Preeclampsia

Placental Vesicles Carry Active Endothelial Nitric Oxide Synthase and Their Activity is Reduced in Preeclampsia

Carolina Motta-Mejia, Neva Kandzija, Wei Zhang, Vuyane Mhlomi, Ana Sofia Cerdeira, Alexandra Burdujan, Dionne Tannetta, Rebecca Dragovic, Ian L. Sargent, Christopher W. Redman, Uday Kishore, Manu Vatish

Abstract—Preeclampsia, a multisystem hypertensive disorder of pregnancy, is associated with increased systemic vascular resistance. Placentae from patients with preeclampsia have reduced levels of endothelial nitric oxide synthase (eNOS) and, thus, less nitric oxide (NO). Syncytiotrophoblast extracellular vesicles (STBEV), comprising microvesicles (STBMV) and exosomes, carry signals from the syncytiotrophoblast to the mother. We hypothesized that STBEV-bound eNOS (STBEV-eNOS), capable of producing NO, are released into the maternal circulation. Dual-lobe ex vivo placental perfusion and differential centrifugation was used to isolate STBEV from preeclampsia (n=8) and normal pregnancies (NP; n=11). Plasma samples of gestational age–matched preeclampsia and NP (n=6) were used to isolate circulating STBMV. STBEV expressed placental alkaline phosphatase, confirming placental origin. STBEV coexpressed eNOS, but not inducible nitric oxide synthase, confirmed using Western blot, flow cytometry, and immunodepletion. STBEV-eNOS produced NO, which was significantly inhibited by l-arginine methyl ester (eNOS inhibitor; P<0.05) but not by N\(^{-}\)-(3-(aminomethyl) bezyl) acetamidine (inducible nitric oxide synthase inhibitor). STBEV-eNOS catalytic activity was confirmed by visualizing eNOS dimerization. STBEV-eNOS was more abundant in uterine vein compared with peripheral blood, indicating placentental origin. STBEV isolated from preeclampsia-perfused placentae had lower levels of STBEV-eNOS (STBMV; P<0.05) and overall lower NO activity (STBMV, not significant; syncytiotrophoblast extracellular exosomes, P<0.05) compared with those from NP. Circulating plasma STBMV from preeclampsia women had lower STBEV-eNOS expression compared with that from NP women (P<0.01). This is the first observation of functional eNOS expressed on STBEV from NP and preeclampsia placentae, as well as in plasma. The lower STBEV-eNOS NO production seen in preeclampsia may contribute to the decreased NO bioavailability in this disease. (Hypertension. 2017;70:372-381. DOI: 10.1161/HYPERTENSIONAHA.117.09321.) ● Online Data Supplement

Key Words: endothelial nitric oxide synthase ■ hypertension ■ nitric oxide ■ preeclampsia ■ syncytiotrophoblast extracellular vesicles

Early in normal pregnancy (NP), maternal blood volume expands while systemic vascular resistance and systemic blood pressure both decline. These changes alter significantly in preeclampsia, a pregnancy-specific syndrome (affecting 5% to 10% of all pregnancies worldwide), which is defined by maternal new-onset hypertension and proteinuria or organ dysfunction, developing after 20 weeks of gestation. Preeclampsia is thought to originate as a result of poor placentation, causing endothelial dysfunction, disordered angiogenic balance and resultant hypertension, glomerular lesions, and hepatic failure.

Nitric oxide (NO) is a potent vasodilator, considered to have major effects on gestational endothelial function. NO is synthesized by nitric oxide synthases (NOS), namely endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS. Studies investigating circulating levels of NO in preeclampsia have reported conflicting results. This is in contrast to studies that have shown that plasma from women with preeclampsia elicits reduced endothelium-dependent vasodilatation in isolated vessels. NO availability may be decreased because of oxidative stress, vascular endothelial growth factor deficiency, or endogenous inhibitors, such as asymmetric dimethylarginine. It is assumed that most of the circulating NO derives from maternal endothelium. But the placenta may also contribute.

In the placenta, NOS is predominantly expressed in the syncytiotrophoblast, villous endothelium, and macrophages; the predominant isoform being eNOS. The multinucleated syncytiotrophoblast layer lining the chorionic villi is the...
interface between the maternal and fetal vascular systems\textsuperscript{16,17} and could contribute to circulating NO. Here we investigate another possibility, namely, that syncytiotrophoblast-derived NOS could be exported to the mother in syncytiotrophoblast extracellular vesicles (STBEV) with the potential of systemically modulating maternal vascular response. STBEV are shed into the maternal circulation, detectable from week 10 of gestation and in increasing amounts through pregnancy.\textsuperscript{18,19} STBEV comprise 2 subgroups: microvesicles (STBMV, 100–1000 nm), which are shed directly from the plasma membrane in response to cell activation or death, and exosomes (STBEX, 20–200 nm), which are released by exocytosis from multivesicular bodies of the endosome.\textsuperscript{20} There is evidence that STBEV could allow communication between the mother and the fetus, in a manner dependent on the content of their cargo.\textsuperscript{21}

Our hypothesis was whether STBEV might carry eNOS as part of their cargo. To investigate this, we examined whether eNOS was present on STBEV derived from NP and preeclampsia-perfused placental lobules, as well as circulating STBMV from NP and preeclampsia peripheral vein blood (PB) and uterine vein blood (UV). We wanted to assess whether STBEV-bound eNOS (STBEV-eNOS) was functional and capable of producing NO, and whether its expression and activity was altered in preeclampsia.

