In Preeclampsia, the Circulating Factors Capable of Altering In Vitro Endothelial Function Precede Clinical Disease

Jenny Myers, Gary Mires, Maureen Macleod, Philip Baker

Abstract—The pathophysiology of preeclampsia involves the release of a circulating factor(s) from a hypoperfused placenta that activates the maternal endothelium. This study investigated the effect on in vitro endothelial function of plasma taken from women in whom preeclampsia subsequently developed. Women at increased risk for an adverse pregnancy outcome were identified using Doppler waveform analysis. Plasma samples (22 and 26 weeks) were incubated with myometrial vessels taken from women with uncomplicated pregnancies. Wire myography was used to study the effect of plasma on the endothelium-dependent vessel behavior. Incubation of vessels from normal pregnant women with plasma from women in whom preeclampsia subsequently developed (n=19) significantly reduced endothelium-dependent relaxation, compared with vessels incubated with plasma from normal pregnant women (n=48). This effect was demonstrable for plasma taken at 22 weeks (residual constriction 47.1±6.6% versus 32.0±4.4%, P=0.004 at 1-hour incubation; and 59.1±8.4% versus 42.3±5.9%, P=0.001 at 18-hour incubation) and 26 weeks (59.2±5.2% versus 29.1±5.6%, P<0.001 at 1 hour; and 63.3±7.6% versus 31.9 +/−7.2%, P<0.0001 at 18 hours). Endothelial-dependent relaxation was unaltered after incubation with plasma taken from women in whom normotensive intrauterine growth restriction subsequently developed (n=19). This study supports the hypothesis that plasma, from women in whom preeclampsia develops, collected weeks before diagnosis is capable of altering endothelial function.

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Key Words: pregnancy ■ preeclampsia ■ hypertension, pregnancy ■ endothelium ■ vasorelaxation

The multisystem disorder of preeclampsia continues to be a leading cause of maternal and perinatal morbidity and mortality. The condition has been the most important cause of maternal death over recent decades and the condition is responsible for the occupancy of ≈20% of special care baby unit cots.1 Growth-restricted babies born from pregnancies affected by preeclampsia have an increased risk of hypertension, heart disease, and diabetes in adulthood.2

There is accumulating evidence for a pathogenic model of preeclampsia whereby deficient trophoblast invasion of the maternal spiral arteries leads to a poorly perfused feto-placental unit.3 This results in secretion of a factor(s) by the placenta into the maternal circulation, which activates the vascular endothelium.4 A number of in vitro studies have supported this hypothesis.5-6 Impaired endothelium-dependent relaxation has been demonstrated in omental,7 subcutaneous,8,9 and myometrial10 arteries. In myometrial vessels isolated from normal pregnant women, the endothelial-dependent agonist bradykinin produced concentration-dependent relaxation; responses were dependent on an intact functional endothelium. In contrast, endothelium-dependent responses to bradykinin were markedly attenuated in myometrial vessels from patients with preeclampsia.10

Studies demonstrating markedly altered function in cultured endothelial cells after exposure to plasma or serum from preeclamptic women11,12 provided convincing evidence of the presence of a circulating factor(s). We have shown that plasma from women with preeclampsia is also capable of altering ex vivo endothelium-dependent relaxation of myometrial vessels from normal pregnant women.13 Using wire myography as an ex vivo model, preliminary characterization studies demonstrated that the active factor is reversible, heat-labile, partially removed by charcoal stripping, maintained within a plasma protein concentrate, and removed by protease digestion.14 It is unknown whether the plasma effect is an epiphenomenon of the established disease process or whether this effect is apparent before the onset of clinically apparent disease.

Here we report results of a blinded study investigating longitudinal plasma samples taken from women at increased risk for an adverse pregnancy outcome. The primary objective was to determine whether plasma, from pregnant women...
in whom preeclampsia subsequently develops, taken before the onset of clinical symptoms/signs is capable of inducing aberrant endothelial function in myometrial vessels. Additionally, we assessed whether this effect was specific to women in whom preeclampsia developed (ie, whether a similar effect was demonstrable in pregnancies complicated by intrauterine growth restriction [IUGR]) and determined whether the effects of plasma from women with preeclampsia were dependent on abnormal Doppler waveform analysis in mid-gestation or the development of associated IUGR.

Methods
The Tayside Medical Research Ethics and Manchester Local Research Ethics Committees gave approval for this work. Written informed consent was obtained for all plasma and tissue samples obtained. An outline of the experimental design is shown in Figure 1.

