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Cytochrome P450 Subfamily 2J Polypeptide 2 Expression and Circulating Epoxyeicosatrienoic Metabolites in Preeclampsia

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Background—Preeclampsia is a multisystem disorder of pregnancy, originating in the placenta. Cytochrome P450 (CYP)-dependent eicosanoids regulate vascular function, inflammation, and angiogenesis, which are mechanistically important in preeclampsia.

Methods and Results—We performed microarray screening of placenta and decidua (maternal placenta) from 25 preeclamptic women and 23 control subjects. The CYP subfamily 2J polypeptide 2 (CYP2J2) was upregulated in preeclamptic placenta and decidua. Reverse-transcription polymerase chain reaction confirmed the upregulation, and immunohistochemistry localized CYP2J2 in trophoblastic villi and deciduas at 12 weeks and term. The CYP2J2 metabolites, 5,6-epoxyeicosatrienoic acid (EET), 14,15-EET, and the corresponding dihydroxyeicosatrienoic acids, were elevated in preeclamptic women compared with controls in the latter two thirds of pregnancy and after delivery. Stimulating a trophoblast-derived cell line with the preeclampsia-associated cytokine tumor necrosis factor-α enhanced CYP2J2 gene and protein expression. In 2 independent rat models of preeclampsia, reduced uterine-perfusion rat and the transgenic angiotensin II rat, we observed elevated EET, dihydroxyeicosatrienoic acid, and preeclamptic features that were ameliorated by the CYP epoxygenase inhibitor N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MsPPOH). Uterine arterial rings of these rats also dilated in response to MsPPOH. Furthermore, 5,6-EET could be metabolized to a thromboxane analog. In a bioassay, 5,6-EET increased the beating rate of neonatal cardiomyocytes. Blocking thromboxane synthesis reversed that finding and also normalized large-conductance calcium-activated potassium channel activity.

Conclusions—Our data implicate CYP2J2 in the pathogenesis of preeclampsia and as a potential candidate for the disturbed uteroplacental remodeling, leading to hypertension and endothelial dysfunction. (***Circulation***. 2012;126:2990-2999.)

Key words: hypertension ■ pregnancy ■ preeclampsia ■ cytochrome P450

Preeclampsia, hypertension, and proteinuria developing after the 20th gestational week affect 3% to 10% of pregnancies and causes >50 000 maternal deaths annually.[1] Preeclamptic women have higher angiotensin II (Ang II) sensitivity than nonpreeclamptic women.[2] Altered uteroplacental perfusion triggers events leading to endothelial and vascular smooth muscle cell dysfunction, increased vascular resistance, and increased blood pressure. The rat reduced

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uterine-perfusion pressure (RUPP) model mimics this state of affairs.[3] Placental villous deterioration promotes the release of tumor necrosis factor-α (TNF-α) and reactive oxygen species.[4] TNF- α promotes hypertension and proteinuria.[5] Endothelial dysfunction, diminished nitric oxide, prostacyclin, and altered hyperpolarizing relaxing factor pathways contribute,[6–8] as do cytochrome P450 (CYP)–dependent metabolites.[3]

Clinical Perspective on p 2999

Epoxyeicosatrienoic acids (EETs) occur as 4 different regio-isomers (5,6-, 8,9-, 11,12-, and 14,15-EET) and are produced by the CYP epoxygenases from arachidonic acid (AA). These epoxy compounds can be further metabolized by the soluble epoxide hydrolase to the corresponding dihydroxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-DHET).[9] 5,6-EET can be further metabolized to 5,6-epoxy-thromboxane (5,6-epTXA1) by cyclooxygenases and thromboxane synthase.[10] Preeclampsia is associated with increased urinary DHET excretion, indicating enhanced endogenous EET formation in the affected women.[11] EETs are the main products of AA metabolism in placenta, fetal membranes, decidua, trophoblasts, and myometrium.[12,13] Based on these findings, we tested the hypothesis that human preeclampsia is associated with increased intrauterine expression of CYP epoxygenases and enhanced circulating EET levels. We used pharmacological inhibition of CYP epoxygenases to prove the pathophysiological significance of enhanced EET biosynthesis in rat models of preeclampsia.

Methods

Uteroplacental Biopsies

The study includes a biobank collection of patient samples at Oslo University Hospital, approved by the Eastern Norway Regional Committee of Medical Research Ethics. Informed written consent was obtained from each participant. Placental, fat, and muscle biopsies were obtained after cesarean sections from 25 preeclamptic women and 23 women with normotensive and uncomplicated pregnancies. Decidual tissue was collected through vacuum suctioning of the placental bed.[14] The uncomplicated pregnancy group consisted of healthy, normotensive women undergoing cesarean section because of breech presentation or other reasons (online-only Data Supplement Table S1).

Longitudinal Study Population

Pregnant women were recruited between 1997 and 2001 as part of an ongoing investigation of preeclampsia at the University of Pittsburgh, Magee-Womens Research Institute, and Magee-Womens Hospital. The University of Pittsburgh Institutional Review Board approved the study, and written informed consent was obtained from all of the subjects. The study population consisted of 10 nulliparous women with uncomplicated normotensive control pregnancies and 10 nulliparous women who developed preeclampsia (online-only Data Supplement Table S2).

Single Nucleotide Polymorphism Analysis

Our single nucleotide polymorphism (SNP) analysis was done with samples of the Finnish Genetics of Preeclampsia Consortium (FINNPEC) cohort after due approval. The sample consisted of 94 Finnish preeclamptic women and 95 frequency-matched (age, body mass index, and ethnicity) controls. The controls were nonpreeclamptic women (online-only Data Supplement Table S3).

mRNA Isolation, Reverse-Transcription Polymerase Chain Reaction, and Microarray Analysis

Total mRNA was isolated with a combined protocol of QIAzol lysis reagent and Qiagen RNeasy mini kit (including the RNase-Free DNAse set; Qiagen) according to the manufacturer's protocol. RNA quantity and quality were confirmed by RNA 6000 Chip in the 2100 Bioanalyzer (Agilent Technologies) and NanaDrop UV/VIS-Spectrometer (PeqLab). RNA was reverse transcribed into cDNA by using the Transcriptor First Strand cDNA synthesis kit from Roche Diagnostics and analyzed by real-time quantitative polymerase chain reaction on ABI 7500 Fast Sequence Detection System (PE Biosystems). Primers and probes were designed with PrimerExpress 3.0 (Applied Biosystems) and shown in the online-only Data Supplement Methods section.

