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BBA Research Letter

Dysregulation of vascular endothelial growth factor receptor 2 phosphorylation is associated with disruption of the blood-brain barrier and brain endothelial cell apoptosis induced by plasma from women with preeclampsia

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ABSTRACT

Disruption of the blood-brain barrier (BBB) is central in the pathophysiology of acute cerebral complications in women who have preeclampsia. Underling mechanisms are unclear.

Using female human brain endothelial cells as an in vitro model of BBB, we show that plasma of women with preeclampsia increases cell apoptosis and permeability via activation of the vascular endothelial growth factor receptor 2 (VEGFR2).

Since plasma of women with preeclampsia also enhanced VEGFR2 phosphorylation in the tyrosine 951 but decreased phosphorylation at the tyrosine 1175, we propose the former would be the more likely active form of VEGFR2 responsible for BBB alterations.

Preeclampsia is a pregnancy-related hypertensive syndrome that affects 3–5% of all pregnancies worldwide and is one of the most common causes of maternal and perinatal morbidity and mortality. About 60 to 70% of maternal deaths associated with preeclampsia are due to cerebral complications, including eclampsia, cerebral edema, and ischemic or hemorrhagic stroke [1]. Despite that, the pathophysiology behind cerebrovascular complications in preeclampsia is largely unknown. However, evidence suggests disruption of the blood-brain barrier (BBB) and alterations in cerebral blood flow as underlying pathophysiological pathways.

Circulating harmful components, such as anti-angiogenic and pro-inflammatory proteins or placental-derived extracellular vesicles, present in the circulation of women with preeclampsia can potentially disrupt the BBB. In agreement with previous evidence in cerebral rat veins [2], we found that plasma of women with preeclampsia can disrupt the BBB using a female human brain endothelial cell line (i.e., hCMEC/d3) [3,4]. Moreover, we demonstrated upregulation of mRNA levels of the vascular endothelial growth factor receptor type 2 (VEGFR2) and its phosphorylation at the tyrosine residue Y951 (pY951-VEGFR2), while phosphorylation at the Y1175 (pY1175-VEGFR2) residue was reduced. Notably, the cellular outcome depends on which tyrosine is phosphorylated. Thus, the phosphorylation at Y951 regulates permeability and cell migration, while pY1175 is involved in cell proliferation [5]. In addition, reduction in both pY951 and pY1175 has been involved in cell death due to apoptosis [6] or endothelial dysfunction generated by circulating factors in preeclampsia [4]. However, whether these outcomes associated with these specific phosphorylation sites of VEGFR2 are involved in disrupting the BBB observed in preeclampsia is still unclear.

We hypothesized that dysregulation of VEGFR2 phosphorylation is

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associated with reduced cell proliferation and apoptosis, contributing to the BBB disruption in women with preeclampsia.

A subset of plasma samples was obtained from women with preeclampsia (n = 11) and women with normal pregnancies (n = 12), as described previously [7]. We used the International Society for Studies on Hypertension in Pregnancy (ISSHP) guidelines to diagnose preeclampsia, defined as de novo hypertension (of ≥140 mm Hg and/or diastolic blood pressure of ≥90 mm Hg measured on two subsequent occasions at least 6 h apart) after 20 weeks of gestation and proteinuria (≥ 2+ on a dipstick or ≥300 mg/24 h) [8]. The research was carried out following the principles expressed in the Declaration of Helsinki and under the authorization of the respective Ethical Review Boards. All participants gave their informed consent prior to sample collection, as described previously [7].

Transendothelial electrical resistance (TEER) and cell permeability to a high-molecular-weight fluorescent dye (Fluorescein-5-isothiocyanate FITC-dextran 70 kDa) were analyzed using monolayers of hCMEC/D3 cells (Merck Millipore, Darmstadt, Germany) exposed for 12 h to women's plasma diluted 1:10 v/v in EndoGro-MV culturing media (Merck Millipore, Darmstadt, Germany) [3,4]. All experiments were performed in the presence (12 h) or absence of the VEGFR2 inhibitor, ZM-323881 (Sigma-Aldrich, San Louis, MO, USA). In addition, plasma-treated cells were used to analyze caspase 3/7 activity using NucView 488 kit (Biotium, Germany). Also, Western blot analyses of Bax, Bcl-2, total, and pY951-VEGFR2; and ELISA for pY1175-VEGFR2 (Cell Signaling, Danvers, MA, USA) were performed following manufacture instructions. Data and statistical analyses were performed using SPSS version 25, GraphPad Prism 6.00 (GraphPad Software, CA, USA).