Study Group and Outcome Definitions
Uterine arterial Doppler waveform analysis was used as a method of screening the obstetric population at mid-gestation to identify a prospective cohort of antenatal patients at significant risk for preeclampsia and IUGR. This technique is performed routinely at the Tayside Medical Research Ethics and Manchester Local Research Ethics Committees gave approval for this work. Written informed consent was obtained for all plasma and tissue samples obtained. An outline of the experimental design is shown in Figure 1.

Myometrial Samples
A single myometrial biopsy sample was taken from women undergoing planned caesarean section with uncomplicated pregnancies at mid-gestation or the development of associated IUGR. This technique is performed routinely at the Tayside Medical Research Ethics and Manchester Local Research Ethics Committees gave approval for this work. Written informed consent was obtained for all plasma and tissue samples obtained. An outline of the experimental design is shown in Figure 1.

Experimental Protocol
Vessels (200 to 500 μm) were dissected from myometrial biopsy samples in ice-cold physiological salt solution (PSS) under a stereomicroscope and mounted on a 4-chamber Danish Myotechnology M610 wire myograph as described elsewhere. Plasma samples were prepared as previously described.

1-Hour Protocol
Plasma was added to the baths (2% final concentration) and 1 U/mL heparin was added to prevent coagulation, warmed to 37°C, gassed with 95% O2/5% CO2, and incubated for 1 hour. Before normalization, the plasma solution was removed and replaced with 7 mL PSS. Sustained vasoconstriction was obtained with arginine vasopressin (AVP; 10−6 M) followed by the addition of incremental doses of the endothelial-dependent vasodilator bradykinin (10−10 to 10−8). Vessels were washed to basal tension and the protocol was repeated. Relaxation was expressed as a percentage of the tonic constriction immediately before bradykinin application. Experiments were excluded if the residual constriction in the control vessel was >70% of maximal constriction.

18-Hour Protocol
After dissection, myometrial vessels were incubated overnight for 18 hours in PSS (2% final plasma concentration; 1 U/μL heparin) at 4°C. Vessels were then mounted in ice-cold PSS, and the baths were warmed to 37°C and gassed with 95% O2/5% CO2. Vessel normalization was followed by the experimental protocol described.

Plasma Incubation
For each experiment (4 vessels run simultaneously), a control vessel (incubated with heparin only) was run alongside 3 vessels incubated with individual plasma samples. Plasma samples were randomly assigned to vessels and myography was performed blinded to the patient outcome; data were retrospectively decoded after analysis. In some instances, enough vessel segments were isolated from biopsy specimens to enable up to 4 experiments to be performed (ie, 16 vessels).

Statistical Analysis
Demographic data were analyzed using SPSS version 10. Myodata (Danish Myotech, Denmark) and Graphpad Prism (San Diego, Calif) version 3.0 was used to analyze the myography data. All myography data were tested for normality and are represented as mean±SEM. Relaxation curves were compared using repeated measures ANOVA. From our previous study, it was anticipated that screening 50 women with abnormal Doppler studies with an equivalent number of women with normal Doppler studies would yield 16 women with preeclampsia and 20 whose pregnancies were complicated by IUGR. On the basis of previous studies, such sample sizes were in excess of those necessary to detect significant differences between the groups at P<0.05 with 90% power.
Demographic Data for Patients From Whom Plasma Samples Were Taken

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal Outcome</th>
<th>Preeclampsia (≤ IUGR)</th>
<th>IUGR (Normotensive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>48</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Abnormal doppler, n</td>
<td>15</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Delivered ≤ 34 weeks, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum SBP, mm Hg</td>
<td>124 (96–141)</td>
<td>150* (140–199)</td>
<td>120 (100–148)</td>
</tr>
<tr>
<td>Maximum DBP, mm Hg</td>
<td>80 (60–92)</td>
<td>104.5* (80–126)</td>
<td>78 (60–98)</td>
</tr>
<tr>
<td>Delivery gestation, wk + d</td>
<td>40 + 3 (37 + 1–42 + 0)</td>
<td>35 + 1* (26 + 5–40 + 5)</td>
<td>39 + 3 (26 + 5–41 + 1)</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>3440 (2600–4900)</td>
<td>2120* (610–4780)</td>
<td>2400* (500–3160)</td>
</tr>
<tr>
<td>IBR, percentile</td>
<td>38 (10–99)</td>
<td>13* (1–100)</td>
<td>2* (0–9)</td>
</tr>
</tbody>
</table>

Median (range) shown.

DBP indicates diastolic blood pressure; IBR, individualized birth weight ratio; IUGR, intrauterine growth restriction; SBP, systolic blood pressure.

*P < 0.05, preeclampsia or IUGR vs normal outcome.