Methods

Human Subjects

This project was approved by the Central Oxfordshire Research Ethics Committee C (REFS 07/H06077/4 & 07/H0606/148). All mothers undergoing elective caesarean section gave written informed consent for the use of their blood and placentas. Blood samples were collected in four 5-mL EDTA/citrate tubes using a 21-gauge needle. Samples were centrifuged at 1500 g for 15 minutes at room temperature, and supernatants were centrifuged again at 13000 g for 2 minutes to produce platelet-free plasma and stored in aliquots at −80°C. PB samples were from the left antecubital fossa, while UV samples were taken at caesarean section (after bladder reflexion before the uterine incision was made). Placentae were collected within 10 minutes of delivery. Placentae from NP were from singleton pregnancies with no current or previous history of preeclampsia or hypertensive disorders. Preeclampsia patients were identified according to the criteria of the International Society for the Study of Hypertension in Pregnancy; hypertension was defined as new hypertension in the second half of pregnancy >140/90 mm Hg accompanied with new proteinuria >300 mg per 24 hours. Clinical characteristics of preeclampsia patients and controls (NP) are described in Table. STBMV from plasma samples were matched for gestational age.

Cell Culture

Human umbilical vein endothelial cells were used as eNOS-positive control. Detailed Methods section is available in the online-only Data Supplement.

Isolation and Characterization of STBEV

STBEV were prepared using a modified dual-lobe placental perfusion system and differential centrifugation as previously described by us.\textsuperscript{22} Fresh STBMV were phenotyped by flow cytometry, using a BD LSRII flow cytometer (BD Biosciences), and STBEX were phenotyped by nanoparticle tracking analysis, using a NanoSight NS500 (Malvern, UK). The phenotype of STBEV was additionally assessed by SDS-PAGE and Western blotting analysis for placental alkaline phosphatase (PIAP) positivity (PIAP is syncytiotrophoblast specific) combined with the exosome markers ALIX, Syntenin, and CD9 to confirm exosomal phenotype because STBEX cannot be resolved by flow cytometry because of their size. STBMV and STBEX were assayed for protein using bicinchoninic acid assay before storage at −80°C.

Flow Cytometry Analysis

Perfusion-isolated STBMV from NP and preeclampsia placentae were interrogated by multicolor flow cytometry using the protocol and settings previously described\textsuperscript{22,23} and specifically interrogated for eNOS. Detailed Methods section is available in the online-only Data Supplement.

Circulating plasma STBMV from PB and UV were analyzed by flow cytometry using a previously described protocol,\textsuperscript{24} with additional modifications to exclude potential nonplacental extracellular vesicle (EV) contaminants. Detailed Methods section is available in the online-only Data Supplement.

Table. Clinical Data of Human Subjects

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Placental STBEV</th>
<th>Plasma STBEV</th>
<th>P Value</th>
</tr>
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<tr>
<td></td>
<td>NP (n=11)</td>
<td>PE (n=8)</td>
<td>NP (n=6)</td>
</tr>
<tr>
<td>Age, y</td>
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<td>32.4±1.6</td>
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<td>Gestation age, wk</td>
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<td>35±5±0.3</td>
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<td>Mean no. of pregnancies</td>
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<td>0.5±0.2</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>32.1±3.5</td>
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<td>Max. proteinuria, PCR, mg/mmol</td>
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<td>Max. systolic pressure, mm Hg</td>
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<td>168.8±8.3</td>
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</tr>
<tr>
<td>Max. diastolic pressure, mm Hg</td>
<td>75.5±2.4</td>
<td>104.4±4</td>
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<tr>
<td>New-born weight, g</td>
<td>3999±2±152.5</td>
<td>1985±205.1</td>
<td>3641±198</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. NP indicates normal pregnancy; PCR, protein creatinine ratio; PE, preeclampsia; and STBEV, syncytiotrophoblast extracellular vesicles. Significant difference shown as P<0.05 (*, †), P<0.01 (**, ††), P<0.001 (***), P<0.0001 (****), or not significant (NS). Statistical comparison between NP and PE’s placental STBEV shown as * and plasma STBEV shown as †.
Flow cytometry data were analyzed using FACS Diva software 8.0 (BD Biosciences), and figures were generated using FlowJo version 10 (Tree Star Inc, Ashland, OR). Given the issues described above about STBEX and flow cytometry, we investigated STBEV coexpression of eNOS and PIAP using paramagnetic immunobead depletion and Western blotting, described below.

**Western Blotting**

Western blots were performed using standard protocols with placental lysate, along with isolated STBMV and STBEX from NP and preeclampsia placentae. Detailed Methods section is available in the online-only Data Supplement.