Cell Culture and Functional Assay

SGHPL-4 cells derived from primary human first trimester extravillous trophoblasts (*EVT*) transfected with the early region of SV40, known previously as MC418, were a kind gift from Judith E. Cartwright (St George's University of London, London, United Kingdom). These cells show similar invasive capabilities to primary EVTs and retain features of normal EVTs.[15] The effect of 72 hours of U-46619 (Cayman Europe) on epidermal growth factor (Sigma-Aldrich)–induced invasion and of 5,6-EET (Cayman Europe) on tube formation and first-trimester placental outgrowth were determined and are described in detail in the online-only Data Supplement Methods section.

Western Blot, Immunohistochemistry, and High-Performance Liquid Chromatography

Cells were processed for protein isolation by radioimmunoprecipitation assay buffer, and 40 μg of protein were boiled with Laemmli solution and processed for SDS-gel electrophoresis, followed by semidry blotting on membrane. The membranes were stained specifically for human CYP2J2 (Abnova) and β-actin (Cell Signaling). Five-microgram sections of HOPE-fixed, paraffin-embedded placental tissues were deparaffinized according to standard procedures. Nonspecific background was blocked by incubation with Ultra V Block (Laboratory Vision/Thermo Fisher Scientific) containing 10% human AB serum for 7 minutes. Primary antibodies were diluted in combination (1/200 for CYP2J2, Abnova; 1/2000 for CK7) in Antibody Diluent (Dako). Secondary antibodies (goat antimouse IgG Alexa Fluor 555 and goat antirabbit IgG Alexa Fluor 488, Invitrogen, Molecular Probes, Eugene, OR) were used (1/200 each). Nuclei were stained in term tissues with 4',6-diamidino-2-phenylindole (Invitrogen, Molecular Probes), slides were mounted with ProLong Gold antifade reagent (Invitrogen, Molecular Probes), and fluorescence microscopy was performed using a Leica DM 6000B Microscope and an Olympus DP 72 camera. CYP-dependent eicosanoids (EETs and DHETs) were analyzed by high-performance liquid chromatography (Agilent 1200SL series) followed by triple quadruple tandem mass spectrometry as described previously.[16] Briefly, 0.5 mL of plasma were hydrolyzed with sodium hydroxide. The solution was extracted with Varian Bond Elute Certify II solid-phase column. The analysis was done on an analytic column Zorbax Eclipse Plus-C18, 4.6×150 mm 1.8-μm column using gradient solvent system Acetonitrile/0.01 mol/L of ammonium acetate. 5,6-Epoxy thromboxane was measured on AGILENT 6490/1290 (please see the online-only Data Supplement Methods section).

Cardiomyocyte Contraction and Patch-Clamp Experiments

The bioassay was described previously.[17] Briefly, neonatal rat cardiomyocytes were dissociated from minced ventricles from 1- to 2-day-old Wistar rats and cultured as monolayers. Spontaneously beating cell clusters occurred after 5 to 7 days. The beating rates were determined for 6 to 8 individual clusters before and after the addition of substances. Whole cell patch-clamp electrophysiology

was carried out as described before in detail.[18] Briefly, whole cell currents were recorded using an EPC-9 patch-clamp amplifier at voltage ramps between -100 and 100 mV. For the activation of Ca^{2+}-activated K^+ channels (K_{Ca}) currents the cells were dialyzed with $CaCl_2$ (0.5 $\mu mol/L$). The inhibitor TRAM-34 was applied in a concentration of 1 $\mu mol/L$ to inactivate the $K_{Ca}3.1$ channel. The selective inhibitor paxilline was used in a concentration of 1 $\mu mol/L$ to identify the $K_{Ca}1.1$. The 5,6-EETs were applied to cells in PBS medium. The measurements were carried after the third passage of cultured vascular smooth muscle cells (VSMCs). Data were analyzed using the software Pulse Fit (HEKA).

Animal Model and Isometric Contraction Measurements

Local regulatory committees approved all of the animal studies according to American Physiological Society standards. All of the animal studies were performed in timed pregnant Sprague-Dawley rats (Harlan Sprague Dawley Inc). We used the RUPP model, which relies on the application of a constrictive silver clip (0.203 mm) to the aorta superior to the iliac bifurcation performed while ovarian collateral circulation to the uterus is reduced with restrictive clips (0.100 mm) to the bilateral uterine arcades at the ovarian end.[19] Rats were treated by MsPPOH (N-[methylsulfonyl]-2-[2-propynyloxy]-benzenehexanamide; Cayman Chemicals) or SC58236 (Sigma Aldrich). We also processed the transgenic rat with preeclamptic phenotype (PE) described previously.[20] Rats were treated with MsPPOH (Cayman Chemicals) as indicated. The isometric contraction measurements have been described previously.[21] The bifurcation of the iliac artery into the main branch of the uterine artery was prepared from pregnant control rats (Sprague Dawley rats [SD]) and PE rats. Models and methods are described in detail in the online-only Data Supplement Methods section.

Statistical Analysis

Data are presented as mean±SEM when normally distributed or median with interquartile range when nonnormally distributed. Normal distribution was assessed by Kolmogorov-Smirnov tests. Groups were compared using the unpaired t test, Mann–Whitney U test, 1-way ANOVA, or Kruskal-Wallis test as appropriate; techniques for each analysis are specified in the Figure legends. Multigroup comparisons were followed by post hoc testing, including the Scheffe test, Dunnett T3, and Mann–Whitney U tests with a Bonferroni correction. Detailed information for or our SNP analysis are described in online-only Data Supplement Methods section.