Results

Patient groups are illustrated in Figure 1. Demographic data are presented in the Table. Women with abnormal pregnancy outcomes were identified in both Doppler groups, but of the women in whom preeclampsia and/or IUGR developed who required delivery before 34 weeks, 10 of 11 had abnormal Doppler studies at 22 weeks gestation. Women in whom preeclampsia developed had significantly elevated blood pressures (by definition) and 12 of 19 delivered low-birth-weight babies (individualized birth weight ratio <10). In the preeclampsia group, plasma samples taken at 26 weeks were obtained 54 days (median; range, 9 to 93) before a diagnosis of preeclampsia. Preeclampsia was diagnosed in 1 patient at 23 weeks and delivered at 26 weeks gestation; therefore, plasma taken from this patient at 26 weeks was excluded from the analysis.

Wire Myography

Addition of AVP evoked maximal constrictions in myometrial vessels, which were not different at 1 hour and 18 hours. Relaxation curves are represented as a mean of the 2 bradykinin relaxation curves, because the relaxation was significantly greater after the second bradykinin-induced relaxation curve after 1 hour (residual constriction 38.3% ± 8.8% run 1 versus 20.1 ± 7.1% run 2; paired t test P = 0.05) and 18 hours (residual constriction 35.5% ± 6.8% run 1 versus 19.6 ± 4.6% run 2; paired t test P = 0.05) plasma-free incubations. In plasma-free control vessels, relaxation curves were not significantly different between 1-hour and 18-hour incubations (residual constriction 29.2 ± 6.9% versus 27.6% ± 4.6%, n = 16; unpaired t test P = 0.84). Endothelial-dependent relaxation of vessels incubated with heparin only and vessels incubated with plasma from normal pregnant patients were not different.

Analysis on the Basis of Pregnancy Outcome

Maximal constrictions after the addition of plasma from women in whom preeclampsia subsequently developed or from women with a normal pregnancy outcome were unchanged. Incubation of vessels from normal pregnant women with plasma from women in whom preeclampsia subsequently developed produced significant attenuation of endothelial-dependent relaxation compared with vessels incubated with plasma taken from women who had normal pregnancies (Figure 2). This effect was demonstrable for plasma taken at 22 weeks (residual constriction 47.1 ± 6.6% versus 32.0 ± 4.4%, P = 0.004, repeated measures ANOVA at 1-hour incubation; and 59.1 ± 8.4% versus 42.3 ± 5.9%, P = 0.001, at 18-hour incubation) and 26 weeks (59.2 ± 5.2% versus 29.1 ± 5.6%, P < 0.001 at 1 hour; and 63.3 ± 7.6% versus 31.9 ± 7.2%, P < 0.0001 at 18 hours). The subsequent development of IUGR did not alter the effect of plasma from normotensive pregnancies (Figure 2a to 2d; P > 0.8) or from pregnancies complicated by preeclampsia (Figure 3a and 3b; P > 0.7).

Analysis on the Basis of Doppler Studies

Endothelial-dependent relaxation in vessels incubated with plasma taken at 22 weeks or 26 weeks from women who had normal versus abnormal Doppler studies at 22 weeks gestation, regardless of pregnancy outcome, was unchanged (Figure 4a and 4b; P > 0.8). Subdivision of the effect of plasma from women who had a normal pregnancy outcome or in whom preeclampsia developed on the basis of Doppler studies showed no differences when vessels were incubated for 1 hour (Figure 4c; P > 0.1). In vessels incubated with plasma from women with preeclampsia for 18 hours, there was a trend toward increased relaxation in the abnormal Doppler group (Figure 4d; P = 0.04).

Discussion

This study provides evidence of a vasoactive circulating factor(s) capable of altering myometrial vessel endothelial function in plasma samples taken weeks or months before the diagnosis of preeclampsia. Previous studies using identical methodology have demonstrated a significant attenuation in endothelial-dependent relaxation after incubation with plasma collected from women with established disease. This effect was not demonstrable in endothelial-denuded vessels and there were no plasma-induced changes in the endothelial-independent behavior of the vascular smooth muscle. Similar effects have been reported in mouse mesenteric arteries using isobaric myography techniques. Recently, VanWijk et al reported an absence of plasma-induced changes in endothelial-dependent relaxation in vessels incubated with whole plasma from women with preeclampsia, but a striking effect of microparticles isolated from the plasma of
The use of different protocols in this study and previous studies as compared with those used by VanWijk et al are likely to account for these disparate findings. These studies confirm the presence of a vasoactive factor in the plasma or in isolated microparticles taken from women with active disease.

