Preeclampsia affects 3% to 8% of all pregnancies and represents a major cause of maternal and perinatal morbidity. Although the pathophysiology behind the development of preeclampsia remains elusive and highly debated, one theory is that abnormal remodeling of uteroplacental bed spiral arteries leads to placental hypoperfusion, prompting release of factors into the maternal circulation. These circulating factors include angiogenic and antiangiogenic molecules like soluble fms-like tyrosine kinase 1 receptor (sflt-1) and select cytokines. For example, sflt-1, the soluble receptor for vascular endothelial growth factor (VEGF) receptor (VEGFR) 1, is elevated in preeclamptic plasma and has been shown to inhibit certain actions of VEGF. In this manner, sflt-1 is thought to provoke endothelial dysfunction resulting in decreased endothelial-dependent vasodilation and increased vessel reactivity. Such changes may increase total peripheral vascular resistance, contributing to a major feature of preeclampsia, namely, hypertension.

One of the most serious sequelae of preeclampsia are eclamptic seizures, a leading cause of maternal and fetal morbidity and mortality. Clinical and experimental findings suggest that the pathophysiology behind eclampsia involves a failure of autoregulation leading to decreased cerebral vascular resistance, transmission of increased pressure to the microcirculation, and blood-brain barrier (BBB) disruption. Increased BBB permeability can result in the passage of damaging plasma constituents and protein into brain parenchyma, causing vasogenic edema and the neurological complications of severe preeclampsia and eclampsia. Despite intensive investigation into the involvement of circulating factors in the etiology of preeclampsia, there is a paucity of data regarding their role in the promotion of cerebrovascular damage, including enhanced BBB permeability and changes in vascular reactivity that could affect cerebral vascular resistance and local perfusion.

The first goal of this study was to evaluate the effect of normal pregnant and preeclamptic plasma on BBB permeability by measuring hydraulic conductivity (L<sub>p</sub>) of blood vessels exposed to plasma. L<sub>p</sub> was evaluated as this parameter relates the filtration of water across the BBB in response to...
hydrostatic pressure. Our second goal was to investigate the involvement of VEGF and related signaling in mediating changes in BBB permeability in response to plasma exposure. This cytokine was chosen because it is an angiogenic molecule with potent vascular permeability properties and has been shown to increase BBB permeability. In addition, whereas pre-eclampsia is associated with elevated sflt-1 levels that are thought to inactivate the actions of VEGF, the residual biological activity of VEGF in preeclamptic plasma is not known, especially with regard to BBB function. Finally, we investigated the effect of circulating factors in the plasma of normal pregnant and preeclamptic women on cerebral artery reactivity and endothelium-dependent vasodilator responses, two components that can affect cerebral vascular resistance.

Methods

Patients and Plasma Samples

Blood samples were collected from patients enrolled in a simultaneous ongoing institutional review board–approved study at the University of Vermont. Institutional review board exemption was granted to use these previously frozen plasma samples, for which patients had given informed consent. Plasma was pooled from 2 groups: a control group of normotensive pregnant women with uncomplicated pregnancies and a group of preterm pregnant preeclamptic women. The control group (n=12) had an average age of 33.4 years (range: 20.0 to 41.0 years) and had no history of hypertension, diabetes mellitus, or infection. The average gestational age at venipuncture was 34.4 weeks (range: 31.9 to 36.3). The preeclamptic group (n=5) was composed of women diagnosed with severe preeclampsia using American College of Obstetricians and Gynecologists criteria of having >3 g of protein measured in a 24-hour urine collection or the presence of intrauterine growth restriction as defined by fetal weight <5% on the Vermont Hybrid growth curve in addition to blood pressure readings >140 mm Hg systolic and >90 mm Hg diastolic on ≥2 occasions, 6 hours apart. The preeclamptic group had an average age of 28 years (range: 23 to 32 years), and average gestational age at venipuncture was 32.2 weeks (range: 28.3 to 36.4). Effort was taken to use plasma from preterm preeclamptic women, because the earlier development of this disease is thought to represent a unique phenotype. Blood samples were collected from patients into Vacutainer tubes containing either ethylenediaminetetraacetic acid or lithium heparin. Blood was centrifuged at 1400 to 1600 g, the plasma removed and aliquoted, and the pooled samples frozen at −80°C until experimentation.

