Microvascular endothelial cells from preeclamptic women exhibit altered expression of angiogenic and vasopressor factors

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Lee DK, Nevo O. Microvascular endothelial cells from preeclamptic women exhibit altered expression of angiogenic and vasopressor factors. Am J Physiol Heart Circ Physiol 310: H1834–H1841, 2016. First published May 3, 2016; doi:10.1152/ajpheart.00083.2016.—Preeclampsia (PE) is a severe complication of pregnancy associated with maternal and fetal morbidity and mortality. The underlying pathophysiology involves maternal systemic vascular and endothelial dysfunction associated with circulating antiangiogenic factors, although the specific etiology of the disease remains elusive. Our aim was to investigate the maternal endothelium in PE by exploring the expression of genes involved with endothelial function in a novel platform of maternal primary endothelial cells. Adipose tissue was sampled at the time of caesarean section from both normal and preeclamptic patients. Maternal microvascular endothelial cells were isolated by tissue digestion and CD31 magnetic Dynabeads. Cell purity was confirmed by immunofluorescence microscopy and flow cytometry. Western analyses revealed VEGF activation of VEGF receptor 2 (VEGFR2) and ERK in primary cells. Quantitative PCR analyses revealed significantly altered mRNA levels of various genes involved with angiogenesis and blood pressure control in preeclamptic cells, including soluble fms-like tyrosine kinase-1, endoglin, VEGFR2, angiotsin receptor 1, and endothelin compared with cells isolated from normal pregnancies. Overall, maternal endothelial cells from preeclamptic patients exhibit extensive alteration of expression of factors associated with antiangiogenic and vasoconstrictive phenotypes, shedding light on the underlying mechanisms associated with the vascular dysfunction characteristic of PE.

angiotensin; blood pressure; cardiovascular diseases; endothelin; hypertension

NEW & NOTEWORTHY

This study is the first to show that maternal primary-isolated microvascular endothelial cells from preeclamptic women exhibit altered gene-expression levels that reflect shifts toward antiangiogenic and vasopressor phenotypes compared with normal pregnancies.

A common complication of pregnancy, preeclampsia (PE) is characterized by high blood pressure (BP) after 20 wk of gestation (7). Severe PE may be associated with substantial maternal morbidity, including excessively high BP, renal failure, respiratory failure, stroke, leukoencephalopathy, and seizures (eclampsia). If not treated promptly, severe PE can be lethal. The underlying pathophysiology of PE is attributed to systemic vascular and endothelial dysfunction due to circulating antiangiogenic factors and syncytiotrophoblast microparticles originating from the placenta (7). The two best characterized antiangiogenic factors are soluble fms-like tyrosine kinase-1 (sFlt1) and soluble endoglin, both shown in vitro to affect endothelial cells negatively and induce the classical signs of PE in small animal models. It is hypothesized that these antiangiogenic factors bind circulating VEGF and placental growth factor (PIGF), thereby decreasing their availability for activating membrane-bound receptors on endothelial cells (7).

Pregnancy is associated with a substantial modification of the maternal vasculature and endothelium. Hormonal changes that occur during pregnancy, such as high levels of steroid hormones (estrogen, progesterone) and growth factors, likely originate in the placenta, elevate blood volume, and directly affect the maternal endothelium (34). The physiological changes in maternal vascular/endothelial function are evident by lowered maternal BP during pregnancy, despite an increased maternal blood volume. The mechanisms underlying these changes are not completely understood, although serum levels of various factors implicated in BP control, such as angiotensin and prostacyclin, have been observed to change during normal pregnancy, whereas others, such as endothelin, change only in PE (26). Whereas information regarding serum levels of factors associated with maternal vasculature adaptation to pregnancy has become increasingly available, information regarding the physiology of maternal endothelial cells, which play a crucial role in the maintenance of vascular tone and in the development of PE, is very limited. Rodent models have proven insightful in delineating some of the potential mechanisms underlying the pathophysiology of PE. However, PE is a solely human disease and requires human subjects, tissues, and cells to investigate fully the origins and molecular pathways of the disease. Due to the complexities and limitations of performing studies in pregnant women, isolated endothelial cells offer an attractive alternative for studying the maternal endothelium, which plays a crucial role in normal pregnancy and in PE. To date, the first and most extensively investigated endothelial cells isolated from humans are human umbilical vein endothelial cells (HUVECs) (19). However, these cells represent macrovascular endothelial cells of fetal origin. There is a large degree of heterogeneity in endothelial cellular expression and function dictated by their location in the vasculature. Subsequently, distinct responses have been observed between HUVECs and endothelial cells isolated from human microvasculature (21). As the majority of the endothelium is located in the microvasculature, this led us to develop a novel platform for the exploration of maternal endothelium in PE compared with normal pregnancies by isolating microvascular endothelial cells (MVECs) from maternal adipose tissue.
In the present study, we first isolated MVECs, examined their purity, and explored the differences in gene expression patterns between MVECs from preeclamptic and normal pregnancies. Our hypothesis is that maternal endothelial cells from preeclamptic patients will exhibit altered gene expression and signaling pathways, favoring antiangiogenic and vasopressor phenotypes that can be the target to assess future treatments.

