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Original Paper

Mechanism of vascular dysfunction due to circulating factors in women with pre-eclampsia

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Abstract

Circulating factors have been proposed to play a major role in the pathophysiology of endothelial dysfunction in pre-eclampsia (PE), which is defined as new-onset hypertension with proteinuria after 20 weeks of gestation. However, the mechanisms leading to altered vascular reactivity remain unclear. We hypothesized that circulating factors lead to endothelial dysfunction by increasing oxidative stress and reducing nitric oxide (NO) and prostaglandin (PG) bioavailability. Pregnant rat uterine and mesenteric arteries were incubated overnight with 3% normotensive (NP) or PE plasma collected from women upon admission to hospital. Responses to methacholine (MCh) were obtained using wire myography to assess endothelial function pathways. Vascular superoxide level was measured via dihydroethidium staining and nitric oxide synthase (NOS) expression via Western blots. PE plasma significantly increased superoxide levels and impaired endothelial dysfunction in uterine arteries (E_{max} 79.9±5.6% compared with 44.9±6.3%, $P = 0.0004$), which was restored in the presence of oxidant scavengers or PG synthesis inhibition. Uterine artery vasodilation was abolished in the presence of pan-NOS inhibitor ($P<0.0001$) in both NP- and PE-treated vessels, but inducible nitric oxide synthase (iNOS)-dependent vasodilation was present only in NP-treated arteries. Uterine arteries exposed to PE plasma exhibit an increased endothelial NOS expression and a decreased iNOS expression. PE plasma did not alter endothelial function in mesenteric arteries, suggesting that the effect of circulating factors was vascular-bed-specific. We have shown that circulating factors lead to endothelial dysfunction via altered oxidative stress and vasodilator pathways. The present study contributes to our understanding of the pathophysiology and finding a potential target for intervention in PE.

Key words: circulating factors, endothelium, nitric oxide, pre-eclamptic plasma, prostaglandin, uterine artery.

INTRODUCTION

Pre-eclampsia (PE), as defined by new-onset hypertension after 20 weeks of gestation with concurrent proteinuria, affects 5–10% of pregnant women worldwide [1]. Globally, it accounts for 16.1% of maternal deaths in developed countries, 25.7% in Latin America and the Caribbean, 9.1% in Africa and 9.1% in Asia. Not only does PE negatively affect maternal health and cause major complications such as eclampsia, liver failure, pulmonary oedema and disseminated intravascular coagulation, but also it increases the risk of intrauterine growth restriction, iatrogenic preterm delivery and perinatal mortality [2,3], all of which adversely affect immediate fetal health as well as long-term sequelae. Unfortunately, there is currently no cure for PE except for the expedited delivery of the infant and the placenta, making understanding its pathophysiology of paramount importance.

Systemic endothelial dysfunction is thought to play a central role in the development of PE. Placental hypoperfusion results

Abbreviations: AUC, area under the curve; DHE, dihydroethidium; EDHF, endothelium-dependent hyperpolarization factor; E_{max}, maximal response; eNOS, endothelial nitric oxide synthase; HBSS, Hanks balanced salt solution; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MCh, methacholine; meclo, meclofenamate; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; NP, normotensive pregnant; OCT, optimal cutting medium; oxLDL, oxidized low-density lipoprotein; PE, pre-eclampsia; pEC_{50}, effective concentration required to achieve 50% of maximal response; PG, prostaglandin; PGHS, prostaglandin H synthase; PGI_2, prostacyclin; PlGF, placental growth factor; ROS, reactive oxygen species; sEng, soluble endoglin; SOD, superoxide dismutase; TG, triacylglycerol; TXA_2, thromboxane; VLDL, very-low-density lipoprotein.

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from a lack of spiral artery remodelling in early placental development, which leads to local oxidative stress and the release of circulating factors into the maternal vasculature [4]. Previous studies have shown that plasma from pre-eclamptic women reduces endothelium-dependent vasodilation in healthy resistance vessels [5–8], although this finding is not universal [9]. However, the mechanisms by which circulating factors lead to endothelial dysfunction remain unclear. Two key regulatory mechanisms in vascular function during pregnancy are the nitric oxide (NO) and prostaglandin (PG) vasodilator pathways. NO, a potent vasodilator, is produced from L-arginine by nitric oxide synthase (NOS), which exists in different isoforms: endothelial (eNOS or NOS-3), inducible (iNOS or NOS-2) and neuronal (nNOS or NOS-1). In the endothelium, the majority of NO production is associated with eNOS, whereas in diseased states such as hypertension and aging, iNOS and nNOS can also contribute to NO bioavailability in the cardiovascular system [10,11]. NO is the major vasodilator in the uterine artery vasculature and it therefore functions to support the increase in blood flow and fetal growth which occurs during pregnancy. Indeed, studies have shown that stable metabolites of NO and eNOS expression are both increased in pregnancy [12]. NO is also essential for embryo development, placenta implantation and trophoblast invasion [13] and regulates placental angiogenesis and maturation throughout pregnancy [14]. However, the bioavailability of NO is intricately affected by levels of reactive oxygen species (ROS), such as superoxide, which scavenge NO to produce peroxynitrite and decrease NO-dependent relaxation of vascular smooth muscle [13]. Therefore, it is of interest to determine how circulating factors in women with PE interact with ROS and the NO system in the maternal vasculature.

In addition, PGs, produced from arachidonic acid by prostaglandin H synthase (PGHS), are also intrinsically related to endothelial dysfunction in PE. Prostacyclin (PGI_2), a vasodilator, is an abundant metabolite of PGHS in the endothelium and its biosynthesis is increased locally in the uterine and renal circulations during pregnancy. Thromboxane (TXA_2), a potent vasoconstrictor, is mainly derived from platelets and its production is also increased 3–5-fold during pregnancy [15]. Although both PGHS-dependent vasodilators and vasoconstrictors have both been shown to increase in pregnancy, a delicate balance between them is required to properly maintain vascular tone. A number of studies have reported a decrease in the PGI_2/TXA_2 ratio in PE as early as the first trimester and throughout pregnancy [16]. Given the ubiquitous nature of NO and PGs in the vasculature, it is of interest to study how circulating factors in plasma from women with PE could interact with these pathways.