Animals

All of the procedures were approved by the University of Vermont Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female late-pregnant Sprague-Dawley rats (day 20; 390 to 402 g) were used for all of the experiments and housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility. Animals had access to food and water ad libitum and maintained a 12-hour light/dark cycle.

Venous Permeability Studies

The effect of normal pregnant and preeclamptic plasma on the Lp of cerebral veins was determined as described previously with slight modification. Veins were perfused with physiological HEPES saline solution only (n=9), normal pregnant plasma (n=5), or preeclamptic plasma (n=5). Veins were first exposed intraluminally to 20% plasma in HEPES buffer from each group for 3 hours at 10±0.3 mm Hg. Plasma was then flushed out of the venous lumen and Lp determined without the presence of plasma in the lumen. This procedure avoided potential differences in colloid oncotic pressure in the different pools of plasma that could influence Lp measurements by having a common perfusate and suffusate.

Arterial Reactivity Studies

To determine the effects of normal pregnant and preeclamptic plasma on vascular reactivity, vessels were perfused with either normal pregnant (n=6) or preeclamptic plasma (n=6). The protocol for measuring vessel reactivity was as described previously. Briefly, a third-order branch of the posterior cerebral artery (PCA) was carefully dissected, mounted on glass cannulas in an arteriograph chamber, and perfused with 20% plasma from either pregnant or preeclamptic women in HEPES buffer. The suffusate consisted of HEPES solution only. All of the vessels were exposed to 20% plasma in HEPES buffer intraluminally for 3 hours as the plasma was left in the vessel for the entire reactivity protocol.

Drugs and Solutions

HEPES physiological salt solution was made fresh daily and consisted of (in mmol/L): 142.00 NaCl, 4.70 KCl, 1.71 MgSO4, 0.50 EDTA, 2.80 CaCl2, 10.00 HEPES, 1.20 KH2PO4, and 5.00 dextrose. N-nitro-l-arginine (L-NNA), indomethacin, and papaverine were made fresh weekly at 10−3 mol/L or 10−4 mol/L stock solutions and stored at 4°C. VEGF was purchased from Sigma and kept frozen until use. HEPES and indomethacin were purchased from Fisher Scientific; papaverine, L-NNA, and A23187 (calcium ionophore) were purchased from Sigma. VEGFR-II was purchased through Calbiochem.

Statistical Analysis

All of the data are presented as mean±SE. Differences in blood pressures, Lp, and between control and preeclamptic plasma, as well as between different vessels with the same plasma, were determined using Student t test or 1-way ANOVA, where appropriate. A post hoc analysis for multiple comparisons was performed with Student-Newman-Keuls test where appropriate. Differences were considered significant at P<0.05.

Results

Effect of Plasma on Hydraulic Conductivity

Changes in Lp versus time for cerebral veins are shown in Figure 1. Cerebral veins exposed to plasma from both normal
pregnant and preeclamptic women had significant increases in $L_P$ versus no plasma exposure. In addition, veins exposed to HEPES buffer only (no plasma exposure) had a constant $L_P$ during the duration of the experiment, whereas veins exposed to pregnant or preeclamptic plasma had $L_P$ that increased over time, suggesting loss of barrier properties over time with plasma exposure. The increase in BBB permeability was significantly greater in veins exposed to preeclamptic versus normal pregnant plasma, suggesting that circulating factors or other properties of plasma in preeclampsia have a greater influence on permeability than normal pregnant plasma.

