**ABSTRACT**

**INTRODUCTION:** Preeclampsia (PE) is a hypertensive disorder, characterized by a wide spectrum of maternal and fetal vascular dysfunction. Aryl hydrocarbon receptor (AhR) ligands induce hypertension and endothelial dysfunction in pregnant rats and impair the function of human umbilical vein endothelial cells (HUVECs) in vitro. However, the transcriptional and phosphoproteomic signaling mechanisms underlying endogenous AhR ligand’s regulation in endothelial cells remains unknown. **METHODS:** We examined AhR activities in human maternal and umbilical vein sera from normotensive (NT) pregnancies and PE by monitoring serum-induced changes in CYP1A1/B1 mRNA levels in HUVECs using RT-qPCR. HUVECs were treated with 1 μM of 2-(1’H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE, a triptophan-derived AhR endogenous ligand) in vitro. ITE-regulated cell proliferation and monolayer integrity were assessed. ITE-altered transcriptomes and phosphoproteomes were determined using RNA-seq and bottom-up phosphoproteomics analyses. **RESULTS:** Compared to NT, PE elevated AhR activities in serum of umbilical, but not maternal vein. The AhR activity in serum was confirmed using CH223191, a specific AhR antagonist. ITE dose-dependently decreased cell proliferation and monolayer integrity in HUVECs. RNA-seq analysis revealed that ITE differentially regulated transcriptomes in female and male HUVECs: up-regulating 53 genes and down-regulating 47 genes in female cells, while up-regulating 57 genes and down-regulating 23 genes in male cells. ITE time-dependently dysregulated phosphoproteins in HUVECs. At 4 hr, ITE increased the phosphorylation of 72 proteins, while suppressing the phosphorylation of 36 proteins. At 24 hr, ITE enhanced the phosphorylation of 84 proteins and inhibited phosphorylation of 27 proteins. Functional enrichment analyses of RNA-seq and phosphoproteomic data showed that these ITE-disrupted transcriptomes and phosphoproteomes are associated with heart, liver, lung, kidney and vascular dysfunction, and defects in inflammatory responses, cell death, and kinase inhibition. **CONCLUSIONS:** Dysregulation of endogenous AhR ligands during pregnancy may contribute to PE-induced endothelial dysfunction via disrupting endothelial transcriptomes and phosphoproteomes. These AhR ligands activated genes and phosphoproteins might represent promising therapeutic targets for PE-impaired endothelial function.

**METHODS**

**HYPOTHESIS**

- AhR activities in maternal and umbilical vein sera
  - Maternal and umbilical vein blood samples were collected from singleton pregnant women.
  - Sub-ethnic HUVECs were treated with 20% serum samples in ECM for 12 or 24 hr.
  - Additional HUVECs were treated with 20% umbilical vein serum in the presence of 10 μM CH23191 (an AhR antagonist) for 12 hr to confirm activation of AhR.
  - RT-qPCR was conducted to determine mRNA expression of cytochrome P450A1 and B1 (CYP1A1/B1, indexes of AhR activity) in HUVECs.

- **HUEVCs**
  - Primary female and male HUVECs were isolated from umbilical veins of women with NT.

- **Functional assays**
  - HUVECs were daily treated with ITE (1 μM) or DMSO in ECM for up to 40 hr.
  - Cell monolayer integrity was determined using the ECIS 2896-well array assay (Applied Physics).
  - Cell proliferation was accessed using the crystal violet method.

- **RNA-seq**
  - HUVECs were daily treated with ITE (1 μM) or DMSO in ECM for 48 hr.
  - 150 million reads per sample from HUVECs (UMW-Biotechnology Center).
  - Data were verified by RT-qPCR.

- **Bottom-up phosphoproteomics**
  - Sub-ethnic HUVECs were treated with ITE (1 μM) or DMSO in ECM for 4 or 24 hr after 16 hr of serum starvation.
  - Bottom-up phosphoproteomic analysis was done in UMW-Biotechnology center.
  - ITE-regulated phosphoproteins were identified using the PANTHER.
  - Data were verified by Western blotting.

