Supplementary Materials

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

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Supplemental Methods

Cell Isolation and Culture

The cord collection and endothelial cell isolation protocols were approved by the Institutional Review Board of Meriter Hospital, and the Health Sciences Institutional Review Boards of the University of Wisconsin–Madison. After 3-4 days of isolation and culture, cells were sorted by flow cytometry based on their expression of CD31. The purity of cells was further verified by uptake of acetylated low density lipoprotein labeled with 1,10-dioctadecyl-3,3,30,30-tetramethyl-indocarbocyanineperchlorate (DiI-Ac-LDL) (Jiang et al., 2013a,b).

Cell Proliferation and Migration

To examine the effects of MEK1/2 and PI3K kinase pathways on cell proliferation, subconfluent cells were seeded in 96-well plates (5000 and 8000 cells/well for HUVECs and HUAECs, respectively). Cell proliferation was assayed as described (Li et al., 2014; Wang et al., 2013). After 16 hr of culture, cells were treated with 1.25 – 5.0μM of LY294002 (a selective PI3K inhibitor, Millipore), 10-50 μM of PD98059 (a selective MEK1/2 inhibitor, Millipore, Billerica, MA) or DMSO (0.1% v/v, the control) in CGM for 2 and 4 days with daily change of media containing LY294002, PD98059 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). Briefly, cells were rinsed with PBS (5 mM phosphate, 145 mM NaCl, 5 mM KCl, pH 7.5), fixed in methanol for 30 min, air-dried for 5 min, and stained with 0.1% (w/v) crystal violet for 15 min. After staining, wells were rinsed with distilled water, and air dried again. Once dry, cells were solubilized with 2% (w/v) sodium deoxycholate solution for 30 min with gentle agitation. Absorbance was measured at 570 nm on a microplate reader (Synergy HT, Biotek, Winooski, VT). Wells containing known cell numbers (0, 1250, 2500, 5000, 10,000, 20000 and 40,000 cells/well; n = 6/cell density) were treated in the similar fashion to establish standard curves for each individual cell line.

To examine the effects of MEK1/2 and PI3K pathways on cell migration, 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). After reaching 70–80% confluence, cells grown on 60 mm culture dishes were treated with 1.25 - 5.0μM of LY294002 (a selective PI3K inhibitor, Millipore), 10-50 μM of PD98059 (a selective MEK1/2 inhibitor, Millipore, Billerica, MA) or DMSO (the control) in CGM for 4 days with daily change of CGM containing PD98059, LY294002 or DMSO. Cells were lifted using trypsin, resuspended in CGM containing LY294002, PD98059 or DMSO, 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.

Western Blot Analysis

Western blot analysis was conducted as described (Li et al., 2014; Wang et al., 2013). We first confirmed the CGM-induced activation of protein kinases. Cells were serum-starved in BM for 16 hr, followed by treating with CGM or BM for 60, 30, 20, 10, or 0 min. Subsequently additional cells were pre-treated with TCDD or DMSO (the control) in the basal media without serum for 16 hr and then treated with CGM in the present of TCDD or DMSO (the control) for 10 min to determine the TCDD's effects on activation of protein kinases. To determine the TCDD's effect on prolonged activation of protein kinases, additional cells were also treated without or with a single dose of TCDD (10 nM) in CGM for 0, 1, 2, 8, 24, 48 hr.

Proteins were subjected to Western blotting (Li et al., 2014; Wang et al., 2013). The membranes were probed with the anti-phospho-p44/42 MAPK (pERK1/2) (Thr202/Tyr204) (cat#9101, Cell Signaling Technology, Danvers, MA), anti-total-ERK1/2(tERK1/2; cat# 9102, Cell Signaling Technology), anti-phospho-AKT1 Ser 473 (pAKT1; cat# sc-7985-R, Santa Cruz Biotechnology, Santa Cruz, CA), anti-total-AKT1 (tAKT1; cat# 9272, Cell Signaling Technology), anti-cleaved caspase-3 (indicative of apoptosis, cat# 9661, Cell Signaling Technology), anti-caspase-3 (Cat# 9665, Cell Signaling Technology), followed by reprobing with a mouse GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) 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.

References

Li, Y., Wang, K., Jiang, Y.Z., Chang, X.W., Dai, C.F., and Zheng, J., 2014. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibits human ovarian cancer cell proliferation. Cell Oncol (Dordr). 37, 429-437.

Jiang, Y.Z., Wang, K., Li, Y., Dai, C.F., Wang, P., Kendziorski, C., Chen, D.B., and Zheng, J., 2013a. Transcriptional and functional adaptations of human endothelial cells to physiological chronic low oxygen. Biol. Reprod. 88, 114.