METHODS

Isolation of human MVECs from adipose tissue. MVECs were isolated from subcutaneous adipose tissue sampled at the time of caesarean section from both normal patients \((n = 6)\) and patients with severe PE \((n = 6)\); Table 1. The PE group was selected to represent classic, severe early-onset PE, based on criteria by the American College of Obstetricians and Gynecologists \((2)\). PE patients with pre-existing conditions, such as diabetes, infection, chronic hypertension, or renal dysfunction, were excluded from this study. Patients with premature rupture of membranes or preterm labor were also excluded. Normal patient-inclusion criteria included healthy pregnancies with delivery by elective caesarean section with no history of high BP or other underlying medical conditions mentioned above. This study was approved by Sunnybrook Health Sciences Centre Research Ethics Board, and informed consent was given by all patient subjects. Human MVECs were isolated using CD31 magnetic Dynabeads (Thermo Fischer Scientific Life Sciences, Waltham, MA), as described previously \((15)\). Briefly, subcutaneous adipose fat was dissected from connective tissue, chopped finely, washed in PBS, and incubated in PBS containing type II collagenase \((2,000 \text{ U/ml})\) at 37°C for 1 h. Digests were washed and cells collected with repeated centrifugation in PBS. Cell pellets were washed further and collected with repeated centrifugation in 10% BSA \((\text{in PBS})\), washed a final time in PBS, and incubated in 2.5% trypsin at 37°C for 10 min. HBSS with 5% FBS was added to neutralize the trypsin and the suspension filtered through a 100-μm nylon mesh to remove remaining connective tissue and debris. The suspension was resuspended with CD31 Dynabeads for 20 min on ice with occasional agitation. MVECs conjugated with Dynabeads were isolated by four rounds of magnetic attraction and washing in HBSS \((5\% \text{ FBS})\) and plated and maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) and 8% O\(_2\) to approximate physiological conditions, an important consideration, as oxygenation levels. MVECs were grown in endothelial basal medium MCDB 131 (US Biological Life Sciences, Salem, MA), supplemented with 10% FBS, penicillin-streptomycin \((100 \text{ U/ml})\); Thermo Fisher Scientific Life Sciences), hydrocortisone \((1 \text{ μg/ml})\); Sigma-Aldrich, St. Louis, MO), human EGF \((10 \text{ μg/ml})\; R&D Systems, Minneapolis, MN), human VEGF \((500 \text{ ng/l})\; Cell Signaling Technology, Danvers, MA), R3-IGF-I \((20 \text{ μg/l})\; Sigma-Aldrich), and ascorbic acid \((1 \text{ μg/l})\; Sigma-Aldrich).