Results

Uteroplacental and Circulating Dysregulation

We examined uteroplacental tissue of 25 preeclamptic women and 23 women with uneventful pregnancies in microarray studies. Of 58 CYP genes represented on Illumina chips, solely 7 genes in decidual tissue and 3 in placental tissue, respectively, were upregulated or downregulated >1.3-fold in the preeclamptic group as compared with the control group. Of these genes, only CYP2J2 was significantly upregulated in both tissues. We confirmed this finding by real-time reverse-transcription polymerase chain reaction (Figure 1A). The CYP2J2 mRNA was significantly upregulated by a factor of 3.90 ($P<0.0001$) in preeclamptic decidual tissue and 1.55 ($P<0.001$) in preeclamptic placenta. The expression level of CYP2J2, measured in different tissues collected by caesarian section of women with uneventful pregnancies, human umbilical vein cells, placenta, and trophoblasts, was the highest in placenta and trophoblasts. Muscle and fat tissues of preeclamptic women did not show statistically significant differential CYP2J2 expression com-

pared with the control group (see online-only Data Supplement Figure S1). We then localized the CYP2J2 protein in placental villous and decidual tissue by immunohistochemistry (Figure 1B). In both placental compartments, collected at term of gestation, we detected a strong colocalization of CYP2J2 to the trophoblast marker cytokeratin 7. The CYP2J2 staining was positive for cytotrophoblasts of the placental villous and extravillous trophoblasts of the decidua. The trophoblasts of placental villous and decidual tissue were also positive for CYP2J2 at week 12 of gestation (Figure 1B, bottom).

We were interested in the consequences of the uteroplacental CYP2J2 upregulation in preeclampsia on circulating metabolites in the maternal circulation. We therefore analyzed the metabolites of the CYP2J2 in plasma of a longitudinal study population, comparing preeclamptic women with women with uneventful pregnancies (Figure 1C). The 5,6-(EET+DHET) concentrations were increased in pregnancy and subsequently decreased after birth. Beginning with the first trimester of pregnancy, we detected higher levels of the 5,6- and 14,15-eicosanoids in the preeclamptic circulation. These increases were significant during the second and third trimesters. One day after delivery, the 5,6-(EET+DHET) levels were still significantly elevated in the preeclamptic samples as compared with controls. However, the levels of 14,15-(EET+DHET) were no longer statistically significant elevated. Because the level of 11,12- and 8, 9-EETs and -DHETs were not elevated in preeclamptic plasma compared with control plasma, the levels of total EET and DHET were not elevated. Circulating levels of total EETs and DHETs increased during pregnancy (data not shown) when the first and third trimesters were compared. The CYP hydroxylase metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) levels were not dysregulated. The 5,6- EET/DHET ratios were significantly lower in the second trimester in the preeclamptic group compared with the control group. In the third trimester and postdelivery the lowered ratios failed to show statistical significance. The ratios of all of the other metabolites were not statistically significantly changed (online-only Data Supplement Figure S2).

Regulation of CYP2J2

To elucidate the regulation of the CYP2J2, we screened for an obvious genetic influence and performed an SNP analysis, focusing on regulatory elements in CYP2J2. The genotypes of the SNPs located in the area upstream of the CYP2J2 gene were determined for the preeclamptic and control subjects by sequencing (Table). Altogether, 7 SNPs were found, including 1 previously unknown SNP. When Hardy-Weinberg equilibrium was tested in control subjects, the genotype frequencies for all except 1 SNP were in equilibrium. We tested for association between the SNPs and preeclampsia but found none. We could therefore not identify polymorphisms to account for the altered transcriptional expression level of the CYP2J2 gene in preeclampsia.

We next analyzed the regulation of CYP2J2 expression in trophoblast cells. The human first-trimester trophoblast–derived cell line SGHPL-4 was treated by different protocols, reflecting the preeclamptic situation. Ang II (10^{-7} M) and
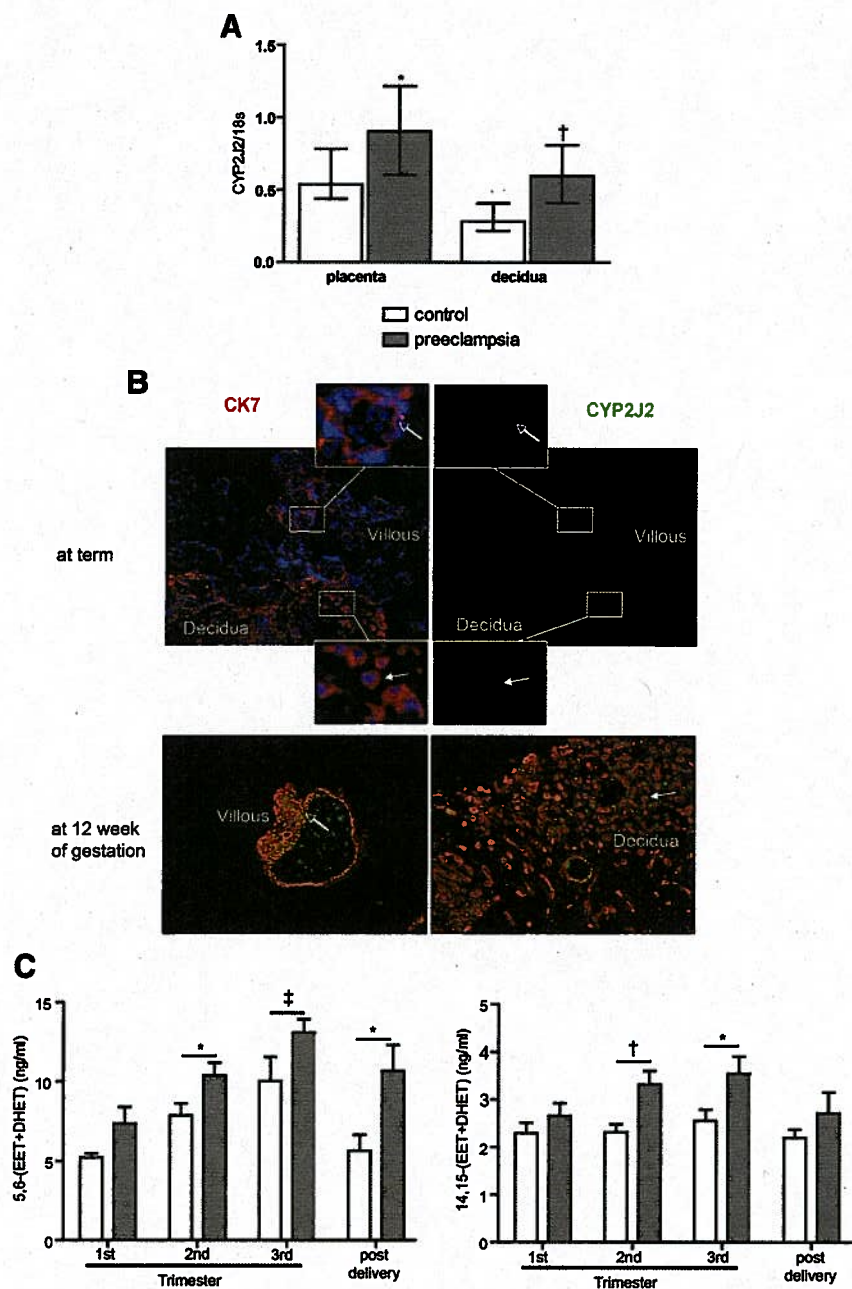
Figure 1. Cytochrome P450 (CYP) 2J2 expression and metabolites in uteroplacental unit and maternal circulation. **A,** CYP2J2 expression was upregulated in placental and decidual tissue in preeclamptic patients (gray bars; n=23 each group; *$P<0.001$; †$P<0.0001$; Mann–Whitney U test; median±IQR). **B,** CYP2J2 (green) was strongly colocalized to the trophoblast marker cytokeratin 7 (CK7; red) at both gestational states. For better localization in the diffuse structure of villous tissue at term, nuclei were stained by 4′,6-diamidino-2-phenylindole. Open arrows are indicating CYP2J2 positive cytotrophoblasts; white arrows indicate CYP2J2 positive extravillous trophoblasts. **C,** CYP2J2 metabolite levels are shown in maternal plasma of a longitudinal study population. 5,6-(Epoxyeicosatrienoic acid [EET]+dihydroxyeicosatrienoic acids [DHET]) levels (left) were elevated in preeclamptic plasma (black bars) vs controls (open bars). 14,15-(Epoxyeicosatrienoic acid [EET]+dihydroxyeicosatrienoic acids [DHET]) levels were also elevated in preeclampsia (n=10 each group; *$P<0.05$; †$P<0.01$; ‡$P<0.001$; unpaired t test).