Here we used 10^{-9} M AVP, which produces a consistent vasoconstriction in human myometrial vessels. Observations in our laboratory have demonstrated that a proportion of vessels exposed to lower doses of AVP exhibit only a transient constriction, which would complicate the analysis of plasma-evoked changes on agonist-induced endothelial-dependent relaxation. Other studies have demonstrated that the plasma-evoked effect on endothelial-dependent relaxation is reversible, ie, can be removed with multiple washes. In an attempt to minimize this effect, the experimental protocol used in this study involved a limited number of changes of the bathing PSS. This may have accounted for the enhanced bradykinin-induced relaxation seen during the second curve. However, it did not influence the plasma-evoked changes we report because vessels from the same biopsy sample were studied after parallel incubation with heparin alone, plasma from normal pregnant women, or plasma from women with preeclampsia.

When the dose-dependent effect of plasma on endothelial-dependent relaxation of isolated vessels was previously investigated, there was an enhancement of plasma-evoked changes between 1% and 2%, but no significant differences between 2% and 5%; 2% plasma concentration was therefore...
used in this study.\textsuperscript{13} Dilution of the plasma 50-fold not only dilutes vasoactive circulating factors but also dilutes other factors that may modulate vascular responses to this factor(s). Although the limitations of this in vitro setting are fully appreciated, the effects of plasma from women in whom preeclampsia subsequently developed was always compared directly with the effects of plasma from women with uncomplicated pregnancies, taken at the same gestational time point.

Our study is the first to our knowledge to examine the effect on endothelial function of plasma taken before diagnosis. Previous in vivo studies have demonstrated changes in angiotensin II pressor sensitivity as early as 22 weeks gestation,\textsuperscript{22} changes in resting peripheral blood flow,\textsuperscript{23} and impaired endothelial-dependent dilatation\textsuperscript{24} in women at risk for preeclampsia before the onset of clinically apparent disease. Similarly, evidence of activation of the clotting cascade\textsuperscript{25} and other markers of endothelial activation, including fibronectin, factor VIII antigen, tissue plasminogen activator, and endothelin-1, have also been shown to be increased in patients with preeclampsia and predate clinical signs.\textsuperscript{26–30} These early pathophysiological features may reflect the circulating factor(s) implicated in this study. Chappell et al\textsuperscript{31} reported increased levels of leptin and changes in the plasminogen activator inhibitor-1/plasminogen activator inhibitor-2 ratio in prediagnosis samples. Thus far, none of these markers has been proven as useful screening tools in this condition.

The incidence of complications in the normal Doppler group was higher than previously reported; however, this study was not designed to assess the sensitivity or specificity of Doppler waveform analysis as a screening test. This has been addressed in previous studies\textsuperscript{15,32} and our experimental design may have contributed to the increased incidence. We used Doppler analyses as a method of identifying a cohort of women at risk for pregnancy complications to facilitate longitudinal plasma sampling. Interestingly, plasma from women whose pregnancies were subsequently complicated by IUGR (in the absence of hypertension) did not attenuate endothelial-dependent relaxation in myometrial vessels. Preeclampsia and IUGR are thought to share similar pathogenesis that involves a placental trigger attributable to deficient trophoblast invasion of the maternal spiral arteries.\textsuperscript{33,34} Inadequate modification of these vessels by trophoblasts results in the development of a high-resistance uteroplacental circulation. The presence of a uterine arterial notch during Doppler studies is considered to be the result of a reflected wave of high amplitude returning from a uteroplacental bed with increased vascular resistance.\textsuperscript{15,35,36} Findings from this study suggest that the pathogenesis of these conditions differ in the maternal response to this initial placental trigger. Plasma from women in whom preeclampsia develops contained a demonstrable vasoactive factor(s) that was not apparent in the plasma of women in whom IUGR develops, or who have an uncomplicated pregnancy. The presence of this factor, as determined by this ex vivo bioassay, was not affected by the presence of associated IUGR or Doppler studies. Therefore, the circulating factor(s) present in the prediagnosis plasma of women in whom preeclampsia develops is not necessarily related to the degree of placental compromise quantified by fetal weight or by mid-trimester Doppler waveform analysis.
Identification of this circulating factor(s) was beyond the scope of this study; however, the presence of a circulating factor(s), demonstrable in vitro, long before the development of clinical disease adds new impetus to pursue the characterization of this substance(s). The use of isolated myometrial vessels as a bioassay may be one method by which these studies could be executed.

**Perspectives**

The demonstration of a plasma-borne vasoactive factor(s) in the plasma of women destined for preeclampsia, long before the development of clinical symptoms and signs, has enormous clinical implications. The use of a vessel bioassay, based on the techniques described in this study, to facilitate characterization and identification of these factor(s) in pre-eclampsia has the potential to guide the development of sensible screening tools and therapeutic strategies for this important pregnancy condition.

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**References**

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