Jiang, Y.Z., Wang, K., Li, Y., Dai, C.F., Wang, P., Kendziorski, C., Chen, D.B., and Zheng, J., 2013b. Enhanced cellular responses and distinct gene profiles in human fetoplacental artery endothelial cells under chronic low oxygen. Biol. Reprod. 89, 133.

Wang, K., Li, Y., Jiang, Y.Z., Dai, C.F., Patankar, M.S., Song, J.S., and Zheng, J., 2013. An endogenous aryl hydrocarbon receptor ligand inhibits proliferation and migration of human ovarian cancer cells. Cancer Lett. 340, 63-71.

Supplemental Table

Supplemental Table S1. Primers used for RT-qPCR Analysis.

Gene	Forward (5'-3')	Reverse (5'-3')	Size (bp)	GenBank Accession No.
CYP1A1	CAGAAGATGGTCAAGGAGCA	GACATTGGCGTTCTCATCC	114	NM_000499.3
CYP1B1	CGGCCACTATCACTGACATC	CTCGAGTCTGCACATCAGGA	105	NM_ 000104.3
VEGFA	TCTTCAAGCCATCCTGTGTG	ATCCGCATA ATCTGCATGGT	112	NM_001287044.1
VEGFR1	CAGGCCCAGTTTCTGCCATT	TTCCAGCTCAGCGTGGTCGTA	82	NM_002019.4
VEGFR2	CCAGCAAAAGCAGGGAGTCTGT	TGTCTGTGTCATCGGAGTGATATCC	87	NM_002253.2
NP-1	CAGAAAACACCAGGTCGAATCC	CGCGCTGTCGGTGTAAAAA	69	NM_001244973.1
NP-2	GCATGGCAAAAACCACAAGGTAT	TGGAGCGTGGAGCTTGTTCA	76	NM_201267.1
TBP	CATACCGTGCTGCTATCTGG	TCCCTCAAACCAACTTGTCA	85	NM_001172085.1
ACTB	CATTCCAAATATGAGATGCATTG	TGCTATCACCTCCCCTGTGT	120	NM_001101.3
GAPDH	CCAGGCGCCCAATACG	CCACATCGCTCAGACACCAT	87	NM_001256799.1

Supplemental Figures

Fig. S1

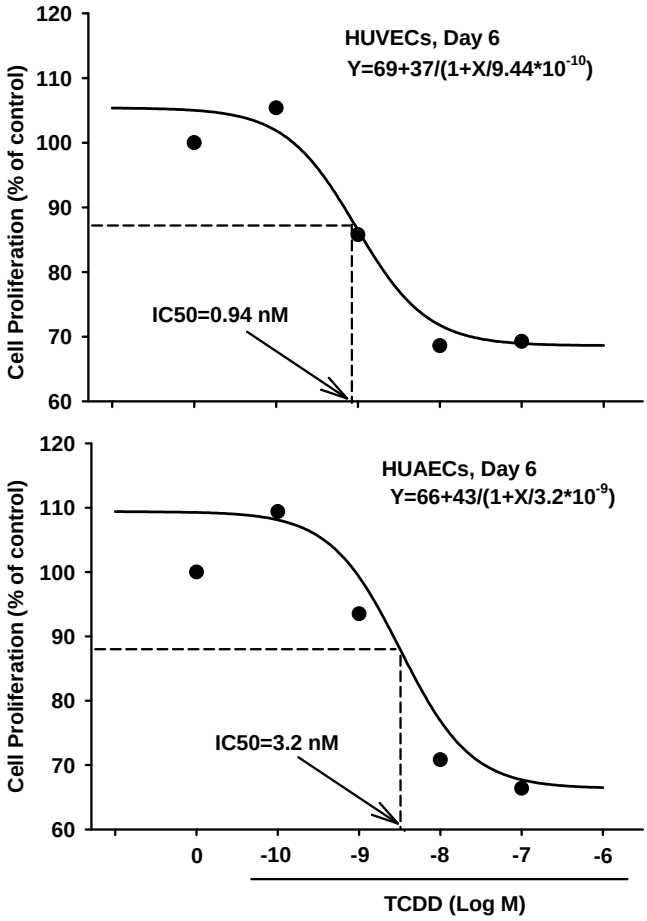

Fig. S1. Estimation of Relative IC50 Values of TCDD on Proliferation of HUVECs and HUAECs on Day 6. Subconfluent cells seeded in 96-well plates were treated with DMSO (the 0 control) or TCDD in CGM for 6 days with a daily change of CGM containing DMSO or TCDD. Cell numbers were determined by the crystal violet. Data are expressed as means ± SEM % of the control (n =3-4). Relative IC50 values were calculated using Sigma plot graphing and statistical analysis software.

