2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Differentially Suppresses Angiogenic Responses in Human Placental Vein and Artery Endothelial Cells

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Abstract

Placental angiogenesis is dramatically increased during pregnancy in association with the elevated placental blood flows to support the rapidly growing fetus. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental toxicant and a ligand of aryl hydrocarbon receptor (AhR). Herein, we investigated the effects of TCDD on proliferation, migration, and viability of fetoplacental endothelial cells in response to a complete growth medium which contained serum and growth supplement using human umbilical cord vein (HUVECs) and artery (HUAECs) cells as models. We found that TCDD dose- and time-dependently inhibited ($p < 0.05$) proliferation of HUVECs and HUAECs. Treatment with TCDD at 10 nM for 6 days inhibited ($p < 0.05$) migration (by ~30%) of HUAECs, but not HUVECs. TCDD at 10 nM also attenuated ($p < 0.05$) viability of HUVECs and HUAECs. Interestingly, specific AhR siRNA blocked ($p < 0.05$) the TCDD-inhibited cellular responses in HUAECs, but not HUVECs. Nonetheless, TCDD at 10 nM neither affected the cell cycle process, nor did it induce cell apoptosis in HUVECs and HUAECs. In addition, TCDD at 10 nM also did not alter activation of ERK1/2 and AKT1 in HUVECs and HUAECs. Collectively, TCDD suppresses proliferation and/or migration (two key steps of angiogenesis) of HUVECs and HUAECs independent and dependent of AhR, respectively. These data suggest that TCDD inhibited growth of HUVECs and HUAECs via decreasing cell viability. Thus, TCDD may inhibit fetoplacental angiogenesis, leading to negative pregnancy outcomes.

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1. Introduction

During pregnancy, fetoplacental angiogenesis is dramatically increased in association with the elevated placental blood flows to support the rapidly growing fetus (Magness and Zheng, 1996, Reynolds et al., 2005; Wang and Zheng, 2012). In addition, it is well established that defective placental angiogenesis is linked to severe pregnancy complications such as preeclampsia and intrauterine growth restriction, leading to negative pregnancy outcomes (Gilbert et al., 2008; Khankin et al., 2010; Mayhew et al., 2004). Thus, any factor that impairs fetoplacental angiogenesis will jeopardize pregnancy and fetal development.

2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) is a ubiquitous environmental pollutant and toxicant, which belongs to the halogenated aromatic hydrocarbons family (Denison et al., 2011; Pocar et al., 2005). Exposure of humans to TCDD could cause severe intoxication including chloracne, hepatotoxicity, neurotoxicity, and cardiotoxicity (Denison et al., 2011; Pelclová et al., 2006; Pocar et al., 2005). Humans are primarily exposed to TCDD by ingesting contaminated food and by industrial accidents (Pelclová et al., 2006; Pocar et al., 2005). After ingested, TCDD is fairly stably stored in the fatty acid and resistant to metabolic degradation, potentially posing chronic adverse effects on humans (Denison et al., 2011; Pelclová et al., 2006; Pocar et al., 2005). In the general population, average TCDD equivalents (TEQ) concentrations are estimated at 58 ng/kg serum lipid, corresponding to 13 ng TEQ/kg body weight (DeVito et al., 1995). TCDD concentrations in human infant tissues (0.16-0.38 ng/kg lipid of adipose tissue and 0.29-0.49 ng/kg lipid of liver) (Kreuzer et al. 1997), which are believed to be lower than or comparable to those in adults (Kreuzer et al. 1997; Leung et al., 1990; Neubert, 1988). However, when humans are accidently exposed to moderate to high levels of TCDD, the serum lipid TCDD concentrations can reach much high levels, ranging from 130-144,000 ppt (Aylward et al., 2005).

During pregnancy, TCDD can easily cross the placental barrier and enter the fetus (Bell et al., 2007; Nau and Bass, 1981). Thus, repeated prenatal exposure to TCDD can induce fetotoxicity, leading to increased prenatal mortality in many species including mouse, rat, monkey, and perhaps human too (Hernández-Ochoa et al., 2009; Peterson et al., 1993). Importantly, the TCDD-induced fetotoxicity may be partially attributed to abnormal vascular formation, growth, and function. For example, prenatal exposure of chicks to TCDD impairs coronary vasculogenesis in chicken embryos by attenuating responsiveness of embryos to angiogenic factors including vascular endothelial growth factor-A (VEGFA; Ivnitski-Steele et al., 2005). In addition, TCDD exposure suppresses vascular remodeling in the labyrinth zone of rat placentas (Ishimura et al., 2006; Wu et al., 2014) and also induces vascular inflammatory response, contributing to the development of atherosclerosis in adult mice (Wu et al., 2011).

The majority of TCDD's activities is mediated via activation of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (Denison et al., 2011). While most of

unliganded AhR resides in the cytoplasm of cells (Denison et al., 2011), upon binding to its ligands, AhR will transfer to the nucleus, where it binds to aryl hydrocarbon nuclear receptor translocator to form a heterodimer (Denison et al., 2011). This heterodimer subsequently binds to the dioxin response element, activating expression of its downstream genes such as CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1) and CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1) (Denison et al., 2011). The AhR-induced downstream signaling also includes the MEK/ERK1/2 and PI3K/AKT1 pathways (Tan et al., 2002, 2004; Wu et al., 2007). After activation, AhR is translocated back to the cytoplasm rapidly degraded by the proteasome (Denison et al., 2011). In addition, AhR activation can also be suppressed by the AhR repressor (Denison et al., 2011). Intriguingly, TCDD may exert its actions independent of AhR (Ahmed et al., 2005; Kondraganti et al., 2003; Park et al., 2003; Park et al., 2005; Sanders et al., 2005). For example, TCDD induces an antiproliferative response in human breast cancer cells and reduces expression of p16[ink4a] (a cell cycle regulator and tumor suppressor) in human endothelial cells via an AhR independent manner (Akintobi et al., 2007; Yoshioka et al., 2012).

We have recently reported that AhR is present in human placentas, primarily in endothelial cells within villous cords, and in endothelial cells of umbilical cord veins, and arteries (Jiang et al., 2010). We have also shown that one dose of TCDD at 1.6 μg/kg body weight interferes with remodeling of rat placentas (Wu et al., 2014). However, the role of TCDD in human fetoplacental angiogenesis is not known, although 3-methylcholanthrene, another exogenous AhR ligand, has been shown to inhibit cell proliferation of human umbilical vascular endothelial cells *in vitro* (Juan et al., 2006). Therefore, in this study, we hypothesize that TCDD inhibits the angiogenic responses of fetoplacental endothelial cells via AhR, which may ultimately lead to adverse pregnancy outcomes. Specifically, we examined the effect of TCDD on the fetoplacental endothelial proliferation and migration, two key steps of angiogenesis, in response to a complete growth medium containing 10% FBS and growth supplement using human umbilical vein (HUVECs) and artery (HUAECs) endothelial cells as cell models since endothelial cells in vein and artery are different tremendously in their global gene expression profiles (Chi et al., 2003; Jiang et al., 2013a,b), possibly leading to different responses to TCDD. We have also attempted to explore the mechanisms underlying TCDD's effects on these cells.

2. Materials and Methods

2.1. Cell Isolation and Culture

HUVECs and HUAECs, two endothelial cell types widely used as fetoplacental endothelial cell models were isolated from umbilical cords of patients with normal term pregnancies using a standard collagenase enzyme digestion as described (Jiang et al., 2013a,b). After isolation, cells were cultured in basal RPMI 1640 media (BM; Life Technology, Grand Island, NY) supplemented with 10% FBS (Thermo Scientific, Waltham, MA), 1% penicillin/streptomycin, 100 mg/L heparin (EMD Chemicals, San Diego, CA), and 37.5 mg/L endothelial cell growth supplement (Millipore, Billerica, MA) under 37°C, 5% CO_2, and 95% air. This supplemental medium was designated as the complete growth medium

(CGM). After verification of their endothelial phenotypes, cells were pooled from five individual cell preparations and passaged. Cells at passages 4-5 were used for all studies described below.