hypoxic conditions (3% oxygen) did not alter the expression of CYP2J2 at several time points (data not shown). However, TNF-α, which is elevated in preeclampsia,[4,22] stimulated the upregulation of CYP2J2 expression in SGHPL-4 cells (Figure 2). On mRNA level, the expression was significantly upregulated dose (5–10 ng/mL) and time dependent (6–120 hours), reflecting an acute and a chronic inflammation. We confirmed this finding by Western blotting with 5 and 10 ng/mL of TNF-α for 48 hours (Figure 2B).

CYP2J2 Metabolite Function

CYP2J2 activity leads to metabolite formation, including the 5,6-EET.[23] Exclusively, 5,6-EET can be further metabolized by the cyclooxygenase (COX) 1 and 2 and by thromboxane A synthase to an thromboxane analog, the 5,6-epoxy-thromboxane

A1 (5,6-epTXA1)[10] This thromboxane analog can induce signaling via the thromboxane receptor (Figure 3A). To study the functional capabilities of CYP2J2 metabolites, we used a cardiomyocyte contraction bioassay. The model stems from our original observation that agents in preeclampsia could be identified by this model.[17,24] We found that the metabolites had a chronotropic influence on the neonatal cardiomyocyte beating rate (Figure 3B). The metabolite 5,6-EET (30 nmol/L) increased the spontaneous beating rate by 24 beats per minute ($P<0.01$) compared with control. This effect was abolished by furegrelate (0.1 nmol/L), a potent thromboxane synthase inhibitor, and the thromboxane receptor blocker SQ29548. The thromboxane analog U-46619 also induced a strong increase in the spontaneous beating rate, which was blocked by SQ29548. The Ang II type 1 receptor antagonist

Table. SNP Analysis

SNP	Genomic Position*	Major Allele	Minor Allele	MAF in Preeclampsia	MAF in Controls	P Value	OR (95% CI)
rs890293	60392494	G	T	0.0479	0.0737	0.39	0.632 (0.267 to 1.50)
New SNP	60392601	C	G	0.0372	0.0474	0.80	0.778 (0.284 to 2.13)
rs11572188	60392970	C	G	0.0479	0.0737	0.39	0.632 (0.267 to 1.50)
rs10889162†	60393005	G	A	0.0851	0.0947	0.86	0.889 (0.439 to 1.80)
rs7533359	60393416	A	G	0.3670	0.3740	0.92	0.972 (0.640 to 1.48)
rs11572185	60393720	A	C	0.0000	0.0052	1.00	0.000 (0 to NA)
rs11572182	60393849	T	G	0.1490	0.1370	0.77	1.10 (0.620 to 1.97)

Shown are known SNPs of CYP2J2 promoter region from n=94 preeclamptic women and n=95 women with uneventful pregnancies. By sequencing, 1 new SNP was detected and analyzed. SNP indicates single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; and NA, not available.
*Data show genome build 37.1.
†Data were not in Hardy-Weinberg equilibrium.

losartan could not block the positive chronotropic effect of 5,6-EET, although a synergistic effect was observable by costimulation with Ang II. For further electrophysiological characterization of the large-conductance $K_{Ca}1.1$ in cultured VSMCs, we performed whole-cell voltage-clamp experiments (Figure 3C). The activation of the $K_{Ca}1.1$ current in the whole-cell mode was carried out with 0.5 μmol/L of Ca^{2+}. In VSMCs, the $K_{Ca}1.1$ channel showed a calcium-sensitive and voltage-dependent outward current with a maximal current of 32.4 pA/pF at a holding potential of −100 mV (n=24). We examined the influence of 5,6-EETs on the activity of the $K_{Ca}1.1$ channel. The direct application of 1 μmol/L of 5,6-EET to the bath solution led to a downregulation of the channel activity by >70% compared with the control current (n=15). The pharmacological characterization of this macroscopic current was performed with the selective $K_{Ca}1.1$

inhibitor paxilline. Paxilline led to a 74% inhibition of the potassium current at a concentration of 1 μmol/L (data not shown). Importantly, the inhibitory effect of 5,6-EET on $K_{Ca}1.1$ channel activity was completely prevented by preincubating the VSMCs with the thromboxane synthase inhibitor furegrelate (Figure 3C). We further investigated the tube formation of the trophoblast-derived cell line SGHPL-4 in a Matrigel assay. The process of tube formation was enhanced by 5,6-EET. Migration of trophoblasts from first-trimester villous explants was unaffected by 5,6-EET (online-only Data Supplement Figure S3). In a fibrin gel invasion assay, the invasive process was strongly induced by epidermal growth factor, and this effect was reduced by costimulation with the thromboxane analog U-46619 (online-only Data Supplement Figure S4).

