Hypoxia-induced small extracellular vesicle proteins regulate proinflammatory cytokines and systemic blood pressure in pregnant rats.

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ABSTRACT

Small extracellular vesicles (sEVs) released from the extravillous trophoblast (EVT) are known to regulate uterine spiral artery remodeling during early pregnancy. The bioactivity and release of these sEVs differ under differing oxygen tensions and in aberrant pregnancy conditions. Whether the placental cell-derived sEVs released from the hypoxic placenta contribute to the pathophysiology of preeclampsia is not known. We hypothesize that, in response to low oxygen tension, the EVT packages a specific set of proteins in sEVs and that these released sEVs interact with endothelial cells to induce inflammation and increase maternal systemic blood pressure. Using a quantitative MS/MS approach, we identified 507 differentially abundant proteins within sEVs isolated from HTR-8/SVneo cells (a commonly used EVT model) cultured at 1% (hypoxia) compared with 8% (normoxia) oxygen. Among these differentially abundant proteins, 206 were upregulated and 301 were downregulated ($p < 0.05$), and they were mainly implicated in inflammation-related pathways. *In vitro* incubation of hypoxic sEVs with endothelial cells, significantly increased ($p<0.05$) the release of GM-CSF, IL-6, IL-8, and VEGF, when compared to control (*i.e.,* cells without sEVs) and normoxic sEVs. *In vivo* injection of hypoxic sEVs into pregnant rats significantly increased ($p < 0.05$) mean arterial pressure with increases in systolic and diastolic blood pressures. We propose that oxygen tension regulates the release and bioactivity of sEVs from EVT and that these sEVs regulate inflammation and maternal systemic blood pressure. This novel oxygen-responsive, sEVs signaling pathway, therefore, may contribute to the physiopathology of preeclampsia.

List of abbreviations

Small extracellular vesicles (sEVs); Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH); mass spectrometry (MS/MS); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Interleukin 6 (IL-6); Interleukin 8 (IL-8); Vascular endothelial growth factor (VEGF); extravillous trophoblast (EVT); Preeclampsia (PE); small non-coding RNA (miRNAs); antiphospholipid antibody (aPL); syncytiotrophoblast derived extracellular vesicles (STBEVs); cluster of differentiation 63 (CD63); Tumor susceptibility gene 101 (TSG101); Code of Federal Regulation (CFR); National Association of Testing Authorities (NATA); A Short Tandem Repeat (STR) ; Deoxyribonucleic acid (DNA); Phosphate-buffered saline (PBS); size-exclusion chromatography (SEC); Gene Set Enrichment Analysis (GSEA); Ingenuity Pathway Analysis (IPA); Information-dependent acquisition (IDA); Placental growth factor (PlGF); Soluble fms-like tyrosine kinase-1 (sFLT-1); endothelial nitric oxide synthase (eNOS); nitric oxide (NO); Soluble endoglin (sEng); Glucocorticoids (GC); Human umbilical vein endothelial cell (HUVECs)

Clinical Perspectives:

- Preeclampsia is a common obstetric complication that results in significant maternal and neonatal morbidity and mortality. The underlying pathophysiology of preeclampsia is poorly understood.

- Low oxygen tension (i.e., hypoxia) predominates in preeclamptic placentae and drives the excessive release of small extracellular vesicles (sEVs), thought to be exosomes, into the maternal circulation, causing vascular endothelial cell dysfunction.

- This study demonstrated that hypoxia modified the content and bioactivity of sEVs in vitro and in vivo, leading to inflammation and an increase in systemic blood pressure in pregnant rats, mimicking the hypertensive changes seen in preeclampsia.

- Our finding suggests that the extracellular trophoblast derived sEVs might have a role in the pathophysiology of preeclampsia.

INTRODUCTION

Optimal pregnancy outcome is dependent upon successful fertilization, endometrial implantation, and placentation to support blastocyst development (1). Extravillous trophoblast plays a significant role in establishing feto-maternal circulation via remodeling of the uterine spiral arteries and placentation (2). During early pregnancy (< 10-12 weeks), endovascular extravillous trophoblasts occlude uterine spiral arteries to maintain a low oxygen environment (~2-3% O_2), which is essential for normal embryogenesis and organogenesis (2). Subsequently, extravillous trophoblast replaces the vascular endothelial and smooth muscle cells to remodel the uterine spiral arteries with the formation of high capacitance and low resistance vessels, enabling adequate placental perfusion (3). In addition, extravillous trophoblast invades the uterine glands and veins and connect all these luminal structures to form the inter-villous space (4). When extravillous trophoblast invasion fails to occur or is dysfunctional, uterine spiral arterial remodeling is inadequate, and placental function is suboptimal, resulting in placental hypoxia and the development of pregnancy pathologies such as preeclampsia (5).