Fig. S2

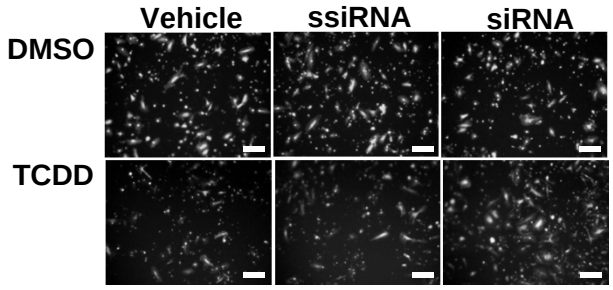

Fig. S2. Effects of AhR siRNA on the TCDD-inhibited Migration of HUAECs. Cells were treated with the vehicle or transfected with the scrambled (ssiRNA) or AhR siRNA (siRNA) for 2 days. After transfection, cells were treated with DMSO (the control) TCDD (10 nM) in CGM for 2 days with a daily change of CGM containing DMSO or TCDD, followed by the cell migration assay.

Fig. S3

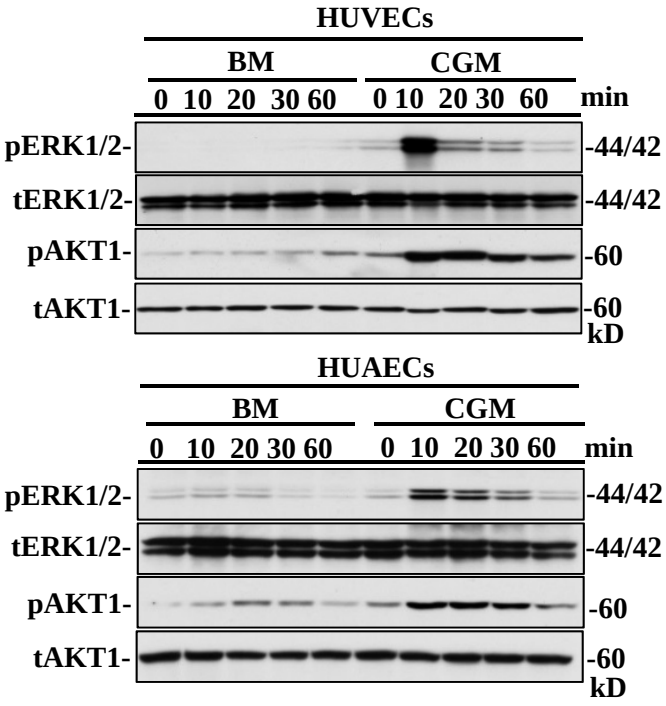

Fig. S3. Effects of CGM on Phosphorylation of ERK1/2 and AKT1 in HUVECs and HUAECs. Cells were serum-starved in basal media (BM) for 16 hr, followed by treating with BM or CGM up for 0, 10, 20, 30, or 60 min. Proteins were subjected to Western blotting for pERK1/2, tERK1/2, pAKT1/2, and tAKT1.