**Co-Immunoprecipitation Using Magnetic Dynabeads**

3.25×10⁷ Dynabeads (Life Technologies) were separately coated with 6 μg/mL of the following antibodies: (1) anti-eNOS (NOS3 A9; Santa Cruz Biotech); (2) eNOS isotype control (IgG2a Clone DAK-GO5; Dako); (3) anti-PIAP antibody (NDO2G); and (4) PIAP isotype control (IgG1 Clone MOPC-21; BioLegend). STBMV or STBEX pooled from 4 NP (1 mg/mL) were incubated with anti-human Fc receptor blocking reagent (10 μL; Miltenyi Biotec) for 10 minutes at 4°C to block any nonspecific antigen binding (same pool was used at each experiment). Next, antibody-coated Dynabeads were incubated overnight at 4°C with 25 μg of protein from either STBMV (=2.1×10⁹ EV/mL) or STBEX (=3.2×10⁹ EV/mL) pools in 1 mL filtered PBS, as per manufacturer’s instructions. STBEX or STBEX bound to antibody-coated Dynabeads were separated and washed with PBS using a magnetic particle concentrator (Dynal MPC-S; Thermofisher) and processed for Western blotting. See Methods section is available in the online-only Data Supplement for percentage calculations.

**Dimerization Analysis**

To investigate eNOS dimerization in STBMV and STBEX, low-temperature SDS-PAGE was performed as previously described.²⁴ NP samples at 15 μg were loaded under nonreducing conditions onto a 4% to 12% SDS-PAGE gel and run at 5 mA overnight at 4°C. Separated proteins were then transferred onto polyvinylidene difluoride membranes and blocked with 3% wt/vol BSA dissolved in Tris-buffered saline and Tween 20 for 1 hour prior to overnight incubation at 4°C with 0.6 μg/mL of anti-eNOS (NOS3-A; Santa Cruz) diluted in blocking buffer. Protein visualization was then performed on Western blot as described earlier.

**NOS Activity Assay**

NOS activity by STBMV and STBEX was determined using an ultrasensitive colorimetric NOS assay kit, which converts all NO metabolites to nitrite using nitrate reductase (NB78 and NB70; Oxford Biomedical Research). We followed the manufacturer’s protocol and depicted nitrite accumulation as NOS activity (ie, production of NO). STBMV and STBEX pools of 4 NP patients (different pools used at each experiment) were first incubated in different concentrations (0, 10, 25, 50, and 100 μg). NOS activity was also measured with or without specific eNOS inhibitor, N⁶-nitro-L-arginine methyl ester (L-NAME; 1 mM/L; Sigma-Aldrich), and the highly specific iNOS inhibitor, N-(3-aminothiophenyl) benzyl acetamide (2 μM/L; Enzo Life Sciences).²⁶ Inhibitors were incubated with STBMV and STBEX for 1 hour prior to analysis. Reactions were measured in triplicate, and absorbance was read at 540 nm.

**Statistical Analysis**

The data were analyzed using GraphPad 5 software. Normality testing was performed using the Kolmogorov–Smirnov test and visual observation. The data were analyzed using paired or unpaired t test with Welch’s correction or 1-way analysis of variance, and data were presented as mean±SEM.

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**Flow Cytometric Analysis of Ex Vivo and In Vivo–Derived STBMV Reveal Coexpression of eNOS and PIAP**

STBMV derived from NP and preeclampsia placentae were analyzed by flow cytometry for eNOS and PIAP.

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**Results**

**Isolated STBEV Confirmed Microvesicular and Exosomal Phenotype**

We first reconfirmed the expected microvesicular and exosomal phenotypes and sizes for STBEV (see Figure S1 in the online-only Data Supplement ). Immunoblots of STBEV showed an enrichment of the STBEV marker, PIAP, relative to placental lysates. This enrichment was seen in both STBMV and STBEX (Figure S1A). STBEX were enriched for the exosomal markers ALIX, Syntenin, and CD9 (Figure S1A). Freshly isolated STBEV were analyzed on nanoparticle tracking analysis for size and particle number profiles. STBMV showed modal size of 323.2±7.1 nm with a broad size distribution, while STBEX were smaller (189.3±9.7 nm) with narrower size distribution (Figure S1B). Representative transmission electron micrographs confirmed the nanoparticle tracking analysis, and Western blot data showing STBMV was enriched for heterogeneous EV>200 nm, while STBEX contained a more homogenous population with a range of 30 to 200 nm EV (Figure S1C). These data confirmed that STBMV and STBEX had been successfully isolated.

**Syncytiotrophoblast and STBEV Express eNOS but Not iNOS**

Immunohistochemical analysis of placental tissue revealed eNOS expression primarily localized in the syncytiotrophoblast cell layer of NP samples in comparison to negative staining of isotype control IgG2a (n=3; Figure 1A), confirming findings made previously by others.¹³–¹⁵ Similarly, eNOS expression was also shown in the placental lysates. We were also able to visualize eNOS expression in STBMV and STBEX isolated from NP (Figure 1B) and from preeclampsia (Figure 1C) placentae. iNOS expression was not detectable in the syncytiotrophoblast cell layer (n=3; Figure 1D). Additionally, no expression of iNOS in either placental lysates, STBMV, or STBEX from NP (Figure 1E) or preeclampsia placentas (Figure 1F) was detected (n=3). Nevertheless, expression of iNOS was seen in positive control RAW 264.7 cells, reassuring the antibody’s quality (Figure 1E and 1F). Positive PIAP expression indicated that the EV analyzed were derived from syncytiotrophoblast. The absence of iNOS prompted us to perform mass spectrometry.