The role of VEGF signaling in mediating changes in BBB permeability was investigated by measuring $L_P$ of veins exposed to pregnant and preeclamptic plasma with the addition of VEGFR-II, a VEGFR tyrosine kinase inhibitor. The permeability of veins exposed to normal pregnant plasma was unaffected by VEGFR-II (Figure 2). However, addition of VEGFR-II to preeclamptic plasma completely prevented the increase in $L_P$, suggesting that VEGF tyrosine kinase signaling has an important role in increasing BBB permeability in response to preeclampsia. To determine whether increased VEGFR activation was attributed to higher VEGF levels in preeclamptic plasma, total peripheral circulating VEGF levels in both groups of plasma were measured via ELISA. Although the concentration of peripheral VEGF was similar between normal pregnant and preeclamptic plasma (62.0 versus 61.4 pg/mL, respectively), it was considerably higher than the level of VEGF found in nonpregnant women (15.0 pg/mL).

The determine whether the level of VEGF measured in plasma affected $L_P$ alone without plasma, a separate set of experiments ($n=4$) was done in which 60 pg/mL were perfused in cerebral veins and $L_P$ measured. We found that 60 pg/mL of VEGF perfused in cerebral veins produced modest permeability that was lower than both plasmas but was only significantly different from preeclamptic plasma.

**Effect of Plasma on Myogenic Reactivity and Tone**

The active responses of PCAs perfused with plasma from either normal pregnant or preeclamptic women to stepwise increases of intravascular pressure are shown in Figure 3, together with their respective passive diameters. As seen in the active diameter versus pressure curves, all of the vessels dilated at pressures below the myogenic pressure range, from 25 to 50 mm Hg, then constricted and exhibited myogenic reactivity as pressure was increased to 75 mm Hg. PCAs perfused with pregnant plasma demonstrated considerable myogenic activity, as demonstrated by the amount of vasoconstriction maintained in response to increased intravascular pressure. PCAs perfused with preeclamptic plasma had similar overall reactivity, although they had nonsignificant increases in lumen diameters at higher pressures (125 to 150 mm Hg). All of the PCAs had similar passive diameters.
In addition, all of the arteries had considerable pressure-induced myogenic tone within the autoregulatory range between 75 and 150 mm Hg that was similar regardless of the type of plasma perfusate (Figure 4).

Influence of NO, Cyclooxygenase, and Endothelium-Derived Hyperpolarizing Factor on Vascular Tone

PCAs perfused with both pregnant plasma and preeclamptic plasma constricted in response to NO synthase inhibition with L-NNA, suggesting that the basal influence of NO to inhibit vascular tone was present in both groups (Figure 5). There was no difference in the amount of constriction between arteries perfused with different plasma types. The addition of indomethacin to inhibit cyclooxygenase caused minimal changes to vessel diameters regardless of the type of plasma perfusate (Figure 5). The influence of endothelium-derived hyperpolarizing factor in PCAs perfused with normal pregnant or preeclamptic plasma was assessed by measuring vasodilation to the addition of A23187 in the presence of NO synthase and cyclooxygenase inhibition (Figure 6). This is a common approach for assessing endothelium-derived hyperpolarizing factor–related mechanisms.17 Under these conditions, A23187 caused dilation in all of the vessels that was not different between groups.

Discussion

The major finding from this study was that BBB permeability was significantly increased by circulating factors in plasma from normal pregnant women, an effect that was further amplified in plasma from severely preeclamptic women. Although plasma from normal pregnant women caused increased Lp of cerebral veins compared with no plasma, exposure to preeclamptic plasma led to an even greater disruption of BBB properties. Furthermore, the finding that VEGF receptor tyrosine kinase inhibition prevented the increase in permeability of veins exposed to preeclamptic but not normal pregnant plasma suggests that VEGF receptor tyrosine kinase activity is involved in increasing BBB permeability after exposure to preeclamptic plasma only. In contrast, we found that acute exposure to circulating factors during preeclampsia did not affect cerebral artery reactivity, myogenic tone, or several endothelium-dependent responses.