Fig. S4

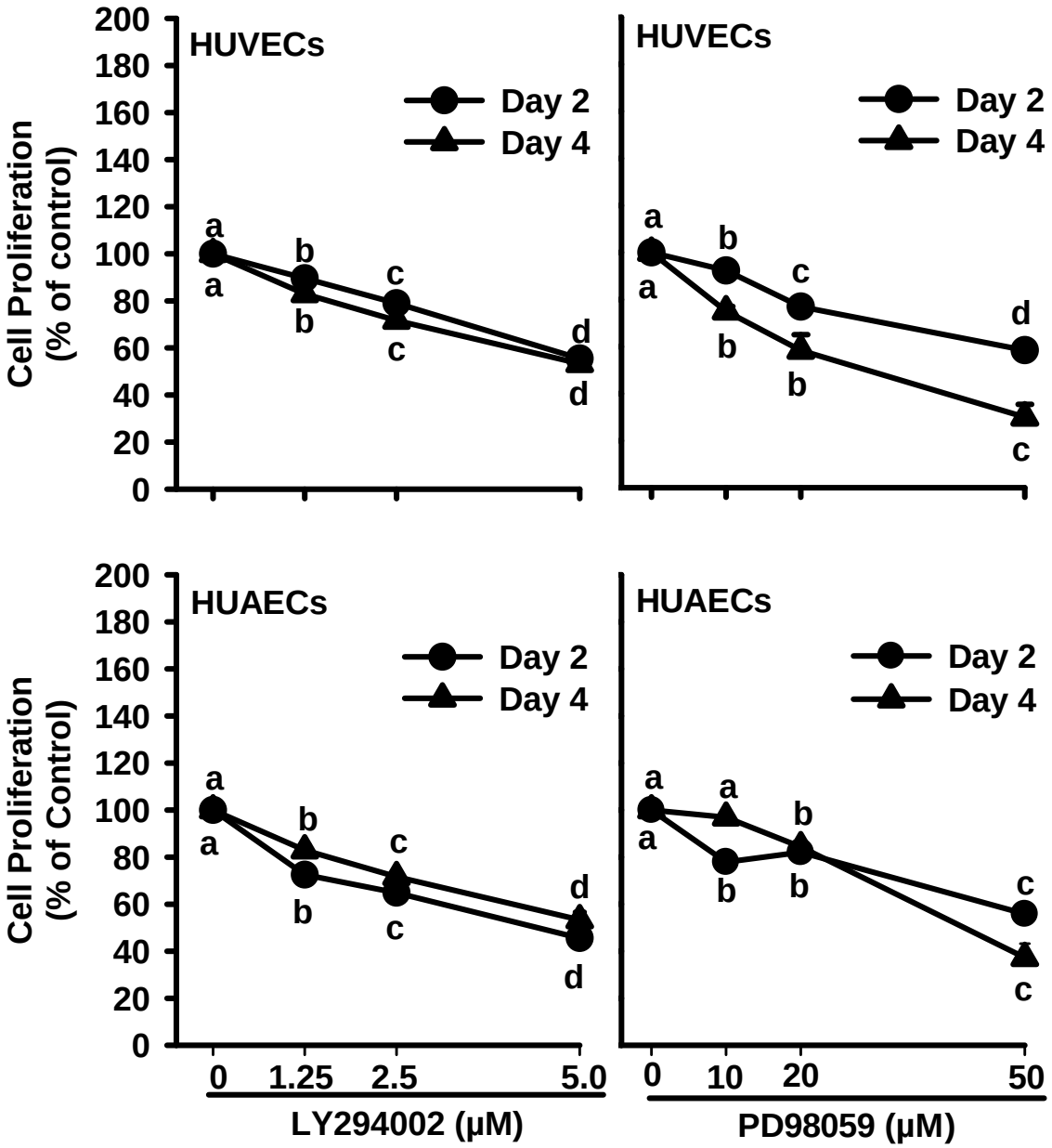

Fig. S4. Effects of LY294002 and PD98059 on Proliferation of HUVECs and HUAECs. Cells were treated DMSO (vehicle control), LY294002 or PD98059 in CGM for 4 days with a daily change of CGM containing DMSO, LY294002 or PD98059. Data are expressed as means ± SEM % of the control (n = 4). ^{a,b,c,d}Means with different letters differ in each corresponding treatment ($p < 0.05$).

Fig. S5

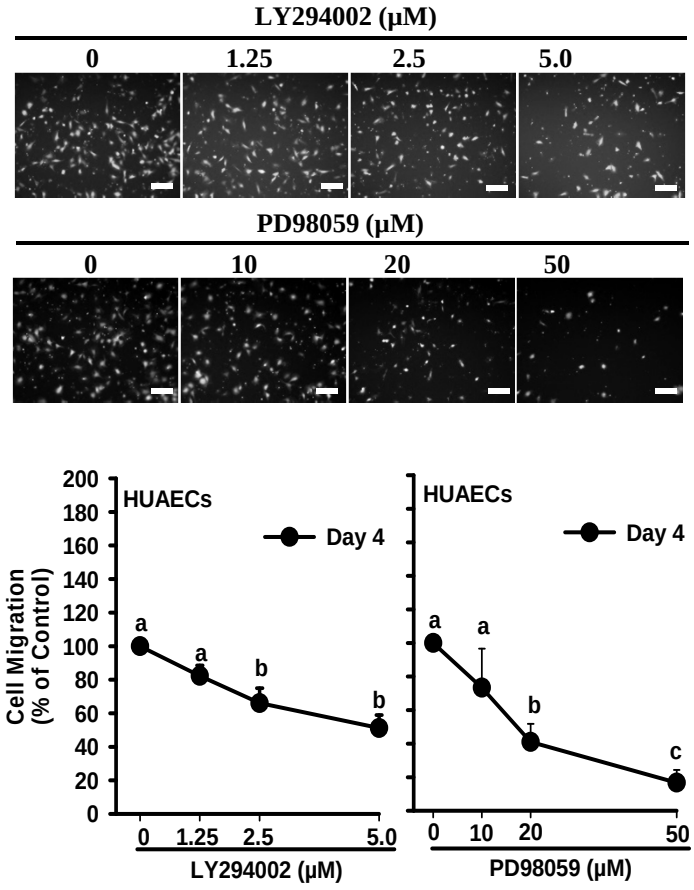

Fig. S5. Effects of LY294002 and PD98059 on Migration of HUAECs. Cells were treated with DMSO (vehicle control), LY294002 or PD98059 in CGM for 4 days with a daily change of CGM containing DMSO, LY294002 or PD98059. The migrated cells were stained and counted. Data are expressed as means ± SEM % of the control (n = 4). [a,b,c]Means with different letters differ in each corresponding treatment ($p < 0.05$). Bars, 200 μm.