**Mass Spectrometry Data**

We assessed a mass spectrometry analysis we have performed on placental lysate, STBMV and STBEX from NP (n=6) and preeclampsia (n=8) patients (Tannetta et Al, unpublished data), and specifically interrogated it for the presence of NOS isoforms (see Table S1). The mass spectrometry was clearly able to identify eNOS, but iNOS was not identified at the protein level. Given these confirmatory findings, we, therefore, focused on STBEV expressing eNOS.
coexpression (n=6). Using the corresponding fluorescence minus one controls, our data revealed double eNOS and PlAP positivity of 30.3±8.5% in NP-derived STBMV and 6.3±3.3% in preeclampsia-derived STBMV (**P<0.05; Figure 2A).

Circulating STBMV were also analyzed by flow cytometry in plasma prepared from paired PB and UV samples (n=8). PIAP and eNOS double-positive events per milliliter were significantly higher in UV (49686±28162) compared with PB (7723±2823) plasma samples, confirming STBMV-bound eNOS consistent with their origin from the placenta (***P<0.01; Figure 2B).

Similarly, STBMV-bound eNOS was measured in gestational age–matched plasma PB samples from NP and preeclampsia patients (n=6). After exclusion of EV derived from other cells rather than the syncytiotrophoblast, the results showed a significant reduction in PIAP and eNOS double-positive events per milliliter of plasma-derived STBMV from preeclampsia (12798±7121) compared with NP (62838±15246; **P<0.01; Figure 2C). These data suggest that there is less plasma-derived STBMV-bound eNOS in preeclampsia compared with NP.

**eNOS and PIAP Are Expressed on the Same EV Population**

Immunodepletion was used to further confirm STBMV and STBEX coexpression of eNOS and PIAP and exclude potential aggregation.

For Western blot analysis, we used anti-eNOS–coated beads to immunoprecipitate eNOS-positive STBEV, which were interrogated for the presence of PIAP. We also conversely

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**Figure 1.** Immunohistochemical staining of normal pregnancy (NP) placenta tissue and Western blot of NP and preeclampsia (PE)-derived placental lysate (PL), syncytiotrophoblast extracellular microvesicles (STBMV) and syncytiotrophoblast extracellular exosomes (STBEX; n=3). A, Placental tissue showing endothelial nitric oxide synthase (eNOS) staining (brown; left) on syncytiotrophoblast (STB) layer and IgG2a-negative staining (right). Immunoblot showing eNOS (140 kDa) and placental alkaline phosphatase (PIAP; 60 kDa) expression in PL, STBMV, and STBEX derived from NP (B) and PE (C) similar to expression of positive control, human umbilical vein endothelial cells (HUVEC). D, Placental tissue demonstrating lack of inducible nitric oxide synthases (iNOS) staining (left) and isotype control IgG1-negative staining (right). Immunoblot showing no expression for iNOS (131 kDa) though positive control, RAW, is expressed and PIAP (60 kDa) is also expressed in PL, STBMV, and STBEX derived from NP (E) and PE (F). Scale bar set at 100 mm.
used anti-PIAP–coated beads to immunoprecipitate PIAP-positive STBEV, which were then interrogated for eNOS. Figure 3A reveals that anti-eNOS–coated magnetic beads could pull out STBMV-bound eNOS, which were positive for PIAP. The converse experiment using anti-PIAP–coated beads revealed similar results, confirming that both species were bound on the same population of STBMV (Figure 3A).

Interestingly, PlAP pull out yielded a greater signal for eNOS compared with the eNOS pull out, which may indicate that some eNOS is intravesicular. To assess the relative quantities of STBMV-bound eNOS or PIAP, we used nanoparticle tracking analysis. The total concentration of STBMV was compared with the supernatant samples from the bead depletion experiments. This data showed that 35.5±4.2% of STBMV were eNOS positive and 64.3±0.4% were PlAP positive (n=3; Figure 3B), while IgG2a control was positive for 11.3±5.7%, and IgG1 control was positive for 11.4±3.4% (n=3; Figure 3C).

Analysis of the STBEX fraction (Figure 3D) revealed similar results. STBEX-bound eNOS comprised 36.7±6.6% while STBEX-bound PIAP was 39.2±5.8% of the initial preparation (n=3; Figure 3E), while IgG2a control was positive for 10.8±4.5% and IgG1 control was positive for 15.1±5.8% (n=3; Figure 3F).

**STBEV-eNOS Is Functional and Produce NO**

eNOS dimer (260 kDa) and monomer (140 kDa) were separated and visualized on Western Blot from ex vivo–derived STBMV and STBEX (n=3; Figure 4A), suggesting STBMV- and STBEX-bound eNOS are active. This was confirmed by demonstrating increasing dose-dependent NOS activity in terms of nitrite accumulation per hour (n=6 pooled STBEV; **P<0.001; Figure 4B) as the protein concentration of STBMV and STBEX was increased. Nitrate accumulation was reduced in STBMV and STBEX incubated with L-NAME (an eNOS inhibitor) compared with control (STBMV, *P<0.05; STBEX, *P<0.05, n=3; Figure 4C). STBMV and STBEX incubated with selective iNOS inhibitor N-(3-(aminomethyl)benzyl) acetamidine) (2 μM) showed no change compared with control (both ns, n=3; Figure 4D).