<table>
<thead>
<tr>
<th>Clinical parameters of participants</th>
<th>Normal</th>
<th>Early Preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Maternal age, yr</td>
<td>40.2 ± 1.6</td>
<td>33.5 ± 0.9</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>39.2 ± 0.4</td>
<td>30.7 ± 0.6*</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.2 ± 0.9</td>
<td>28.0 ± 0.5</td>
</tr>
<tr>
<td>Gravidity/parity, mean</td>
<td>2.2/1</td>
<td>2.8/1.1</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>121 ± 1.7</td>
<td>181.6 ± 10.4*</td>
</tr>
<tr>
<td>Systolic</td>
<td>75.8 ± 2.7</td>
<td>106.6 ± 3.1*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>0</td>
<td>2.8 ± 0.3*</td>
</tr>
<tr>
<td>Proteinuria (plus protein)</td>
<td>3,377.4 ± 201.3</td>
<td>1,141.5 ± 143.9*</td>
</tr>
</tbody>
</table>

Values are mean ± SE; *P < 0.05.

Cell lines and reagents. Primary human microvascular endothelial cells (HMVEC), immortalized by engineering the human telomerase catalytic protein into the cells, were a generous gift from Dr. Rong Shao (Biomedical Research Institute, Baystate Medical Center/University of Massachusetts at Amherst, Springfield, MA) \((42)\). The von Willebrand factor \((\text{vWF})\) antibody \((\text{A0082, 1:100})\) was purchased from Dako (Troy, MI). The VEGF receptor 2 \((\text{VEGFR2}; 2479, 1:10,000)\), phospho-VEGFR2 \((2478, 1:1,000)\), and phospho-ERK \((9106, 1:2,000)\) antibodies were purchased from Cell Signaling Technology. The β-actin \((\text{sc-1616, 1:5,000})\), angiotsins receptor 1 \((\text{AT1R}; \text{sc-1173, 1:200})\), endothelin A receptor \((\text{ETAR}; \text{sc-33535, 1:200})\), and endothelin B receptor \((\text{ETBR}; \text{sc-33537, 1:200})\) antibodies were purchased from Santa Cruz Biotechnology \((\text{Dallas, TX})\). The APC-conjugated mouse antibody for human CD31 \((55544, 1:100)\) and allophycocyanin \((\text{APC}-\text{IgG2b and FITC-IgG1 mouse control antibodies were purchased from BD Biosciences (San Jose, CA)\). The APC-conjugated mouse antibody for human vWF \((\text{IC27641A, 1:100})\) was purchased from R&D Systems.

Immunofluorescence and flow cytometry. For immunofluorescence analyses, cells were grown in supplemented MCDB 131 media on gelatin-treated coverslips. The cells were washed once in media, fixed in media containing 3.7% formaldehyde at 37°C for 15 min, rinsed three times in PBS, permeabilized in 0.2% Triton X-100/PBS for 5 min, and rinsed again in PBS. Cells were incubated for 1 h at room temperature in blocking solution \((\text{PBS, 5% horse serum, 0.2% Triton X-100})\), followed by 2 h incubation at room temperature in blocking solution containing vWF antibody \((1:100)\). Cells were washed three times in PBS and incubated for 1 h at room temperature in blocking solution containing a DyLight 649-conjugated anti-rabbit IgG antibody \((1:100)\). Cells were washed again three times in PBS and incubated with 4',6-diamidino-2-phenylindole \((300 \text{ nM})\) in PBS for 5 min. After a final PBS rinse, coverslips were mounted in 90% glycerol/PBS on glass slides. Fluorescent images were acquired and processed using a Zeiss Axiovert 200M microscope and Zeiss AxioVision imaging software \((\text{Carl Zeiss, Oberkochen, Germany})\). For flow cytometry, live cell suspensions were prepared in fluorescence-activated cell-sorting buffer and stained with either FITC-labeled anti-CD31 or APC-labeled anti-vWF at 1:100 dilution or with controls \((\text{APC-labeled mouse IgG2b or FITC-labeled mouse IgG1})\). Analyses were performed using a BD Biosciences FACScalibur system, and all data were analyzed using FlowJo software \((\text{Tree Star, Ashland, OR})\).