Preeclampsia affects approximately 8% of pregnancies worldwide and is recognized to cause 60,000 maternal deaths and 500,000 neonatal deaths from preterm delivery each year (6). This condition is characterized as early-onset (that develops before 34 weeks of gestation), or late-onset (develops at or after 34 weeks of gestation). Early-onset preeclampsia is associated with impaired spiral artery remodeling and placental ischemia and excessive release of bioactive molecules implicated in the development of maternal vascular dysfunction (7, 8).

Cell-to-cell communication between the placental and maternal tissues is essential for the establishment of normal pregnancy. In recent years, the role of extracellular vesicles (EVs) and, in particular, small EVs called exosomes in cell-to-cell communication has been recognized (9). Exosomes are nanometer-sized lipid-bilayer extracellular vesicles that contain bioactive molecules, including proteins, lipids, and small non-coding RNAs (*e.g.,* miRNAs). They are released from a wide range of cells (including placental cells) and are taken up by target cells to modify their functions. Exosomes have been identified as important mediators in feto-maternal communication (10, 11)

The concentrations of circulating exosomes in plasma are higher in pregnant compared with non-pregnant women (12). Exosomes are released from placental cells (*e.g.,* syncytiotrophoblasts) into the maternal systemic circulation as early as 6 weeks of gestation (13), and their concentration increases through gestation (12). Interestingly, higher concentrations of placental exosomes in maternal circulation are associated with complications of pregnancies, such as preeclampsia (14), gestational diabetes mellitus (15), intrauterine growth restriction (16), preterm birth (17) and maternal obesity (18) compared with the concentrations observed during normal pregnancy.

The potential role of exosomes in the development of preeclampsia has been investigated by determining the effects of placenta-derived exosomes on various target cells. Hypoxia increases the release of exosomes from placental cells and exosomes isolated from cells incubated under low oxygen tension induce the release of pro-inflammatory cytokines and decrease cell migration in their target cells (19,

20). Interestingly, the miRNA content of exosomes isolated from HTR-8/SVneo cells (commonly used EVT model) changes in response to low oxygen tensions and regulate endothelial and vascular smooth muscle cell migration (21). These data support a role for exosomes from EVT in the remodeling of uterine spiral arteries under both normal and pathological pregnancies (5, 21, 22).

The capacity of exosomes to induce changes in the target cells is mediated by the specific delivery of bioactive molecules, such as proteins and miRNAs (23, 24). Recently, using a longitudinal study design, we reported that the miRNA content within exosomes changes in preeclamptic compared to normotensive pregnancies (14). In addition, oxygen tension regulates the miRNA profile of EVT-derived exosomes (21). The biological effects of oxygen tension on the protein profile of EVT-derived exosomes, however, have yet to be described. Sammar *et al*. investigated the level of expression of placental protein 13 in syncytiotrophoblast-derived extracellular vesicles (STBEVs) isolated from preeclamptic and normal pregnancy placental perfusate and reported low expression in preeclamptic placentae (25). Tong *et al.* described a novel mechanism by which placental extracellular vesicles can attenuate the pathogenesis of preeclampsia in the presence of antiphospholipid antibody (aPL) that can induce the synthesis of toll-like receptors on placental extracellular vesicles to increase the level of expression of mitochondrial DNA in these vesicles (26). Thus, these data suggest that placenta-derived EVs are involved in gene regulation, placental homeostasis, and cellular function that overall reflect the placental-maternal crosstalk.

Poor placentation associated with a failed invasion of the EVT is a feature of preeclampsia and is associated with hypoxia and oxidative stress. We hypothesize that, in response to low oxygen tension, the EVT packages a specific set of proteins in sEVs and that these released sEVs interact with endothelial cells to induce inflammation and increase maternal systemic blood pressure. To test this hypothesis, small EVs were isolated from a transformed extravillous trophoblast cell line (HTR8/SVneo, commonly used as EVT model) cultured under different oxygen tensions to mimic normal and pathological conditions. sEVs were isolated from HTR8/SVneo cell-conditioned media and the protein profile was identified using quantitative mass spectrometry. The effect of sEVs on the secretion of GM-CSF, IL-6, IL-8, and VEGF from endothelial cells was evaluated. Finally, sEVs were injected in pregnant rats and the systemic blood pressure was monitored. The results obtained in this study are consistent with the hypothesis that oxygen tension regulates the release and bioactivity of sEVs from HTR8/SVneo cells and that these sEVs regulate maternal systemic blood pressure. Extracellular vesicles are a heterogenic population of vesicles, and there is considerable debate about the definition and nomenclature of the different populations of extracellular vesicles. In this study, the term small extracellular vesicles (sEVs) refers to extracellular vesicles with a median diameter of ~ 100 nm, which are CD63 and TSG101 positive and of cup-shape morphology.