It has been hypothesized that circulating factors in preeclampsia contribute to the development of brain edema and the neurological complications of severe preeclampsia.17–19 However, an effect of preeclamptic plasma on BBB permeability has not been examined previously. Results from this study indicate a 3-hour intraluminal exposure to preeclamptic plasma significantly increased BBB permeability compared with normal pregnant plasma and no plasma exposure (Figures 1 and 2). Permeability was measured by determining the Lp of cerebral veins, a critical parameter relating flux of water in response to hydrostatic pressure attributed to both transcellular and paracellular transport across the BBB.10 Cerebral veins were used for these experiments because they have been shown to be a primary site of BBB disruption during acute hypertension20,21 and in response to VEGF.22 Although this is the first report that we know of showing that preeclamptic plasma increased Lp of cerebral veins, Neal et al23

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found exposure to preeclamptic plasma increased $L_p$ of mesenteric microvessels from frogs. In that study, there was increased permeability of vessels exposed to plasma from women with severe but not mild preeclampsia. Preeclamptic plasma used in our study was pooled from women who also had the diagnosis of severe, preterm preeclampsia, suggesting that circulating factors during this disease increase permeability of the BBB, as well as systemic vessels, and may promote vasogenic brain edema seen with severe preeclampsia.

Our current results suggest that circulating factors in the plasma from preeclamptic women increase BBB permeability and that VEGFR tyrosine kinase activity is involved, because VEGFR-II, a specific inhibitor of VEGFR tyrosine kinase activity, prevented the increase in permeability. This result was distinctly different from what was found with normal pregnant plasma, which produced a more moderate increase in permeability and no change with VEGFR-II. However, the differential response of the BBB to pregnant versus preeclamptic plasma and the apparent involvement of VEGFR tyrosine kinase activity in preeclamptic plasma only, occurred despite similar levels of VEGF (60 pg/mL) in the 2 plasmas. These findings suggest that elevated levels of VEGF alone may not be responsible for the increase in permeability in response to preeclamptic plasma. In fact, we tested the response of 60 pg/mL of VEGF alone on the $L_p$ of cerebral veins and found that this amount of VEGF caused modest permeability, lower than both plasmas. Together, these results suggest that VEGF alone is not the only circulating factor during either pregnancy or preeclampsia that affects BBB permeability.

There are at least 2 possibilities to explain our findings. First, there may be circulating factors present in preeclamptic plasma that enhance effects of VEGF on permeability, as has been shown in other studies.24–27 PlGF, a member of the VEGF family that binds and activates VEGFR1, has been shown to enhance $L_p$ compared with VEGF alone,24,25 PlGF is significantly elevated in both normal pregnant and preeclamptic plasma; however, it is unlikely that PlGF is acting in this manner to enhance VEGF-induced permeability, because studies have consistently found that levels of PI GF are decreased in preeclamptic plasma compared with normal pregnancy.28,29 However, there may be another yet-unidentified factor that is elevated in preeclamptic plasma that may be acting to sensitize the BBB to VEGF-induced permeability. There are numerous cytokines and growth factors produced in preeclampsia2–4 that could activate downstream pathways and/or increase endothelial cell calcium and in this way sensitize the BBB to VEGF-induced permeability.

Second, there may be differences in VEGF isoforms present in preeclamptic versus normal pregnant plasma. VEGFs are a family of 6 growth factors, including VEGF-A, -B, -C, -D, and -E, as well as PlGF.13,30 VEGF-A, the most commonly secreted form, exerts its actions via the tyrosine kinase activity of VEGFR1 and VEGFR2, whereas other growth factors in this family are receptor specific.13,30 For example, PlGF only binds and activates VEGFR1, whereas VEGF-C specifically activates VEGFR2.25,30,31 Although total VEGF was found to be similar in normal pregnant and preeclamptic plasma, the ELISA that we used does not distinguish between different VEGF isoforms. Thus, it is possible that receptor-specific isoforms are present in higher concentrations in preeclamptic plasma and activating different VEGFRs, compared with normal pregnant plasma, to increase $L_p$. In addition, a primary mechanism of hypertension and proteinuria in preeclampsia is thought to be attributed to diminished VEGF activity resulting from enhanced sflt-1 competitive binding that inhibits the interaction between VEGF and VEGFR1.25 Our finding that preeclamptic plasma appears to increase $L_p$ through activation of VEGF receptors is somewhat contrary to these studies. However, because sflt-1 is specific for inhibiting VEGFR1 signaling, other isoforms that specifically bind VEGFR2, such as VEGF-C, may be elevated in preeclamptic plasma and cause an increase in permeability. It is worth noting that, despite the importance of VEGF signaling in vascular function, the mechanisms by which VEGF increases vascular permeability and the receptors involved are still largely unknown. One limitation of this study was that the inhibitor that we used was nonselective for VEGFR1 versus VEGFR2, and further studies are needed to determine which receptors are involved in increasing permeability of the BBB in response to preeclamptic plasma and the exact cellular mechanisms by which this occurs.

