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Arachidonic acid metabolites of CYP4A and CYP4F are altered in women with preeclampsia

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ABSTRACT

Few studies exist on cytochrome P450 (CYP450) metabolites of arachidonic acid (AA) pertaining to the pathophysiological events in pregnancy. We hypothesized that metabolism of AA via the CYP450 pathways is altered within the placenta in women with preeclampsia (PE) and contributes to the pathophysiology of the disease. Thus, placental vascular CYP450 enzyme expression and activity were measured in normal pregnant (NP) and preeclamptic (PE) patients. CYP450 isoform expression (CYP4A11, CYP4A22, CYP4F2, and CYP4F3) was found to be elevated within the placenta of women with PE compared to normal pregnant (NP) women and chronic hypertensive (CHTN) pregnant women. In addition, placental production of 20-HETE was significantly increased in PE women compared to both NP and CHTN women. Moreover, there was an imbalance in circulating 20-HETE:EETs in PE women. To examine whether alterations in CYP450 AA metabolism contribute to the altered placentation in PE, trophoblast function, proliferation and migration were assessed in the presence of exogenous 20-HETE and a 20-HETE specific synthesis inhibitor, HET0016. Trophoblast proliferation was significantly increased in the presence of 20-HETE (1 μM) and reduced with 20-HETE blockade by HET0016 (1 mM, 5 mM, and 10 mM). On the contrary, administration of exogenous 20-HETE (1 μM) significantly reduced trophoblast migration. In conclusion, metabolism of AA via CYP450 is altered in PE, and increased placental production of 20-HETE may contribute to the pathophysiology of the disease.

1. Introduction

Preeclampsia (PE) is a pregnancy specific disease defined by new-onset hypertension and proteinuria after the 20th week of gestation. Recent updates to diagnostic guidelines also define severity of the disease with findings of two or more severe ranged blood pressures with systolic values ≥160 mmHg and/or diastolic values ≥110 mmHg taken four hours apart, renal insufficiency, cerebral or visual disturbances, pulmonary edema, impaired liver function, and/or thrombocytopenia [1–4]. PE affects approximately 5–10% of pregnancies and attributes to increased maternal and neonatal mortality rates in this population. Although the exact etiology of the disease is unknown, it is believed to be caused by shallow trophoblast migration and spiral artery remodeling during placentation. Research over the past 20 years has identified many contributing factors, such as altered inflammatory factors and anti-angiogenic imbalance, both of which could contribute to decreases in vascular remodeling within the placenta [5–14]. In addition, numerous studies have investigated the metabolism of arachidonic acid (AA) through the cyclooxygenase (COX) pathway in normal and compromised pregnancies.[15] However, few studies exist on cytochrome P450 (CYP450) metabolites of AA pertaining to the pathophysiological events in pregnancy [16–18]. Thus the goal of our study was to investigate the role of AA metabolites of the CYP450 pathway in placental tissue during pregnancy.

One of the major metabolites of the CYP4A and CYP4F enzyme families, 20-hydroxyeicosatetraenoic acid (20-HETE), a potent vasoconstrictor, has been found to play an important role in the regulation of blood pressure and vascular tone in the brain, kidney, heart and splanchnic beds [19–21]. 20-HETE has also been reported to contribute to migration and proliferation of vascular smooth muscle cells (VSMC) *in vitro* [22–29], and recent reports suggest 20-HETE contributes to constrictive vascular remodeling following endothelial cell injury [30]. Studies also suggest the vasodilatory CYP450 metabolites, epoxyeicosatrienoic acids (EETs) play a permissive role in hypertension and inflammation [31], and women with PE have increased placental EET production [16,18,32–34]. Furthermore, disease states characterized by

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an imbalanced production of 20-HETE compared to EETs display vascular dysfunction [35]. However, very few studies have evaluated the role of CYP450 metabolites in the placenta and their potential role in the pathophysiology of PE. Previous studies have shown that women with PE have increased placental EET production and that production of EETs in PE is location dependent [16,18,33,35]. Therefore, this study tested the hypothesis that the CYP450 pathway, specifically 20-HETE and EET production, are altered during PE. Additionally, we hypothesized that increases in 20-HETE production contribute to the pathophysiology of the disease through changes in trophoblast function and increases in maternal blood pressure. We tested our hypothesis by evaluating CYP450 expression and 20-HETE production in isolated placental vessels, in addition to circulating eicosanoids in normal pregnant (NP) women and women with PE. Due to limitations in evaluating the functional role for 20-HETE in early gestation in human subjects, we used BeWo cell cultures, which are trophoblast-like choriocarcinoma cells, to evaluate trophoblast growth and migration in response to different 20-HETE concentrations.

2. Methods

2.1. Patient population

Blood and placentas were collected from women undergoing scheduled Cesarean sections who were enrolled in an institutional review board-approved study at the University of Mississippi Medical Center. PE was diagnosed in accordance with the American College of Obstetrics & Gynecologists criteria [36]: systolic ($\geq$140 mmHg) with a diastolic ($\geq$90 mmHg) blood pressures observed at least 4 h apart, proteinuria ($\geq$300 mg/24 hr collection) or dipstick proteinuria $\geq$3+. Pregnant, chronic hypertensive women are defined as patients having a diagnosis of hypertension before pregnancies or elevated blood pressures with systolic pressure $\geq$140 mmHg and/or diastolic pressures $\geq$90 mmHg prior to 20 weeks gestation. NP women were used as control patients.