METHODS

Cell culture

All experimental procedures were conducted within an ISO17025 accredited National Association of Testing Authorities (NATA, Australia) research facility. All data were recorded within a 21 Code of Federal Regulation (CFR) part 11 compliant electronic laboratory notebook (Lab Archives, Carlsbad, CA 92008, USA). The project was approved by the Human Research Ethics Committees of the University of Queensland and Royal Brisbane and Women Hospital (HREC/11/QRBW/342). The HTR-8/SVneo cell line was kindly provided by Dr. Charles H. Graham (Queen's University, Ontario, Canada). The HTR-8/SVneo cell line was established by the transfection of the first-trimester trophoblasts with the Simian virus 40 large T antigen (27). HTR-8/SVneo cells are commonly used as a model for extravillous trophoblast function. Authentication of HTR-8/SVneo cells was performed with authentication by STR DNA Profiling Analysis. HTR-8/SVneo cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained at 37 ^{0}C and humidified under an atmosphere of 5% CO_2-balanced N_2 and either an 8% or 1% oxygen in an automated PROOX 110-scaled hypoxia chamber (BioSpherics™, Lacona, NY, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS-exosomes depleted for 48h before exosome isolation. Cells were sub-cultured with dissociation media, TrypLE^{TM} Express (Life Technologies, USA), and cellular viability was determined using the Trypan Blue exclusion solution and Countess® Automated cell counter (Life Technologies, USA).

Isolation and characterization of small extracellular vesicles

Small extracellular vesicles (sEVs) were isolated from cell-conditioned media, as previously described with slight modification (21). In brief, cell-conditioned media was centrifuged at 2,000 x *g* for 10 min at 4°C (Sorvall®, high-speed microcentrifuge, 90^0 fixed rotor angle, Thermo Fisher Scientific Inc., Asheville, NC, USA,). The 2,000 x *g* supernatant fluid was then centrifuged at 12,000 x *g* for 15 min at 4°C (Sorvall, high-speed microcentrifuge, 90° fixed rotor angle). The resultant supernatant fluid was filtered through a 0.22 µm filter (Steritop™, Millipore, Billerica, MA, USA) and then subjected to size-exclusion chromatography (SEC). Briefly, Pierce™ Disposable Columns, 10 mL (Thermo Scientific), were packed with 10 ml of Sepharose® CL-2B (Sigma) and sorted overnight at 4°C. The packed bed was equilibrated with ice-cold PBS and topped with a column filter. The 500 µl of the concentrated sample was overlaid on top of the filter and followed by elution with PBS. Five-hundred-microliter of 12 fractions were collected, and particle concentration determined using nanoparticle tracking analysis (NAT, NanoSight). High particle fractions were pooled and stored at -80°C until sEVs analysis. sEVs were characterized by size distribution, the abundance of proteins associated with sEVs (i.e., CD63, sc15363 [1:1000] and TSG101, EPR7130 [1:1000]) and morphology using Nanoparticle Tracking Analysis (NTA), Western blot analysis and electron microscopy, respectively as previously described (16). sEVs were quantified using an electrochemical exosome detection method, as we previously described (28). Samples were suspended in PBS and divided into several aliquots after the isolation and stored immediately at -80°C. To thaw the sEVs, samples were taken out from -80°C and maintained at 4°C in ice until completion of the thawing process. The protein concentration and the number of vesicles were quantified immediately

after the isolation and also after thawing at 4°C to evaluate the stability and yield of the vesicles under the storage conditions. No differences were observed in the protein concentration and yield (i.e., vesicles/protein) after the thawing process. This data is consistent with our previously published studies in which no significant differences were observed using fresh or frozen plasma in exosome quantification, exosomal marker expression, microRNA expression and protein content(13). All samples were stored and thawing with the same procedure, discarding that the differences observed at the endpoint experiments are due differences to stored and thawing protocols.

Quantitative Mass spectrometry analysis of exosomes

In-gel Digestion. A local ion library was generated to use in the Sequential Window Acquisition of All Theoretical mass spectra (SWATH) mass spectra analysis using an in-gel digestion method. Briefly, two protein pools were prepared from exosomes obtained from 8% and 1% oxygen. The samples were mixed with Bolt™ LDS sample buffer (ThermoFisher), sonicated for 5 min and heated at 95°C for 5 min. Samples were resolved on a Bolt™ Bis-Tris Plus polyacrylamide gel (ThermoFisher) at 160 V until full separation. The gel was stained with SimplyBlue™ SafeStain (ThermoFisher Scientific), and a total of 12 gel fractions were excised for each pooled sample. The fractions were washed firstly with 50 mM of ammonium bicarbonate/acetonitrile (ABC/ACN) followed by ACN. 50 µl of 100 mM DTT was added to each sample and incubated at 56°C for 30 min. DTT was removed, and 70 µl of iodoacetamide (IAA) was added and incubated at room temperature (RT) for 20 min. The samples were washed with 300 µl ACN and incubated with 50 mM ABC/ACN for 30 min at room temperature. Then, 300 µl of ACN was added and left for 2 min. ACN was removed and air-dried for 5 min. 50 µl