We hypothesize that circulating factors in PE plasma contribute to an increase in oxidative stress and result in an impairment of endothelial vasodilation in uterine (responsible for supplying the uterus and the fetus) and mesenteric (responsible for peripheral resistance and blood pressure control) arteries. Specifically, we hypothesize that circulating factors increase superoxide production in the endothelium, which scavenges and decreases the bioavailability of NO, resulting in endothelium-dependent vascular dysfunction. We also hypothesize that circulating factors will lead to reduced PGI_2 production resulting in impaired vasodilation.

MATERIALS AND METHODS

Ethics approval

The animal use protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee, in accordance with the Canadian Council on Animal Care guidelines. The study protocol for human blood sample collection was approved by the Foundación Cardiovascular de Colombia Institutional Ethics Review Board and conducted in Colombia. Written informed consent was obtained from all study participants. The blood sample collection protocols were also approved by the University of Alberta Ethics Committee to be used for studies in our laboratory.

Pooled plasma preparation

The plasma samples were obtained from the authors of a previously published multi-centre case–control study conducted in Colombia [17]. Briefly, subjects with PE were identified upon admission to the obstetrical services and recruited if blood pressure was $\geqslant$140/90 mmHg after the 20th week of gestation with concurrent proteinuria (24-h urinary protein $\geqslant$300 mg or dipstick protein $\geqslant$1+ in at least two random urine samples at least 4–6 h apart). The control normotensive pregnant (NP) group was recruited consecutively from the same hospital after matching by age and ethnicity. Women with a history of chronic hypertension, cardiovascular disease, endocrine disease, autoimmune disease, renal disease, mental illness, human immunodeficiency virus and cancer were excluded. Blood samples were collected from the antecubital vein using EDTA-coated tubes, centrifuged at 1200 ***g*** for 15 min and transferred to Canada while frozen at -80°C. Prior to the experiments, plasma samples were randomly selected (NP group $n = 10$; PE group $n = 12$), pooled and stored at -80°C until use. The pooled samples were matched by maternal age and body mass index in both groups and none of the subjects smoked during pregnancy (Table 1).

In our study, we have chosen 3% plasma treatment to study the impact of PE circulating factors on pregnant rat arteries. This concentration is based on several previous studies [5–8,18,19]. In particular, Hayman et al. [6] have shown that bradykinin-dependent vasodilation was not different between human myometrial arteries exposed to either 0% or 1% PE plasma, but was significantly impaired by 2% plasma which was comparable to those exposed to 5% plasma.

Measurement of biochemical markers

After blood collection from the subjects, the measurement of the biochemical markers was completed in Colombia. An aliquot of the whole blood was used to determine haemoglobin, haematocrit and leucocyte. The lipid profile was measured using enzyme colorimetric test (Biosystems BTS-303 Photometric). Among the PE subjects, the levels of haematocrit and platelets were decreased and the levels of leucocytes, total

Table 1 Characteristics of subjects from whom plasma samples were collected and pooled

*Gestational age and proteinuria were not normally distributed. They are presented as medians (range). A non-parametric Mann–Whitney test was used for statistical comparison. †None of the subjects smoked during pregnancy. All 12 subjects in the PE group and all ten but one subject in the NP group were lifelong non-smokers. One subject in the NP group was a casual smoker who quit at least 1 year before conception.

Characteristics	NP ($n = 10$)	PE ($n = 12$)	*P*-value
Diagnostic criteria			
Systolic blood pressure (mmHg)	113.2 ± 2.1	150.3 ± 4.3	<0.0001
Diastolic blood pressure (mmHg)	68.9 ± 1.9	95.1 ± 2.9	<0.0001
24 h proteinuria (mg/24 h)*	–	360 (305–1184)	–
Patient characteristics			
Maternal age (years)	27.5 ± 2.5	23.7 ± 1.4	0.17
Gestational age (weeks)*	38 (30–40)	36 (25–38)	0.01
Height	160.6 ± 1.9	162.0 ± 1.5	0.57
Weight	67.7 ± 12.0	73.2 ± 3.7	0.31
BMI (kg/m^2)	26.3 ± 1.5	28.0 ± 1.6	0.45
Smoking in pregnancy (%)†	0	0	–
Biochemical markers			
Haemoglobin (g/dl)	122.9 ± 2.9	112.3 ± 4.7	0.08
Haematocrit (%)	37.4 ± 1.1	33.2 ± 1.5	0.04
Platelets ($\times 10^9$/l)	260.3 ± 25.4	188.1 ± 12.2	0.02
Leucocytes ($\times 10^9$/l)	8.2 ± 1.3	12.1 ± 1.0	0.02
Total cholesterol (mg/ml)	241.4 ± 16.9	220.9 ± 12.8	0.34
VLDL (mg/ml)	47.7 ± 4.5	69.0 ± 7.0	0.02
Triacylglycerol (mg/ml)	238.4 ± 22.7	345.0 ± 35.2	0.02
HDL (mg/ml)	60.2 ± 5.3	52.6 ± 3.6	0.23

cholesterol, very-low-density lipoprotein (VLDL) and triacylglycerol (TG) were increased compared with NP subjects (Table 1).

Animal and vessel preparation

Three-month-old female Sprague–Dawley rats were bred after acclimatization in 10-h light/14-h dark cycle cages with free access to standard rat chow and tap water. A total of 26 animals were used for vascular function experiments (NP: $n = 14$, PE: $n = 12$). Day 0 of pregnancy was confirmed by the presence of sperm in a vaginal smear following an overnight mating. On day 20 of gestation, the animals were killed by exsanguination while under anaesthesia by isoflurane and a laparotomy was performed to harvest the mesenteric and uterine arteries. The arteries were isolated under a microscope to ensure that all connective tissues and adipocytes were removed. The arteries were then incubated overnight with 3% plasma (3 μl) from either NP or PE women with 1 unit/ml heparin (2 μl of 50 units/ml stock) in 95 μl of physiological saline solution at 4°C. The concentration of plasma incubation was chosen based on previous studies [6,8,19]. The arteries were mounted for myography experiments on the following day after 22–24 h of incubation.