**Ex Vivo STBEV-eNOS Reduced Activity in Preeclampsia**

NOS activity was compared between STBEV from NP (n=11) and preeclampsia (n=8) samples. For STBMV, the overall data showed no statistical difference in NOS activity (Figure 5A). When we analyze the same data taking into account gestational age (Figure 5B), we are able to see that preeclampsia samples from patients with a gestational age most similar to the available controls show a reduction in NOS activity (>34 weeks preeclampsia versus <40 weeks NP samples; *P<0.05). The earlier gestational ages are more difficult to interpret because of the lack of age-matched NP controls.

The STBEX showed an overall significant reduction in NOS activity (*P<0.05; Figure 5C), which persisted when we compared preeclampsia samples from patients with a gestational age most similar to controls (Figure 5D).

Taking STBMV and STBEX together, there is an overall reduction in NOS activity in preeclampsia compared with control.

**Discussion**

We have demonstrated for the first time that eNOS is coexpressed with PIAP, the syncytiotrophoblast marker, on both STBMV and STBEX isolated from ex vivo NP placentas by Western blot, flow cytometry, and paramagnetic immunoprecipitation. Ex vivo–derived STBMV- and STBEX-bound eNOS exists as dimers, a feature required for NOS catalytic activity, and confirmed that these are capable of producing NO, which can be inhibited by L-NAME (an eNOS inhibitor). In addition, flow cytometry evaluation of in vivo–derived circulating STBMV from matched PB and UV plasma revealed that STBMV-eNOS are released by syncytiotrophoblast and circulate in the maternal blood.

NO plays an important role in mediating NP vasodilatation, while defective endothelial NO synthesis and bioavailability has...
been associated with preeclampsia. This is the first study to reveal ex vivo–derived STBMV and STBEX isolated from placental perfused lobes to have less eNOS activity in preeclampsia in comparison to controls. Similarly, in vivo–derived plasma STBMV analyzed by flow cytometry showed less STBMV-bound eNOS expression in preeclampsia compared with NP.

We were unable to observe iNOS expression in NP syncytiotrophoblast and its derived STBEV either by Western blotting or mass spectrometry. iNOS expression and activity has been described by some in syncytiotrophoblast and placenta lysate. iNOS differences between normal and hypertensive pregnancies have been reported, while other studies have reported the absence of iNOS in the syncytiotrophoblast and a debatable relationship between iNOS and preeclampsia. Nevertheless, we think eNOS is the principal isoform bound to STBMV and STBEX.

Several studies corroborate our findings. It has been reported that isolated EV from plasma exert various effects

Figure 3. Immunobead depletion and nanoparticle tracking analysis (NTA) profiles of syncytiotrophoblast extracellular microvesicles (STBMV) and syncytiotrophoblast extracellular exosomes (STBEX) pools from normal pregnancy (NP; n=3). A. Representative immunoblot showing endothelial nitric oxide synthase (eNOS) and placental alkaline phosphatase (PIAP) coexpression on STBMV pool (Total) and STBMV pulled out (PO) with anti-eNOS Dynabeads, anti-PIAP Dynabeads, anti-IgG2a Dynabeads (isotype control for eNOS), and anti-IgG1 Dynabeads (isotype control for PIAP). B. Representative NTA size vs number profiles of STBMV pool (solid line), supernatant from post-incubation with anti-eNOS Dynabeads (dashed line) and anti-PIAP Dynabeads (dotted line). C. STBMV pool (solid line), supernatant of post incubation with anti-IgG2a Dynabeads (dashed line) and anti-IgG1 Dynabeads (dotted line). D. Representative immunoblot showing eNOS and PIAP coexpression on STBEX pool (Total) and STBEX PO with anti-eNOS, anti-PIAP, anti-IgG2a, and anti-IgG1 Dynabeads. E. Representative NTA size vs number profiles of STBEX pool alone (solid line), supernatant from post-incubation with anti-eNOS (dashed line) and anti-PIAP (dotted line) Dynabeads. F. STBEX pool (solid line), supernatant of post incubation with anti-IgG2a (dashed line) and anti-IgG1 (dotted line) Dynabeads.
Figure 4. Syncytiotrophoblast extracellular microvesicles (STBMV) and syncytiotrophoblast extracellular exosomes (STBEX) express functional endothelial nitric oxide synthase (eNOS) and produce nitric oxide (NO).

A, Dimerization of syncytiotrophoblast extracellular vesicles (STBEV) immunoblot image showing eNOS dimer (260 kDa) and eNOS monomer (140 kDa) expressed in human umbilical vein endothelial cells (HUVEC), STBMV, and STBEX (n=3). B, STBMV (**P<0.001) and STBEX (**P<0.001) pools showed NO production in a dose-dependent manner (n=6). C, 25 μg pool of STBMV and STBEX preincubated for 1 hour with 1 mmol/L of N\textsuperscript{G}-nitro-l-arginine methyl ester (L-NAME), NOS inhibitor, showed significant reductions in NO production compared with controls (both *P<0.05; n=3). D, 25 μg pool of STBMV and STBEX preincubated for 1 hour with 2 μmol/L N-(3-(aminomethyl)benzyl)acetamidine) (1400W), inducible nitric oxide synthases (iNOS)–specific inhibitor, showed no changes in NO production compared with controls (both ns; n=3).
on endothelial and trophoblast cells according to the physiological/pathological state of the pregnant woman. A decrease in expression and activity of eNOS bound to circulating nonpregnant EV has also been associated with cardiovascular diseases. Kao et al showed a significant increase in superoxide levels and impaired endothelial dysfunction in rat uterine arteries incubated with human preeclampsia plasma. Interestingly, L-NAME abolished this uterine artery vasodilation in both NP- and preeclampsia-derived plasma-treated vessels, suggesting that this effect was because of eNOS. Their findings support ours, although circulating factors rather than STBEV were described.