2.2. Collection of placental samples

Placentas were collected immediately after delivery, and tissue samples were excised from the maternal/fetal interface for isolation of villous vessels by microdissection. Isolated vessels where then rinsed in ice-cold PBS for removal of blood and used for microsome isolation for eicosanoid measurements (described below). Chorionic villi were extracted from the placental basal plate, rinsed in phosphate buffered saline (PBS) solution, and fixed in 10% buffered formalin for paraffin processing for immunohistochemistry.

2.3. Isolation of trophoblast cells

Placental pieces were washed twice with warm PBS and minced into 10–5 mm^2 pieces. Under sterile conditions the tissue was digested while mixing in a warm Hank's Balanced Salt Solution (HBSS), 10% trypsin, and 0.25U DNase I solution for 8–20 min. At the end of each digest, the tissue solution was allowed to settle for 5 min and the supernatant was collected in 50 mL centrifuge tubes containing 2.5 mL of fetal bovine serum (FBS). The collected digests were centrifuged at 4 °C for 8 min at 1600RPM. The supernatant was then aspirated, and resulting cells were kept on ice before being placed over a percoll gradient (70/50/30/10% percoll layers). Trophoblast cells in layers 50 and 30 were collected and lysed for 10 min with 15% ammonia chloride lysis solution to lyse any residual red blood cells. Cells were then washed twice with warm PBS and placed on FBS coated petri-dishes for 20 min under normal incubating conditions. After 20 min non-adherent trophoblast cells were collected, washed, and stored at −80 °C until homogenized for eicosanoid measurements.

2.4. Determination of CYP4A expression by immunohistochemistry

After deparaffinization, tissue sections (3 μm thick) were subjected to antigen retrieval with Proteinase K (Dako) for 10 min at room temperature followed by 30 min incubation in blocking solution (serum-free, ready-to-use, Dako) and incubated overnight with CYP4A22 antibody ((1:100; Santa Cruz Biotechnology). Sections were washed and incubated for an hour with 1:200 secondary antibody (Alexa Fluor 488, Life Technologies), counterstained with 0.001% Evans blue, cover slipped with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories), and stored at 4°C in the dark until analysis. Images were captured using a Nikon fluorescence microscope interfaced with NIS-Elements D 3.0 software (Nikon Instruments Inc.). 3–5 images were captured per stained slide. The percent staining was determined by the percent area of determined staining (color) per field/image as defined by the manufacturer's instructions. Images were captured for CYP4A staining (green), tissue auto-fluorescence (red), and DAPI (blue) under the same magnification and exposure but under respective excitation wavelengths. Yellow staining indicates an overlap of CYP4 staining and tissue auto-flourescence. Because significant auto-flourescence was observed within the vessel lumen despite quenching methods, images were merged in order to aid in the quantitation of CYP4A staining. Tissues were also stained with Harris' hematoxylin and eosin stain for tissue identification.

2.5. Measurement of CYP4A and CYP4F isoform expression

Microdissected placental microvessels were placed into ice cold RNA*later* solution (Life Technologies, Grand Island, NY) overnight, and were homogenized in TRIzol solution (Life Technologies, Grand Island, NY) using a FastPrep-24 homogenizer (MP Biomedical, Santa Ana, CA). RNA was extracted according to manufacturer's instructions. Aliquots of the RNA (1 μg) were added to a 20 μL reverse transcription reaction using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The reactions were incubated at 25°C for 5 min, 42°C for 30 min followed by inactivation at 85 °C for 5 min. The 25 μL PCR reactions contained 25 ng of the forward and reverse primers, 20 mM Tris-HCl buffer (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 M of each dNTP, 0.5 U Taq DNA Polymerase (QIAGEN, Valencia, CA). The reaction mixtures were initially denatured at 94 °C for 5 min and then cycled 35 times between 94 °C (denaturation) for 30 s, 64 °C (annealing) for 30 s, and 72 °C (elongation) for 30 s followed by extension for 7 min at 72 °C. The RT-PCR products were separated on 1% agarose gel in a 1X Tris-borate-EDTA (TBE) buffer containing ethidium bromide (Sigma, St. Louis, MO) and the band intensity analyzed using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). The primer sequences for CYP4A11 corresponded to 5′-ATGTCTCTGGAATCCTCCAAGCGG-3′ and 5′-ATTTCTG AATCCGTTGTAGCTCCTGG-3′. The final PCR product size is 191 bp. The primer sequences for CYP4A22 corresponded to 5′-CAATGACATC CTGAAGCCATACG-3′ and 5′-CATGAAAGGCATTCCTCATACAGC-3′. The final PCR product size is 236 bp. The primer sequences for CYP4F2 corresponded to 5′-CAACCCCACAGAGGAGGGCSTGAG-3′ and 5′-GAG GCGTTGATGACAGACCG-3′, the final PCR product size is 139 bp. The primer sequences for CYP4F3 corresponded to 5′-AGGGGAGAGGAGG TTGTGTGGGACA-3′ and 5′-CAGGTGACCCAAGAACCAATTCCGTT-3′, the final PCR product size is 235 bp. The primer sequences for human GAPDH corresponded to 5′-AAGGTGAAGGTCGGAGTCAA-3′ and 5′-AATGAAGGGGTCATTGATGG-3′, the final PCR product size is 120 bp.

2.6. Isolation of placental vessel microsomes

Microsomes were isolated from placental vessels as previously described [37–39], and incubated in 1 mL 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 M K_2HPO_4, 0.1 M KH_2PO_4, 0.5 M EDTA, 10 mM $MgCl_2$, 20 mM cold AA, 10 mM NADPH, 20 mM indomethacin,