Superoxide detection

Superoxide level was measured by staining with dihydroethidium (DHE; $n = 5$). DHE reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates red fluorescence. After overnight incubation in plasma, segments of uterine arteries were embedded in optimal cutting medium (OCT) and snap-frozen in liquid nitrogen for storage. Sections of the arteries were cut at 10 μm, mounted on glass slides at −20°C and stored at −80°C until use. On the day of analysis, the slides were thawed, washed with Hanks balanced salt solution (HBSS) three times at 2 min each, and incubated with fresh HBSS for 10 min at 37°C in a humid chamber. The slides were then incubated with DHE for 30 min at 37°C, washed with HBSS three times at 2 min each, coverslipped and visualized under an IX81 Olympus fluorescence microscope. The total intensity of fluorescence was quantified using ImageJ software (NIH) and the intensity of PE-plasma-treated arteries was compared with that of NP-plasma-treated arteries and expressed as a relative mean fluorescence intensity.

Western blot analysis for NOS expression

Following an overnight incubation in plasma, uterine arteries ($n = 5$–8) were snap-frozen in liquid nitrogen and stored at −80°C until use. On the day of analysis, uterine arteries were thawed and homogenized in lysis buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 1 mM PMSF; 1x protease inhibitor cocktail tablet (SIGMA*FAST* Protease Inhibitor Cocktail Tablets, EDTA-free) and 2 mM sodium orthovanadate]. Total protein was determined using the BCA assay (Pierce Thermo Scientific) and 50 μg of total protein was loaded on to SDS/7.5% acrylamide gels. After electrophoresis, the proteins were transferred on to 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories) and blocked with 50%

blocking reagent (Rockland). The membranes were then incubated overnight with primary antibodies against iNOS (mouse monoclonal; 1:500 dilution; BD Biosciences catalogue number 610432), eNOS (mouse monoclonal; 1:500 dilution; BD Biosciences catalogue number 610297), nNOS (mouse monoclonal; 1:500 dilution; BD Biosciences catalogue number 610309) and β-actin (rabbit polyclonal; 1:1000 dilution; Abcam catalogue number ab75186). On the following day, the membranes were incubated with secondary antibodies for 1 h: donkey anti-mouse IgG (IRDYe 800CW; 1:10 000 dilution; Li-Cor catalogue number 926-32212) or goat anti-rabbit IgG (IRDYe 680RD; 1:10 000 dilution; Li-Cor catalogue number 926-68071). Blots were imaged using an Odyssey infrared imaging system and densitometry was measured with Odyssey software version 3.0.021 (Li-Cor Biosciences). Results were normalized to β-actin as a loading control.

Myography

Following an overnight incubation in 3% plasma and 1 m-unit/ml of heparin, mesenteric and uterine arteries were mounted on two 40-μm wires and attached to a wire myograph (DMT) for isometric tension recordings. The vessels were normalized and equilibrated for a minimum of 30 min before they were exposed to two wake-up doses of phenylephrine (10 μmol/l) and a single dose of methacholine (MCh; 3 μmol/l) to ensure endothelial function and smooth muscle integrity. A cumulative concentration–response curve to phenylephrine (0.001–100 μmol/l) was created to determine the concentration that produces 80% of maximal constriction. This concentration was then used to preconstrict the vessels for endothelium-dependent relaxation curves using cumulative doses of MCh (0.0001–10 μmol/l).

Pharmacological agents

To study the involvement of different vascular function pathways, specific inhibitors were added to the myograph baths 30 min prior to creating the MCh concentration–response curve. We used a pan-NOS inhibitor, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μmol/l; Sigma N5751), to study the contribution of NO to endothelial vasodilation. We also used a highly selective inhibitor for iNOS, 1400W (10 μmol/l; Sigma W4262), which is at least 5000-fold more potent against iNOS ($K_i \leqslant 7$ nM) than against eNOS ($K_i = 50$ μM) [20]. To study the effect of NO scavenging by superoxide, we used superoxide dismutase–PEG (50 units/ml; Sigma S9549) and catalase as a downstream enzyme (500 units/ml; Sigma C1345). To study the involvement of PGs, we used a non-selective PGHS inhibitor, meclofenamate (meclo; 1 μmol/l; VWR J60484).

Statistics

Statistical analyses were performed using GraphPad Prism software. The Kolmogorov–Smirnov normality test was used for testing the characteristics of human subjects (Table 1): normally distributed data were compared using a two-tailed Student's *t* test and non-normally distributed data were compared using a Mann–Whitney test. Myography data were summarized using maximal response (E_{max}), the effective concentration required to achieve 50% of the maximal response (pEC_{50}) or area under the curve (AUC) values. The effect of plasma (NP or PE) or inhibitors within a group were compared using a two-way ANOVA and a Bonferroni post-hoc test. A *P*-value <0.05 was considered statistically significant.

RESULTS

The effect of circulating factors on nitric oxide bioavailability in the uterine artery

Endothelium-dependent vasodilation of uterine arteries was significantly impaired after overnight incubation with PE plasma when compared with those in NP plasma (Figure 1). SOD significantly restored endothelium-dependent vasodilation in arteries exposed to PE plasma to a level comparable with those treated with NP plasma; however, SOD had no effect on