We hypothesize that decreased systemic eNOS in the form of circulating STBEV-eNOS may contribute to the reduced bioavailability of NO seen in preeclampsia, possibly affecting vascular functions. We are uncertain as to whether eNOS is donated to endothelial cells or whether NO is produced close to the endothelial cell and work to elucidate this is ongoing. We therefore interpret our data with some caveats. Although we show decreased NO production by STBEV-eNOS, there may also be a few other factors that contribute to the reduction of NO bioavailability. There may be (1) variation in NOS gene expression and activity; (2) lower substrate levels for NOS; (3) elevated inhibitors of NOS; or (4) increased breakdown of NO per se. Decreased NO release would not necessarily be limited by the shedding of less STBEV-eNOS from placenta. Other cell types expressing eNOS, such as platelets, endothelial progenitor cells, or circulating EV derived from endothelial cell and red blood cell, may contribute to overall NO bioavailability.

Our study has some limitations. Exosomes cannot be readily examined by routine flow cytometry because their size is below the limit of resolution. Thus, we were unable to measure STBEX-bound eNOS expression as we did with STBMV. Notwithstanding, we did see decreased levels of NO production in preeclampsia-derived STBEX. It is difficult to age match placental tissue in a disease which typically occurs earlier in pregnancy. We have attempted to mitigate this by using placenta from patients who developed preeclampsia later in pregnancy, but this limitation is common to all preeclampsia studies. Also, despite measuring STBMV-bound eNOS expression in plasma, attempts to measure NO production from plasma-derived STBMV and STBEX were unsuccessful because we were below the NOS assay limits of detection.

In conclusion, our data show that both STBMV and STBEX carry functionally active eNOS. Also, iNOS is not expressed in either STBMV or STBEX, thus, making STBEV-eNOS the principal moiety. Furthermore, STBEV-eNOS activity is reduced in preeclampsia, significantly in STBEX-bound eNOS. These findings suggest that STBEV-eNOS might contribute to the overall decreased NO bioavailability seen in preeclampsia.
Perspectives
This study demonstrates that STBEV carry active eNOS into the maternal circulation. Importantly, the lower circulating STBEV-eNOS seen in preeclampsia might contribute to the diminished NO reported in this disease.

Acknowledgments
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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- Ex vivo and in vivo circulating–derived placental extracellular vesicles express functional endothelial nitric oxide synthase (eNOS), not inducible nitric oxide synthase.
- The plasma circulating levels of syncytiotrophoblast extracellular microvesicles–bound eNOS is significantly reduced in patients with pre-eclampsia compared with gestational age–matched controls.
- The circulating syncytiotrophoblast extracellular microvesicles–bound eNOS is produced by the placenta and elevated in uterine vein blood compared with peripheral blood.
- Ex vivo syncytiotrophoblast extracellular microvesicles and syncytiotrophoblast extracellular exosomes–bound eNOS activity is significantly decreased overall in pre-eclampsia compared with controls.

**What Is Relevant?**

- Preeclampsia is a major cause of maternal morbidity and fetus mortality.
- Decreased circulating syncytiotrophoblast extracellular vesicles–bound eNOS might contribute to the overall decreased nitric oxide bioavailability seen in preeclampsia.
- Placental syncytiotrophoblast extracellular vesicles may play a role in signaling from the placenta to the maternal vascular system.

**Summary**

The presence of trophoblast-derived circulating eNOS potentially links abnormal placental function to altered maternal vascular status.
Placental Vesicles Carry Active Endothelial Nitric Oxide Synthase and Their Activity is Reduced in Preeclampsia

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ONLINE SUPPLEMENTARY DATA

PLACENTAL VESICLES CARRY ACTIVE ENDOTHELIAL NITRIC OXIDE SYNTHASE AND THEIR ACTIVITY IS REDUCED IN PREECLAMPSIA

Authors

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Short title: Reduced eNOS activity in STBEV from PE patients

Keywords: preeclampsia, hypertension, nitric oxide, eNOS, syncytiotrophoblast extracellular vesicles

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SUPPLEMENTARY METHODS

Cell Culture

HUVEC were isolated from NP umbilical cords, as previously described \(^1\), grown at 37°C with 5% v/v CO\(_2\) using endothelial cell medium (EGM-2 SingleQuot Kit CC-4176, Lonza). Cell lysates were obtained with cold RIPA buffer, subjected to BCA protein assay and stored at -20°C.