RNA isolation and quantification using real-time PCR. RNA isolation and quantitative PCR \((\text{qPCR})\) were performed as described previously \((31)\). Briefly, total RNA was extracted from cultured cells \((\text{from passage 4 to 5})\) using TRizol reagent \((\text{Thermo Fisher Scientific Life Sciences})\), treated with DNase \((\text{Thermo Fisher Scientific Life Sciences})\) to remove genomic DNA contamination, and purified further by column purification \((\text{EZ-10 Spin Column RNA; Bio Basic, Amherst, NY})\). Total RNA \((1 \mu\text{g})\) was reverse transcribed using the SuperScript VILO cDNA synthesis kit with random hexamers \((\text{Thermo Fisher Scientific Life Sciences})\). The resulting templates were quantified by real-time qPCR using SYBR Select Master Mix \((\text{Thermo Fisher Scientific Life Sciences})\) and a StepOnePlus Real-Time PCR machine \((\text{Thermo Fisher Scientific Life Sciences})\). Custom primers were purchased from Thermo Fisher Scientific Life Sciences \((\text{listed in Table 2})\). Relative quantification of data was performed using logarithmic curves. Expression levels were normalized with 18S expression using the comparative threshold cycle formula, as described previously \((45)\).

Western blot analysis. Western analyses were performed as described previously \((31)\). Briefly, MVECs at passages 4–5 were serum starved for 2 h and incubated for various time points in the presence or absence of VEGF \((20 \mu\text{g/l})\), supplemented only with penicillin-streptomycin \((100 \text{ U/ml})\). Cells for basal expression studies were not serum starved. Cells were collected in radioimmunoprecipitation as-
say buffer containing cOmplete ULtra protease inhibitor cocktail and PhosSTOP (Roche Diagnostics, Indianapolis, IN) and homogenized, and 25 μg total protein lysates were subjected to 8% (w/v) SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubation in 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 60 min. Membranes were incubated with antibodies specific for VEGFR2, phospho-VEGFR2, phospho-ERK, AT1R, ETAR, ETBR, or β-actin in 5% milk or 5% BSA (per their respective manufacturers’ instructions) at 4°C overnight. Membranes were washed in TBST and incubated for 60 min at room temperature with 1:2,000 diluted horseradish peroxidase-conjugated anti-rabbit, -goat, or -mouse IgG (Santa Cruz Biotechnology) in 5% milk in TBST. After washing with TBST, blots were visualized by enhanced chemiluminescence substrate Western Lightning Plus-ECL (PerkinElmer, Waltham, MA) using a MicroChemi 4.2 chemiluminescent imager (DNR Bio-Imaging Systems, Jerusalem, Israel) and band intensities quantified using GelQuant software (DNR Bio-Imaging Systems) and normalized to β-actin levels.

**Statistics.** Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). For comparisons between normal and PE samples, we used the Mann-Whitney test. Significance was defined as *P* < 0.05. Results are expressed as the means ± SE.

**RESULTS**

**Isolation of MVECs.** Following isolation from adipose tissue, primary MVECs from both normal and PE patients grew to confluence within 2 wk after Dynabeads selection. Whereas these primary MVECs were typically observed to grow to passage 10 without observable alterations to their morphology, we routinely used them for our experiments between passages 4 and 5. We first ascertained whether the isolated cells were homogeneous and functional primary MVECs. Cells grown in culture exhibited a typical endothelial cell distribution when viewed by phase contrast microscopy (Fig. 1A). To visualize the homogeneity and purity of these cell populations, MVECs were stained with APC-conjugated antibodies for human vWF, an endothelial cell marker. All isolated MVECs from both normal and PE populations revealed uniform staining with vWF (Fig. 1B). These results were consistent with those seen from a positive control HMVEC cell line and in stark contrast to a negative control HeLa cell line, where vWF expression was absent. To confirm what appeared from fluorescent microscopy to be homogeneous MVEC populations, we performed flow cytometry on the primary MVEC to determine the percentage of CD31 and vWF-positive populations (Fig. 1C). Whereas the negative control HeLa cell line revealed no expression of either endothelial cell marker, positive control HMVEC and the MVEC isolated from both normal and PE adipose tissue yielded cell populations that expressed both CD31 and vWF throughout, indicated by clear bimodal expression patterns by histogram and purity of nearly 100% by flow cytometry counts.