of 13 ng/µl of trypsin (Promega, Australia) in ABC was added to the alkylated gels and stored on ice for 30 min. Then, 20 µl of 50 mM ABC/H_2O (v/v) was added and incubated overnight at 37°C. Following overnight incubation, the supernatant containing peptides was reserved. A mix of 100 µl of extraction buffer (0.25 ml 5% (v/v) formic acid, 0.25 ml water and 0.5 ml ACN) was added to the gel pieces and sonicated for 10 min. The resulting supernatant fluid was collected and combined with the reserved supernatant fluid. The combined supernatant fluid was dried in a vacuum centrifuge. The dried samples were resuspended in 200 µl 0.1% TFA.

Filter Aided Sample Preparation: For SWATH analysis, individual exosome samples were processed using the Filter Aided Sample Preparation (FASP) method (29). A total of 15 µg of exosome protein from each sample was reduced with an equal volume of lysis buffer containing 8% SDS, 100 mM Tris, pH 7.6, and 0.2 M DTT followed sonication and heating of samples at 95°C, each. Samples were allowed to cool down completely before adding 8 M urea in 100 mM Tris, pH 8.5. Samples were transferred into a Nanosep® filter unit with a 30K molecular weight cut off and centrifuged for 10,000 *g* for 15 min. Then, filter units were washed with 400 µl of urea buffer and centrifuged for 10,000 *g* for 15 min. Samples were alkylated by the addition of 100 µl of 50mM IAA in 8M urea buffer and incubated in the dark for 20 min. The filter units were washed with 8 M urea buffer followed by ABC. Proteins were digested using 0.3 µg of trypsin and incubated overnight at 37°C.

Desalting: The solubilized peptides from pooled and individual samples were desalted using SOLAµ HRP SPE 96 well plate (Thermo Fisher Scientific) according to the manufacturer's instruction. ***Analysis of peptides:*** Tryptic digest was loaded onto a reversed-phase trap column (CHROMXP C18CL 5um, 10 x 0.3mm;

Eksigent, Redwood City) and on-column wash was performed for 15 min (3 ul/min) followed by peptide separation on reversed-phase CHROMXP C18CL 3 um, 120 A^0, 150 x 0.075mm; (Eksigent, Redwood City) analytical column. The LC gradient started with 95% mobile phase A (H_2O/ 0.1% FA), 5% B (ACN/ 0.1% FA) at 0 min and increase to 10% B over for 2 min and then a 58-min linear gradient to 40% B followed by 50% B for 5 min. Mobile phase B was then increased from 50% to 95 % over 10 min, followed by a column wash at 95% B for 15 min and re-equilibrated with 5% Buffer B for 6 min. The flow rate was kept at 250 nl/min during the entire LC run. The resulting peptide samples were processed in IDA on an AB Sciex 5600 TripleTOF mass spectrometer with the top 18 precursor ions automatically selected for fragmentation. The data obtained were combined to establish a peptide ion database. For SWATH acquisition, the TripleTOF® 5600 System was configured as described by Gillet at al. (30). Using an isolation width of 26 Da (25 Da of optimal ion transmission efficiency and 1 Da for the window overlap), a set of 32 overlapping windows was constructed covering the mass range 400 to 1200 m/z.

Data Processing: To generate a local ion library, a protein database search was conducted using the ProteinPilot version 4.5b Software (AB SCIEX) and the Paragon™ Algorithm. The search was performed against the SwissProt Homo sapiens database with a global false discovery rate (FDR) of 1% was used as the threshold for the number of proteins for import. The SWATH Acquisition Microapp 2.0 in PeakView 2.2 (SCIEX) was used to create a spectral library file. This local library was extended using the R package SwathXtend (version 2.3) (31) with a published SWATH dataset of healthy human plasma (32). The extended library was used for all subsequent SWATH analysis. Processing settings for the SWATH Microapp: 2 peptides per protein, 3 transitions per peptide, peptide confidence

threshold corresponding to 1% global FDR and FDR threshold of 1% was used. The retention time was then manually realigned with a minimum of 5 peptides with high signal intensities and distributed along the time axis. The resulting peak area for each protein after SWATH processing was exported to MarkerView 1.3.1 (SCIEX) for statistical analysis. The resulting data were normalized using the Total Area Sums (TAS) approach. The coefficient of variation in the abundance of peptides across the samples was established by comparing SWATH peptide ion against the IDA library. For independent samples, *t*-tests were used to compare protein expression between exosomes from cells cultured to 8% and 1% oxygen. The proteins with $p<0.05$ were considered as statistically significant.

Ingenuity Pathway Analysis (IPA) and Gene set enrichment analysis (GSEA).