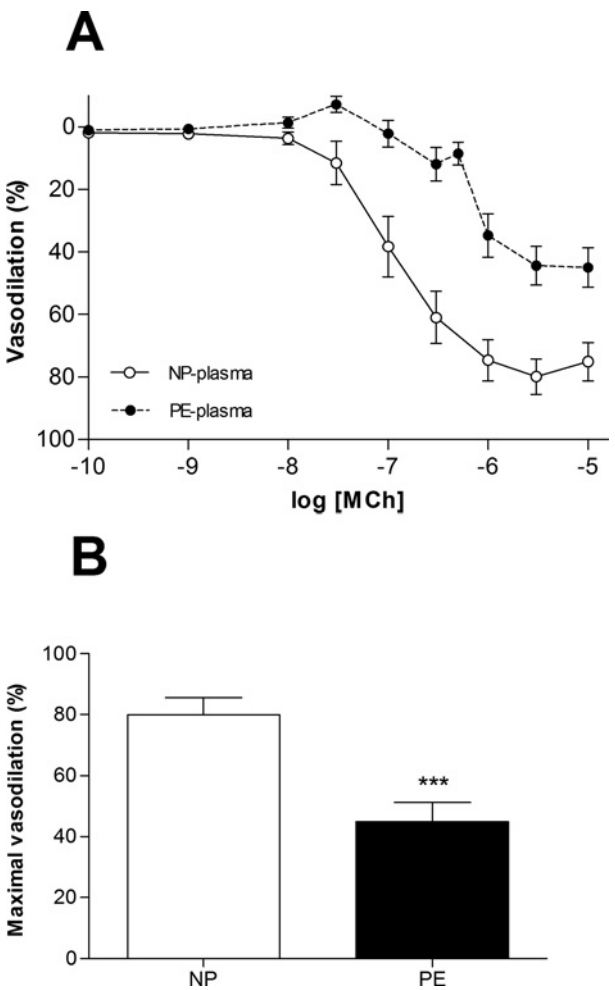

Figure 1 Exposure to PE plasma impairs uterine vascular responses

Uterine vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator (NP: $n = 12$; PE: $n = 14$). (**A**) Uterine endothelial vasodilation was significantly impaired by exposure to circulating factors in PE plasma (closed circles) compared with NP plasma (open circles). (**B**) Percentage maximal vasodilation. By Student's *t* test: $***P < 0.001$.

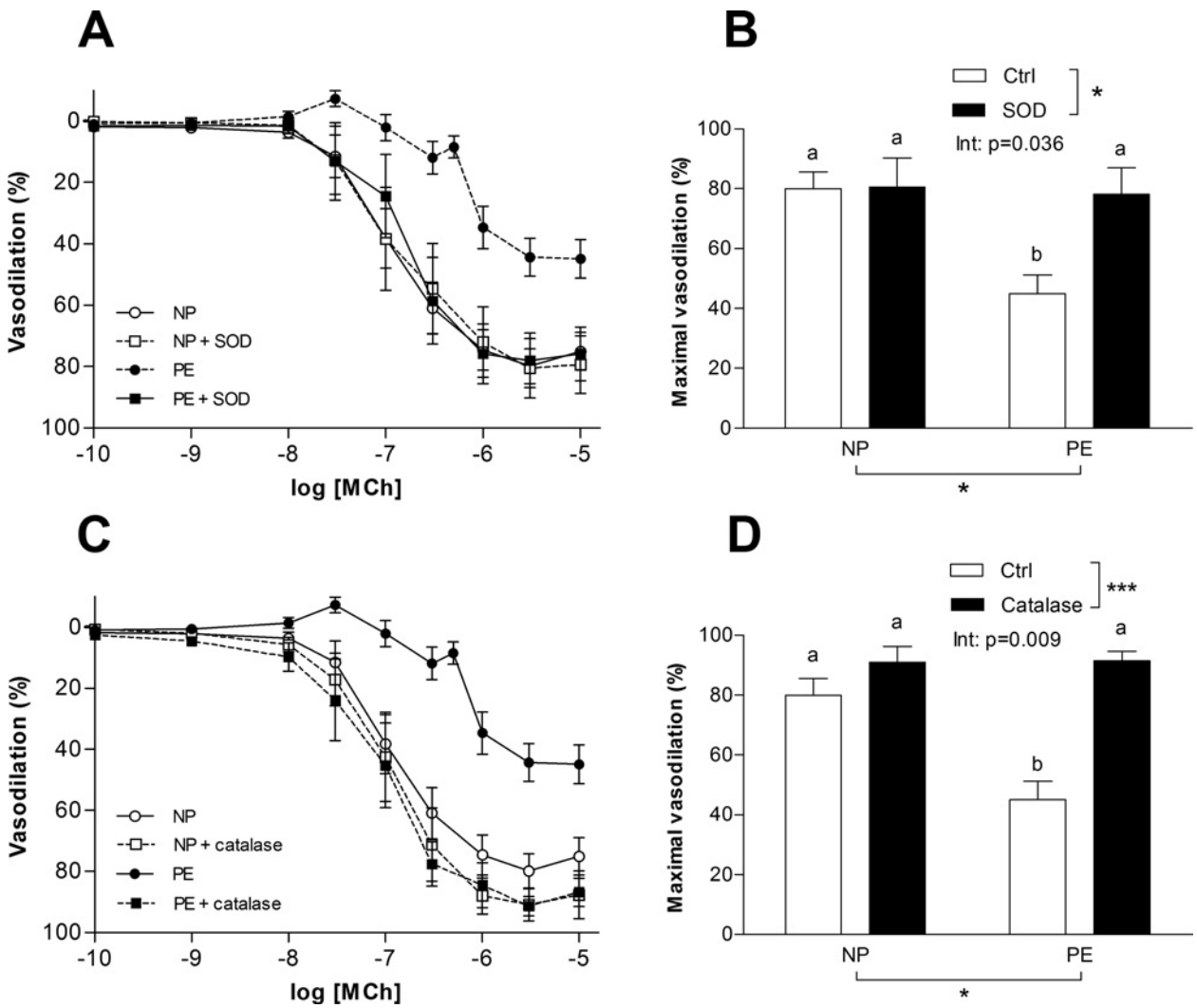

Figure 2 Oxidant scavengers improve uterine endothelial function in PE plasma-exposed vessels

Uterine vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator, in the absence or presence of oxidant scavengers ($n = 7$ per group). (**A**) Scavenging of superoxide with SOD significantly restored vasodilation in arteries exposed to PE plasma, but had no effect in arteries exposed to NP plasma. (**B**) Percentage maximal vasodilation. (**C**) Treating PE-plasma-exposed arteries with catalase significantly restored vasodilation responses but had no significant effect in NP-plasma-treated arteries. (**D**) Percentage maximal vasodilation. Using two-way ANOVA: $*P<0.05$, $***P<0.001$, and 'a' denotes a statistical significance from 'b' with $P<0.05$. Ctrl, control.

vasodilation in arteries exposed to NP plasma (Figures 2A and 2B). Similarly, catalase restored vasodilator responses in PE-plasma-treated uterine arteries and had no effect on NP-plasma-treated vessels (Figures 2C and 2D). Uterine vasodilation was largely abolished in the presence of L-NAME, a pan-NOS inhibitor, in both NP- and PE-plasma-treated vessels (Figure 3A). However, the ΔAUC was significantly reduced in PE-plasma-treated arteries, suggesting that the contribution of NO to endothelial vasodilation was decreased in PE-exposed vessels (Figure 3B). Interestingly, inhibition of iNOS with 1400W significantly impaired endothelial function in uterine arteries exposed to NP plasma, but not in those exposed to PE plasma (Figures 4A and 4B).