**Primary MVEC response to VEGF.** We assessed the ability of the MVECs to respond to stimuli by treating them with VEGF (20 ng/ml) for 10 min, 1 h, and overnight (17 h). Protein lysates were prepared from cell culture and subjected to Western analysis (Fig. 2). VEGF treatments at 1 h or longer appeared to decrease VEGFR2 protein, suggesting that chronic treatment with VEGF resulted in a downregulation of the VEGFR2 receptor in primary MVECs. Acute treatment with VEGF (10 min) resulted in a rapid increase in phosphorylated VEGF and ERK levels, indicating a clear response and activation of the VEGFR2 receptor and downstream signaling. These findings suggest that the isolated primary cells are functional endothelial cells, suitable for further study in comparing endothelial cell function between normal and PE populations.

**MVECs from preeclamptic patients exhibit altered gene expression.** We compared the basal expression levels of various factors associated with PE, hypertension, or endothelial cell function (Figs. 3 and 4). MVECs of normal and PE origin were cultured and collected for RNA isolation. RNA was subjected to qPCR quantification analysis, comparing target levels between normal and PE populations. For angiogenic factors, we observed a significant increase of sFlt1 and endoglin mRNA expression in PE populations compared with normal, with a significant decrease in PIGF and VEGFR2 levels (Fig. 3). The full-length, membrane-bound VEGFR1 also appeared to be increased in PE samples, although the difference was not found to be significant. No changes in expression levels were detected for VEGF or sFlt14, a splice variant of sFlt1. For BP-related factors, AT1R mRNA expression levels were significantly increased ~30-fold in PE samples, whereas the ETAR was also significantly increased (Fig. 4). Conversely, the RNA expression levels for both ETAR and ETBR were significantly decreased in PE MVECs. These findings suggest that the expression of endothelial cell markers and proangiogenic factors in preeclamptic MVECs is altered compared with normal MVECs, which may contribute to the development of PE.

**Table 2. Primer list for qPCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>18s</td>
<td>5′-GTAAAGCCCGTTGGAAGCATT-3′</td>
<td>5′-CCATCCAACTCGTGATAGGC-3′</td>
</tr>
<tr>
<td>Apelin</td>
<td>5′-GTCCTGCTGCTATAAGCTGTCG-3′</td>
<td>5′-GGATTACCTAAAAGACTCAACAG-3′</td>
</tr>
<tr>
<td>Api</td>
<td>5′-GTCCTGCTGCTATAAGCTGTCG-3′</td>
<td>5′-GGATTACCTAAAAGACTCAACAG-3′</td>
</tr>
<tr>
<td>AT1R</td>
<td>5′-ATTTAGAAGCTGCTATGGATTGCC-3′</td>
<td>5′-CAGCGTGATTATCATGATCTG-3′</td>
</tr>
<tr>
<td>Endoglin</td>
<td>5′-GTCGACTGCTGCTACAATGTCACCA-3′</td>
<td>5′-AGCTGGCCACCTAAGAAGCTG-3′</td>
</tr>
<tr>
<td>Endothelin</td>
<td>5′-AGAGTGGTGCCTACTCTCTCCCA-3′</td>
<td>5′-CTTCAACGAGCTGTTGATCTC-3′</td>
</tr>
<tr>
<td>ETAR</td>
<td>5′-GTCGTTTGCTATTGTATGCTGCC-3′</td>
<td>5′-GAGGACCAAGCTGTTGATCTC-3′</td>
</tr>
<tr>
<td>ETBR</td>
<td>5′-GTCGTTTGCTATTGTATGCTGCC-3′</td>
<td>5′-GAGGACCAAGCTGTTGATCTC-3′</td>
</tr>
<tr>
<td>PIGF</td>
<td>5′-GAACGGGCTGCTCAAGGTTGGA-3′</td>
<td>5′-GAGACCTAGGAAGAAACAGCTTT-3′</td>
</tr>
<tr>
<td>Prostacyclin synthase</td>
<td>5′-CTCGGTTGGCTGCTGCTGCC-3′</td>
<td>5′-CTTCAACGAGCTGTTGATCTC-3′</td>
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<tr>
<td>sFlt1</td>
<td>5′-GGAAAGAATTGCTCCCCCCAGGAA-3′</td>
<td>5′-GAGACTCGAGAAGAAAAGCTTT-3′</td>
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<tr>
<td>sFlt14</td>
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<td>VEGFR2</td>
<td>5′-ATAGAAGGCTGCTCAAGGAAAGTTG-3′</td>
<td>5′-GCTTCCAGATGCTGCCAGATTG-3′</td>
</tr>
</tbody>
</table>

