 1, or 10 nM TCDD for 24 h. Total RNA was analyzed for *CYP1A1* mRNA expression by Northern blots.

Figure 3. Reporter gene activity and AhR binding are suppressed in TCDD-treated Huh.8 cells. (A) Transient transfection assays. Huh7 (open bars) and Huh.8 cells (closed bars) were transiently transfected with the 1A1Luc plasmid, cells treated with TCDD (10 nM) or MC (2 μ M) for 24 h, and lysates assayed using the Dual Luciferase Assay System as described under "Materials and Methods." Luciferase activity is expressed as the ratio of relative light units of *Firefly* to *Renilla* activities. Values shown are for three independent transfection experiments performed in four replicates. Error bars represent standard

deviation from the mean (SD). Statistical differences between group mean values (Huh7 vs. Huh.8) were determined using the unpaired t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** Representative EMSA. Nuclear extracts from cells treated with 10 nM TCDD or 10 μ M β NF for 24 h were incubated with radiolabelled human CYP1A1 XRE ds-oligonucleotide in a binding buffer, as described under "Materials and Methods." Protein-DNA complexes representing the AhR/Arnt complex were quantified by phosphorimaging. Autoradiograph is representative of three nuclear preparations. ns, non-specific complex. Free probe is not shown in this autoradiograph.

Figure 4. AhR and Arnt protein expression is similar between Huh7 and Huh.8. **(A)** Western blot analysis of AhR expression was performed on 30 μ g of cytosolic and 15 μ g of nuclear proteins prepared, as described under "Materials and Methods," from cells exposed to TCDD (10 nM) for 1, 3, 6, and 24 h. **(B)** Analysis of Arnt expression was performed on 15 μ g of nuclear protein isolated from untreated cells. Hepa-1 nuclear extract was used as a positive control.

Figure 5. CYP1A1 protein expression and enzyme activity are suppressed in the Huh.8 cells. **(A)** Representative Western blot. Cells were treated with vehicle control (C), TCDD (T)(10 nM), β NF (B)(10 μ M), or MC (2 μ M) for 24 h. Whole cell lysates were prepared as described under "Materials and Methods" and 20 μ g per lane were analyzed. Blots were probed with CYP1A1 and β -actin antibodies and immune complexes were visualized using chemiluminescence. Positive controls: recombinant CYP1A1 (rCYP1A1) and lysates from TCDD-treated HepG2. Representative image shown for 4-8

independent experiments. **(B)** CYP1A1 enzyme activity was measured using the P450-Glo assay. Cells were plated in 96-well plates and treated with 10 nM TCDD for 24 h. The assay was performed using 50 μ M of the CYP1A1 specific substrate, and luciferase activity measured. Data shown are the mean \pm SD for three independent experiments performed in triplicate. Statistical differences among groups (control, closed bars; TCDD, open bars) were determined using one-way ANOVA followed by Bonferroni's test ($*p < 0.001$).

Figure 6. The suppression of TCDD-induced *CYP1A1* mRNA in Huh.8 cells is partially reversed by antioxidants. Huh.8 cells were pretreated with various concentrations of NAC **(A)** or 15 μ M NDGA **(B)** for 60 min prior to the addition of 10 nM TCDD. After 24 h exposure to TCDD, total RNA was isolated and *CYP1A1* mRNA assayed by quantitative real-time PCR. Shown is the mean \pm SD of 3-5 experiments. For NAC treatments (closed bars), statistical differences from TCDD control (open bar) were determined using one-way ANOVA followed by Dunnett's test ($*p < 0.05$, $** p < 0.01$). For NDGA treatment (closed bar), statistical differences from the TCDD control (open bar) were determined using the unpaired t test ($*p < 0.05$).

Figure 7. Effects of AhR ligands on intracellular H₂O₂ production. Cells were plated on 96-well black plates. The next day, the DCF-DA probe was added to a final concentration of 5 μ M, cells incubated for 30 min, rinsed with PBS, and media containing xenobiotics added. Fluorescence readings were taken after various times of treatment from 15 min to 24 h. Data points shown were obtained from Huh7 (open bars) and Huh.8 (closed bars)

exposed to xenobiotics for 24 h. Fluorescence intensities for the DCF measurements were divided by DNA fluorescence using a DNA assay kit (Molecular Probes) to normalize cell number. The error bars represent the SEM of 8 replicate measurements of an individual experiment, $n = 1$.