Fig. S6

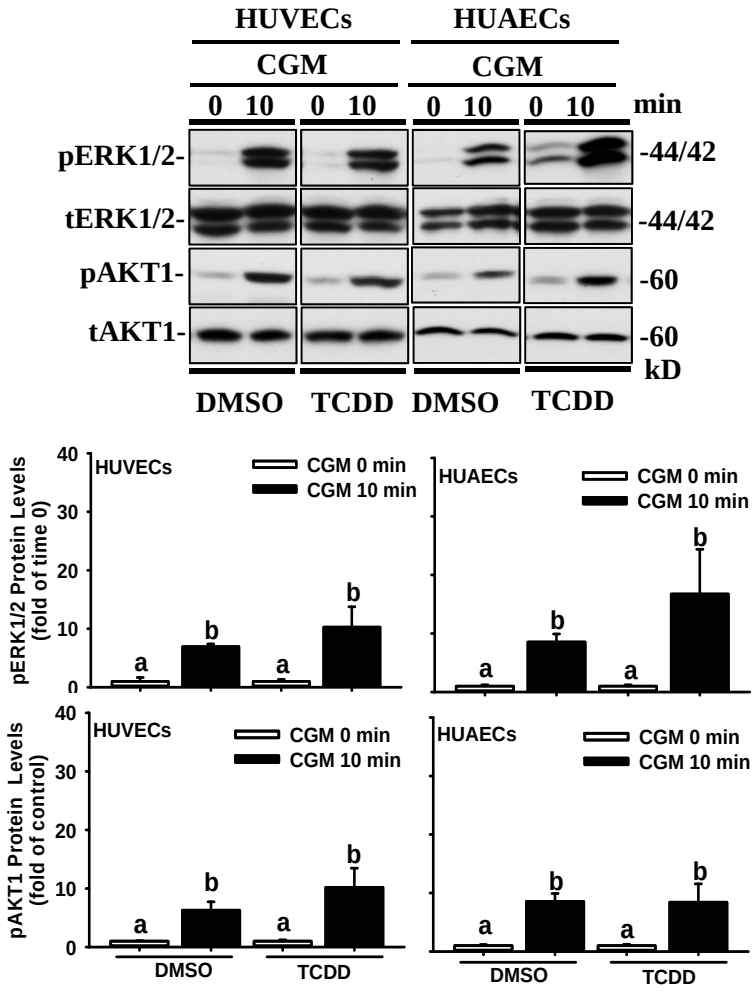

Fig. S6. Effects of TCDD on CGM-induced Phosphorylation of ERK1/2 and AKT1 in HUVECs and HUAECs. Cells were serum-starved for 16 hr in BM containing DMSO (0.01% v/v, vehicle control) or TCDD (10 nM) followed by treating with CGM containing DMSO (0.01% v/v) or TCDD (10 nM) for 10 min. Proteins were subjected to Western blot analysis. Data are expressed as means ± SEM fold of the time 0 control (n =3). *Differ from the time 0 control ($p < 0.05$).