**Adjusted expression of preeclamptic endothelium**

**Table 2. Primer list for qPCR**

For comparisons between normal and PE samples, we used the Mann-Whitney test. Significance was defined as *P* < 0.05. Results are expressed as the means ± SE.
levels of ETBR, prostacyclin synthase, and apelin were significantly decreased in PE samples. No significant changes in expression levels were detected for thromboxane synthase or the apelin receptor (APJ). MVECs of normal and PE origin were also cultured and collected for Western analyses (Fig. 5).

**DISCUSSION**

We have developed a novel tool in PE research based on primary isolated endothelial cells from maternal tissue. This is
the first report to show expression differences between maternal MVECs isolated from normal and PE pregnancies, particularly with genes involved with angiogenesis, which mirrors expression differences observed in PE placenta, such as the upregulation of sFlt1 and endoglin and the downregulation of PlGF and VEGFR2. sFlt1, a circulating factor associated with maternal endothelial dysfunction in PE, is thought to originate mainly from the placenta. The identification of endothelial cells as a potential source of elevated sFlt1 suggests another origin point but one that may act in an autocrine/paracrine manner, in addition to effects from circulating placental sFlt1. Interestingly, endothelial cells from preeclamptic women also exhibit altered gene expression that includes overexpression of genes that are associated with high BP (including ET1, ETAR, and AT1R) and decreased levels of genes associated with vasorelaxation (including ETBR, prostacyclin synthase, and apelin). The findings reveal that endothelial cells of preeclamptic women have altered gene expression in culture compared with cells from normal pregnancies and are a new tool for PE research with exceptional opportunities for further research.

We present evidence that the primary isolated cells reported here are homogeneous and functional endothelial cells, evident by both flow cytometry and immunofluorescence and activation by VEGF, with increased levels of phosphorylated VEGFR2 and ERK. There is considerable heterogeneity of the endothelium that resides in large or small vessels, dependent on their organ-specific location and microenvironment. Whereas endothelial cells have been used in PE research, such investigations have primarily used HUVECs for in vitro experiments. HUVECs are macrovascular endothelial cells from fetal origin, reported to have a distinct phenotype compared with MVECs (20). Therefore, our work focused on primary isolated MVECs (which comprise the vast majority of endothelial cells in the body) as a suitable physiological model to explore the maternal endothelial alterations that arise in PE. Primary endothelial cells from human tissues have been used before to explore other diseases, such as type 2 diabetes, although the number of reports is limited, and have not been used in the context of PE (13, 44, 46). The most commonly reported isolation method is by immunomagnetic beads (16), although others have reported endothelial cell isolation by density gradient (6). We chose the positive-selection isolation method using immunobeads and showed high cell purity levels by endothelial cell marker detection.

Expression analyses of isolated MVECs from PE and normal pregnancies revealed an overall shift favoring antiangiogenic factors in PE cells. VEGF is an important angiogenic factor, whose effects are mediated through VEGFRs—VEGFR1 and VEGFR2. VEGF is thought to be a key modulator of the maternal systemic vasculature by inducing the upregulation of vasodilators nitric oxide and prostacyclin (17, 47). PlGF is another important angiogenic factor, which like VEGF, is a known vasodilator and is implicated in decreasing BP during pregnancy (33). sFlt1, a soluble splice variant of VEGFR1, which binds VEGF and PlGF, is observed to have increased maternal circulating levels in normal pregnancy with earlier onset and greater levels in PE pregnancies (23). In this report, we determined that whereas VEGF mRNA expression levels
were similar in normal and PE MVECs, we observed increased levels of sFlt1 and endoglin (the soluble version, which is known to be increased in maternal circulation in PE) (22) and decreased levels of PIGF and VEGFR2 in PE MVECs, suggesting a shift to an antiangiogenic phenotype and in agreement with current evidence of angiogenic factor levels in PE.