The level of DHE staining, which reacts with superoxide to produce fluorescent ethidium, was increased 2-fold in PE-plasma-treated arteries when compared with NP-plasma-treated arteries (Figure 5), suggesting an increase in oxidative stress in arteries exposed to PE circulating factors. The uterine arterial expression of eNOS was significantly increased in arteries exposed to PE plasma when compared with NP controls (Figure 6A). However, the expression of iNOS was decreased in PE-plasma-treated uterine arteries (Figure 6B). The expression of nNOS in uterine arteries was negligible and below the level of detection by Western blot (results not shown).

The effect of circulating factors on prostaglandin bioavailability in the uterine artery

In uterine arteries, inhibition of PG synthesis with meclo restored endothelium-dependent vasodilation in PE-plasma-treated vessels to a level comparable with that of the controls, although

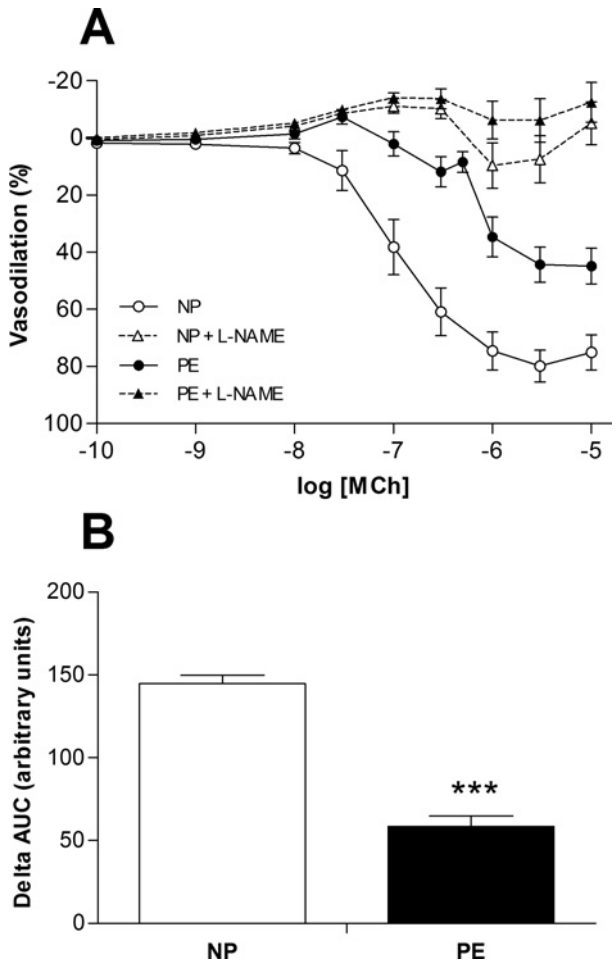

Figure 3 The effect of NOS inhibition on uterine endothelial function

Uterine vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator, in the absence or presence of a pan-NOS inhibitor, L-NAME ($n = 8$ per group). (**A**) Endothelial vasodilation was completely abolished in the presence of L-NAME in both NP- and PE–plasma-treated arteries. (**B**) The contribution of NO to vasodilation was decreased in arteries exposed to PE plasma. Results are ΔAUC in arbitrary units. Using two-way ANOVA: ***$P<0.001$.

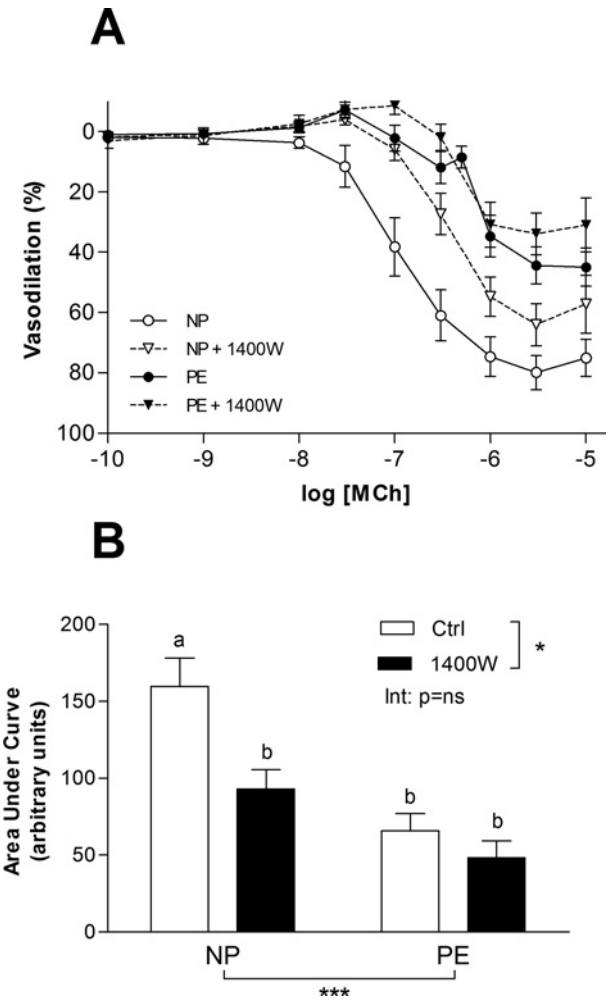

Figure 4 The effect of inducible NOS inhibition on uterine endothelial function

Uterine vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator, in the absence or presence of an iNOS inhibitor, 1400W ($n = 8$ per group). (**A**) Inhibition of iNOS with 1400W resulted in impaired vasodilation in NP-plasma-treated arteries but not in PE-plasma-treated arteries. (**B**) AUC in arbitrary units. Using two-way ANOVA: *$P<0.05$, and 'a' denotes a statistical significance from 'b' with $P<0.05$. Ctrl, control.

meclo had no effect on NP-plasma-treated arteries (Figure 7). This suggests that circulating factors in PE plasma contribute to an increase in PGHS-dependent vasoconstrictors.