We next used the RUPP model to test the role of CYP epoxygenases in preeclampsia development under in vivo conditions.[19] The CYP epoxygenase inhibitor MsPPOH ameliorated the preeclamptic syndrome in the RUPP rats. The mean arterial pressure (MAP) measured on day 18 of pregnancies was normalized by MsPPOH administration from 126 mm Hg in RUPP to 111 mm Hg in the MsPPOH-RUPP group (Figure 4A). Similar to Zhou et al,[25] we observed that MsPPOH treatment in normal pregnant rats elevated MAP. Normal pregnant rats had an MAP of 101 mm Hg, and MsPPOH treatment increased MAP to 110 mm Hg. MsPPOH also improved the RUPP-related pregnancy outcomes. We found that the pup weight was partially restored by the treatment, compared with controls (Figure 4A). The COX inhibitor SC58236 also improved the preeclamptic features in RUPP rats. The MAP was lowered to 115 mm Hg, and the pup weight increased to 2 g in the RUPP+SC58236 group. The 5,6-(EET+DHET) levels were enhanced in RUPP rats compared with controls and were decreased to control levels after MsPPOH administration ($P<0.01$; Figure 4B). All of the other metabolites (8,9-, 11,12-, and 14,15-EETs+ DHETs) were not statistically significant elevated in RUPP rats, although a trend toward elevation was observable. However, levels of 11,12- and 14,15-(EET+DHET) significantly decreased after MsPPOH treatment. The RUPP procedure and the MsPPOH treatment did not statistically significant affect the CYP hydroxylase metabolite 20-HETE, the
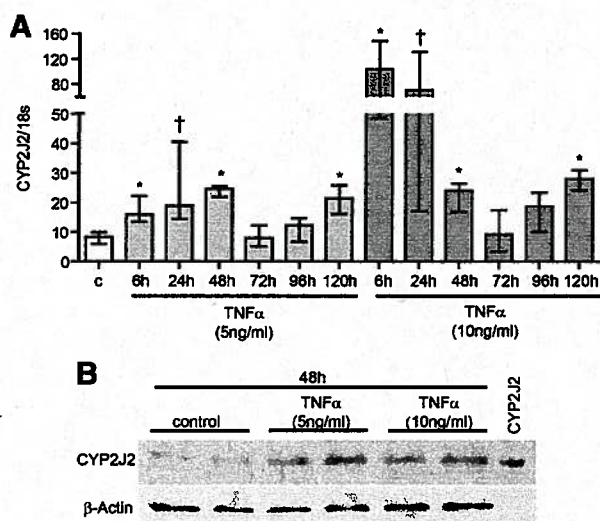
Figure 2. Tumor necrosis factor-α (TNFα) regulates cytochrome P450 (CYP) 2J2 expression in a trophoblast-derived cell line. **A**, CYP2J2 expression in a trophoblast-derived cell line is shown on mRNA level. TNFα stimulated the CYP2J2 expression dose and time dependently (n=4; *$P<0.05$; †$P<0.001$; Mann–Whitney U test with Bonferroni correction; median±IQR). **B**, Western blot results confirmed upregulation of CYP2J2 by TNFα stimulation. Recombinant CYP2J2 served as control.

Figure 3. Activity and inhibition of cytochrome P450 (CYP) 2J2 metabolites. **A**, CYP pathway is shown. The phospholipase A2 (PLA2) releases arachidonic acid (AA) from the cell membrane, which is further metabolized by the enzyme CYP2J2 to its epoxyeicosatrienoic acids (EETs) and by the soluble epoxide hydrolase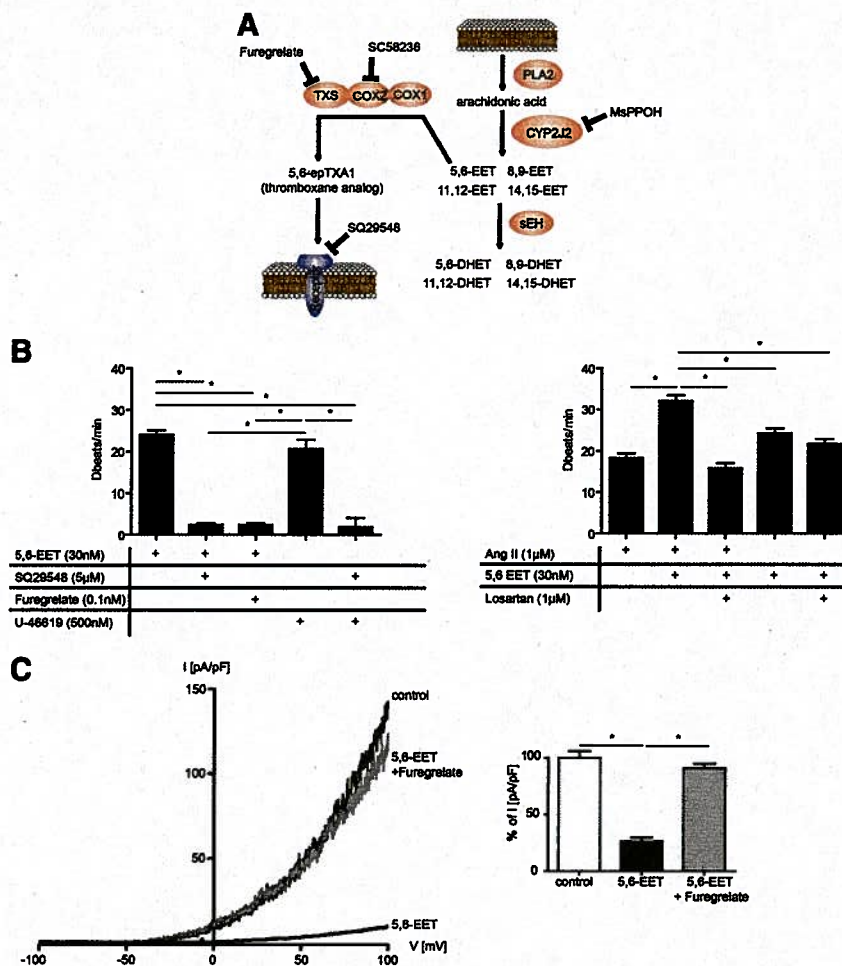 (sEH) to the dihydroxyeicosatrienoic acids (DHETs). MsPPOH inhibits CYP epoxy-genases. 5,6-EET can be metabolized by the cyclooxygenases 1 and 2 (COX1 and COX2) and the thromboxane synthase (TXS) to the thromboxane analog 5,6-epoxy-thromboxane A1 (5,6-epTXA1) that can induce signaling via the thromboxane receptor. Furegrelate (TXS-inhibitor) and the thromboxane A2 receptor antagonist SQ29548 inhibit the cascade. **B**, The effect of 5,6-EET on cardiomyocyte contraction bioassay is shown. At **left**, 5,6-EET significantly enhanced the beating rate of cardiomyocytes. This effect was blocked by SQ29548 and furegrelate. The thromboxane analog U-49919 also enhanced beating rate, blocked by SQ29548. **Right**, Synergistic effect of 5,6-EET to angiotensin II (Ang II). Losartan blocks the Ang II–dependent effect but not the 5,6-EET effect (n=6–8; *$P<0.05$; ANOVA with Scheffe post hoc test; mean±SEM). **C**, The large-conductance calcium-activated potassium channel $K_{Ca}1.1$ is shown (**left**, representative picture; **right**, graph of 15 experiments). Activation of the $K_{Ca}1.1$ current in the whole cell mode by 0.5 μmol/L of Ca^{2+} led to a maximal current of 31.5 pA/pF at a holding potential of −100 mV. Direct application of 5,6-EET (1 μmol/L) led to a downregu-lation of the channel activity by 73.7% vs the control current. Furegrelate abolished the effect of 5,6-EET (n=15; *$P<0.001$; ANOVA with Dunnett T3; mean±SEM).