IPA (Qiagen, Hilden, Germany) was performed to identify canonical pathways, diseases and functions, and protein networks. Significantly enriched pathways for the proteins and pathways were identified with the criterion p-value < 0.05. To determine the genes associated with changes in the protein in sEVs in response to oxygen tensions, GSEA (version 3.0) was performed. Normalized SWATH results from cells and exosomes were used in the GESA. The protein expression data were processed using the hallmark gene sets within the MSigDB database v6.2 with permutations set at 1000 and Signal2Noise metric for ranking genes. Default values were chosen for all other parameters.

Effect of sEVs on cytokines release from endothelial cells

To determine the effect of exosomes on cytokine release from target cells, exosomes were isolated from extravillous trophoblast cell-condition media and

incubated with cells under either 8% or 1% O_2. sEVs (20, 40, 80 and 100 µg protein/ml equivalent to 1 to 10 x 10^8 vesicles per ml) were then incubated with endothelial cells (HMEC-1, from Lonza) in medium containing 5 mM d-glucose under an atmosphere of 8% O_2 to mimic the physiological conditions for 24 h. Cytokine release, defined as the accumulation of immunoreactive cytokine in cell-conditioned medium, was quantified using a protein solution array assay, as previously described *(20)*.

In vivo experiments

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the University of Wisconsin at Madison. All the animal experiments were performed at the University of Wisconsin-Madison (USA). Timed pregnant Sprague-Dawley rats (day 4 of gestation; copulation plug on day 1; Charles River, Wilmington, MA) were used in the experiment. On the gestational day (GD) 6 (after two days of acclimatization), dams were anesthetized with 2.5% isoflurane, and a flexible catheter attached to a radio transmitter (TA11PA-C10, Data Sciences, and Minneapolis, MN) was inserted into the left femoral artery. After surgery, rats were given housed in individual cages and allowed to recover for a week. On GD 16, dams were randomly divided into 2 groups. Dams in the treatment group were injected intravenously through the tail vein once daily with sEVs from 1% hypoxic group (exosome protein amount-10 µg/day) for 4 days from GD 16–19. The other group received sEVs form 8% normoxic group. A subset of control animals was treated with saline. Blood pressures were recorded continuously from GD 14 until GD 21. Blood pressure measurements obtained with a 10-s sampling period were

averaged and recorded every 10 minutes, 24 hours a day using the software (Dataquest 4.0) provided by the manufacturer. All acquired blood pressure, and heart rate data were averaged into 12-hour blocks paralleling the light-dark cycle.

Statistical analysis

All data are presented as mean ± SEM and calculated using Graph Pad Prism (La Jolla, CA). Repeated measures ANOVA (treatment and time as factors) with a Bonferroni post hoc were used for comparisons of blood pressures between the hypoxic (1% oxygen) and control (8% oxygen) groups. Statistical significance was defined as $p < 0.05$.

RESULTS

Isolation of sEVs from extravillous trophoblasts

sEVs were isolated from HTR-8/SVneo cell-conditioned media and enriched using differential centrifugation and SEC (Figure 1A). The NTA analysis identified vesicles with a diameter between 50 to 150 nm, with enrichment of vesicles of around 100 nm (Figure1B and C), consistent with sEVs. Vesicles were positive for proteins known to enriched in sEVs, *i.e.,* CD63 and TSG101 (Figure 1D). There were no differences in exosome size distribution and abundance of sEVs-associated protein markers between sEVs isolated from EVT cultured under normoxic and hypoxic conditions, indicating that hypoxia does not impact upon the size distribution of sEVs. The morphology and size of the sEVs were confirmed by electron microscopy (Figure 1E). Interestingly, the levels of EVT-derived sEVs from cells cultured under hypoxic conditions were around 3-fold higher ($p<0.05$) compared with the values observed in normoxic conditions (Figure 2A).

Proteomic contents of extravillous trophoblast sEVs

Information-dependent acquisition (IDA) and SWATH profile were generated from sEVs from HTR-8/SVneo cells cultured at 1% (hypoxic) or 8% (normoxic) oxygen concentrations. The IDA library was used to identify peptide ions that were present in SWATH ion profiles. Proteins were identified and quantified by comparing SWATH-generated peptide ion profiles for each sample against the IDA library (PeakView). IDA of mass spectra from sEVs samples was initially performed and identified 727 total proteins (Table S1) and analyzed using IDA and SWATH. To evaluate whether hypoxia changes the protein profile within sEVs from HTR-8/SVneo cells, we