Clinical characteristics have been previously published for the whole group of pregnant women [4,7]. In this randomly selected group, women with preeclampsia and women with normal pregnancy were similar regarding age (mean ± SD, 30 ± 6.7 versus 32.5 ± 4.5 years), body mass index (BMI, 25.8 ± 4.1 versus 25.0 ± 3.2 kg/m^2), and gestational week of examination (37.2 ± 2.0 versus 37.3 ± 1.4 weeks, respectively). Women with preeclampsia had a higher mean arterial pressure at examination (35–37 weeks) (MAP, 111.4 ± 8.1 versus 85.9 ± 8.6 mm Hg, respectively) than women with normal pregnancies. Preeclampsia was diagnosed at a mean of 37.0 ± 2.1 gestational week. Two of the 12 women were diagnosed with severe features at the time of examination, but none developed eclampsia.

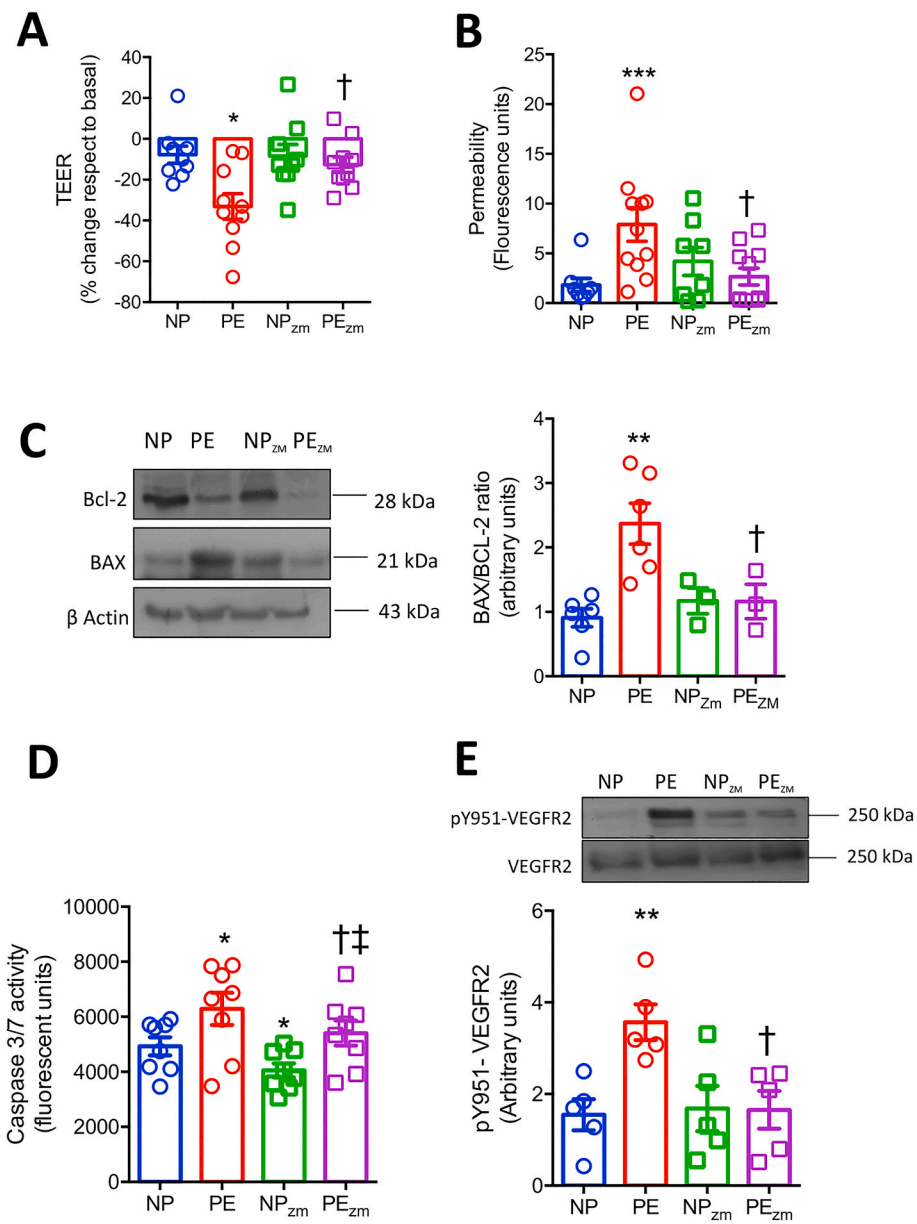

Fig. 1. The plasma of women with preeclampsia disrupts the in vitro blood-brain barrier model associated with VEGFR2 phosphorylation. Effect of plasma from women with normal pregnancies (NP), women with preeclampsia (PE), and co-treatments with the VEGFR2 inhibitor ZM-323881 ($_{ZM}$) on (A) TEER, (B) permeability, (C) expression of Bcl-2 and Bax, (D) caspase 3/7 activity, and (E) phosphorylation of tyrosine 951 (pY951) of VEGFR2, on hCMEC/d3 cells. Results are presented as mean ± standard deviation. Every dot represents individual plasma. *$p < 0.05$ or **$p < 0.01$ versus normal pregnancy. $^{\dagger}p < 0.05$ versus preeclampsia. $^{\ddagger}p < 0.05$ versus NP_{ZM}. Kruskal-Wallis test was used for comparison among the studied groups, followed by a Dunn's multiple comparisons test in case of statistical significance ($p < 0.05$).