Fig. S7

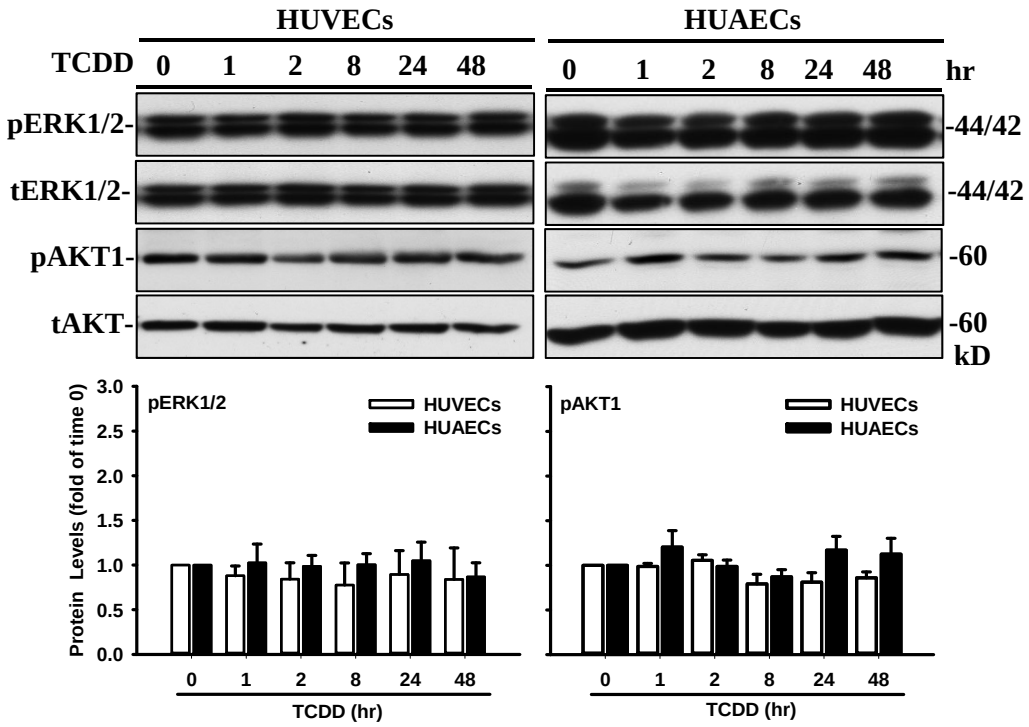

Fig. S7. Effects of TCDD on Phosphorylation of ERK1/2 and AKT in HUVECs and HUAECs. Cells were treated without or with a single dose of TCDD (10 nM) in CGM up to 48 hr. Proteins were subjected to Western blot analysis. Data are expressed as means ± SEM fold of the time 0 control (n =5).

Fig. S8

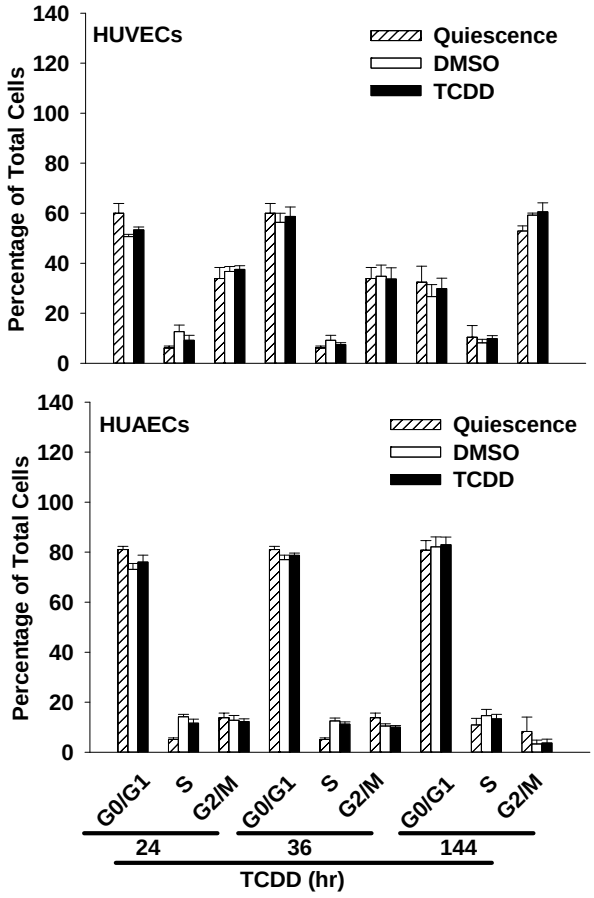

Fig. S8. Effects of TCDD on the Cell Cycle Progress in HUVECs and HUAECs. After serum starvation in BM for 24 hr, cells were treated with DMSO (0.01%, vehicle control) or TCDD (10 nM) in CGM for 24, 36 or 144 hr, and then stained with propidium iodide, followed by flow cytometry assay Data are expressed as means ± SEM % of the control (n=3).

