BASIC SCIENCE: OBSTETRICS

Evidence for multiple circulating factors in preeclampsia

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OBJECTIVE: The purpose of this study was to enrich vasoactive factors that are present in the plasma of women with preeclampsia by the application of sequential fractionation and determination of the activity of each fraction in a bioassay.

STUDY DESIGN: Pooled plasma from women with preeclampsia (n = 23) and matched control subjects (n = 23) was subjected to fractionation with ultrafiltration, targeted immunodepletion, or size exclusion chromatography. Myometrial arteries that were isolated from healthy cesarean section biopsy specimens (n = 28) were incubated with plasma fractions (2%, volume/volume), and their endothelial function was assessed by wire myography.

RESULTS: Incubation of arteries with preeclampsia plasma or immunodepleted preeclampsia plasma had a deleterious effect on endothelial-dependent relaxation. Bioactivity of the plasma factors was absent in all fractions after either plasma ultrafiltration or separation with the use of size exclusion chromatography; however, activity was restored after recombination of these fractions.

CONCLUSION: This study provides the first conclusive evidence that multiple synergistic factors, with a combined vasoactive effect, are present in the plasma of women with preeclampsia.

Key words: endothelium, hypertension, plasma, preeclampsia, pregnancy, relaxation

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

There is accumulating evidence for a pathogenic model of preeclampsia, whereby deficient trophoblast invasion of the maternal spiral arteries leads to a poorly perfused fetoplacental unit. This abnormal placentation is thought to lead to the secretion of factors by the placenta into the maternal circulation that activates the vascular endothelium. The concept of a circulating factor in preeclampsia is not new. Blood-letting was advocated by Mauriceau in 1694; at the 1901 Giessen symposium, there was almost unanimous opinion that the disease was caused by a toxin. A number of in vitro observations have supported this hypothesis. Bioactivity of the circulating factors in the plasma of women with preeclampsia can be demonstrated by the assessment of the endothelial function of isolated myometrial arteries as a bioassay.

Arteries that are taken from women with uncomplicated pregnancies that are mounted on a wire myograph and incubated with plasma from women with preeclampsia exhibit significantly attenuated endothelial-dependent relaxation. This vasoactive factor has been demonstrated recently in the plasma of women who are destined to experience preeclampsia, long before the development of clinical symptoms and signs. This finding has important clinical implications, because characterization and subsequent identification of this factor in preeclampsia has the potential to guide the development of new screening and treatment options for this condition.

Previous studies have used this technique as a bioassay in the determination of some preliminary biologic characteristics of the circulating factors that are responsible for the alterations in endothelial function. The vasoactive factors were demonstrated to be reversible and heat labile and were removed partially by charcoal stripping, maintained within a plasma protein concentrate, and removed by protease digestion. These findings suggest that the circulating factor is likely to be a protein or closely as-
associated proteinaceous component of plasma.

The characterization and subsequent identification of the circulating factors in preeclampsia relies on appropriate fractionation; human plasma contains thousands of distinct proteins that span a large dynamic range. To conduct characterization experiments with the myography bioassay, the separation techniques that are used must not alter the bioactivity of the proteins of interest, because the presence or absence of the circulating factors will be determined subsequently by its effect on endothelial function. Separation techniques that expose plasma proteins to denaturing chemical conditions may reduce the efficacy of such proteins in the bioassay. A few abundant proteins make up >85% of the plasma proteome, and the removal of these proteins would facilitate improved analysis of the proteins that are expressed in smaller quantities.

The aim of this study was to perform sequential fractionation steps on plasma from women with preeclampsia to enrich the vasoactive factors that are capable of inducing aberrant endothelial function in vitro to facilitate future identification efforts.

**Methods**

The Manchester Local Research Ethics Committees gave approval for this work. Written informed consent was obtained for all plasma and tissue samples that were obtained.

**Study group**

Patients were recruited into the study in the previously described manner; blood samples were taken at diagnosis from women who had preeclampsia (n = 23) and from normotensive control subjects (n = 23) who were matched for gestation at sampling and parity. Women with preexisting medical disease were not recruited, and preeclampsia was diagnosed with the use of standard definitions. Individualized birthweight ratios were calculated for each pregnancy with a standard computer package, and women in the control group delivered babies with an individualized birthweight ratio of >10th percentile.

**Plasma samples**

Blood samples that were collected in precooled EDTA vials were centrifuged for 15 minutes at 1500 rpm at 4°C; the plasma was removed and stored at −80°C until used. Plasma samples were defrosted on ice and divided into 50-μL aliquots; aliquots of pooled plasma from the preeclampsia and control groups were created and stored at −80°C. Aliquots were also defrosted on ice and were always maintained at <4°C during preparation of experiments. Protein concentration was measured in triplicate with a protein assay (Cat no. 500-0111; Bio-Rad Laboratories, Epsom, Hempstead, UK).