In vitro results confirm previous studies [3,4], showing reduced TEER and increased permeability to FITC-dextran 70 kDa in hCMEC/d3 cell monolayers exposed to plasma from women with preeclampsia (Fig. 1A and B). This phenomenon occurred without changes in cell proliferation (Fig. S1A). Remarkable, co-treatment with the VEGFR2 inhibitor ZM-323881 reverted the effects on TEER and permeability (Fig. 1A and B), endorsing the participation of this receptor in those outcomes. No significant changes were observed in TEER and permeability in cells exposed to plasma from women with normal pregnancies in the absence or presence of the VEGFR2 inhibitor.

Treatment with plasma from women with preeclampsia also reduced the protein expression of the anti-apoptotic protein Bcl-2 while increasing the pro-apoptotic protein Bax (Fig. 1C). Therefore, a decreased Bcl-2/Bax ratio was found in cells exposed to plasma from women with preeclampsia compared to those exposed to plasma from women with normal pregnancies (Fig. 1C), suggesting increased apoptosis. This outcome was reinforced by increased caspase 3/7 activity in cells exposed to plasma from women with preeclampsia (Fig. 1D). Inhibition of VEGFR2 with ZM-323881 restored the Bcl-2/Bax ratio and decreased caspase 3/7 activity generated by plasma of women with preeclampsia (Fig. 1C and D).

We further investigated the effect of VEGFR2 inhibition on pY951-VEGFR2. We found that plasma of women with preeclampsia increased the protein amount of pY951-VEGFR2 in hCMEC/D3 cells compared to the treatment with plasma from women with normal pregnancies, and the co-treatment with ZM-323881 reverted this effect (Fig. 1E).

Plasma from women with preeclampsia also reduced the protein amount of pY1175-VEGFR2 in the cell membrane fraction (Fig. S1B) and the whole-cell extractions (Fig. S1C). However, the effect of ZM-323881 on pY1175-VEGFR2 was not further studied since treatment with plasma from women with preeclampsia already exerted an inhibitory action.