Although preeclamptic plasma increased cerebral vein permeability, it had a negligible effect on cerebral artery reactivity, myogenic tone, or endothelium-dependent responses. This lack of effect may have been secondary to the acute exposure to plasma, because PCAs were exposed to plasma for a total duration of ∼3 hours. Vascular function of these arteries may differ with chronic exposure to preeclamptic plasma. Another possible explanation for the lack of effect of preeclamptic plasma on arterial reactivity may be because of the discrepancy between the concentrations of plasma that we used as a perfusate (20%) compared with physiological values of ∼55%. This concentration was used because of limited plasma availability. However, a previous study comparing 20% versus 40% normal pregnant plasma as a luminal perfusate found no differences in vascular reactivity.17 However, permeability was not measured in that study, and thus, the use of 20% plasma may have underestimated effects at normal physiological levels. Nevertheless, these experiments suggest that acute exposure to circulating factors in preeclamptic plasma influence cerebrovascular function primarily by affecting the BBB and increasing the permeability of cerebral veins rather than the reactivity of cerebral arteries.

**Perspectives**

This is the first study that we are aware of that examined the effect of circulating factors during preeclampsia on cerebral vascular function. The findings that factors during preeclampsia, but not normal pregnancy, significantly increase BBB permeability through a mechanism that involves VEGFR tyrosine kinase activity challenges current dogma that the pathophysiology underlying preeclampsia is invariably linked to decreased VEGF activity secondary to elevated sflt-1 levels. In addition, although investigators have administered recombinant VEGF to animal models of preeclampsia in an attempt to reverse the phenotypic features of this disease,22 our study cautions such treatment, because this has the potential to increase BBB permeability. However, we are also not suggesting the use of VEGFR tyrosine kinase inhibitors for treatment of
preeclampsia, because these compounds are used extensively as anticancer agents and are known to promote hypertension and renal damage. The VEGFR inhibitor used in this study was for mechanistic purposes only. However, our results raise questions regarding the potential mechanisms behind VEGFR tyrosine kinase activity in response to preeclamptic plasma, and further studies evaluating the effects of specific VEGFR-1 versus VEGFR-2 activation or other circulating factors that enhance VEGF-induced permeability are warranted. The lack of effect of preeclamptic plasma on vascular reactivity, myogenic tone, and endothelium-dependent vasodilation suggests that increased BBB permeability from circulating factors may be the dominant factor behind the development of vasogenic edema and the neurological complications of severe preeclampsia and eclampsia.

Sources of Funding

We gratefully acknowledge the generous support of the Preeclampsia Foundation (to Ó.A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to Ó.A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to O¨ .A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to O¨ .A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to O¨ .A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to O¨ .A.A. and M.J.C.), the National Institute of Neurological Disorders and Stroke (NS045940 to M.J.C.), the American Heart Association (to O¨ .A.A. and M.J.C.).

Disclosures

None.

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Plasma From Preeclamptic Women Increases Blood-Brain Barrier Permeability: Role of Vascular Endothelial Growth Factor Signaling

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*Hypertension.* 2010;56:1003-1008; originally published online September 20, 2010;
doi: 10.1161/HYPERTENSIONAHA.110.158931

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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