Figure 8. Expression of the HCV protein NS5A down-regulates TCDD-induced *CYP1A1* expression in Huh7 cells. **(A)** Expression of *CYP1A1* in Ava.1 cells. Cells were treated with 10 nM TCDD for 24 h and total RNA and protein analyzed for *CYP1A1* expression by Northern (left panel) and Western (right panel) blotting, respectively. **(B)** Adenovirus expression of NS5A. Huh7 cells were infected with 2×10^3 adenovirus particles per cell, containing either Ad-NS5A or Ad-GFP as a negative control. After 24 h post-infection, cells were exposed to 10 nM TCDD for an additional 24 h, and 10 μ g of cellular protein analyzed for *CYP1A1* expression by Western blotting.

Figure 9. **(A)** RT-PCR products of *UGT1A1* and *UGT1A6* from Huh7 and Huh.8 cells. RNA was extracted from cells and used in semi-quantitative RT-PCR reactions. PCR primers and conditions were exactly as described in (Strassburg et al., 1997). The experiment shown is representative of three separate analyses. **(B)** Comparison of constitutive *UGT1A1* and *CYP1A1* mRNA expression. Arrow denotes position of amplified products. Molecular weight markers are shown in the first lane of each blot. Numbers at side of gels represent PCR product sizes.

Figure 1

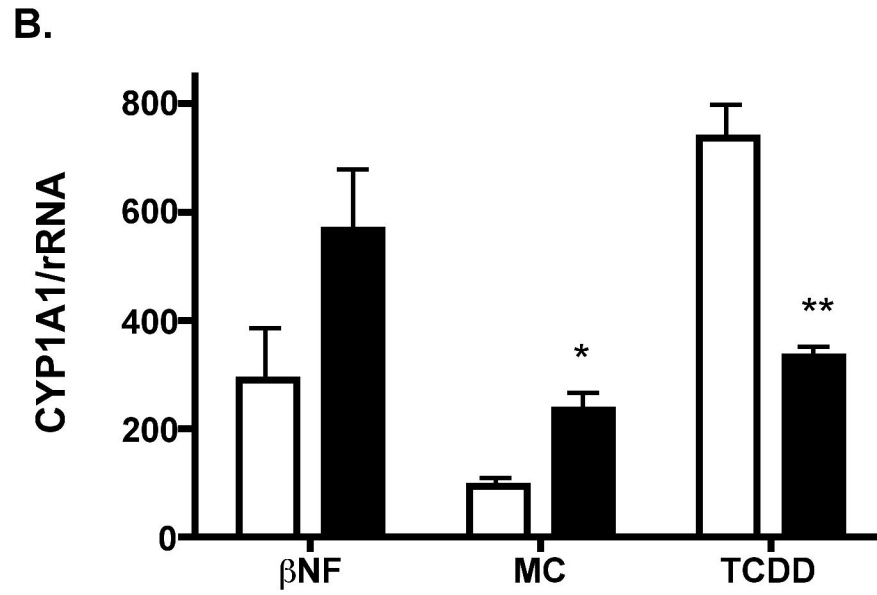
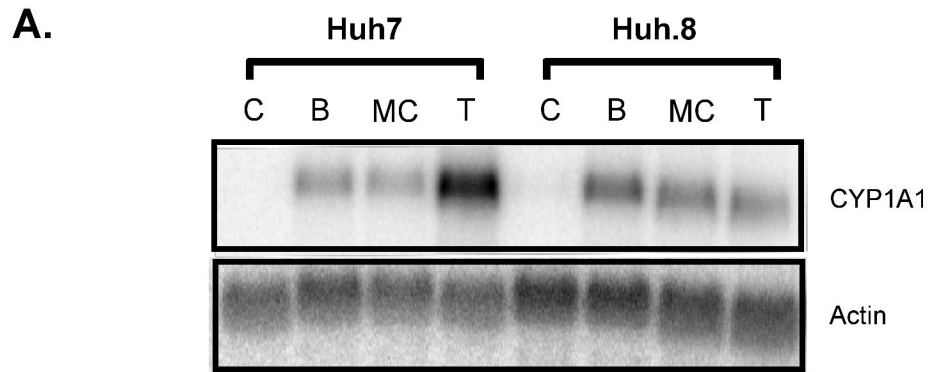
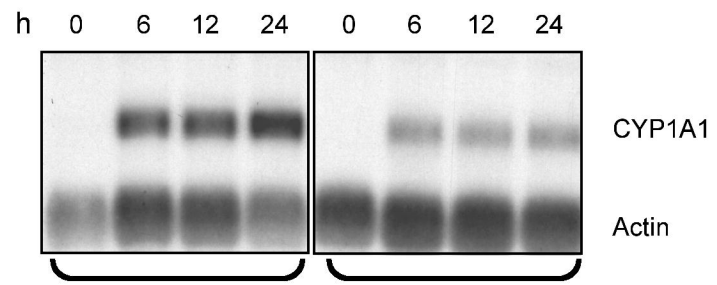