analyzed data using an unsupervised principal component analysis (PCA) with Qlucore Omics Explorer. With the first three PCA components explaining >90% of the total variance, the generated PCA plot revealed that the sEVs from hypoxic and normoxic groups had distinct protein contents (Figure 2B). The variation in the relative abundance of exosomal proteins between sEVs from hypoxic and normoxic cell lines was established by comparison with the SWATH profile against the IDA library and presented as a volcano plot (Figure 2C). A total of 507 statistically significant proteins (206 up- and 301 down-regulated) were differentially expressed. Among all the proteins, alpha-2 macroglobulin, alpha-fetoprotein, apolipoproteins A1 and E, chaperonin, gelsolin, heat shock proteins (Hsp90, Hsp70, Hsp60, and Hsp10), inter alpha trypsin inhibitor, gamma-glutamyl transferase, lactotransferrin, serpin, thrombospondin, tubulin, vitrin, vitronectin, annexin family of proteins, fibronectin, histone, haptoglobin, syndecan-1, galectin 3 binding protein, glyceraldehyde 3 phosphate dehydrogenase, and alpha 2 HS glycoprotein were identified that are likely to be associated with preeclampsia pathogenesis.

To investigate the potential functions of the differentially expressed proteins, pathway analysis of the exosomal proteomic profile was performed. The top canonical pathways identified by IPA are presented in the Figure 3A; with the most significant difference in the sEVs protein profiles between these groups were associated with Eukaryotic Initiation Factor 2 (EIF2; a signaling pathway that activates vascular endothelial growth factor, VEGF signaling, and with glucocorticoid receptor signaling pathway). Interestingly, the majority of the pathways were associated with inflammation, and the top 25 canonical pathways with the common genes (network/overlap) are presented in Figure 3B. Many of the differentially

expressed genes are present in multiple pathways related to inflammation. Finally, GSEA of the total protein profile revealed several gene sets that were significantly enriched in sEVs derived from hypoxic compared with normoxic cells. This is illustrated by the normalized enrichment score. There was an enrichment of proteins involved in MYC targets, hypoxia, and epithelial to mesenchymal transition suggesting that these biological processes might be regulated by the hypoxic sEVs (Figure 3C).

Effect of HTR-8/SVneo cells-derived sEVs on cytokines releases from endothelial cells

The effect of hypoxic and normoxic sEVs on the release of IL-6, IL-8, VEGF, and GM-CSF from endothelial cells is presented in Figure 4. sEVs derived from hypoxic EVT dose-dependently increased ($p < 0.05$) the release of all cytokines from endothelial cells when compared to controls (without sEVs) or sEVs from cells cultured at 8% oxygen (normoxic control).

Effect of EVT-derived exosome in systemic blood pressure in pregnant rats.

The mean litter size and maternal weights were similar between hypoxic (1% oxygen) and control (8% oxygen) groups. Fetal weights (8% O_2: 2.59 ± 0.06 g; 1% O_2: 2.47 ± 0.05 g), placental weights on GD 21 (1%: 0.48 ± 0.09 g; 8%: 0.50 ± 0.05 g) were comparable between the two groups. Rats are nocturnal animals, and continuous monitoring of blood pressure by telemetry revealed a characteristic circadian pattern with higher arterial pressure and heart rate values during the dark cycle (active phase) compared to the light cycle. In animals injected with normoxic sEVs, MAP progressively decreased from GD 16 and reached a nadir on GD21,

which was comparable to the MAP in the saline-injected group. Pregnant rats injected with hypoxic sEVs had significantly higher MAP starting from GD18 to GD21 compared to the respective time point in the control group (Figure 5A; n = 6 rats in each group; $P < 0.05$). The changes in MAP correlated with a significant increase in systolic blood pressures in the hypoxic compared to the control group (Figure 5B; n= 6 rats in each group). The diastolic blood pressure increased only in the later part of gestation (i.e., GD 20-21) in the hypoxic compared to the control group (Figure 5C; n= 6 rats in each group). No differences in heart rate were observed between the hypoxic and control groups (Figure 6; n = 6 rats in each group).

DISCUSSION

The data obtained in this study are consistent with the hypothesis that the protein content of EVT sEVs is programmed by low oxygen tension to be pro-inflammatory (*i.e.*, increasing the release of the IL-6, IL-8, VEGF and CS-GMS from target cells) and to promote hypertension.

The bioinformatic analysis revealed that in normoxic (8% oxygen) conditions, the proteins in EVT sEVs are associated with EIF2 signaling that activates the VEGF signaling pathway. VEGF is a protein mediator that is synthesized and secreted by placental macrophages. VEGF binds as a ligand with the soluble fms-like tyrosine kinase (sFLT-1) receptor (also described as VEGF receptor 1) expressed on the surface of vascular endothelial and smooth muscle cells (33). It also binds with the kinase insert domain (KDR) receptor (also described as VEGF receptor 2), which is expressed only on the surface of vascular endothelial cells (33). Activation of these pathways assists in increasing endothelial cell permeability, migration, proliferation, and survival, ultimately leading to proper angiogenesis of the feto-placental vascular tree and contributing to adequate trophoblast development and placental perfusion

(34). VEGF also mediates vasodilatation and increases vessel permeability via the release of nitric oxide from the uterine arterial endothelial cells in pregnancy (35). In preeclampsia, however, maladaptation occurs due to the altered concentrations of VEGF and PlGF, augmented placental secretion of sFLT-1 and soluble endoglin (sEng), polymorphism in the endothelial nitric oxide gene and and reduced bioavailability of nitric oxide secondary to oxidative stress (36). The consequences are inappropriate angiogenesis, endothelial dysfunction and vasoconstriction leading to inadequate placental perfusion to the fetus and maternal hypertension.