2.2. Cell Proliferation and Migration

To determine the effect of TCDD on cell proliferation and migration, two indicatives of angiogenesis were performed. Cell proliferation was assayed as described (Li et al., 2014; Wang et al., 2013). Subconfluent cells were seeded in 96-well plates (5000 and 8000 cells/ well for HUVECs and HUAECs, respectively). After 16 hr of culture, cells were treated with 0.1 to 100 nM of TCDD (cat # ED-901-B Cambridge Isotope Laboratories, Tewksbury, MA) or the vehicle, dimethyl sulfoxide (DMSO, 0.1% v/v; the maximum concentration used in the final TCDD solutions, Sigma, St. Louis, MO) in CGM up to 6 days with daily change of media containing TCDD or DMSO. At the end of treatment, the number of cells was determined using the crystal violet method (Li et al., 2014; Wang et al., 2013). The relative IC50 values of TCDD for cell proliferation on Day 6 were calculated using the SigmaPlot graphing and statistical analysis software (v12.7; San Jose, CA).

Cell migration was evaluated using a FluoroBlok Insert System (8.0 μm pores; cat# 351158, BD Biosciences, San Jose, CA) as described (Li et al., 2014; Wang et al., 2013). Subconfluent cells grown on 60 mm culture dishes were treated with TCDD (10 nM) or DMSO (0.01% v/v) in CGM for 6 days with a daily change of medium containing TCDD or DMSO. This dose of TCDD was widely used for *in vitro* studies and was also chosen based on the dose response of cell proliferation described above. Moreover, this concentration of TCDD is ~ 23 fold higher than that in general population (DeVito et al., 1995) and appears to be within the range of TCDD levels in humans who were accidently exposed to moderate to high levels of TCDD (Aylward et al., 2005). Cells were lifted and seeded into the insert (30,000 cells/insert). The bottom wells were also filled with the same medium. After 6 hr of culture, cells migrated were stained with 0.2 μg/ml of calcein AM (cat# C3100MP, Invitrogen, Carlsbad, CA) and counted using the MetaMorph image analysis software.

2.3. Cell Viability

To examine if the TCDD-inhibited cell proliferation is associated with a decrease in cell viability, cell viability assay was performed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay kit (cat # 10009365, Cayman Chemical Company, Ann Arbor, MI). This method is based on reducing the yellow MTT to violet formazan, which is catalyzed by mitochondrial dehydrogenases. Since these enzymes are active only in living cells, the MTT method is widely used to assess cell viability (Berridge *et al*., 2005). Different from the cell proliferation assay as described above, the confluent cells (40000 cells/well, 6 wells/dose) were seeded in 96-well plates. After 16 hr of culture, cells were treated with TCDD (10 or 100 nM) or DMSO (0.1% v/v, the concentration used in 100 nM of TCDD) in CGM for 4 or 6 days with a daily change of medium containing TCDD or DMSO. At the end of treatment, cells were incubated with the MTT reagent for 4 hr, followed by the cell lysis. The absorbance was read at 570 nm using the microplate reader.

2.4. Western Blot Analysis

Western blot analysis was conducted as described (Li et al., 2014; Wang et al., 2013). To confirm AhR activation induced by TCDD, cells were treated with a single dose of TCDD (10 nM) in CGM for 0, 1, 2, 8, 24, or 48 hr. Proteins were subjected to Western blotting (Li et al., 2014; Wang et al., 2013). The membranes were probed with the rabbit anti-AhR antibody (1: 2000; cat# BML-SA210-0100, Enzo Life Sciences, Plymouth Meeting, PA), followed by reprobing with a mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:10,000; cat# H00002597-M01, Novus, Littleton, CO). Proteins were visualized using the enhanced chemiluminescence reagent (Amersham, Piscataway, NJ). Signals were recorded using an Epson Perfection 4990 Photo Scanner (Long Beach, CA) and analyzed using NIH Image J software.

2.5. Reverse Transcription-Quantitative PCR (RT-qPCR)

To confirm induction of AhR's downstream signaling, and to determine the role of TCDD in mRNA expression of pro-angiogenic factors, RT-qPCR was performed as described (Jiang et al., 2013a,b; Li et al., 2014). Subconfluent cells were treated with a single dose of TCDD (10 nM) in CGM for 0, 24, or 48 hr. Total RNA were extracted using RNeasy Plus Mini Kit (Qiagen, cat # 74134, Valencia, CA) and quantified by using NanoDrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The quality and integrity of total RNA were confirmed using the Agilent RNA6000 NanoChip in the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with RNA integrity number scores larger than 9.0 were used. Samples of total RNA (1 μg) were reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (AB applied Biosystems, cat# 4368814) in a 20 μL volume.

The effects of TCDD on mRNA expression of CYP1A1 and CYP1B1 (two well-known signaling molecules induced by TCDD) were first analyzed. The effects of TCDD on mRNA expression of VEGFA and its major receptors (VEGFR1, VEGFR2, NP1, and NP2) were also examined. In parallel, TBP (TATA-BOX), ACTB (β-actin), and GAPDH were run, serving as controls. All primers were showed as the Supplemental table S1. RT-qPCR was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Amplification was performed using a Roche 480 Lightcycler system. Each cDNA reaction was performed in triplicate. Fluorescence was measured at the end of each annealing step for each cycle. To check the specificity of each primer pair, the predicted PCR amplicon melting temperature was confirmed by a dissociation curve analysis. Relative expression values for the genes studied were normalized to the mean of the relative expression values of two out of three reference genes TBP, ACTB, and GAPHD, selected by Qbase Plus software.

2.6. AhR siRNA Transfection

To determine the AhR role in the TCDD-inhibited cell proliferation, migration, and viability, siRNA transfection was performed as described (Li et al., 2014; Wang et al., 2013). The AhR siRNA targeting human AhR was purchased (Dharmacon, Cat # L-004990-00-0020, Chicago, IL). Scrambled siRNA (siRNA) with 5'-Cy3 (Sense: 5-GAGAGGUCCCUCCCAUCUUTT-3; Antisense: 5-AAGAUGGGAGGGACCUCUCTT-3)

was synthesized (IDT, Coralville, IA). Subconfluent cells were transfected with 20 nM of the AhR or scrambled siRNA (the siRNA control) in the Lipofectamine RNAiMAX transfection reagent (Invitrogen) or treated with the transfection reagent alone (the vehicle control) up to 6 days. After an optimal time point was identified, additional cells were transfected for determining their proliferation, migration, and viability in response to TCDD.

2.7. Cell Cycle and Cell Apoptosis

To determine if the TCDD-inhibited cell proliferation is associated with a decrease in cell cycle progress, cell cycle was analyzed using flow cytometry. After serum-starved for 24 hr, cells were treated with TCDD or DMSO in CGM for 24, 36 or 144 hr and then stained with propidium iodide (PI, 0.033 mg/ml) (cat # 195458, MP Biomedicals, Santa Ana, CA). After staining, cells in each phase (G1, S, G2, and M) were analyzed using a flow cytometer (FACSCalibur).

To examine if the TCDD-inhibited cell proliferation is associated with an increase in cell apoptosis, two apoptosis experimental approaches were taken. First, cell apoptosis was examined using the Annexin V and PI apoptosis detection kit (Cat#:88-8005-72, eBioscience, San Diego, CA), following the manufacturer's instruction. After treatment with TCDD (10 nM) or DMSO (0.01% v/v) for 8, 24, 36 or 144 hr, cells were stained by PI and fluorescein isothiocyanate -conjugated Annexin V and analyzed using a flow cytometer (FACSCalibur). Secondary, apoptosis was determined by analyzing the formation of cleaved-caspase-3 proteins by TCDD using Western blotting. Cells were treated with one single dose of TCDD (10 nM) or DMSO (0.01% v/v) in CGM up to 48 hr. Cells treated with Staurosporine (200 nM, 8 hr; cat# s5921, Sigma) were used as a positive control in both apoptosis experimental approaches.