Figure 2

A.



B.

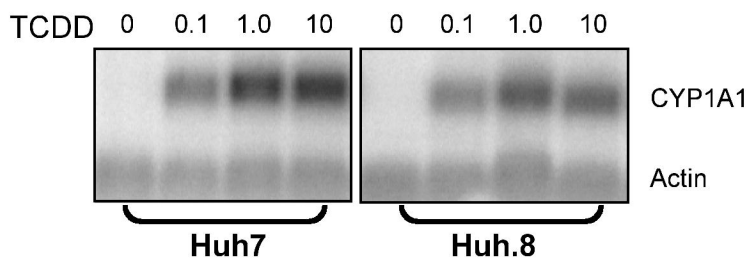


Figure 3

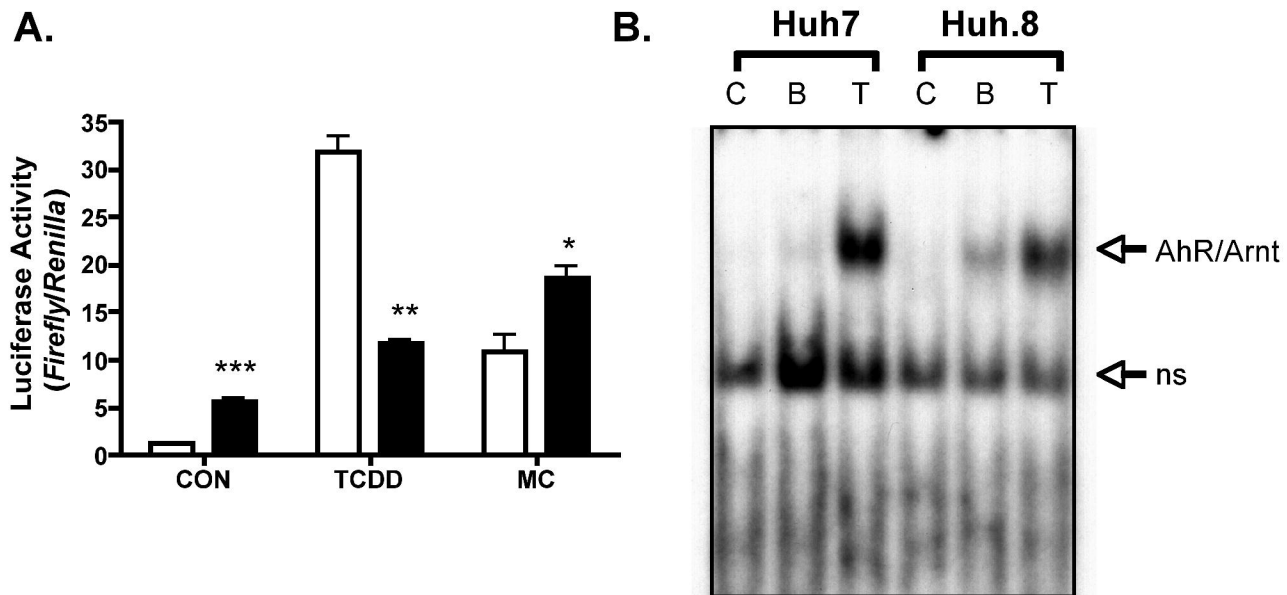


Figure 4

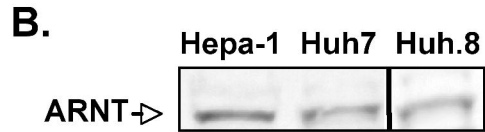
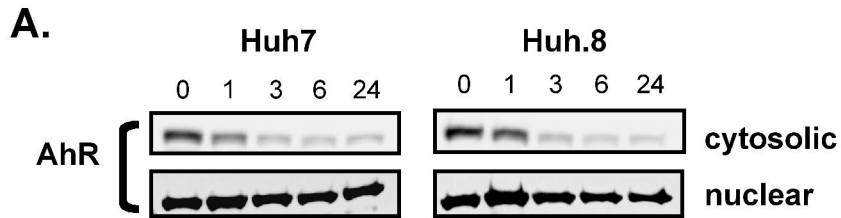