A recent study reported that the gene regulating sFLT-1 receptor (that binds with VEGF) is polymorphic and that some variants increase susceptibility to preeclampsia (37). Virtanen *et al.*, found that the concentrations of angiogenic proteins (VEGF and PlGF) in serum collected from women during the third trimester of uncomplicated pregnancies are increased and that they stimulate angiogenesis. These angiogenic factors were found to be decreased in preeclamptic serum, and they also inhibited tubule formation (38). The findings of our study are consistent with previous data suggesting that VEGF signaling and placental vasculogenesis are regulated by hypoxia (39).

A hypoxic environment (1% oxygen tension for culture) also promotes the packaging of EVT sEVs with proteins involved in glucocorticoid receptor signaling. Glucocorticoids (GC) are steroid hormones that are secreted predominantly from the adrenal gland. These hormones exert diverse effects on vascular function and have an anti-angiogenic effect (40). Ozmen et al. studied the effects of GC on human umbilical vein endothelial cells (HUVEC), where they observed increased expression

of VEGF and VEGFR1 proteins and decreased expression of VEGFR2 protein when HUVECs were treated with GCs (40). Recently, we have reported that the differential expression of exosomal miRNAs in maternal plasma in term and preterm birth are associated with GC receptor signaling (41).

The concentration of maternal cortisol increases with gestation and is significantly correlated with blood pressure rise during pregnancy (42). The GC receptor gene is located on chromosome 5q and encodes a nuclear transcription factor that mediates GC receptor signaling Polymorphism in the (GC) receptor is associated with the development of hypertension (43). Interestingly, genetic variants of stratin, a protein that interacts with the steroid (GC) receptors, is associated with salt-sensitive blood pressure regulation in mice (44). Moreover, the peripheral blood-derived mixed population of exosomal microRNAs can regulate systolic blood pressure in older individuals (45). Placental trophoblast derived exosomal micro RNAs are associated with maternal-fetal immune interaction and the physiologic consequences of placental-maternal communication in a murine model (46). Another study observed that human umbilical cord mesenchymal stem cell-derived sEVs improves the placental tissue morphology in the pregnant rat by inhibiting trophoblast apoptosis and promoting placental angiogenesis (47). These reports support our hypothesis that the proteins encapsulated in sEVs isolated from EVTs cultured under hypoxia can prevent the decrease in blood pressure observed in normal pregnancy mimicking preeclamptic symptoms in an in-vivo model- pregnant rats. This lack of a pregnancy-related fall in blood pressure is considered as a cardinal feature of preeclampsia (48), which is also observed in other rat models of preeclampsia (49, 50). This suggests that the mechanisms controlling blood pressure during pregnancy are perturbed by proteins encapsulated in sEVs isolated from EVTs cultured under

hypoxia. The lack of impact on fetal growth and number in this study suggests that this could be a mild model of preeclampsia. Further studies should examine if higher exosomes concentrations in pregnant rats could negatively impact fetal weight producing a severe form of preeclampsia. The patterns of exosomal protein expression and VEGF and GRS signaling pathways seen in the hypoxic and control groups may give some insights into explaining the hypertensive effect of hypoxia treated EVT sEV proteins.

In this study, we used HTR8/SVneo cell line as EVT model, which is frequently used as a model of physiologically invasive extravillous trophoblast. Previous studies have demonstrated that HTR8/SVneo express KR7, CG, CGR, and HLG (51) consistent with proteins identified in primary EVT. We have previously established that EVT-derived exosomes carry HLA-G (21). However, other studies have reported whether HTR-8/SVneo cells contain a mix of cell populations that differs compared with primary EVT. For example, Abou-Kheir *et al.,* showed that the HTR-8/SVneo cells contained a heterogeneous population of cells including trophoblast and mesenchymal/stromal cells (52). In comparison to other placental cell lines, the abundance of epithelial markers such as cytokeratin 7 (KR7) and e-cadherin was silenced in HTR-8/SVneo cells (52-54) while a higher abundance of vimentin (a marker of epithelial to mesenchymal transition) was observed (52-55). Likewise, Takao *et al.,* observed low expression of HLA-G and integrin alpha-V/beta-3 (56), which are known primary EVT (epithelial) markers (57-59). Furthermore, genome-wide gene expression profiles showed that the molecular signature of HTR8/SVneo cells was vastly different from that of primary EVTs (60). Therefore, results obtained from HTR-8/SVneo cells must be further verified using the appropriate primary EVT cells.