2.8. Statistics

Data were analyzed using one-way ANOVA using the SigmaStat software (Jandel Co., San Rafael, CA). When an F-test was significant, data were compared with their respective control by the Bonferroni's multiple comparison test or Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. TCDD Inhibits Proliferation of HUVECs and HUAECs, and Migration of HUAECs

As compared to the vehicle control (DMSO), TCDD dose- and time-dependently significantly inhibited ($p < 0.05$) proliferation of HUVECs and HUAECs induced by CGM (Fig. 1A and B). In both HUVECs and HUAECs, TCDD at 10 and 100 nM caused a maximal inhibition on Days 4 and 6, respectively, whereas TCDD at 0.1 nM had no effects on Days 2, 4, and 6. On Day 6, TCDD at 100 nM decreased ($p < 0.05$) proliferation of HUVECs and HUAECs by 40% and 35%, respectively. The relative IC50 values for HUVECs and HUAECs on Day 6 were estimated to be 0.94 nM and 3.23 nM, respectively (Supplemental Fig. S1).

Treatment with TCDD at 10 nM for 6 days significantly suppressed ($p < 0.05$) migration of HUAECs, but not HUVECs by ~30% (Fig. 2).

3.2. TCDD Attenuates Viability of HUVECs and HUAECs

Treatment with TCDD at 10 and 100 nM decreased ($p < 0.05$) viability of HUVECs by 12% and 17 %, respectively on Day 4, and by 8% and 13%, respectively on Day 6 (Fig. 3A). Similarly, TCDD at 10 and 100 nM also significantly decreased ($p < 0.05$) viability of HUAECs by 19% and 19%, respectively on day 4, and by 17% and 32%, respectively on Day 6 (Fig. 3B).

3.3. TCDD Activates the AhR/CYP1A1 and AhR/CYP1B1 Pathways

The AhR protein was detected at ~ 95kD in HUVECs and HUAECs (Fig. 4) as reported (Jiang et al., 2010; Juan et al., 2006; Li et al., 2014; Wang et al., 2013). A single dose of TCDD at 10 nM quickly (1 or 2 hr) decreased ($p < 0.05$) AhR protein levels in HUVECs and HUAECs, indicating that TCDD induced activation and degradation of AhR. However, the patterns of changes in AhR protein levels appeared to be slightly different between the HUVECs and HUAECs. For instance, the TCDD-induced decrease in AhR began at 8 hr (68%) in HUVECs, whereas it started at 1 hr (35%) in HUAECs.

To further confirm activation of the AhR downstream signaling, RT-qPCR was conducted for CYP1A1 and CYP1B1. TCDD increased ($p < 0.05$) mRNA levels of CYP1A1 by ~ 52 and 46 fold at 24 and 48 hr, respectively, in HUVECs; and by ~ 94 and 92 fold at 24 and 48 hr, respectively in HUAECs (Fig. 5 A). Similarly, TCDD increased ($p < 0.05$) mRNA of CYP1B1 by ~ 1504 and 1696 fold at 24 and 48 hr, respectively in HUVECs, and by ~ 3131 fold and 5385 fold at 24 and 48hr, respectively in HUAECs (Fig. 5 B). These data demonstrate that TCDD substantially activates the AhR/CYP1A1 and AhR/CYP1B1 pathways in both HUVECs and HUAECs.

3.4. Effects of the AhR siRNA on TCDD-Inhibited Cell Proliferation, Migration and Viability

To further evaluate roles of AhR in the TCDD-inhibited cellular responses in HUVECs and HUAECs, AhR was knocked-down by the AhR siRNA (Fig. 6), followed by conducting the cell proliferation, migration, and viability assays (Fig. 7). As compared with the vehicle control and the scrambled siRNA control, the AhR siRNA at 20 nM decreased ($p < 0.05$) AhR protein levels by 93%, 81%, and 55% in HUVECs (Fig. 6A), while it decreased ($p < 0.05$) AhR protein levels by 89%, 86%, and 58% in HUAECs (Fig. 6B) after 2, 4, and 6 days of AhR siRNA transfection, respectively, indicating the successful knockdown of AhR expression in HUVECs and HUAECs.

The AhR siRNA did not affect the TCDD-inhibited proliferation and viability of HUVECs (Fig. 7A and D, respectively), while it blocked ($p < 0.05$) the TCDD-inhibited proliferation and viability of HUAEC (Fig. 7B and E, respectively). Similarly, the AhR siRNA also blocked ($p < 0.05$) the TCDD-suppressed migration of HUAECs grown in CGM (Fig. 7C and Supplement Fig. S2). These data indicate that TCDD inhibited cell proliferation and viability of HUVECs independent of AhR, whereas TCDD inhibited proliferation, migration, and viability of HUAECs dependent of AhR.

3.5. TCDD Mediates mRNA Expression of VEGFA, VEGFR1, VEGFR2, NP1, and NP2

The effects of TCDD on mRNA expression of VEGFA (one potent angiogenic factor) and its receptors in HUVECs and HUAECs were determined using RT-qPCR. TCDD significantly increased ($p < 0.05$) VEGFA mRNA levels by ~ 210% at 24 hr in HUVECs, and by 40% and 60% at 24 hr and 48 hr, respectively in HUAECs (Fig. 8A). TCDD significantly decreased ($p < 0.05$) VEGFR1 mRNA levels by ~ 20% and 12% at 48 hr in HUVECs and HUAECs, while increased ($p < 0.05$) VEGFR2 by ~ 27% at 24 hr in HUVECs, and by 32% at 24 hr and 48 hr in HUAECs (Fig. 8C). However, TCDD had no effect on the mRNA levels of NP1 and NP2 in HUVECs and HUAECs (data not shown).

3.6. TCDD Does Not Alter CGM-Induced Phosphorylation of ERK1/2 and AKT1

We first confirmed that CGM induced robust activation of the ERK1/2 and AKT1 (Supplement Fig. S3) and the MEK1/2/ERK1/2 and PI3K/AKT1 pathways mediated the CGM-stimulated cell proliferation, and migration (Supplement Fig. S4 and S5). However, we observed that TCDD did not alter the CGM-induced phosphorylation of ERK1/2 and AKT1 at 10 min (Supplemental Fig. S6). TCDD also did not change the phosphorylation levels of ERK1/2 and AKT1 after 1 to 48 hr of TCDD treatment in HUVECs and HUAECs (Supplemental Fig. S7).

3.7. TCDD Does Not Alter Cell Cycle Progression, Neither Does TCDD Increase Cell Apoptosis

To evaluate the TCDD's effects on the cell cycle progression, flow cytometry was conducted. As compared with the vehicle control, TCDD did not alter the percentage of cells in each cell cycle phase (G1, S, G2, and M) in HUVECs and HUAECs (Supplemental Fig. S8).

As compared with DMSO, TCDD at 10 nM did not significantly change the percentage of annexin V and/or PI positive cells in HUVECS and HUAECs up to 144 hr. For example, the percentages of annexin V (indicative of the early apoptosis) positive HUVECs after 8 hr of TCDD and DMSO treatments were similar (10.7% vs. 13.8%). These percentages were significantly ($p < 0.05$) lower than that induced by the positive control staurosporine (40.9 %, Supplemental Fig. S9).

Treatment with TCDD at 10 nM up to 48 hr also did not induce cleavage of caspase-3, neither did it change caspase-3 protein expression in HUVECs and HUAECs (Supplemental Fig. S10). In contrast, staurosporine robustly induced cleavage of caspase-3 (Supplemental Fig. S10). These data suggest that TCDD at the dose and time studied does not induce significant apoptosis of HUVECs and HUAECs.