In vitro, increased sFlt1 levels result in vasoconstriction and endothelial dysfunction (27). sFlt1 serum levels are known to be increased in PE, and their increased expression is attributed to higher placental expression (31) and peripheral blood mononuclear cells (36). This is the first report showing that maternal endothelial cells also express higher levels of sFlt1 in PE. Specifically, we found that only sFlt1 mRNA is increased, as opposed to the sFlt14 isoform, which was similar in both groups. As increased levels of sFlt1 associated with PE are thought to originate from the placenta, we are the first to report sFlt1 expression in MVEC, which provides a previously unreported source of sFlt1 in the pathophysiology of PE. An increase in local sFlt1 may act in a paracrine/autocrine manner, exacerbating the effects of circulating sFlt1, but may also be part of a physiologic mechanism that participates in vessel repair (32). In addition, we previously reported that increased levels of sFlt1 may reduce VEGFR2 expression by direct heterodimer formation (30). Decreased expression of VEGFR2 may substantially affect a spectrum of cellular functions, including proliferation and apoptosis (5). Interestingly, although VEGFR2 mRNA was reduced in PE MVECs, we did not observe a significant reduction of VEGFR2 protein, suggesting a compensatory mechanism stabilizing the VEGFR2 protein population from degradation. VEGFR2 is known to have a complex system of trafficking, including a constitutive recycling of inactive receptors and directed sorting to lysosomes upon receptor activation (40, 43). Given the shift favoring an antiangiogenic phenotype in the PE MVECs, inactive VEGFR2 may be protected from degradation by a recycling trafficking loop between the cell membrane and sorting endosomes.

Isolated MVECs from PE also exhibit a shift in gene expression favoring vasoconstriction in maternal microvessels compared with normal pregnancies, in agreement with increased BP that is a hallmark of PE. The angiotensin system is well established as a primary regulator of BP in the endothe-
lum, and there is a growing body of evidence of increased AT1R activity in women with PE (7). Circulating AT1R autoantibodies, which stimulate AT1R, have been found in preeclamptic patients and are thought to contribute to this observed hyperactivity of the receptor. The activation of AT1R by autoantibodies leads to intracellular cascades, which in addition to physiological effects, such as increased BP, may lead to upregulation of PE-associated factors, such as sFlt1, soluble endoglin, and ET1 (14). Here, we provide direct evidence of elevated AT1R mRNA and protein expression in MVECs from PE patients who may act synergistically with AT1R autoantibodies. Furthermore, the decrease of apelin expression in MVECs from women with PE further supports the notion of dysfunction of the renin-angiotensin system. Apelin (and its receptor APJ) has a number of physiological roles in homeostasis but primarily in the cardiovascular system by mediating and antagonizing the effects of ANG II (10). Apelin exhibits a hypotensive effect through an endothelium and nitric oxide-dependent mechanism, with lower levels ob-
served in patients with hypertension (35). Our data are the first to report that apelin expression in the maternal endothelium in PE is decreased and follows the same pattern of decreased expression in the placenta (18). Additionally, we observed a decrease in prostacyclin synthase expression, which suggests decreased prostacyclin production and supports previous data of decreased circulating prostacyclin levels in PE (8). Together, increased levels of AT1R and decreased levels of apelin and prostacyclin synthase in PE MVECs indicate a disruption of key mediators of BP favoring vasoconstriction.