In our study, the selective inhibition of VEGFR2 counteracted the effect of plasma from women with preeclampsia on TEER, permeability, and apoptosis in the hCMEC/d3 monolayer model, linking VEGFR2 in the disruption of the BBB observed in preeclampsia. The causes and consequences of VEGFR2 activation with plasmas of women with preeclampsia are largely unknown. However, our results suggest that increased pY951-VEGFR2 may contribute to the cellular outcomes.

As brain endothelial cells mainly express VEGFR2 homodimers on the abluminal side of brain vasculature [9], activation of this receptor occurs through VEGF released from brain cells (i.e., neurons and astrocytes) instead of circulating VEGF. Furthermore, research from stroke- and multiple sclerosis models has demonstrated that pharmacological or genetic blockade of VEGFR2 signaling and inhibition of VEGF release from astrocytes improves the clinical outcome by restoring the integrity of the BBB [10]. Therefore, in preeclampsia, the VEGFR2-mediated disruption of BBB and brain endothelial cell apoptosis might require an initial stimulus to activate VEGF release from surrounding neural and glial cells. In this regard, pro-inflammatory cytokines are known for increasing the permeability of the BBB in a rodent model of placental ischemia [11]. Thus TNFα and/or other inflammatory cytokines may initiate the cascade of events that lead to cerebrovascular dysfunction. Furthermore, evidence demonstrates that TNFα induces the secretion of VEGF [12] and transactivates VEGFR2, an effect that can be counteracted through VEGFR2 inhibitors [13]. In our laboratory, we are currently exploring this possibility.

Our results also showed that co-treatment with ZM-323881 reversed the increase in the pY951-VEGFR2 induced through plasma from women with preeclampsia, reinforcing the participation of this critical pro-migratory and pro-permeability residue [14] in the development of cerebral endothelial dysfunction in preeclampsia. No similar results have been described in the field of preeclampsia, so the underlying molecular mechanisms are largely unknown. However, we speculate that pY951-VEGFR2 downstream signaling pathways may involve Akt activation [5] and alterations in cytoskeletal filaments.

We found that plasma of women with preeclampsia increased apoptosis of hCMEC/d3, which was also associated with activation of VEGFR2. We propose that apoptosis may also contribute to disrupting the BBB observed in the preeclampsia setting. Whether pY951-VEGFR2 is responsible for cell apoptosis is unclear. However, results in the cancer field have shown that inhibition of VEGFR2 using bevacizumab (a humanized monoclonal antibody of VEGF) reduces pY951-VEGFR2 and enhances apoptosis in breast cancer [6], which contrasts with our findings. Though, in our study, we also observed a reduction in the pY1175-VEGFR2 that could have a role in activating the pro-apoptotic processes generated by plasma from women with preeclampsia. Our findings are consistent with a study conducted in human umbilical vein endothelial cells (HUVECs), which demonstrated that inhibition of pY1175-VEGFR2 by the tyrosine kinase inhibitor, sunitinib, down-regulates Bcl-2 and increases apoptosis [15]. However, as co-treatment with ZM-323881 reverted the effects of plasma from women with preeclampsia on Bcl-2/Bax ratio and caspase 3/7 activities, the sole reduction in pY1175-VEGFR2 may not be enough to initiate pro-apoptotic events.

In conclusion, in a human-derived in vitro model, we demonstrate that VEGFR2 is involved in the disruption of the BBB generated with plasma from women with preeclampsia. Significantly, dysregulation of VEGFR2 phosphorylation, characterized by increased pY951 but reduced pY1175, is associated with cell apoptosis that may contribute to the BBB disruption in women with preeclampsia. However, the causes and consequences of these alterations in the VEGFR2 phosphorylation are less clear and may involve differential crosstalk with other critical cellular pathways, including those involved in inflammation.

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CRediT authorship contribution statement

CE conceptualized the study. FT, JA, and EK performed most of the experiments. LB and AKW were responsible for patient recruitment. PTV was a consultant in experiments related to the blood-brain barrier and apoptosis and wrote the draft of the manuscript. LB and AKW revised the manuscript. All co-authors approved the final version of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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