4. Discussion

In this study, we have demonstrated that TCDD inhibits proliferation of HUVECs and HUAECs and migration of HUAECs grown in a complete growth medium without significantly altering cell progression and apoptosis. These TCDD-induced inhibitory actions are associated with a decrease in cell viability. Intriguingly, these TCDD-inhibited

cellular responses are mediated independent and dependent of AhR in HUVECs and HUAECs, respectively. These data suggest that TCDD may attenuate proliferation and/or migration of fetoplacental vein and artery endothelial cells via decreasing cell viability. These data also indicate that different mechanisms (AhR dependent or independent) mediates the TCDD-inhibited proliferation, viability, and/or migration of fetoplacental vein and artery endothelial cells. However, it is clear that TCDD inhibits these cellular complete growth medium-induced responses not via suppressing the MEK1/2/ERK1/2 and PI3K/AKT1 pathways. Thus, although mechanisms underlying the TCDD's differential regulation of cellular responses in HUVECs and HUAECs remain elusive, these data suggest that TCDD might impair fetoplacental angiogenesis by decreasing endothelial viability, leading to adverse pregnancy outcomes.

The current observations that TCDD decreased cell proliferation and viability is the first report, as far as we are aware, to directly show the TCDD's inhibitory effects on proliferation and viability of primary HUVECs and HUAECs. These observations are contrary to the previous studies (Beatty et al., 1975, Knutson and Poland, 1980), in which TCDD at 100 nM or 1 μM failed to affect growth and viability of 27 human and animal primary cultures and transformed cell lines (not including HUVECs and HUAECs), derived from tissues and/or species susceptible to TCDD toxicity *in vivo*. Nonetheless, our finding is consistent with a previous report, which showed that benzo[a]pyrene (another AhR exogenous ligand) inhibited angiogenic factors-induced neovasculogenesis and angiogenic activity of HUVECs (Li et al., 2010). It is still not clear why these cell types respond differently to TCDD *in vitro*. However, as the most non-responding cells used in the studies by Beatty et al. (1975) and Knutson and Poland (1980) were derived from adult humans and animals, it is possible that fetoplacental cells are more sensitive to TCDD as suggested (Peterson et al., 1993). Moreover, the TCDD-inhibited cell proliferation of HUVECs and HUAECs (Fig. 1) may partially result from the decreased viability of HUVECs and HUAECs as indicated by the decreased activity of mitochondrial dehydrogenases (intracellular NADPH-oxidoreductases) within the live cells in the MTT assay, possibly due to the increased accumulation of cellular reactive oxygen species (Wan et al., 2014). However, it is obvious that the TCDD-decreased activity of mitochondrial dehydrogenases in HUVECs is not sufficient to suppress migration of HUVECs. Another possibility of the TCDD-inhibited cell proliferation is via necroptosis, a programmed form of necrosis (Linkermann and Green. 2014).

It is noteworthy that TCDD differentially regulated proliferation and migration of HUVECs and HUAECs. We do not know what cause these differential regulation between HUVECs and HUAECs. However, the lack of a migratory response to TCDD in HUVECs obviously does not result from uncoupling TCDD from the AhR/CYP1A1 and AhR/CYP1B1 pathway since TCDD robustly activated both of these pathways in HUVECs and HUAECs, although we cannot exclude the possibility that HUAECs are more sensitive to the activation of AhR/CYP1A1/B1 than HUVECs. Thus, other yet to be identified mechanisms must be involved in such differential regulation. Among these potential downstream signaling molecules, both ERK1/2 and AKT1, possibly JNK and p38 MAPK too (unpublished data from Zheng's lab) can be excluded from the potential candidate list since TCDD did not significantly alter

activation of these four kinases in HUVECs and HUAECs. This is not surprisingly since that another AhR ligand [2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)] also does not inhibit the TGFβ1-induced phosphorylation of ERK1/2 or AKT1 in primary human orbital fibroblasts (Lehmann et al., 2011). Other possible signaling molecules, however, might include p21 and p27 as 3-methycholanthrene (3-MC, another AhR ligand) has been shown to inhibit the proliferation of HUVECs via inducing p21 and p27 (Pang et al., 2008).

Our observation that TCDD did not suppress the cell cycle progression in HUVECs and HUAECs is contrast to the previous report showing that 3MC arrested the cell cycle at G0/G1, even though only one representative data set from the 20 hr of treatment was present in this report (Juan et al., 2006). In addition, the estimated doubling times for HUVECs and HUAECs under the current culture condition are ~ 40 and 52 hr, respectively, which are similar to the previously reported ones (47 and 46 hr) for HUVECs and HUAECs, respectively (Martín et al., 2009). Thus, it is likely that the three time points (24, 36, and 144 hr) run in the current study should be within the optimal range to detect the possible TCDD-induced changes in the cell cycle.

The TCDD-inhibited cell proliferation and migration are also not due to significantly increased apoptosis of HUVES and HUAECs as determined using two widely-used approaches. This is supported by the current observation that the numbers of HUVECs and HUAECs after TCDD treatments were always higher than those initially seeded (5000 and 8000 cells/well for HUVECs and HUAECs, respectively). For instance, the estimated cell numbers in the TCDD (10 nM) treatment on Day 6 was 10000 ± 1947 and 10078 ± 2172 for HUVECs and HUAECs, respectively.

The most striking finding of the current study is that the TCDD-inhibited cellular responses (proliferation, migration, and viability) are mediated via AhR independent and dependent manners in HUVECs and HUAECs, respectively. These data further confirm the regulatory heterogeneity of HUVECs and HUAECs in addition to their tremendous differences in global gene expression profiles (Chi et al., 2003; Jiang et al., 2013a, b). However, these data are not surprising since the AhR independent manner in TCDD mediation has been reported. For example, TCDD induced the antiproliferative response in human breast cancer cells MCF-7 and reduction of p16[ink4a] expression in HUVECs via an AhR independent manner (Akintobi et al., 2007; Yoshioka et al., 2012), and that ITE also inhibited TGFβ1 signaling in human fibroblasts independent of AhR (Lehmann et al., 2011). In addition, since the AhR siRNA only partially suppressed the AhR expression in HUVECs and HUAECs, it is possible that the remaining AhR is sufficient to mediate the cellular responses in HUVECs, but not HUAECs. Moreover, as the AhR siRNA induced similar decreases in AhR protein levels in HUVECs and HUAECs, these data also imply that HUAECs are more sensitive to the altered AhR levels as compared with HUVECs.

VEGFA plays important roles in mediating the fetoplacental angiogenesis which is regulated via activation of its receptors including VEGFR1, VEGFR2, NP1, and NP2 (Magness and Zheng, 1996; Reynolds et al., 2005; Wang and Zheng, 2012). Among these receptors, VEGFR2 mediates most known VEGFA's activities (i.e., cell proliferation, migration, and

permeability), while VEGFR1 may antagonize the VEGFR2's stimulatory effect (Gille et al., 2000; Zeng et al., 2001). Our current observations demonstrated that TCDD increased expression of VEGFA and VEGFR2 mRNA, while decreased expression of VEGFR1 mRNA. To date, it is unclear what is the mechanism controlling such different expression. However, given that TCDD also increased expression of VEGFA mRNA in rat placentas (Wu et al., 2014), we propose that TCDD may compensatorily increase expression of pro-angiogenic factors and decrease expression of anti-angiogenic factor in these fetoplacental endothelial cells upon inhibiting growth of these endothelial cells.