Figure 5

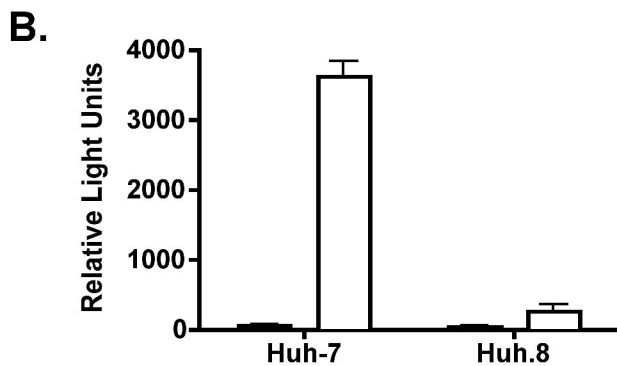
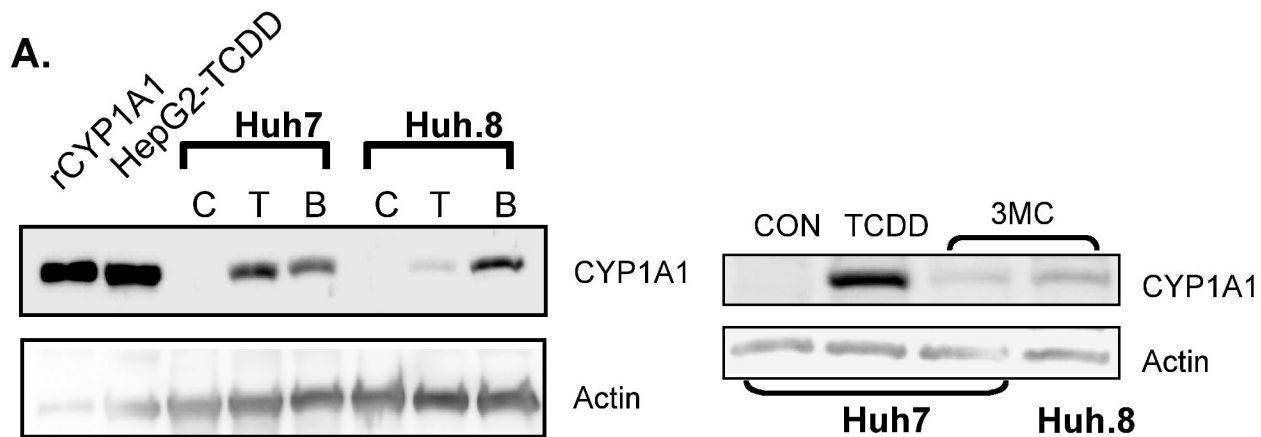
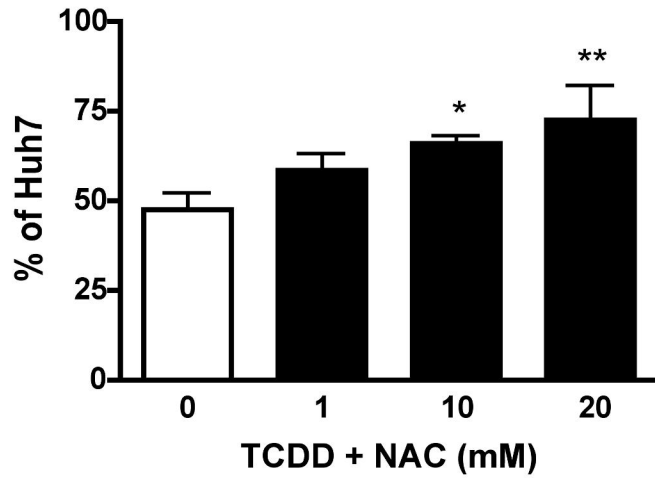


Figure 6

A.