The effect of circulating factors in the mesenteric artery

In contrast with the uterine arteries, endothelial vasodilation was not significantly different in mesenteric arteries exposed to either NP or PE plasma (Table 2). SOD and catalase increased vasodilation in both NP- and PE-plasma-treated mesenteric arteries compared with their respective controls, but the effect appears to be independent of exposure to either NP or PE circulating factors. Inhibition of NOS with L-NAME significantly impaired sensitivity but not maximal vasodilation in both NP- and PE-plasma-treated mesenteric arteries. Inhibition of iNOS also did not alter vasodilation responses in either NP- or PE-plasma-treated mesenteric arteries. Furthermore, inhibition of PG synthesis with meclo did not affect vascular responses in either NP- or PE-plasma-treated mesenteric arteries. These data are summarized in Table 2.

DISCUSSION

In the present study, we have shown that circulating factors in PE plasma contribute to an increase in superoxide production and impairment of endothelium-dependent vasodilation in uterine arteries, but not in mesenteric arteries. Scavenging of superoxide, which increases the bioavailability of NO, results in restoration

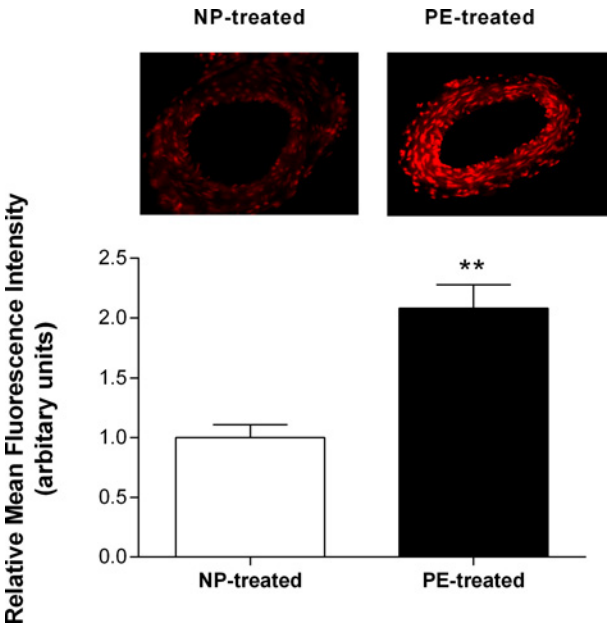

Figure 5 Superoxide levels are increased in uterine arteries exposed to PE plasma

DHE staining in uterine arteries after a 24-h incubation in either NP or PE plasma. DHE staining, as a marker of superoxide levels, was increased in PE-plasma-treated arteries when compared with NP-plasma-treated arteries. Representative images are shown ($n = 5$ per group). Red fluorescence indicates nuclear fluorescence generated by the reaction of DHE with superoxide to produce ethidium. All data are expressed as relative mean fluorescence intensity and analysed using Student's *t* test. $^{**}P<0.01$.

of endothelial vasodilation in arteries exposed to PE plasma. Circulating factors in PE also increase PGHS-dependent vasoconstrictors since meclo significantly improves endothelial function in the exposed uterine arteries. Not only does the present study highlight vascular bed-specific mechanisms through which circulating factors interact with vasodilator pathways, but it also enhances the understanding of how circulating factors contribute to the endothelial dysfunction seen in the pathophysiology of PE.

Previous studies have demonstrated that PE plasma impairs endothelial vasodilation in arteries [5–7]; however, there has been a paucity of data on the underlying mechanism. A recent study has shown that activation of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which is a scavenger receptor frequently implicated in cardiovascular disease and, more recently, PE, contributes to impaired vasodilation in omental arteries exposed to PE plasma [8]. Our group has previously demonstrated that PE plasma increases superoxide levels in isolated endothelial cells through activation of NADPH oxidase by LOX-1 [21]. However, the functional consequence has not been demonstrated. In the present study, we have shown that circulating factors in PE plasma contribute to an increase in superoxide production in uterine arteries and do indeed impair endothelium-dependent relaxation. We have also shown that scavenging superoxide with SOD, which would be expected to increase NO bioavailability, improves endothelial vasodilation in uterine arteries exposed to PE circulating factors. This supports our hypothesis that circulating factors in PE plasma impair endothelial function by contributing to an increase in the production of oxidative stress. Interestingly, the presence of catalase, which breaks down the harmful by-product of SOD, hydrogen peroxide (H_2O_2) into water and oxygen, also improved endothelial vasodilation, suggesting that H_2O_2 acts as a vasoconstrictor in uterine arteries exposed to circulating factors. Although H_2O_2 is known to act as an endothelium-dependent hyperpolarization factor (EDHF) which causes vasodilation in vascular smooth muscle [22,23], it can also act as a vasoconstrictor [24]. The level of H_2O_2 has been shown to be increased in maternal circulation and placenta in term pregnancies [25] as well as in PE pregnancies [26]. H_2O_2 can decrease the production of NO by increasing arginase metabolism [27], resulting in an inverse relationship between the levels of H_2O_2 and NO in pregnancy [25]. This provides an explanation for our findings that catalase increases the breakdown of H_2O_2 which may contribute to an increase in NO bioavailability and improved endothelial vasodilation in arteries exposed to PE plasma.

A myriad of circulating factors have been implicated in the pathophysiology of endothelial dysfunction in PE. In our sample population of Colombian women, it has been shown that (anti-) angiogenic circulating factors such as soluble endoglin (sEng) was increased and placental growth factor (PlGF) was decreased among PE women [17]. We have also shown that modified lipids such as TG and VLDL are increased in PE women and oxidized low-density lipoprotein (oxLDL) is increased among women with severe PE [17]. In addition, English et al. [8] have shown that arteries exposed to PE plasma exhibit further endothelial impairment in the presence of oxLDL which was restored by the inhibition of the LOX-1 receptor. Together, this suggests that abnormal oxLDL uptake by LOX-1 may lead to an increase in NADPH oxidase activation and superoxide production contributing to reduction in NO-mediated endothelial vasodilation as observed in our studies.

Uterine arteries are highly dynamic and adaptive in order to accommodate a 40% increase in maternal blood volume during pregnancy which supplies the enlarging uterus, placenta and fetus [28]. Our finding that the inhibition of NOS completely abolished endothelial vasodilation suggests that uterine arteries rely heavily on NO bioavailability. This is in agreement with studies demonstrating that pregnancy is associated with an increase in NO metabolites in the maternal vasculature and an increase in endothelial eNOS protein and mRNA levels in uterine arteries [12]. Our study shows an increase in oxidative stress in PE-exposed vessels which contributes to a reduction in NO bioavailability for endothelium-dependent vasodilation. This is also associated with an increased uterine eNOS expression, which, by speculation, would compensate for reduced NO availability in order to maintain uterine blood flow to supply the feto-placental unit.