linoleic acid epoxymetabolites 9,10- and 12,13-(EpOME+DiHOME), and the EET/DHET ratios (online-only Data Supplement Figures S5 and S6).

We then performed experiments in a transgenic PE rat model that we developed earlier, which features hypertension and albuminuria from day 13 of pregnancy onward.[20,21] In this model, 5,6-(EET+DHET) levels were elevated com-pared with normal pregnant SD rats, whereas other metabo-lites (8,9-, 11,12-, and 14,15- EETs+DHETs) and EET/DHET ratios were not statistically significant dysregulated (online-only Data Supplement Figure S7). MsPPOH treat-ment in these rats lowered the MAP and the albuminuria significantly (Figure 4C and 4D). As we reported in our earlier study,[21] uterine artery rings from these hypertensive

dams showed a pronounced relaxation to low doses of acetylcholine (1×10^{-9} to 1×10^{-6} mol/L) and a paradoxical contractile response to higher doses (3×10^{-6} to 1×10^{-5} mol/L), compared with rings from SD rats. This effect of paradoxical contractile response was diminished when the thromboxane receptor antagonist SQ29548 and the COX inhibitor indomethacin were applied. Here we found that the expression of thromboxane A synthase 1 and COX 1 was upregulated in these arteries (online-only Data Supplement Figure S8). Furthermore, we found that MsPPOH also abol-ished the contractile response to acetylcholine with doses above 3×10^{-6} mol/L in both the PE and the SD groups (Figure 4E). Expressions of thromboxane A synthase 1, COX1, and COX2 were also upregulated in placenta of PE
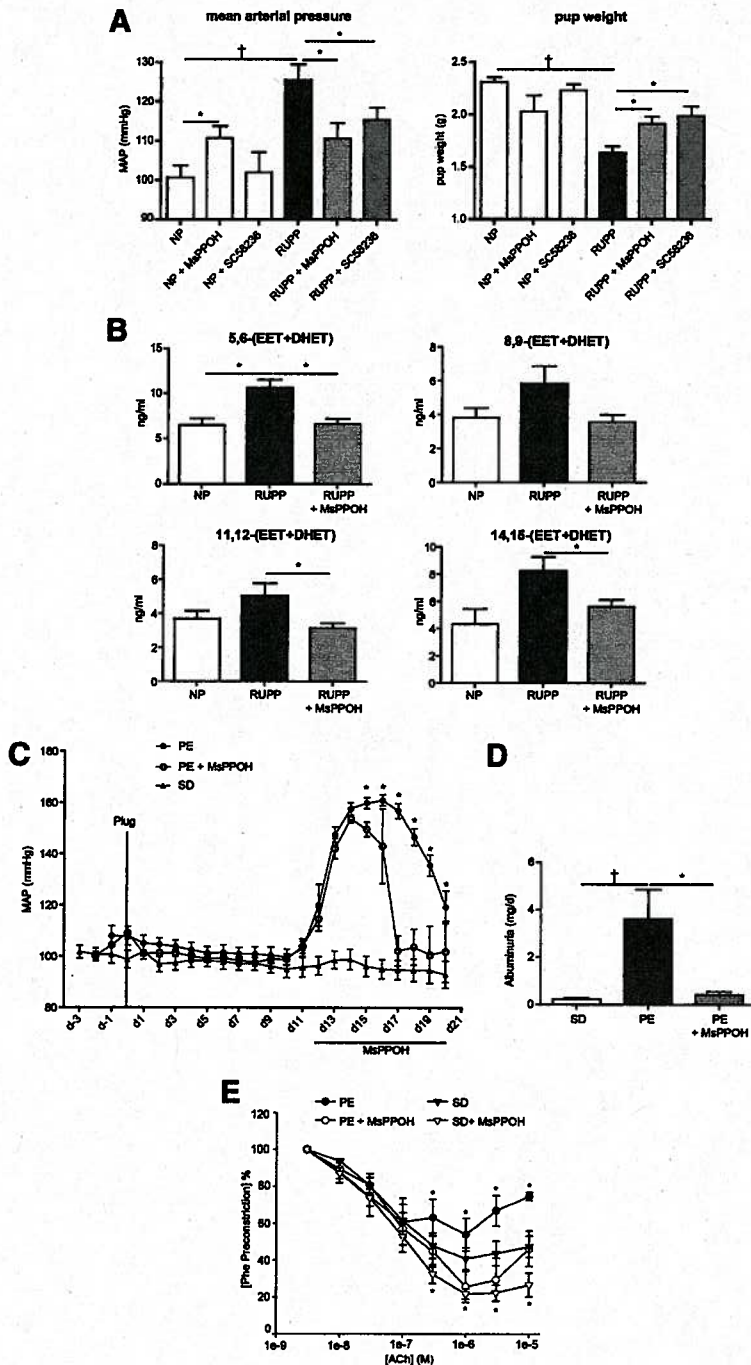
Figure 4. Cytochrome P450 inhibition in pre-eclampsia models. **A,** Mean arterial pressure (MAP) measured on day 18 of pregnancies was enhanced in reduced uterine-perfusion pressure (RUPP) rats vs normal pregnant (NP) rats. MsPPOH and SC58236 abolished this effect (n=5–9; *P<0.05; †P<0.01; ANOVA with Tukey post hoc test; mean±SEM). MsPPOH and SC58236 also abolished the effect of the RUPP procedure on pups. RUPP group litters showed growth restriction vs litters of the normal pregnant group (n=5–9; *P<0.05; †P<0.001 ANOVA with Dunnett T3; mean±SEM). **B,** Cytochrome P450 metabolites are increased in RUPP rats. This effect is lowered by MsPPOH administration (n=5–9; *P<0.01 ANOVA with Dunnett T3; mean±SEM). **C,** Mean arterial pressure measured by telemetry was enhanced in transgenic rats with preeclamptic phenotype (PE) vs normal pregnant rats (Sprague Dawley [SD]). MsPPOH administration abolished this effect (n=4–6; *P<0.05; †P<0.01; Mann–Whitney U test). **D,** Albuminuria was also enhanced in PE vs SD rats and normalized by MsPPOH administration (n=3–11; *P<0.05; †P<0.001; ANOVA with Tukey post hoc test; mean±SEM). **E,** Ex vivo relaxation experiments of uterine artery rings of pregnant rats in response to acetylcholine (ACh) are shown. Uterine artery rings of PE rats showed a paradoxical contractile response to high dose of ACh (3×10^{-6} to 1×10^{-5} mol/L) vs rings of SD rats. MsPPOH (50 μmol/L) enhanced the vasodilatory response to ACh in both groups vs nonstimulated (n=5–6; *P<0.05 for SD vs SD+MsPPOH and PE vs PE+MsPPOH; Mann–Whitney U test).