Immunohistochemical Staining

Placental tissues were fixed in 4% v/v formaldehyde, embedded in paraffin blocks, cut in 8µm thick sections and placed on slides. Slides were deparaffinized in Histo-clear (Company), rehydrated in graded ethanol, and antigens were retrieved to enable detection using 0.01M Citrate buffer. Endogenous peroxidase was reduced with 3% v/v hydrogen peroxide. Tissue sections were blocked in 10% v/v fetal calf serum (FCS) for 1 h. Slides were incubated overnight at 4°C with primary monoclonal antibodies against: eNOS (0.6 µg/mL, NOS3-A9 Santa Cruz), eNOS isotype control IgG2a (0.6 µg/mL, Clone DAK-GO5, Dako), iNOS (1 µg/mL, clone 2D2-B2, R&D) and iNOS isotype control IgG1 (1 µg/mL, IgG1 Clone MOPC-21, BioLegend in 1% v/v FCS in PBS with 0.01M Tween 20 (PBS-T) overnight at 4°C. All sections were then incubated with of 0.2 µg/mL anti-mouse IgG secondary antibody conjugated to
horseradish peroxidase (HRP) in 10% v/v FCS for 1 h at room temperature. After washing with PBS-T, Antigen-specific detection was revealed using DAB kit (Vector laboratories) and counterstained with Shandon Gill 2 Haematoxylin (ThermoFisher). The slides were dehydrated in graded ethanol and mounted with Depex (VWR). Sections were viewed under a Leica DM2500 optical microscope (Leica Microsystems), and photographed using a digital camera linked to a computer hard drive (Micropublisher 5.0 RTV).

Flow Cytometry Analysis

Placental Perfusion Derived STBMV

Multi-colour flow cytometry panel was used to analyse placental perfusion derived STBMV. Flow cytometer and settings used for analysis were the same as previously described\(^2,^3\), to allow comparison between different samples. Fluorochrome compensation was set using BD CompBeads (BD Biosciences), REA CompBeads (Miltenyi Biotech) and a single stain using Bio-Maleimide labelled \textit{ex vivo} STBMV. STBMV were first incubated with 10 µL of FcR blocking reagent for 10 mins at 4ºC prior to labelling with antibodies and isotope matched control antibody for 15 mins at room temperature in the dark. The EV marker Bio-maleimide, which stains membrane proteins, was used to confirm biological material. CD41 and CD235a/b were used to exclude platelet and red blood cell (RBC) EV contamination, respectively. The STBEV specific marker PLAP was used to confirm placental origin of the vesicles. Anti-human eNOS-APC conjugated was used to detect eNOS. The corresponding isotope controls were used as Fluorescence minus one (FMO) controls to discriminate true events from noise and set control gates. (Antibodies’ details in Online Data Supplement Table S1).

Platelet Free Plasma (PFP) derived STBMV

Circulating STBMV from PB and UV were analysed by flow cytometry using a previously described protocol and flow cytometer settings\(^4\), with additional modifications to exclude potential non-placental EV contaminants. Peripheral vein blood (PB) and uterine vein blood (UV) PFP samples were thawed in a water bath at 37 ºC. 200µl from each plasma sample was labelled with the 'Dump Channel' antibodies. 'Dump Channel' includes contaminant markers against Classical HLA class I and II (leukocytes), CD235a/b (RBC) and CD41 (platelet) all conjugated to PEvio770 (Miltenyi Biotec). Sample was also stained against PLAP-PE (STB marker) and eNOS-APC for co-expression analysis. (See Online Data Supplement Table S1 for antibodies’ details.) Plasma sample were incubated for staining for 15 min at 4ºC in the dark. Samples were passed through a durapore-PVDF 0.22µm filter (Ultrafree-MV-GV, Millipore) by centrifugation at 800g for 3 mins (5430R, Eppendorf) in order to concentrate the EV > 200 nm on the filter membrane. The filtrate (pass through) was topped up to 800 µL with sterile PBS (1/5 dilution). The EV (on the filter membrane) were carefully resuspended using 100 µL filtered PBS and stained in the dark with 10 µL Bio-maleimide for 10 min at room temperature. After staining, the EV sample was topped up with filtered PBS up to 500 µL (1/5 dilution), and then analysed for 10 min of data acquisition on the flow cytometer. EV sample was then incubated with 20 µL (1/25 dilution) of neat detergent Nonidet P-40 detergent (Sigma) for 20 min in the dark (to disrupt the vesicle membranes). The EV sample-treated with detergent, together
with the filtrate (pass through), were also analysed for 2 min data acquisition on the
flow cytometer and used to set up the control gates.

Plasma derived STBMV data was then analysed using the following gating strategy
(see Online Data Supplement Figure S3). The filtrate sample (pass through) was used
to set up the ‘Dump Channel’ control gate at 1% cut off to separate EV population
positive for Bio-Maleimide and negative for ‘Dump Channel’ contaminants. The EV
sample-treated with detergent was used to set up the PLAP+ eNOS+ control gates at
1% cut off. Once control gates were set up, plasma derived STBMV double positive
(PLAP+ eNOS+) events/mL were calculated taking into account the dilution factor of
EV sample (1/5) and to standardise all samples to 1mL. The following formula we
used:

\[
\text{PLAP+ eNOS+ STBMV events/mL} = (\text{PLAP+ eNOS+ EV events x 5}) \times 5
\]

**Western Blotting**

HUVEC lysate was used as a positive control for eNOS. Murine macrophage cells
(RAW 264.7 cell lysate; Santa Cruz Biotech) were used as positive control for iNOS
(30ug protein in all samples). (Antibody details below in Table S1). Reactions were
visualized by using an appropriate secondary antibody conjugated to horseradish
peroxidase (HRP) before incubation with HRP substrate enhanced luminescence
(ThermoFisher).