In pathological conditions, an up-regulation of iNOS has been reported in both experimental and clinical hypertension [10]. Pro-inflammatory cytokines that up-regulate iNOS have been associated with PE [29,30] and the inhibition of iNOS attenuates hypertension and oxidative stress seen in an animal model of PE [10]. Contrary to our expectation, NP-plasma-exposed arteries exhibited iNOS-dependent vasodilation that is absent from PE-plasma-exposed arteries. We speculate that prolonged exposure to

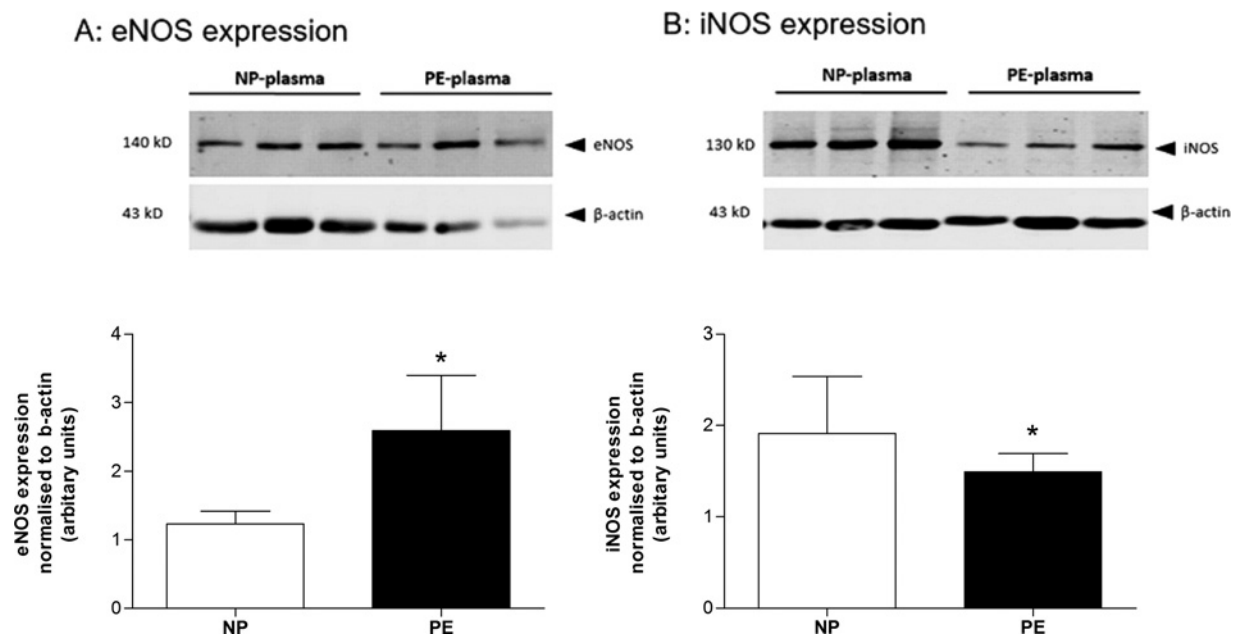

Figure 6 The effect of PE plasma on prtein expression of NOS enzymes in uterine arteries

Protein expression of NO synthase in rat uterine arteries after a 24-h incubation with either NP or PE plasma (n = 5–8 per group). (**A**) eNOS expression was increased in PE-plasma-treated arteries when compared with NP-plasma-treated arteries. (**B**) iNOS expression was decreased in PE-plasma-treated arteries compared with NP-plasma-treated arteries. Representative Western blots are shown. All data are expressed in arbitrary units relative to β-actin protein expression and were compared using Student's t test, $^*P<0.05$.

circulating factors in PE plasma may result in a down-regulation of iNOS to protect the vessels from the deleterious effects of the metabolites, such as peroxynitrite, which can be formed in the presence of abnormally high NO and superoxide [31]. Our finding that uterine arteries exposed to circulating factors in PE plasma exhibit a decreased iNOS expression supports this and is in line with previous studies that show a decrease in placental iNOS mRNA levels in women with PE [32]. This offers an interesting perspective into the current paradigm of PE that, instead of a state of heightened inflammation, we speculate that PE may represent a state of failed compensation for the reduced NO bioavailability to maintain proper vascular function. However, conflicting evidence also exists. Other studies have shown an increase in iNOS protein expression in human umbilical vein endothelial cells (HUVECs) after exposure to serum from PE women [33], an increase in iNOS protein expression in human omental arteries after exposure to microparticles from PE women [34], and no change in iNOS mRNA levels in HUVECs after exposure to plasma from women with umbilical placental vascular disease defined by abnormal umbilical artery Doppler [35]. Whether this discrepancy is due to variations in species or different circulating factors present in different compartments of the blood sample from PE women remains to be elucidated.

In the present study, we have also demonstrated that the inhibition of PG synthesis significantly restored endothelium-dependent vasodilation in uterine arteries exposed to PE plasma, suggesting that circulating factors contribute to an increase in PGHS-dependent vasoconstrictors rather than a reduction in vasodilator PGs, such as PGI_2, as we had originally hypothesized. Although meclo non-selectively inhibits PGHS, it is also known to inhibit TXA_2 synthase activity [36]. Many studies have shown that PE is associated with a decrease in the ratio of PGI_2 to TXA_2 [16]. The present study supports the theory that circulating factors contribute to an increase in PGHS-dependent vasoconstrictors, presumably TXA_2/PGH_2, which reduce endothelial vasodilation and which are inhibited in the presence of meclo. This is also in accordance with clinical trials that support the use of aspirin, a non-reversible PGHS-1 inhibitor which decreases TXA_2 during early pregnancy in high-risk patients to reduce the risk of developing PE [37].