Fig. S9

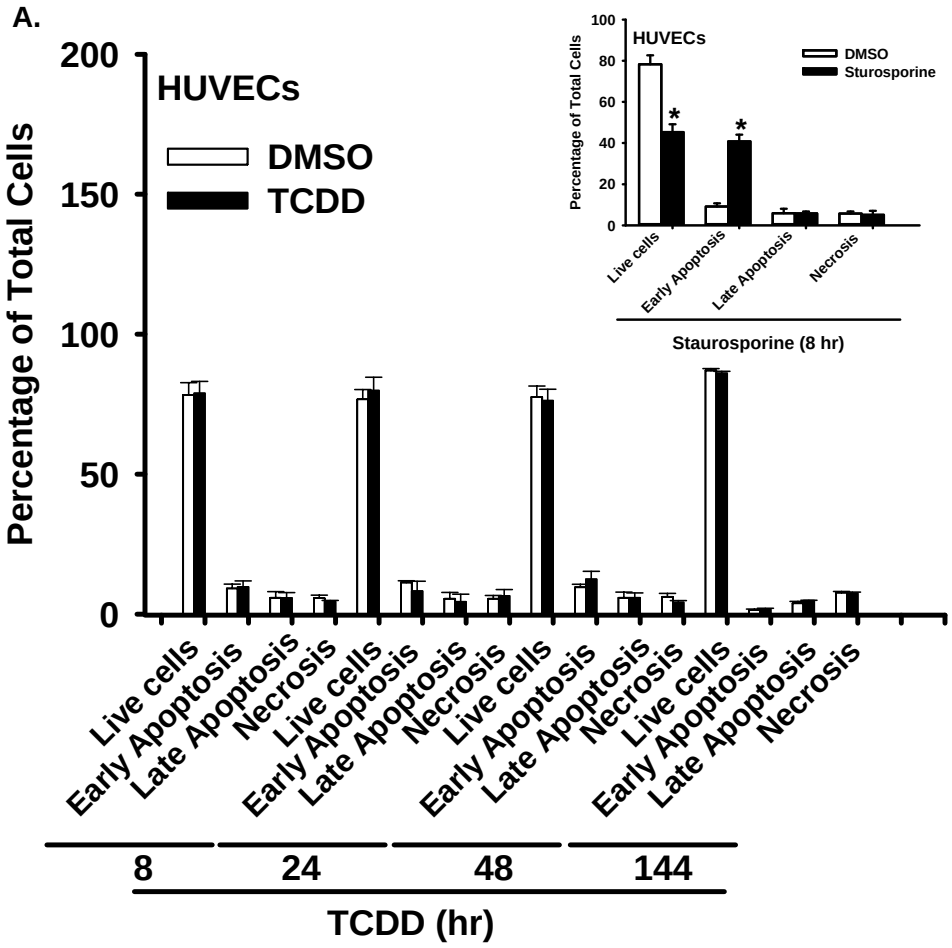
A.
HUVECs
DMSO
TCDD
Percentage of Total Cells
200
150
100
50
0
Live cells
Early Apoptosis
Late Apoptosis
Necrosis
8
24
48
144
TCDD (hr)
HUVECs
DMSO
Sturosporine
Percentage of Total Cells
100
80
60
40
20
0
*
*
Live cells
Early Apoptosis
Late Apoptosis
Necrosis
Staurosporine (8 hr)