Based on the data obtained, we suggest that hypoxia alters the content of EVT sEVs and that these changes contribute to the physiopathology of preeclampsia. The changes in protein and miRNA expression promote an inflammatory environment within uterine spiral arteries and cause hypertension during pregnancy. These changes are likely to occur in pregnancies characterized by compromised placental perfusion and ischemia (expressed as preeclampsia and intrauterine growth restriction) as an adaptive response aiming to improve placentation.

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Author´s contributions: C.S., S.D. conceived and designed the study. S.K., and C.S. designed the in vivo experiments. S.S., A.L., K.S-R., M.S., Y.Y., and J.M. performed the experiments. S.D. and C.S. wrote the manuscript. C.S., G.R., S.K., and J.H. edited the manuscript. All authors reviewed/edited the manuscript and approved the final version. C.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figures

Figure 1. Characterization of sEVs isolated from HTR-8/SVneo cells**.** sEVs were isolated from cell-conditioned media by differential and ultracentrifugation, followed by size exclusion chromatography. (A) Flow chart for the exosome isolation and enrichment procedure (B and C) Representative size distribution of sEVs in NTA of sEVs isolated from cells cultured at 1% oxygen and 8% oxygen, respectively. (D) Representative Western blot for exosome enriched marker CD63 and TSG101. (E) Electron micrograph of sEVs-exo. In E, scale = 100nm.

Figure 2. Comparison of protein enrichment in sEVs from HTR-8/SVneo cells cultured at different oxygen tensions. (A) Quantification of sEVs particles from HTR-8/SVneo cells cultured under 1% or 8% oxygen presented as the normalized number of particles/10^6 cells/48 h using an electrochemical exosome detection method based on biotinylated anti-CD9 (Abcam) onto the surface of a streptavidin-coated screen-printed carbon electrode (SPCE-STR). (B) principal component analysis (PCA) plot of the protein profile within sEVsfrom extravillous trophoblasts (exo-EVT) cultured at 1% and 8%. (C) Volcano plot showing differentially expressed protein in the hypoxic sEVs compared to normoxic sEVs-exo. The horizontal axis represents the $\log_2$ of fold change and the vertical axis represents *p-value*. The horizontal dotted line shows p= 0.05. Each blue dot represents a protein with blue dots on the right above the dashed line are proteins upregulated while on the left are downregulated in hypoxic sEVs.

Figure 3. Bioinformatic analysis of sEVs. (A) IPA canonical pathway analysis of the protein content within sEVs from hypoxic compared with normoxic. (B) The top 25 canonical pathways selected for finding the genes common in more than one canonical pathway (overlap). (C) sEVs protein signatures were analyzed by GSEA using the gene sets (GSE) derived from HTR-8/SVneo cells cultured under 8% and 1% oxygen.

Figure 4. Effect of sEVs on cytokines secretion from endothelial cells. The concentration of GM-CSF, IL-6, IL-8, and VEGF were quantified in sEVs and endothelial cell-conditioned media using an ELISA kit. sEVs were isolated from HTR-8/SVneo cells cultured at 8% and 1% oxygen and the concentration of IL-6 (A), IL-8 (B), VEGF (C), and GM-CSF (D), were quantified in sEVs. Data represents n=4 well for each point (6 different experiments in duplicate). Values are mean ± SEM.

Figure 5. Mean arterial, systolic, and diastolic pressure in pregnant rats treated with 1% and 8% hypoxic sEVs. (A) Mean blood pressure was continuously monitored *via* telemetry catheters in the femoral artery from gestational day (GD) 14 until GD 21. Mean blood pressures are presented in 12-h intervals showing circadian variation; dark periods are shaded. Data represent the mean ± SEM of measurements in 6 rats in each group. *$p \leq 0.05$ 1% *vs* 8% hypoxic group. (B and C) Mean systolic and diastolic pressure were continuously monitored *via* telemetry catheters in the femoral artery from gestational day (GD) 14 until GD 21. Mean systolic and diastolic pressures are presented in 12-h intervals showing circadian variation; nighttime periods are shaded. Data points represent the mean ± SEM of measurements in 6 rats in each group. *$P \leq 0.05$ *vs* 8% hypoxic group.

Figure 6. Mean heart rate in pregnant rats treated with 1% and 8% hypoxic sEVs. Mean heart rates were continuously monitored *via* telemetry catheters in the femoral artery from gestational day (GD) 14 until GD 21. Mean heart rates are presented in 12-h intervals showing circadian variation; nighttime periods are

shaded. Data points represent the mean ± SEM of measurements in 6 rats in each group.