5. Conclusion

In conclusion, our current data indicate that TCDD differentially suppresses angiogenic responses of HUVECs and HUAECs, although the underlying signaling mechanisms remain elusive. Moreover, since that placental angiogenesis is vital for the placental growth (Magness and Zheng, 1996; Reynolds et al., 2005; Wang and Zheng, 2012), and TCDD can easily cross the placenta barrier (Bell *et al*., 2007; Nau and Bass, 1981), maternal exposure of exogenous AhR ligands such as TCDD may impair the fetoplacental angiogenesis, leading to negative pregnancy outcomes. In addition, as the increased number of endogenous AhR ligands have been identified (Nguyen and Bradfield, 2008), further studies are needed to explore their roles in placentas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Ahmed S, Shibazaki M, Takeuchi T, Kikuchi H. Protein kinase Cθ activity is involved in the 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced signal transduction pathway leading to apoptosis in L-MAT, a human lymphoblastic T-cell line. FEBS J. 2005; 272:903–915. [PubMed: 15691325]

Akintobi AM, Villano CM, White LA. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) exposure of normal human dermal fibroblasts results in AhR-dependent and -independent changes in gene expression. Toxicol Appl Pharmacol. 2007; 220:9–17. [PubMed: 17257637]

Aylward LL, Brunet RC, Carrier G, Hays SM, Cushing CA, Needham LL, Patterson DG Jr, Gerthoux PM, Brambilla P, Mocarelli P. Concentration-dependent TCDD elimination kinetics in humans: toxicokinetic modeling for moderately to highly exposed adults from Seveso, Italy, and Vienna, Austria, and impact on dose estimates for the NIOSH cohort. J Expo Anal Environ Epidemiol. 2005; 15:51–65. [PubMed: 15083163]

Beatty PW, Lembach KJ, Holscher MA, Neal RA. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on mammalian cells in tissue cultures. Toxicol Appl Pharmacol. 1975; 31:309–312. [PubMed: 165593]

Bell DR, Clode S, Fan MQ, Fernandes A, Foster PM, Jiang T, Loizou G, MacNicoll A, Miller BG, Rose M, Tran L, White S. Relationships between tissue levels of 2,3,7,8-tetrachlorodibenzo-p-

dioxin (TCDD), mRNAs, and toxicity in the developing male Wistar (Han) rat. Toxicol Sci. 2007; 99:591–604. [PubMed: 17656490]

Berridge MV, Tan AS, Herst PM. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev. 2005; 11:127–152. [PubMed: 16216776]

Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci USA. 2003; 100:10623–10628. [PubMed: 12963823]

Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. Toxicol Sci. 2011; 124:1–22. [PubMed: 21908767]

DeVito MJ, Birnbaum LS, Farland WH, Gasiewicz TA. Comparisons of estimated human body burdens of dioxin-like chemicals and TCDD body burdens in experimentally exposed animals. Environmental Health Perspectives. 1995; 103:820–831. [PubMed: 7498094]

Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am J Physiol Heart Circ Physiol. 2008; 294:H541–H550. [PubMed: 18055511]

Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, Ferrara N. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3′-kinase activation and endothelial cell migration. EMBO J. 2000; 19:4064–4073. [PubMed: 10921887]

Hernández-Ochoa I, Karman BN, Flaws JA. The role of the aryl hydrocarbon receptor in the female reproductive system. Biochem Pharmacol. 2009; 77:547–559. [PubMed: 18977336]

Ishimura R, Kawakami T, Ohsako S, Nohara K, Tohyama C. Suppressive effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on vascular remodeling that takes place in the normal labyrinth zone of rat placenta during late gestation. Toxicol Sci. 2006; 91:265–274. [PubMed: 16495355]

Ivnitski-Steele ID, Friggens M, Chavez M, Walker MK. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) inhibition of coronary vasculogenesis is mediated, in part, by reduced responsiveness to endogenous angiogenic stimuli, including vascular endothelial growth factor A (VEGF-A). Birth Defects Res A Clin Mol Teratol. 2005; 73:440–446. [PubMed: 15880788]

Jiang YZ, Wang K, Fang R, Zheng J. Expression of aryl hydrocarbon receptor in human placentas and fetal tissues. J Histochem Cytochem. 2010; 58:679–685. [PubMed: 20354149]

Jiang YZ, Wang K, Li Y, Dai CF, Wang P, Kendziorski C, Chen DB, Zheng J. Transcriptional and functional adaptations of human endothelial cells to physiological chronic low oxygen. Biol Reprod. 2013a; 88:114. [PubMed: 23536375]

Jiang YZ, Wang K, Li Y, Dai CF, Wang P, Kendziorski C, Chen DB, Zheng J. Enhanced cellular responses and distinct gene profiles in human fetoplacental artery endothelial cells under chronic low oxygen. Biol Reprod. 2013b; 89:133. [PubMed: 24152727]

Juan SH, Lee JL, Ho PY, Lee YH, Lee WS. Antiproliferative and antiangiogenic effects of 3-methylcholanthrene, an aryl-hydrocarbon receptor agonist, in human umbilical vascular endothelial cells. Eur J Pharmacol. 2006; 530:1–8. [PubMed: 16359657]

Khankin EV, Royle C, Karumanchi SA. Placental vasculature in health and disease. Semin Thromb Hemost. 2010; 36:309–320. [PubMed: 20490981]

Knutson JC, Poland A. 2,3,7,8-Tetrachlorodibenzo-p-dioxin: failure to demonstrate toxicity in twenty-three cultured cell types. Toxicol Appl Pharmacol. 1980; 54:377–583. [PubMed: 7394793]

Kondraganti SR, Ferandez-Salguero P, Gonzalez FJ, Ramos KS, Jiang W, Moorthy B. Polycyclic aromatic hydrocarbon-inducible DNA adducts: evidence by 32P-postlabeling and use of knockout mice for Ah receptor-independent mechanisms of metabolic activation in vivo. Int J Cancer. 2003; 103:5–11. [PubMed: 12455047]

Kreuzer PE, Csanády GA, Baur C, Kessler W, Päpke O, Greim H, Filser JG. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and congeners in infants. A toxicokinetic model of human lifetime body burden by TCDD with special emphasis on its uptake by nutrition. Arch Toxicol. 1997; 71:383–400. [PubMed: 9195020]

Lehmann GM, Xi X, Kulkarni AA, Olsen KC, Pollock SJ, Baglole CJ, Gupta S, Casey AE, Huxlin KR, Sime PJ, Feldon SE, Phipps RP. The aryl hydrocarbon receptor ligand ITE inhibits TGFβ1-

induced human myofibroblast differentiation. Am J Pathol. 2011; 178:1556–1567. [PubMed: 21406171]

Leung HW, Wendling JM, Orth R, Hileman F, Paustenbach DJ. Relative distribution of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human hepatic and adipose tissues. Toxicol Lett. 1990; 50:275–282. [PubMed: 2309246]

Li CH, Cheng YW, Hsu YT, Hsu YJ, Liao PL, Kang JJ. Benzo[a]pyrene inhibits angiogenic factors-induced alphavbeta3 integrin expression, neovasculogenesis, and angiogenesis in human umbilical vein endothelial cells. Toxicol Sci. 2010; 118:544–553. [PubMed: 20876236]

Li Y, Wang K, Jiang YZ, Chang XW, Dai CF, Zheng J. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits human ovarian cancer cell proliferation. Cell Oncol (Dordr). 2014; 37:429–437. [PubMed: 25404385]

Linkermann A, Green DR. Necroptosis. N Engl J Med. 2014; 370:455–465. [PubMed: 24476434]

Magness, RR.; Zheng, J. Circulatory changes during gestation. In: Gluckman, PD.; Heymann, MA., editors. Scientific Basis of Pediatric and Perinatal Medicine. Edward Arnold Publishers; London: 1996. p. 762-772.