rats. In addition, we detected the 5,6-epoxy-thromboxane with 2 characteristic transitions of 385.2—229.2 and 385.2—167 (online-only Data Supplement Figure S9).

Discussion

Our results suggest a novel link between CYP eicosanoids and preeclampsia. We found that CYP2J2 expression was significantly upregulated in the uteroplacental unit of pre-eclamptic women compared with women with uncomplicated pregnancies. The CYP2J2 protein was specifically localized to the trophoblasts in these uteroplacental tissues. Our liquid chromatography tandem mass spectrometry analysis revealed

that the development of preeclampsia is associated with increased circulating levels of these metabolites and of 5,6-EET+DHET in particular, even before clinical onset of the syndrome. In trophoblasts, we identified the proinflammatory cytokine TNF-α as a potent stimulator of CYP2J2. We next explored the activity of the CYP2J2 product 5,6-EET in a preeclampsia relevant bioassay. 5,6-EET increased the spontaneous neonatal cardiomyocyte contraction rate, which was blocked completely with either a thromboxane receptor blocker or furegrelate, a potent thromboxane synthase inhibitor. The positive chronotropic effect of 5,6-EET was synergistic with Ang II. Furthermore, 5,6-EET led to a

downregulation of the $K_{Ca}1.1$ channel activity, which could be blocked by inhibiting the thromboxane synthase. In the RUPP preeclampsia rat model, in which TNF-α has been shown to play a prominent role,[26] pharmacological inhibition of CYP epoxygenases or COX reduced hypertension and increased pup weight, indicating that enhanced EET biosynthesis and COX conversion play a detrimental role in the development of preeclampsia. Results could be confirmed in a second, transgenic, rat model with preeclamptic phenotype. Hypertension and albuminuria were lowered by CYP inhibition.

Most of these novel findings may come as a surprise when compared with previous studies demonstrating exclusively beneficial roles of EETs in the regulation of cardiovascular and renal function. EETs mediate vasodilatation and are generally considered as antihypertensive metabolites.[27–31] Normal pregnancy results in enhanced EET formation, and pharmacological inhibition of CYP epoxygenases causes hypertension and renal failure in pregnant rats.[25,32] Moreover, EETs exert anti-inflammatory and antiapoptotic effects that contribute to tissue protection under hypoxic conditions.[28] Prominent examples include EET-mediated protection against ischemia-reperfusion injury in the heart and brain.[33,34] Exogenous EET administration and CYP2J2 overexpression also prevent cytokine-induced activation, as well as hypoxia-induced damage of endothelial cells.[35,36] Thus, the current paradigm of EET action would have predicted CYP2J2/EET upregulation as part of a compensatory mechanism suitable to ameliorate the preeclamptic syndrome. However, our findings are apparently contradictory to this notion. Indicating that the role of EETs has to be specifically defined for the given pathophysiological condition, we found that pharmacological CYP-epoxygenase inhibition indeed raises blood pressure in normal rat pregnancy but exerts the opposite effect in the RUPP model and the transgenic model of preeclampsia. Furthermore, our study shows that trophoblasts are an additional site of EET production and the major site of the uteroplacental unit, where the EET-producing CYP2J2 is upregulated during preeclampsia.

A major difference between normal and preeclamptic pregnancy concerns the sites and mechanisms of enhanced EET biosynthesis. In normal pregnancy, increased EET biosynthesis occurs in the kidney and vasculature,[25,32] where EETs promote antihypertensive and anti-inflammatory mechanisms. In contrast, our study shows that uteroplacental trophoblasts are the major site of EET-producing CYP2J2, which is upregulated during preeclampsia. Although limited in scale, our genetic analysis indicates that common polymorphisms in the CYP2J2 promoter do not contribute to preeclampsia susceptibility. However, we found that CYP2J2 is highly inducible by TNF-α in cultured trophoblasts. This finding suggests that CYP2J2 overexpression proceeds secondary to enhanced TNF-α release in response to placental ischemia. Trophoblasts are key players in spiral artery remodeling; a failure in this process may cause preeclampsia.[37] Trophoblast invasion is triggered by hypoxia but is surprisingly repressed after activation of the hypoxia-inducible factor-1α.[38] Providing a possible link between CYP2J2/EET expression and trophoblast invasion, EETs activate hypoxia-inducible factor-1α as shown in other cell types.[39] In accor-

dance with their vasculoprotective properties,[40] EETs may also prevent the replacement of endothelial cells for trophoblasts during spiral artery remodeling.