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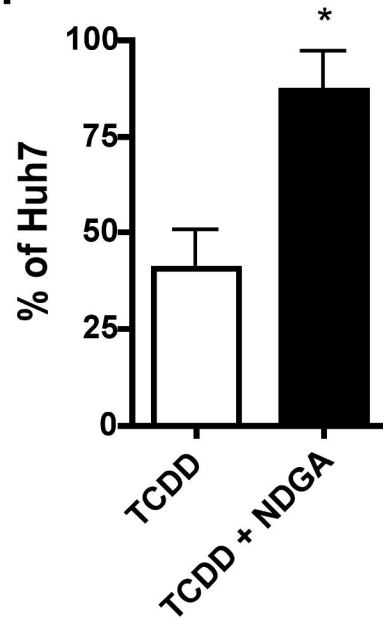


Figure 7

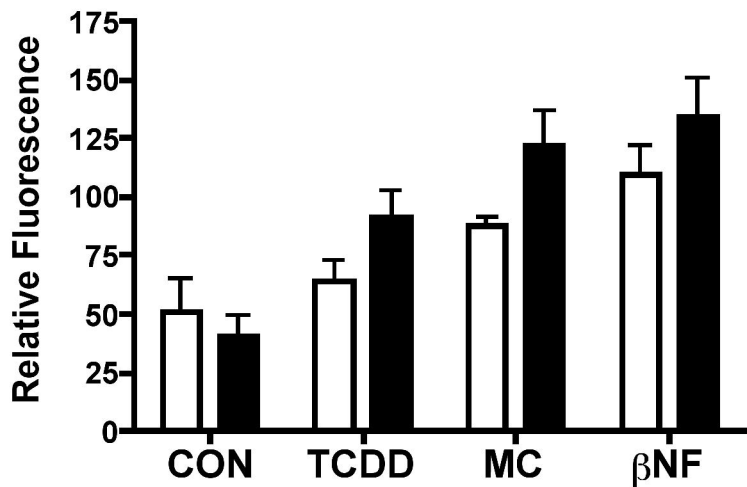
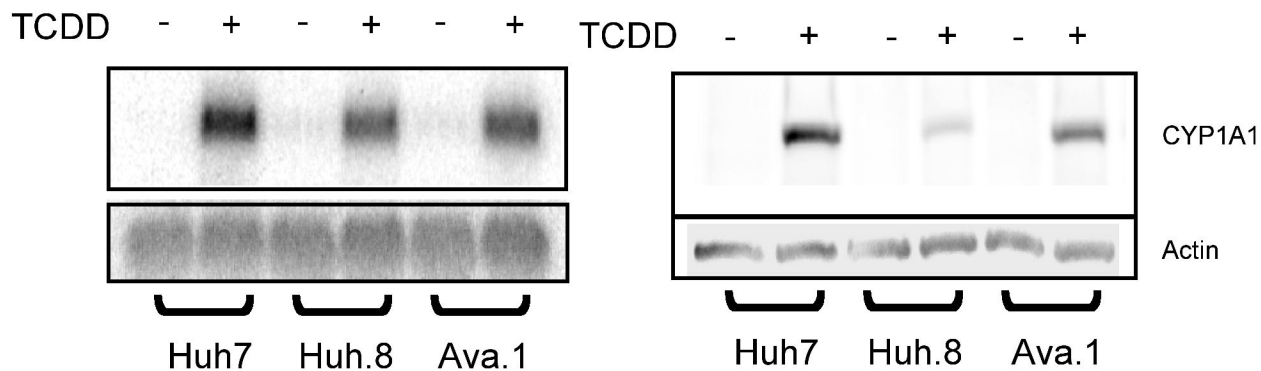


Figure 8

A.



B.

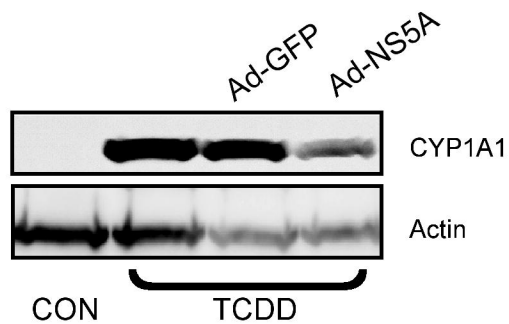


Figure 9