Martín de Llano JJ, Fuertes G, Torró I, García Vicent C, Fayos JL, Lurbe E. Birth weight and characteristics of endothelial and smooth muscle cell cultures from human umbilical cord vessels. J Transl Med. 2009; 7:30. [PubMed: 19393064]

Mayhew TM, Charnock-Jones DS, Kaufmann P. Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies Placenta. 2004; 25:127–139. [PubMed: 14972445]

Nau H, Bass R. Transfer of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to the mouse embryo and fetus. Toxicology. 1981; 20:299–308. [PubMed: 7314120]

Neubert D. Significance of pharmacokinetic variables in reproductive and developmental toxicity. Xenobiotica. 1988; 18(Suppl 1):45–58. [PubMed: 3278490]

Nguyen LP, Bradfield CA. The search for endogenous activators of the aryl hydrocarbon receptor. Chem Res Toxicol. 2008; 21:102–116. [PubMed: 18076143]

Pang PH, Lin YH, Lee YH, Hou HH, Hsu SP, Juan SH. Molecular mechanisms of p21 and p27 induction by 3-methylcholanthrene, an aryl-hydrocarbon receptor agonist, involved in antiproliferation of human umbilical vascular endothelial cells. J Cell Physiol. 2008; 215:161–171. [PubMed: 18022818]

Park JH, Hahn EJ, Kong JH, Cho HJ, Yoon CS, Cheong SW, Oh GS, Youn HJ. TCDD-induced apoptosis in EL-4 cells deficient of the aryl hydrocarbon receptor and down-regulation of IGFBP-6 prevented the apoptotic cell death. Toxicol Lett. 2003; 145:55–68. [PubMed: 12962974]

Park SJ, Yoon WK, Kim HJ, Son HY, Cho SW, Jeong KS, Kim TH, Kim SH, Kim SR, Ryu SY. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin activates ERK and p38 mitogen-activated protein kinases in RAW 264.7 cells. Anticancer Res. 2005; 25:2831–2386. [PubMed: 16080534]

Pelclová D, Urban P, Preiss J, Lukás E, Fenclová Z, Navrátil T, Dubská Z, Senholdová Z. Adverse health effects in humans exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). Rev Environ Health. 2006; 21:119–138. [PubMed: 16898675]

Peterson RE, Theobald HM, Kimmel GL. Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol. 1993; 23:283–335. [PubMed: 8260069]

Pocar P, Fischer B, Klonisch T, Hombach-Klonisch S. Molecular interactions of the aryl hydrocarbon receptor and its biological and toxicological relevance for reproduction. Reproduction. 2005; 129:379–389. [PubMed: 15798013]

Reynolds LP, Borowicz PP, Vonnahme KA, Johnson ML, Grazul-Bilska AT, Wallace JM, Caton JS, Redmer DA. Animal models of placental angiogenesis. Placenta. 2005; 26:689–708. [PubMed: 16226119]

Sanders JM, Burka LT, Smith CS, Black W, James R, Cunningham ML. Differential expression of CYP1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. Toxicol Sci. 2005; 88:127–133. [PubMed: 16107549]

Tan Z, Chang X, Puga A, Xia Y. Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function. Biochemical Pharmacology. 2002; 64:771. [PubMed: 12213569]

Tan Z, Huang M, Puga A, Xia Y. A critical role for MAP kinases in the control of Ah receptor complex activity. Toxicol Sci. 2004; 82:80–87. [PubMed: 15272135]

Wan C, Liu J, Nie X, Zhao J, Zhou S, Duan Z, Tang C, Liang L, Xu G. 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces premature senescence in human and rodent neuronal cells via ROS-dependent mechanisms. PLoS One. 2014; 9:e89811. [PubMed: 24587053]

Wang K, Li Y, Jiang YZ, Dai CF, Patankar MS, Song JS, Zheng J. An endogenous aryl hydrocarbon receptor ligand inhibits proliferation and migration of human ovarian cancer cells. Cancer Lett. 2013; 340:63–71. [PubMed: 23851185]

Wang K, Zheng J. Signaling regulation of fetoplacental angiogenesis. J Endocrinol. 2012; 212:243–55. [PubMed: 22106098]

Wu R, Zhang L, Hoagland MS, Swanson HI. Lack of the aryl hydrocarbon receptor leads to impaired activation of AKT/protein kinase B and enhanced sensitivity to apoptosis induced via the intrinsic pathway. J Pharmacol Exp Ther. 2007; 320:448–457. [PubMed: 17018692]

Wu D, Nishimura N, Kuo V, Fiehn O, Shahbaz S, Van Winkle L, Matsumura F, Vogel CF. Activation of aryl hydrocarbon receptor induces vascular inflammation and promotes atherosclerosis in apolipoprotein E-/- mice. Arterioscler Thromb Vasc Biol. 2011; 31:1260–1267. [PubMed: 21441140]

Wu Y, Chen X, Zhou Q, He Q, Kang J, Zheng J, Wang K, Duan T. ITE and TCDD differentially regulate the vascular remodeling of rat placenta via the activation of AhR. PLoS One. 2014; 9:e86549. [PubMed: 24475139]

Yoshioka H, Hiromori Y, Aoki A, Kimura T, Fujii-Kuriyama Y, Nagase H, Nakanishi T. Possible aryl hydrocarbon receptor-independent pathway of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced antiproliferative response in human breast cancer cells. Toxicol Lett. 2012; 211:257–265. [PubMed: 22521833]

Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. J Biol Chem. 2001; 276:26969–26979. [PubMed: 11350975]
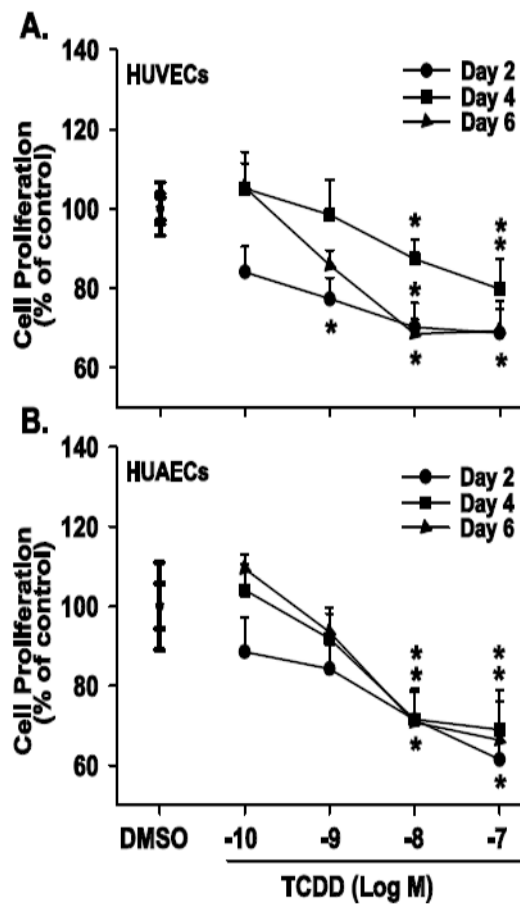
Figure 1.
Effects of TCDD on Proliferation of HUVECs and HUAECs. Subconfluent HUVECs (A) and HUAECs (B) were treated with DMSO (vehicle control) or TCDD in CGM for up to 6 days with a daily change of CGM containing DMSO or TCDD. Cell numbers were determined by the crystal violet. Data are expressed as means ± SEM % of the control (n =3-4). *Differ from the control within each corresponding day ($p < 0.05$).
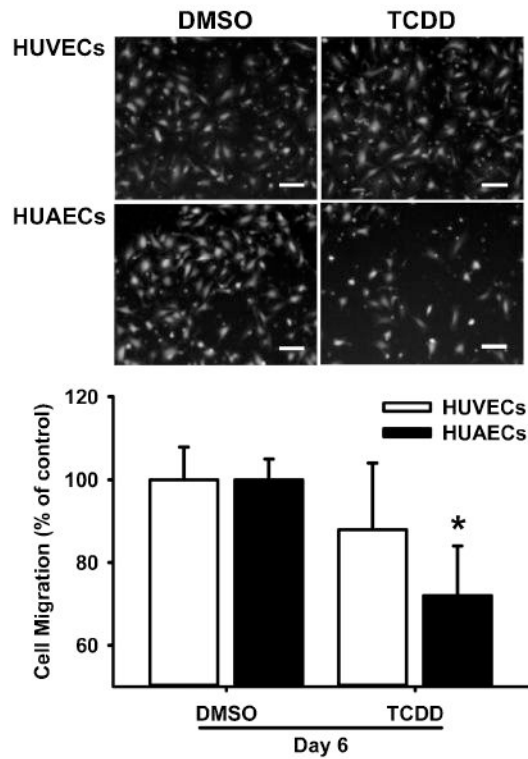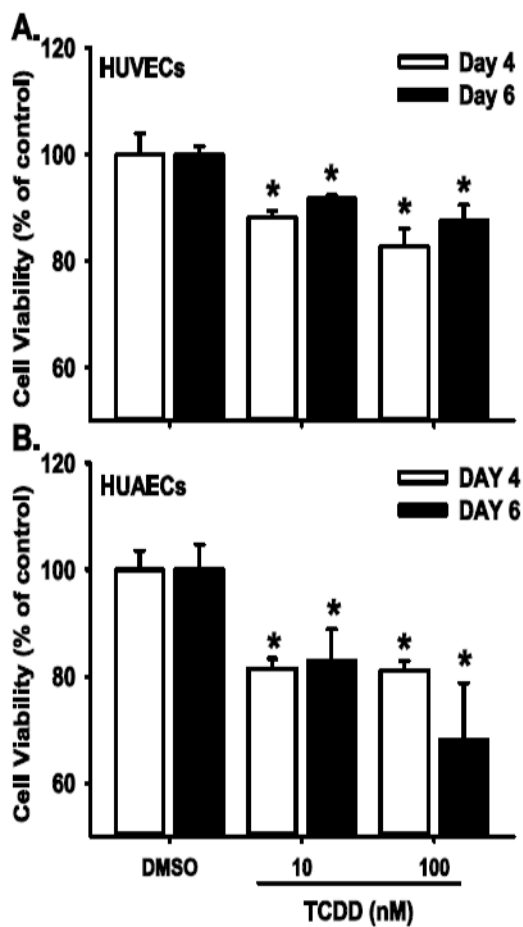
Figure 2.
Effects of TCDD on Migration of HUVECs and HUAECs. Cells were treated with DMSO (vehicle control) or TCDD (10 nM) in CGM for 6 days, followed by the migration assay. The migrated cells were stained and counted. Data are expressed as means ± SEM % of the control (n = 4-5). *Differ from the control within each corresponding day ($p < 0.05$). Bars, 200 μm.