**Fractionation**

**Vivaspin**

Plasma was separated with Vivaspin 500 spin columns (Sartorius, Stockport, UK) with molecular weight cutoffs (MWCO) of 10, 30, and 50 kDa; 300 μL of plasma was added to the column insert and centrifuged at 11,500g at 4°C for 30 minutes. The membrane was washed with 100 μL physiologic salt solution (PSS; 127.76 mmol/L NaCl, 25 mmol/L NaHCO3, 4.69 mmol/L KCl, 2.4 mmol/L MgSO4, 1.6 mmol/L CaCl2, 1.18 mmol/L KH2PO4, 6.05 mmol/L glucose, 0.034 mmol/L EDTA; pH 7.4) and subjected to further centrifugation; this protocol was repeated twice. Both the eluent and retained fractions were recovered and stored at −80°C until use.

**Multiple affinity removal system**

A multiple affinity removal column was used to remove the 6 highest-abundant proteins simultaneously from plasma (albumin, immunoglobulin G, antitrypsin, immunoglobulin A, transferrin, and haptoglobin). This column was run, according to manufacturer’s instructions, using a capillary high-performance liquid chromatography pump (Rheos; CVRx, Minneapolis, MN) with UV absorbance detected at 280 nm. Depletion of albumin was confirmed on 1-dimensional electrophoresis (data not shown). Fresh aliquots of pooled plasma were diluted 1:5 with column loading buffer and 200 μL injected per run. The eluted and retained fractions were collected on ice and pooled. Fractions were re-concentrated to their original volume with 5MWCO spin columns (Agilent Labs), centrifuged at 4000g for 1 hour, washed twice with PSS, and subsequently stored at −80°C until use. Depletion of albumin was confirmed on a linear 12% polyacrylamide 1-dimensional gel that was stained with Coomassie brilliant blue.

**Size exclusion chromatography**

Plasma, that had been depleted of abundant proteins in the aforementioned manner, was separated with a Bioselect 250-5 column (Bio-Rad Laboratories). The elution buffer was standard Kreb’s solution without glucose (140 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L CaCl2, 1.2 mmol/L MgCl2, 10 mmol/L Hepes, adjusted to pH 7.4 with NaOH). Depleted plasma (50 μL) was injected per run, and the column was developed at a flow rate of 1 mL/min; fractions were collected at 2-minute intervals on ice. To rationalize the number of myography experiments, fractions from 2 size exclusion chromatography runs were pooled into 4 aliquots (Figure 1) and concentrated to their original volume in the aforementioned manner.

**Myography**

A single myometrial biopsy specimen was taken from women who underwent planned cesarean section with uncomplicated pregnancies at term (n = 28). Women with known underlying medical disease, fetal abnormality, or fetal compromise were not recruited. Vessels (200–500 μm) were dissected from myometrial biopsy specimens in PSS under a stereomicroscope and mounted on a 4-chamber M610 wire myograph (Danish Myotechnology, Aarhus, Denmark), as described elsewhere.

After dissection, myometrial vessels were incubated for 18 hours in PSS (2% final concentration plasma fraction; 1 U/μL heparin) at 4°C. Vessels were
then mounted in ice-cold PSS; the baths were warmed to 37°C and gassed with air/5% CO₂. Vessels were normalized as previously described and allowed to equilibrate for 20 minutes. Sustained vasoconstriction (2-5 minutes) was observed with arginine vasopressin (10⁻⁸ mol/L), followed by the addition of incremental doses of the endothelial-dependent vasodilator bradykinin (10⁻¹⁰ to 10⁻⁶ mol/L), to create the residual constriction in the negative control vessel. Experiments were excluded if vessels that were incubated with heparin/PSS only did not differ from that of vessels that were incubated with plasma fractions. Untreated pooled plasma was exposed to the same number of freeze/thaw cycles as fractionated plasma. Relaxation was expressed as a percentage of the tonic constriction immediately before bradykinin application, and an average of the 2 bradykinin dose-response curves was taken. Experiments were excluded if the residual constriction in the negative control vessel was >70% of maximal constriction.

Statistical analysis
Demographic data was analyzed with SPSS software (version 10; SPSS Inc, Chicago, IL). Myodata (Danish Myotechnology) and Graphpad Prism (version 3.0; San Diego, CA) was used to analyze the myography data. All myography data were tested for normality and are represented as mean ± SEM. Relaxation curves were compared with the use of repeated measures analysis of variance.