**RESULTS**

**Table 1. ITE dysregulates phosphoproteins in HUVECs**

<table>
<thead>
<tr>
<th>Commonly upregulated phosphoproteins/phosphosites at 4 and 24 hr</th>
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<tbody>
<tr>
<td>1. Death-associated protein 1 (DAP1), S51</td>
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<tr>
<td>2. Microtubule-associated protein 1B (MAP1B), S1396; S1400</td>
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<tr>
<td>3. Ras GTPase-activating protein-binding protein (G3BP1), S</td>
</tr>
<tr>
<td>4. Heat shock protein 22 (HSP22), S434</td>
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<tr>
<td>5. MAP7 domain-containing protein 1 (MAP7D1), S753</td>
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<tr>
<td>6. Tubulin alpha-1 (TUBA1), S520</td>
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<tr>
<td>7. Vimentin (VIM), S430</td>
</tr>
<tr>
<td>8. Nestin (NES), S680</td>
</tr>
<tr>
<td>9. MAP7 domain-containing protein 1 (MAP7D1), S753</td>
</tr>
<tr>
<td>10. MAPK phosphatase (MKP)</td>
</tr>
<tr>
<td>11. AKAP12 (A-kinase anchor protein 12), S696</td>
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</table>

**Fig. 1. ITE elevates AhR activities in fetal serum.** HUVECs were treated with 20% maternal (Mat) or umbilical vein (UV) in ECM for 12 or 24 hr. Compared with control, umbilical, but not maternal vein saw increased CYP1A1/B1 mRNA levels in HUVECs (A and B). Compared with NT, PE further elevated CYP1A1 mRNA levels in umbilical vein serum at 12 hr (A). Additional cells were treated with 20% PE-UV in the presence or absence of 10 μM CH23191 for 12 hr (C). This PE-increased CYP1A1 mRNA level was blocked by CH23191, which vitally activated AhR in serum. The Holm-Sidak test was performed for all pairwise multiple comparisons. In A and B, *P < 0.03 vs. control, *P > 0.05 vs. NT-UV, n = 3 for control, n = 4 for serum samples. In C, *P < 0.005 vs. control, #P < 0.001 vs. PE-UV, n = 3 in control, n = 6 in serum samples. **Fig. 2. ITE decreases cell proliferation and monolayer integrity in HUVECs.** ITE dose-dependently inhibited cell proliferation, starting at 10 μM (0.001<, dose and time-dependent, beginning at 15 and 20 hr for 1 and 16 μM of ITE, respectively, and reaching the maximum effect up to 40 hr, indicating that ITE reduced cell monolayer integrity in HUVECs, n = 4 individual cell preparations/oven sex. (A). The ITE-dose-related electrical resistance was dose- and time-dependent, beginning at 15 and 20 hr for 1 and 16 μM of ITE, respectively, and reaching the maximum effect up to 40 hr, indicating that ITE reduced cell monolayer integrity in HUVECs, n = 4 individual cell preparations/oven sex. (B). The Holm-Sidak test was performed for all pairwise multiple comparisons. Means differ from DMSO (cell proliferation) or DMSO at corresponding time point (monolayer integrity), *P < 0.05. **Fig. 3. ITE dysregulates transcriptomes and pathways in HUVECs.** RNA-seq analysis showed that there were more ITE-dysregulated genes in female (F) HUVECs than male (M) HUVECs (A, B). Differently expressed genes (DEGs) identified by RNA-seq were highly correlated to RT-qPCR data in F and M HUVECs. *Means differ (F-PCR vs. control) from DMSO, **Means differ vs. DMSO (0.00 < P < 0.05), n = 4-6/fetal sex group (C). Biological function enrichment analysis (D). Diseases-associated biological function enrichment analysis (E). Canonical pathways enrichment analysis (F) and Upstream regulator analysis (G) were determined using IPA software (P < 0.05, Fisher exact test). Dotted line: P > 0.05. **Fig. 4. ITE dysregulates phosphoproteomic pathways in HUVECs.** PANTHER analysis revealed that these ITE-dysregulated phosphoproteins were overrepresented (P < 0.05) in biological process and molecular process.

**SUMMARY and CONCLUSIONS**

- PE elevated AhR activities in fetal circulation.
- ITE decreased cell proliferation and cell monolayer integrity in HUVECs.
- ITE dysregulated transcripts and phosphoproteomes in HUVECs.

Dysregulation of endogenous AhR ligands disrupts transcriptomes and phosphoproteomes in fetal endothelial cells, potentially contributing to vascular dysfunction in PE.

**ACKNOWLEDGMENT**

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