Figure 3.
Effects of TCDD on Viability of HUVECs and HUAECs. Confluent HUVECs (A) and
HUAECs (B) were treated with DMSO (vehicle control) or with TCDD in CGM for 4 or 6
days with a daily change of DMSO or TCDD. Cell viability was determined by the MTT
assay. Data are expressed as means ± SEM % of the control (n = 4-5). *Differ from the
control within each corresponding day ($p < 0.05$).

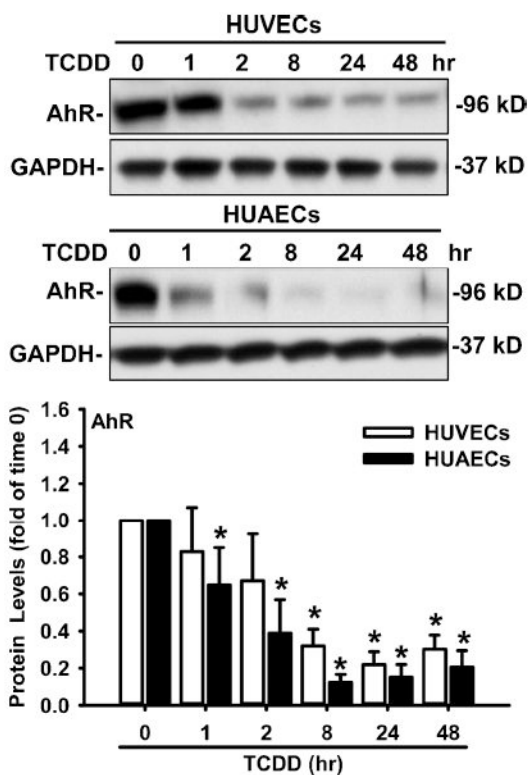**Figure 4.**
Effects of TCDD on Protein Levels of AhR in HUVECs and HUAECs. Cells were treated with a single dose of DMSO (TCDD 0 hr, vehicle control) or TCDD (10 nM) in CGM up to 48 hr. Proteins were subjected to Western blotting. Data are expressed as means ± SEM fold of the control (n =3-5). *Differ from the time 0 control ($p < 0.05$).

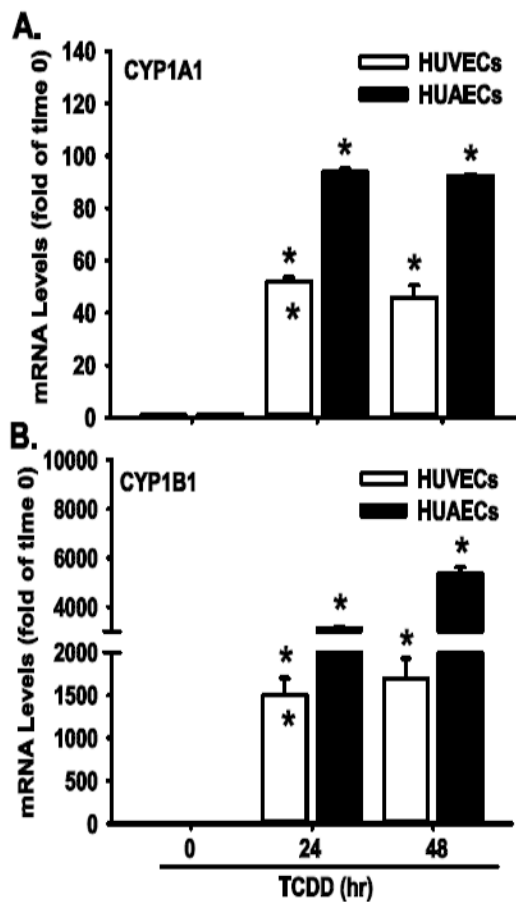**Figure 5.**
Effects of TCDD on mRNA Levels of CYP1A1 and CYP1B1 on HUVECs & HUAECs.
Cells were treated with DMSO (vehicle control, 0.01% v/v)/with TCDD (10 nM) in CGM
for 24 or 48 hr, followed by RT-qPCR assay for CYP1A1 (A) and CYP1B1 (B). Data are
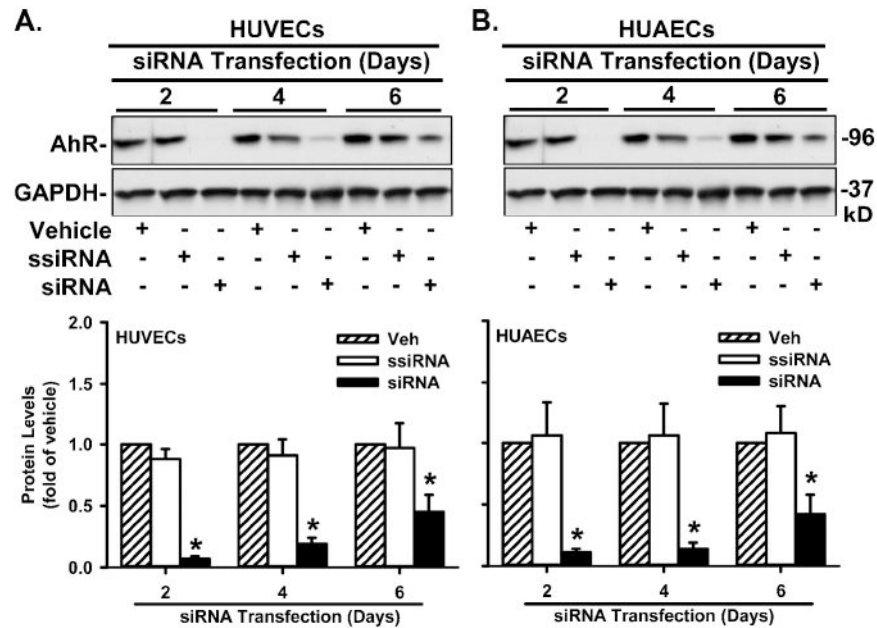expressed as means ± SEM fold of the vehicle (n =3). *Differ from the vehicle ($p < 0.05$).