RESULTS
Patient characteristics are presented in the Table 1. Women who experienced preeclampsia had significantly elevated blood pressures (by definition) and delivered lower birthweight babies. In the preeclampsia group, plasma samples were obtained a median of 5 days (range, -7 to 8 days) within a diagnosis of preeclampsia being made; all samples were taken before delivery. Control samples were obtained from women with uncomplicated pregnancies (n = 23) who were matched for gestation at sampling (±2 weeks) and parity.

Wire myography
Endothelial-dependent relaxation of vessels that were incubated with heparin only did not differ from that of vessels that were incubated with plasma from normal pregnant women (data not shown). There was no difference in the agonist-induced maximal constrictions after incubation with plasma from

<table>
<thead>
<tr>
<th>Variable</th>
<th>Preeclampsia plasma samples (n = 23)</th>
<th>Normal outcome plasma samples (n = 23)</th>
<th>Normal outcome myometrial samples (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum systolic blood pressure (mm Hg)</td>
<td>152* (130-199)</td>
<td>124 (96-140)</td>
<td>120 (100-140)</td>
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<tr>
<td>Maximum diastolic blood pressure (mm Hg)</td>
<td>103* (84-126)</td>
<td>78 (60-90)</td>
<td>72 (60-90)</td>
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<td>Delivery gestation (wk⁺⁵)</td>
<td>36 ±6 (26 ±5-40 ±7)</td>
<td>40 ±5 (38 ±4-41 ±6)</td>
<td>39 (36-41)</td>
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<td>Birthweight (g)</td>
<td>2285* (590-4780)</td>
<td>3480 (2670-4380)</td>
<td>3295 (2860-4280)</td>
</tr>
<tr>
<td>Individualized birthweight ratio¹⁶ (percentile)</td>
<td>16* (1-100)</td>
<td>38 (12-99)</td>
<td>35 (14-94)</td>
</tr>
<tr>
<td>Parity (n)</td>
<td>0 (0-2)</td>
<td>0 (0-2)</td>
<td>1 (0-3)</td>
</tr>
</tbody>
</table>

Data represented as median (range).

* P < .05, preeclampsia vs normal outcome.
women with preeclampsia, compared with incubation with plasma from women with a normal pregnancy outcome (data not shown). Consistently, incubation of arteries that were taken from normal pregnant women with pooled plasma that was taken from women with preeclampsia produced significant attenuation of endothelial-dependent relaxation compared with vessels that were incubated with plasma that was taken from women who had normal pregnancies (Figures 2-5).

**Ultrafiltration**

The bioactivity of the vasoactive factor from plasma from women with preeclampsia was retained by the 10-kd cut-off filter ($P < .001$; Figure 2) but was not retained by 30- or 50-kd cutoff filters ($P > .05$; Figure 3). There was no attenuation in endothelial-dependent relaxation in vessels incubated with plasma fractions after filtration through 10-, 30-, or 50-kd filters and endothelial relaxation in vessels exposed to these fractions was not significantly different from control vessels or vessels that were exposed to untreated control plasma (data not shown). However, recombination of the flow-through material with the retentate resulted in a comparable alteration in endothelial function to that seen with unfractionated plasma ($P > .05$; Figure 3).

**Multiaffinity removal system**

Endothelial-dependent relaxation in vessels that were incubated with plasma after removal of abundant proteins was attenuated significantly in comparison with vessels that were incubated with plasma fractions that contained only abundant proteins ($P = .04$; Figure 4). Dilution of plasma from women with preeclampsia in column buffers at the same concentration did not affect endothelial-dependent relaxation (62% ± 11% buffer A vs 69% ± 13% buffer B vs 54% ± 7% untreated; $P > .05$), and there was no effect of protein depletion in plasma from women with normal pregnancies (38% ± 4% depleted vs 28% ± 11% untreated; $P > .05$).

**Size exclusion chromatography**

For size exclusion chromatography, to reduce the number of myography bioassay experiments, fractions that were collected at 2-minute intervals (11 in total; Figure 1) were combined into 4 pools. Pool A contained the largest proteins, and pool D contained the smallest proteins. The incubation of arteries with individual fractions A-D did not alter endothelial-dependent relaxation; however, incubation of arteries with a combination of fractions A, B, C, and D resulted in significant attenuation in endothelial-dependent relaxation, comparable with unfractionated plasma (Figure 5).
Comment

This study provides the first conclusive evidence that multiple synergistic vasoactive factors are present in the plasma of women with preeclampsia. We have also demonstrated that these factors are retained after affinity stripping of the highly abundant plasma proteins that account for >85% of the plasma proteome, which implies that the factors are not bound strongly to the abundant plasma proteins. When plasma was fractionated on the basis of size, whether by ultrafiltration or size exclusion chromatography, bioactivity of the resultant material was diminished, except when plasma was filtered through a 10-kd cutoff membrane. The diminution of activity was unexpected, but the recovery of activity when different fractions were recombined provided strong evidence for the synergism of multiple factors.