A link between enhanced EET biosynthesis and preeclampsia is suggested by the regio-isomeric composition of the circulating metabolites. Comparing normal and preeclamptic pregnancies, we observed no differences in the plasma levels of 8,9- and 11,12-EET. However, we found a pronounced increase of the 5,6- and 14,15-EET levels. Of note, 5,6-EET has been identified as the predominant EET regio-isomer in trophoblasts and other intrauterine tissues.[13] 5,6-EET is also the major product of cyclic AMP-induced AA metabolism in human first-trimester trophoblasts.[41] Moreover, CYP2J2 is probably unique among the human epoxygenases in generating the whole set of regio-isomeric epoxides including 5,6-EET.[23] Accordingly, the increased circulating EET levels in preeclampsia may indeed reflect enhanced CYP2J2-mediated uteroplacental EET biosynthesis.

In general, the beneficial cardiovascular effects of EETs have been attributed to the whole class of these AA metabolites. However, most of the biological activities are certainly regio-selective and stereo-selective. Importantly, the effects of 5,6-EET can be modified by subsequent metabolism. Unlike other EET regio-isomers, 5,6-EET is readily metabolized by COXs and can be converted into a potent vasoconstrictor by the subsequent action of thromboxane synthase.[10] Given the imbalance of prostacyclin and TXA2 in preeclampsia, thromboxane-analog formation may be an important source of thromboxane synthesis during the development of the syndrome.

In preeclampsia, endothelial dysfunction or inappropriate cell activation, as well as alterations in endothelium-dependent vascular contractile properties, is part of the maternal syndrome. In an earlier study, we had shown that the vasodilatory response is reduced in the uterine artery of our PE rats. Uterine artery rings from PE rats relaxed at low acetylcholine doses, whereas higher doses increase the resistance. Our data suggested that a COX-dependent, thromboxane-like metabolite is responsible.[21] Now we could further show that the paradoxical acetylcholine response is also prevented by MsPPOH. This finding indicates that a CYP epoxygenase-dependent metabolite served as precursor of the thromboxane-like activity. This observation is in line with observations in humans. In vitro studies on isolated microvessels from patients with preeclampsia showed an absence of acetylcholine-mediated vasodilatation in omental microvessels.[42] Thus, our data direct attention to possibly similar mechanisms in preeclampsia.

Ang II is a strong vasoactive peptide. During normal pregnancies, the Ang II responsiveness is reduced compared with the nonpregnant state. However, in preeclampsia, the Ang II sensitivity is elevated.[2] In recent studies, we described an elevated Ang II sensitivity mediated by agonistic autoantibodies to the Ang II receptor type 1.[43] Here, we demonstrated that 5,6-EET also has a synergistic effect on the contractility of cardiomyocytes and could, therefore, be a potent facilitator of the Ang II sensitivity in preeclampsia.

K_{Ca}s modulate the membrane potential and regulate Ca^{2+}-dependent contraction and are therefore important mediators

in the control of vascular tone and blood pressure. They induce membrane hyperpolarization in response to increased intracellular Ca^{2+} and thereby counteract smooth muscle contractility. $K_{Ca}1.1$ channels, also known as the BK channels, are the predominantly expressed K_{Ca} channels in contractile VSMCs and are the presumed targets of CYP-generated AA metabolites.[44,45,50] $K_{Ca}1.1$-deficient mice with targeted disruption of α- and β-subunit genes show a phenotype consisting of hypertension and endothelial dysfunction. The relevance of $K_{Ca}1.1$ for blood pressure and cardiovascular disease has been also demonstrated by SNP analysis.[44] We showed that 5,6-EET led to a down regulation of $K_{Ca}1.1$ channel activity, which was blocked by inhibiting the thromboxane synthase. These results suggest that the effects of EET are mediated by a thromboxane analog, such as the 5,6-epTXA1. Our data agree with the idea that blockade of K_{Ca}s can cause inhibition of endothelium-dependent hyperpolarizing factor and thus contribute to the increase of peripheral vascular resistance. The suggestion that endothelium-dependent hyperpolarizing factor contributes to vasodilatory mechanisms during pregnancy is consistent with this notion.

We propose that uteroplacental CYP2J2 overexpression contributes to preeclampsia. Our data are consistent with the 2-stage theory.[46] In stage 1 (placental dysfunction), placental villous deterioration triggers enhanced TNF-α production that, in turn, induces trophoblastic CYP2J2/EET expression. The consequences might contribute to the reduced trophoblast invasion, insufficient spiral artery remodeling, and eventually impaired placental blood flow. In stage 2 (the clinical maternal disease), enhanced intrauterine CYP2J2 activity may result in increased circulating 5,6-EET levels. Under maternal proinflammatory conditions, 5,6-EET is further converted to metabolites that could aggravate hypertension and target-organ damage via thromboxane-like effects. Future studies should elucidate which CYP2J2-dependent metabolites gain access to the maternal systemic circulation.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Preeclampsia remains the greatest cause of perinatal maternal and fetal morbidity with long-term cardiovascular ramifications for mother and child. New mechanistic insights are desperately needed for improved preventative and therapeutic strategies. We used microarray expression to screen placenta (fetal tissue) and decidua (maternal tissue) from preeclamptic women and controls. We identified upregulation of the cytochrome P450 (CYP) epoxygenase, CYP2J2, and located the protein in trophoblasts, placental cells that invade into the decidua. We next screened maternal plasma for CYP2J2 metabolites and observed an elevation of 5,6-epoxyeicosatrienoic acid (EET) and 14,15-EET, as well as the corresponding dihydroxyeicosatrienoic acids (DHETs) in preeclamptic women compared with controls in the latter two thirds of pregnancy. In a trophoblast-derived cell line, the preeclampsia-associated cytokine, tumor necrosis factor-α, enhanced CYP2J2 gene and protein expression. Furthermore, we observed increased CYP2J2 metabolites in 2 different rat models of preeclampsia, in which pharmacological CYP epoxygenase inhibition ameliorated the preeclamptic condition. Finally, we observed that 5,6-EET is metabolized to a thromboxane analog that stimulated calcium-activated potassium channel ($K_{Ca}1.1$) activity. Our results suggest a novel link between CYP eicosanoids and preeclampsia. The findings support the common hypothesis that the preeclampsia begins in the placenta and is translated to a maternal syndrome by circulating factors. Because 5,6 EET can be metabolized to thromboxane, our data also provide a molecular mechanism linking thromboxane metabolism with preeclampsia. We believe that this complex series of findings may permit various treatment avenues that could conceivably be extended to patients.