Another regulator of BP, ET1, is known to be one of the most potent vasoconstrictors and together with its receptors (ETAR and ETBR), is an important factor in BP control (37). There is a growing body of evidence associating increased levels of ET1 with PE (12), although it is noteworthy that much of this data stems from rodent models of pregnancy or pertains to tissue and cells derived from human placenta, indicating a clear need for a maternal endothelial model for PE. The serum levels of ET1 were reported to increase in PE (11), and thus it was hypothesized that increased ET1 levels are included in the mechanisms contributing to high BP in PE. In general, ETAR and ETBR have opposing effects in modulating BP, with ETAR activation resulting in an increase in BP, whereas ETBR reduces BP through activation of nitric oxide synthase (24). Whereas ETAR is known primarily for its role and expression in the underlying smooth muscle vascular layer in blood vessels, recent reports have highlighted its expression and function in endothelial cells as well, including a role in the secretion of ET1, regulation of intracellular calcium levels, and protective effects against hypoxia-induced cellular injury (1, 4, 25, 39, 41). Recently, a study examined the role of ETBR in pregnant rats, which observed enhanced ETBR-mediated microvascular relaxation compared with virgin rats (28). In a follow-up study, the same group used a rodent model of hypertensive pregnancy (reduction of uteroplacental perfusion pressure rats) and determined that ET1-induced constriction of microvessels was enhanced vs. normal pregnant rats, which was attributed to ETBR disruption/downregulation (29). Here, we report the first evidence that MVECs from preeclamptic women express much higher levels of ET1, with increased mRNA and protein levels of ETAR, with decreased mRNA and protein levels of ETBR, which suggests a disruption of the microvascular endothelial endothelin system toward a vasoconstrictive state. Our findings suggest that the ET1 signaling pathway is profoundly disrupted in patients with severe PE, although the specific mechanism is unclear. However, there is evidence that ET1 levels are affected by various factors known to be elevated in PE. TNF-α, for example, induces ET1 levels in sensitized endothelial cells (9), whereas sFlt1-augmented, ET1-mediated vasoconstriction has been observed in mice (3).

Two primary hypothetical explanations are given to the overarching variations in gene expression observed in our study and others: either that the variations are pre-existing and predispose pregnant woman to abnormal placentation and to PE or that changes in maternal endothelium gene expression occur, due to circulating factors associated with PE. The information provided here cannot directly prove either hypothesis. However, as it is unlikely that healthy individuals would have such a degree of altered endothelial gene expression (~30-fold difference in AT1R and ET1), this does suggest that the sustained expression observed in cultured cells is a result of
the effects of elevated circulating factors in PE. Previous reports lend support to this hypothesis. For example, as mentioned above, the elevation of key circulating factors, such as sFlt1 and TNF-α, in PE has been shown to increase BP, augment ET1 levels and activity, and result in endothelial dysfunction. However, we cannot rule out the contribution of pre-existing differences in endothelial expression profiles between normal and PE patients. A recent study comparing cardiovascular risk factors pre- and postpregnancy observed that in approximately one-half of the cases examined, the positive association between PE and gestational hypertension with the postpregnancy cardiovascular risk factors may be due to pre-existing conditions (38). This poses the suggestion that pregnancy in women at risk for cardiovascular disease acts as a stress challenge that may exacerbate pre-existing conditions to manifest long-term cardiovascular dysfunction.

Whereas our data clearly show a directional bias of PE MVEC in culture toward an antiangiogenic and vasopressor state, we acknowledge a few limitations of this study. As our study deals with a certain population of MVECs from one type of adipose tissue, we cannot conclude whether the differences in expression that we see in PE cells reflect local vs. systemic changes compared with those from normal pregnancies. One possible solution would be sampling and comparisons from different tissue sources, such as omental adipose (for variable pre-existing conditions (38)). This poses the suggestion that the elevation of key circulating factors, such as circulating factors that are secreted by the placenta.

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The hallmark of PE is maternal endothelial dysfunction caused by circulating factors that are secreted by the placenta. We have developed a new tool in PE research based on primary isolated endothelial cells from maternal tissue and discovered that endothelial cells from preeclamptic women exhibit altered gene expression that includes overexpression of genes associated with high BP and decreased levels of genes associated with vasorelaxation. The findings suggest that endothelial cells of preeclamptic women have altered gene expression even when in culture and can be used as a new platform with exceptional opportunities for further research in PE. This observation may also explain the long-term cardiovascular morbidity that is more common in women with a history of PE.
ALTERED EXPRESSION IN PREECLAMPTIC ENDOTHELIUM