These findings have both positive and negative implications for future attempts to identify these plasma factors. Removal of highly abundant proteins reduces the dynamic range of the plasma proteome and improves the possibility of the identification of low-abundance proteins, which may differ in preeclampsia and normal pregnancy. The existence of >1 vasoactive substance that is necessary for aberrant endothelial function in vivo complicates the use of sequential separation techniques to isolate and identify a single plasma factor in this condition.

The myography method used in this study evokes reproducible alterations in the endothelial function of isolated myometrial arteries after exposure to plasma from women with preeclampsia. Deviation from this protocol potentially would mask the effect of plasma from women with preeclampsia on isolated vessel function. In an attempt to reduce the inherent biologic variability of this bioassay, segments from the same vessel were compared where possible; vessel incubations were run in duplicate; an average of 2-dose response curves was taken, and all plasma fractionation steps were repeated multiple times. Previous investigation of the dose-dependent effect of plasma on endothelial-dependent relaxation of isolated vessels demonstrated that there was an enhancement of plasma-evoked changes between dilutions to 1% and 2%, but no significant differences between 2% and 5%. Because of the limited supply of plasma, a 2% final concentration (volume/volume) was used for all plasma fractions in this study. During the fractionation process, plasma was diluted and reconstituted several times in some instances. Because no enhanced effect has been demonstrated previously between 2% and 5% plasma concentration, overnight incubation volumes were always adjusted to allow for significant losses during the fractionation process; where possible, protein concentrations were measured to direct these adjustments.

Dilution of the plasma 50-fold not only dilutes vasoactive circulating factors but also dilutes other factors that may modulate vascular responses to these factors. While the limitations of this in vitro setting are fully appreciated, the presence or absence of vasoactive factors after each separation step was confirmed by a direct comparison of vessel relaxation after exposure to each plasma fraction, with the relaxation of vessels incubated in PSS/heparin alone or with untreated plasma from women with preeclampsia run simultaneously. The effects of plasma/fractions from women with preeclampsia were compared with plasma/fractions from women with uncomplicated pregnancies for the majority of the separation steps (ultrafiltration and immunodepletion). However, in view of tissue limitations and the labor-intensive nature of this bioassay, it was beyond the scope of this study to perform parallel plasma fractionation with normal plasma for the size exclusion chromatography.

Previous studies have used immunodepletion to allow better identification of lower-abundance proteins within the plasma proteome. Albumin accounts for >55% of plasma protein and binds multiple plasma factors; therefore, removal of albumin may also deplete the plasma of many of these bound substances. However, in our bioassay, the effect of albumin-depleted plasma was comparable with untreated plasma from women with preeclampsia. In addition, the removal of albumin had no effect on endothelial-dependent relaxation in arteries that were incubated with plasma from normotensive controls, which suggests that the removal of potentially “protective” factors that were bound to albumin had no effect in this in vitro system. Recombination of plasma fractions after separation by size was important to the methods of this study, because it specifically tested whether the separation process had influenced the bioactivity of the plasma factors directly. Restoration of the effect after recombination of fractions confirmed the validity of the observation concerning a lack of effect that was seen after exposure to individual fractions and, more importantly, demonstrated that >1 plasma factor is necessary to evoke alterations in the endothelial function of isolated arteries. Further studies that would investigate the effect of the recombination of individual size exclusion fractions, generated from plasma from both women with normal pregnancies and preeclampsia, potentially would provide further information regarding the activity of vasoactive factors. Although worthy of investigation, this would require a significant number of bioassays to investigate in detail.

A plethora of plasma markers have been shown to be altered in the plasma of women with preeclampsia, in many cases before the onset of clinical disease. Other studies have also shown that concentration ratios of several factors represent a better method of predicting disease compared to the concentrations of individual factors. Thus far, none of these markers has been shown to have the specificity or sensitivity to allow its use as a screening tool or therapeutic target for this disease. Rather than measuring randomly selected plasma factors, this study aimed to use a biologically relevant bioassay to attempt to isolate a candidate plasma marker that would guide the development of novel screening and therapeutic strategies. Although this technique has reduced the complexity of the plasma proteome by the removal of highly abundant proteins that include albumin, the confirmation that >1 factor is responsible for aberrant en-
endothelial function complicates the use of this approach in future studies. Future studies may need to be guided by an alternative bioassay or utilize biomarker discovery techniques with advanced mass spectrometric or other techniques.

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REFERENCES