Figure 6.
Effects of AhR siRNA on AhR Protein Expression in HUVECs and HUAECs. HUVECs (A)
and HUAECs (B) were treated with the transfection reagent (vehicle) or transfected with the
scrambled (ssiRNA, 20 nM) or AhR siRNA (siRNA, 20 nM) for 2, 4, or 6 days. Proteins
were subjected to Western blotting to determine the degree of AhR knock down. Data are
expressed as means ± SEM fold of the vehicle (Veh, n =4). *Differ from the vehicle (Veh)
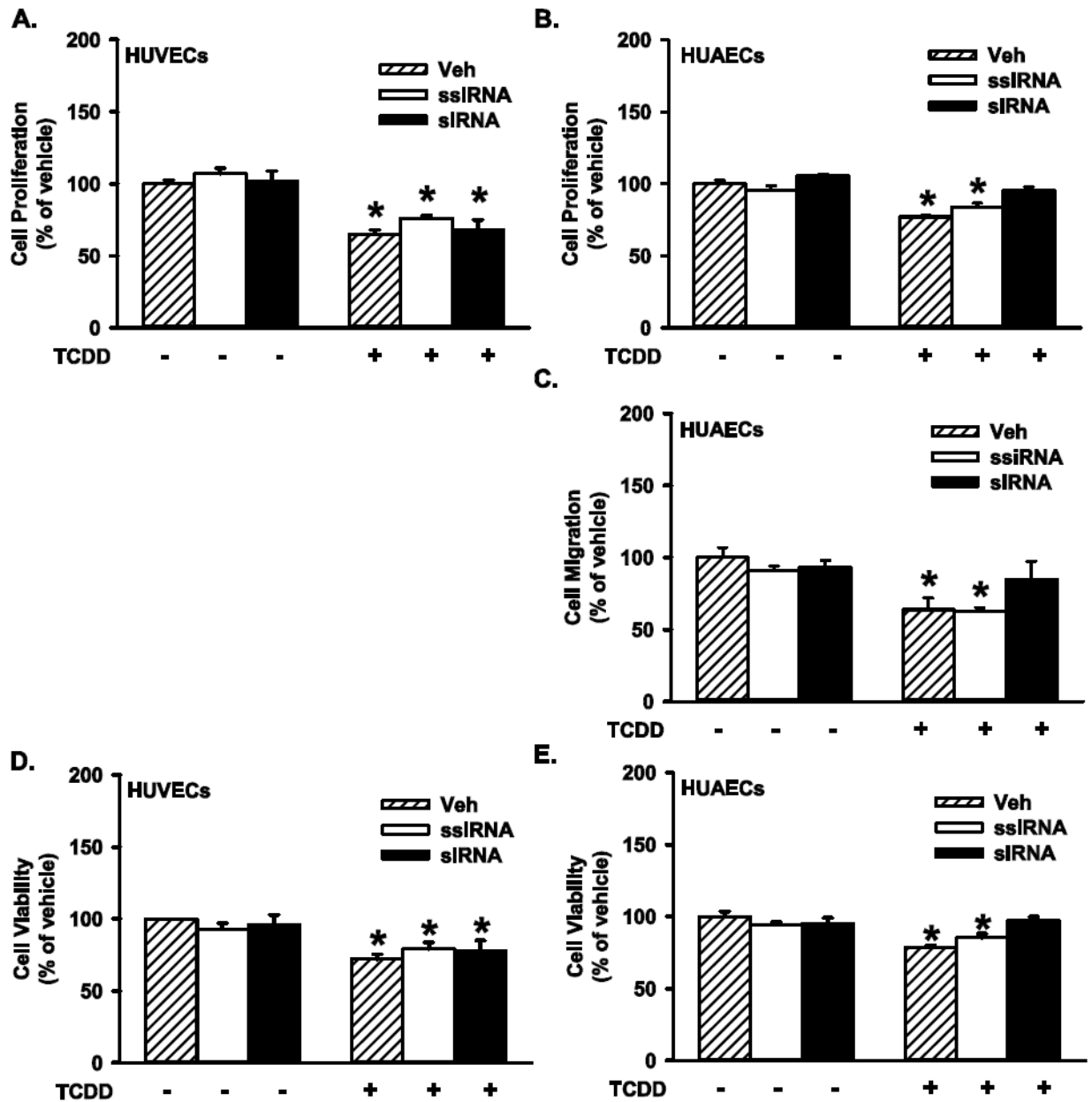($p < 0.05$).

Figure 7.
Effects of AhR siRNA on the TCDD-inhibited Proliferation, Migration, and Viability of HUVECs and HUAECs. Cells were treated with the vehicle control or transfected with the scrambled (ssiRNA) or AhR siRNA for 2 days. After transfection, cells were treated with TCDD (10 nM) for 4 days (for cell proliferation, A and B), with TCDD (10 nM) for 2 days (for cell migration, C), or with TCDD (100 nM) for 4 days (for cell viability, D and E) in CGM with a daily change of TCDD, followed by the cell proliferation, viability, and migration assays. Data are expressed as means ± SEM % of the vehicle (Veh; n = 5-6). *Differ from Veh ($p < 0.05$).
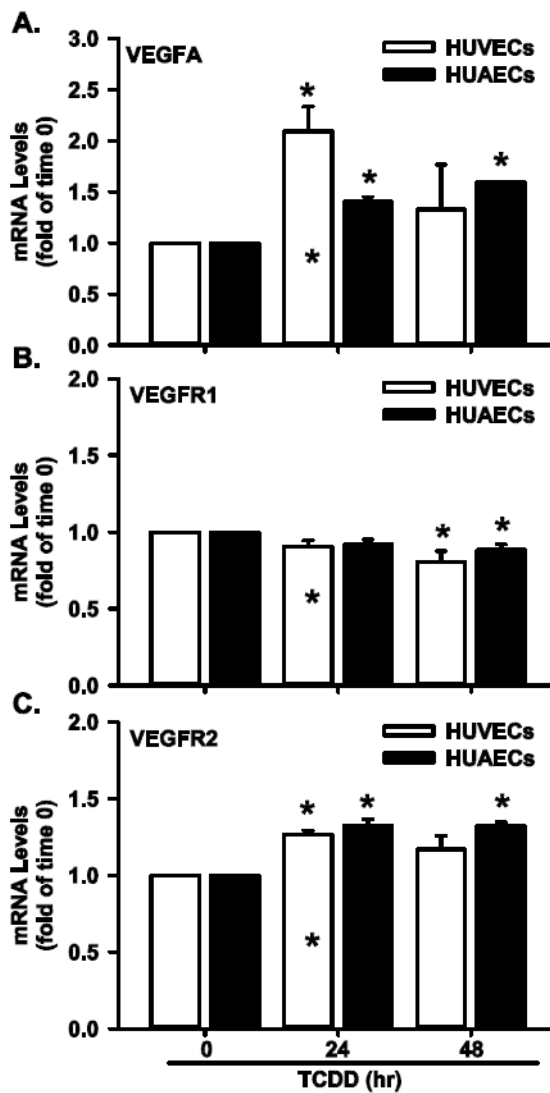
Figure 8.
Effects of TCDD on mRNA Levels of VEGFA, VEGFR1 and VEGFR2 in HUVECs and HUAECs. Cells were treated with DMSO (TCDD 0 hr, the control) or TCDD (10 nM) in CGM for 24 or 48 hrs, followed by real time-qPCR assay. Data are expressed as means ± SEM fold of the control (n=3). *Differ from the control ($p < 0.05$).