Unlike the uterine arteries, our data show that the circulating factors present in 3% PE plasma do not impair endothelial vasodilation in mesenteric arteries. We speculate that different vascular beds may exhibit different sensitivities to the effect of PE circulating factors. This is in agreement with Hayman et al. [7] who have demonstrated that human myometrial arteries exhibit impaired vasodilation after exposure to PE plasma whereas the same dose had no effect on human infracolic omental arteries. It is possible that the sensitivity of a specific vascular bed to the effect of circulating factors is influenced by underlying vascular function mechanisms. In both NP- and PE-plasma-treated arteries, mesenteric vasodilation is increased in the presence of superoxide scavengers and reduced in the presence of NOS inhibition. This suggests that mesenteric arteries rely partially on NO vasodilator pathways and this is independent of the effect of circulating factors in PE plasma. Inhibition of PG synthesis also did not affect endothelial vasodilation in mesenteric arteries

Table 2 Mesenteric vascular responses after exposure to NP and PE plasma

Summary table of log EC_{50} values for mesenteric vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator, in the absence or presence of different inhibitors ($n = 6$–10 per group). The effect of NP and PE plasma on mesenteric arteries was compared using Student's *t* test. The effect of NP and PE plasma and the effect of inhibitors were compared using two-way ANOVA. $^{**}P<0.01$, $^{***}P<0.001$ and 'Int' denotes an interaction between the two factors by two-way ANOVA. NS, not significant.

(a)

Sample	NP plasma	PE plasma	Student's *t* test
Control	7.34 ± 0.86	7.23 ± 0.10	NS

(b)

			Two-way ANOVA		
Inhibitor	NP plasma	PE plasma	Plasma	Inhibitor	Int
SOD	8.12 ± 0.07	8.25 ± 0.11	NS	***	NS
Catalase	7.82 ± 0.08	8.06 ± 0.12	NS	**	NS
L-NAME	6.68 ± 0.15	6.93 ± 0.11	NS	***	NS
1400W	7.47 ± 0.10	7.32 ± 0.04	NS	NS	NS
Meclo	7.42 ± 0.09	7.27 ± 0.10	NS	NS	NS

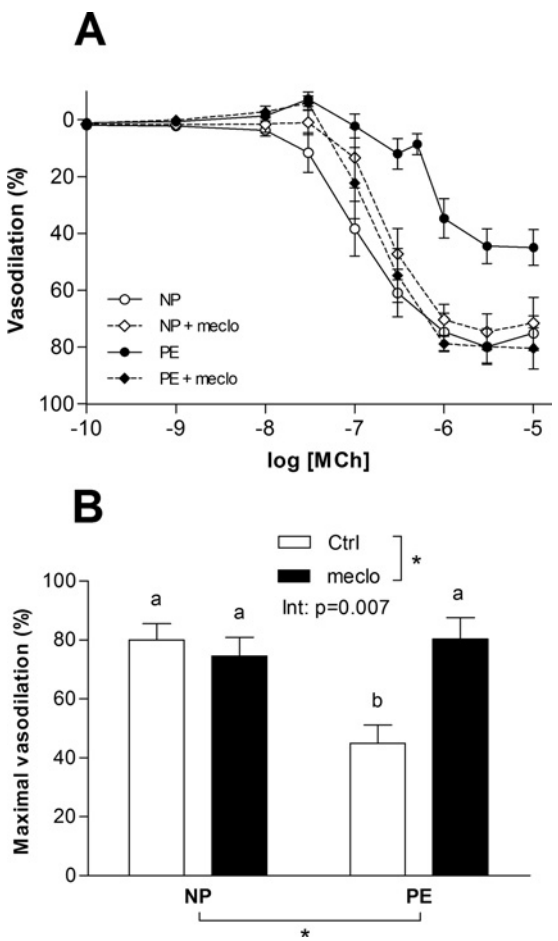

Figure 7 The effect of PG inhibition on uterine endothelial function

Uterine vascular responses after overnight incubation in NP plasma compared with PE plasma to cumulative doses of MCh, an endothelium-dependent vasodilator, in the absence or presence of PG synthesis inhibitor, meclo ($n = 7$ per group). (**A**) Treating PE-plasma-exposed uterine arteries with meclo restored endothelial vasodilation but had no effect in NP-plasma-exposed arteries. (**B**) Percentage maximal vasodilation. Using two-way ANOVA: $^{*}P<0.05$, and 'a' denotes a statistical significance from 'b' with $P<0.05$.

in the presence of circulating factors in PE. This is in agreement with previous literature that other vasodilators, such as EDHF, might play a more important role in mesenteric arteries which contribute to peripheral vascular resistance and blood pressure control [38–40]. The present study highlights that the mechanism of action of the circulating factors present in PE plasma are highly vascular bed-specific.

In conclusion, our studies have shown that circulating factors contribute to endothelial dysfunction in PE by increasing superoxide production and decreasing NO bioavailability. Circulating factors also contribute to an increase in PGHS-dependent vasoconstrictors which are associated with impaired vascular responses. Furthermore, we have shown that the mechanism of action of circulating factors is highly vascular bed-specific. The present study contributes to understanding the pathophysiology and potential target of intervention in improving the vascular dysfunction associated with PE.

CLINICAL PERSPECTIVES

- The multifactorial nature of the pathophysiology seen in PE has contributed to the difficulty in finding an effective treatment. Endothelial dysfunction is one of the most critical convergences in the underlying pathophysiology, hence understanding how circulating factors contribute to vascular dysfunction could potentially lead to finding a better therapeutic intervention.
- We have demonstrated that circulating factors in PE contribute to endothelial dysfunction by increasing oxidative stress, decreasing NO bioavailability and increasing PGHS-dependent vasoconstrictors.
- The present study contributes to the collective knowledge of the mechanisms behind circulating factors and endothelial dysfunction. We hope to further this knowledge and find a potential pharmacological target to treat or prevent PE in order to reduce the high maternal and perinatal morbidity and mortality associated with this disease.

AUTHOR CONTRIBUTION

Cindy Kao designed the experiment, performed the myography experiments, collected the tissue samples for DHE staining and Western blots, analysed the data and wrote the paper. Jude Morton provided assistance for myography techniques and data analysis. Anita Quon performed the DHE staining and Western blots. Laura Reyes and Patricio Lopez-Jaramillo contributed to the collection of plasma samples and measurement of biochemical markers performed in Colombia. Jude Morton and Sandra Davidge supervised and assisted in interpretation of the data and editing of the paper before submission.

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