**Co-Immunoprecipitation using Magnetic Dynabeads**

To calculate STBMV or STBEX particle number bound to antibody coated beads,
STBMV or STBEX without Dynabeads (Total) and nominal unbound STBMV or
STBEX fraction left in supernatant (eNOS and PIAP negative) were analysed using
the NTA. The percentage of STBMV or STBEX bound eNOS or PIAP was calculated
as follows: eNOS and PIAP positive = (([Total] - (eNOS and PIAP negative)) / [Total]) \times
100.
REFERENCES FOR SUPPLEMENTARY METHODS


## SUPPLEMENTARY TABLES

<table>
<thead>
<tr>
<th>Antibody/Dye</th>
<th>Concentration /Dilution</th>
<th>Antigen</th>
<th>EV Specificity</th>
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<td>Anti-eNOS (NOS3-A9)</td>
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<td>R&amp;D</td>
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<td>In-house antibody</td>
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<td>Exosomes</td>
<td>Cell Signalling</td>
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<td>Abcam</td>
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<td><strong>Flow Cytometry</strong></td>
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<td><strong>STBMV Analysis</strong></td>
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<td>Bio-maleimide (BODIFY FL N-(-2-aminoethyl)-maleimide</td>
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<td>All EV</td>
<td>Molecular probes</td>
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Table S1. Antibodies, fluorescent labels, isotype controls and secondary antibodies used for Western blot analysis, immunohistochemistry and flow cytometry.

<table>
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<tr>
<th>NOS Isoforms</th>
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<td>/</td>
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<td>73.98</td>
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<td>PL</td>
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<td>/</td>
<td>/</td>
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<tr>
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<td>STBMV</td>
<td>/</td>
<td>/</td>
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<tr>
<td></td>
<td>STBEX</td>
<td>/</td>
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Table S2. Nitric oxide synthases (NOS) isoforms identified by mass spectrometry analysis of placental lysate (PL), syncytiotrophoblast microvesicles (STBMV) and exosomes (STBEX) samples from 8 normal (NP) and 6 preeclamptic (PE) pregnancies. eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase.
**Figure S1.** Characterisation of normal pregnancy (NP) derived syncytiotrophoblast microvesicles (STBMV) and exosomes (STBEX). **A,** Representative Immunoblot showing an enrichment of STBEV marker, PLAP (60 KDa) on NP placental lysates (PL) and STBEV; and an enrichment of exosomal markers Alix (96 KDa), Syntenin (60 KDa) and CD9 (24 KDa) on syncytiotrophoblast exosomes (STBEX). **B,** Representative Nanoparticle Tracking Analysis (NTA) size vs particle number distribution profiles of STBMV (323.2 ± 7 nm) and STBEX (189.3 ± 9.7 nm). **C,** Representative Transmission Electron Micrographs (TEM) of STBMV and STBEX. Scale bars represent 200 nm.
Figure S2. Representative flow cytometric multicolour gating strategy used to analyse STBMV. A, EV displayed on Forward Scatter (FSC) versus Side Scatter (SSC) plot with 1µm cut off gate, ≤ 1µm EV displayed on SSC vs Bio-maleimide (BioM) plot and stained with: (B) Bio-Maleimide fluorochrome minus one (FMO) to draw 1% cut off gate for BioM positive; and (C), BioM (EV marker) to include BioM positive
EV population. CD235a/b-PECy5 vs CD41-PECy7 plots displaying ≤ 1µm BioM positive EV stained with: (D) CD235a/b FMO to draw 1% cut off gate for CD235a/b positive (Q1 and Q2); (E) CD41 FMO to draw the 1% cut off gate for CD41 positive (Q2 and Q3) and (F) CD235a/b and CD41 contaminant markers to exclude EV positive for CD235ab and CD41 (Q1, Q2 and Q3). ≤ 1µm BioM positive and negative for CD235a/b and CD41 EV displayed in a PLAP-PE vs eNOS-APC plot stained with: (G) PLAP FMO to draw 1% cut off gate for PLAP positive (Q1 and Q2); (H) eNOS FMO to draw 1% cut off gate for eNOS positive (Q2 and Q3) and (I) PLAP (STBMV maker) and eNOS (antigen of interest marker) to show final analysis of double positivity for PLAP and eNOS (Q2) on STBMV.
Figure S3. Representative flow cytometric multicolour gating strategy used to analyse STBMV derived from platelet free plasma (PFP). A, The filtrate sample (pass through) used to draw the ‘Dump Channel’ gate at 1% cut off using Dump channel-PeCy7 vs. Bio-Maleimide-FITC dot plot. Dump Channel includes contaminant markers such as CD231a/b, CD41, HLA-ABC and HLA Class II; all conjugated with PEvio770 labelling. B, Dump channel negative and Biomaleimide positive STBMV stained sample was treated with detergent to draw the 1% cut off gates for PLAP+ (Q1 and Q2) and eNOS+ (Q2 and Q3), using PLAP-PE vs eNOS-APC dot plot. C, ‘Dump Channel’ positive population from the STBMV sample was excluded (outside dump channel gate), while ‘Dump Channel’ negative EV and Bio-Maleimide positive EV was included (inside dump channel gate). D, STBMV population double positive for PLAP and eNOS (Q2) showed circulating plasma.
derived STBMV co-expressed PLAP and eNOS. STBMV event number per mL was calculated.