Fig. S9

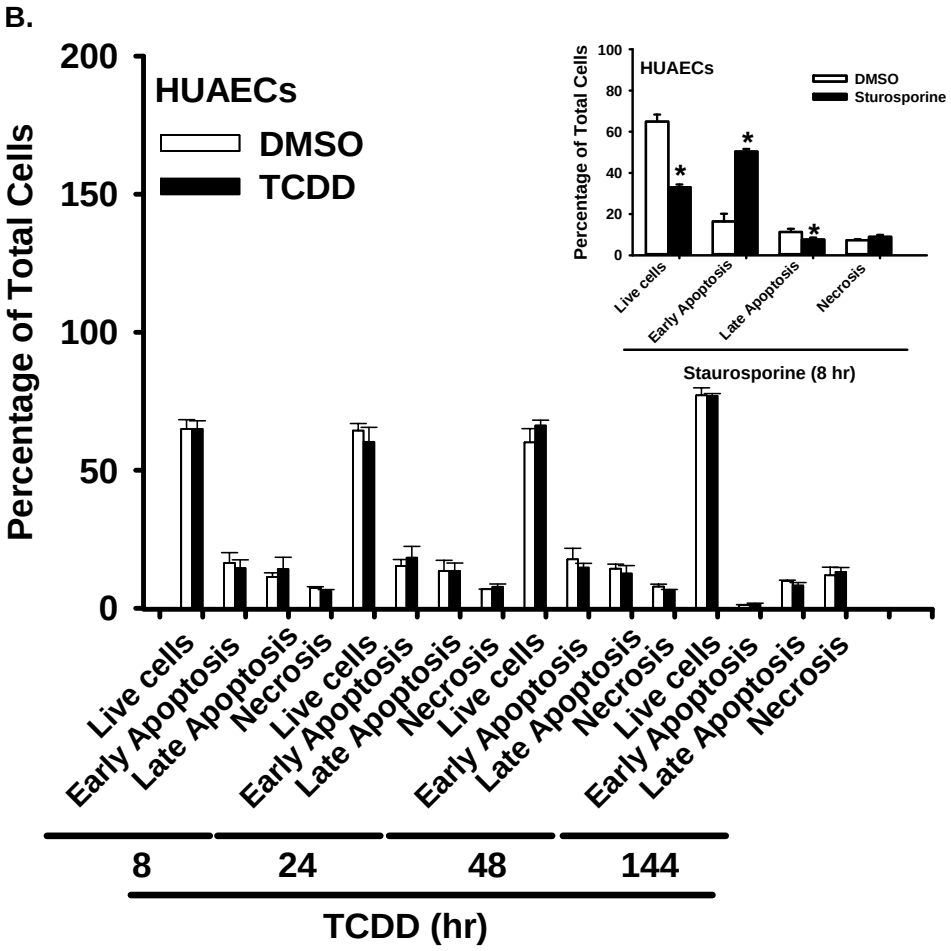

Fig. S9. Effects of TCDD on Cell Apoptosis in HUVECs and HUAECs. After serum starvation in BM for 24 hr, cells were treated with DMSO (0.01% v/v, vehicle control) or TCDD (10 nM) in CGM for 8, 24, 36 or 144 hr. Cells were stained with annexin V and PI, followed by flow cytometry assay. Staurosporine (200 nM) serves as a positive control. Data are expressed as means ± SEM % of the control (n = 3).

Fig. S10

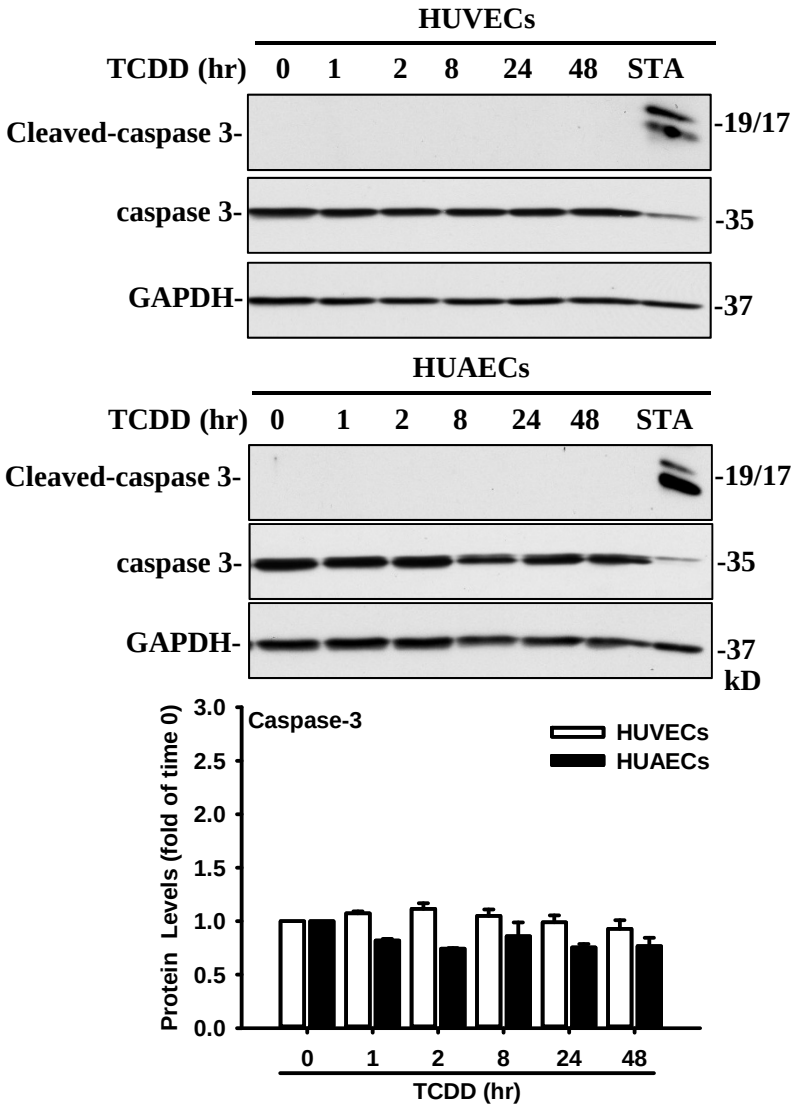

Fig. S10. Effects of TCDD on Cleavage of Caspase-3 in HUVECs and HUAECs. Cells were treated without or with a single dose of TCDD (10 nM) in CGM up to 48 hr. Proteins were subjected to Western blotting. Data are expressed as means ± SEM fold of the control (n =4-5). STA: staurosporine (200 nM; a positive control for apoptosis).