Placenta 85 (2019) 63–68

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Heparanase regulation of sFLT-1 release in trophoblasts *in vitro*

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ARTICLE INFO

Keywords:
sFlt-1
Heparanase
Trophoblasts
Preeclampsia

ABSTRACT

Introduction: Preeclampsia is a common pregnancy disorder which is characterized by new onset hypertension and endothelial dysfunction. Despite efforts to determine the causal factors of this disease, little progress has been made in discerning the etiology. The hypoxic and ischemic placenta, however, is generally accepted as the source for secreted factors in the maternal circulation, such as sFLT-1, which drive the maternal syndrome.
Methods: Using BeWo placental trophoblast cells, we measured the role of hypoxia on *sFLT*-1 mRNA as well as protein production. We also exposed the cells to treatment with heparin and heparanase inhibitor OGT-2115.
Results: We found that under hypoxic conditions mRNA levels of *sFLT-1* were unchanged compared to normoxic controls. Although the message level did not differ under hypoxic conditions, the sFLT-1 release into the media was significantly greater in hypoxia. Additionally, we found that sFLT-1 is able to bind heparan strands in the extracellular matrix with its heparin binding site. These heparan strands can be cleaved by the extracellular enzyme heparanase. We found that heparanase expression was significantly increased in hypoxia, and inhibiting the actions of heparanase attenuated the release of sFLT-1 into the media.
Discussion: While the placenta remains a source of sFLT-1, the mechanism of increased circulating sFLT-1 may differ than simple upregulation of the protein. These data demonstrate the potential importance of the role heparanase may play in releasing previously made sFLT-1 into the maternal circulation.

1. Introduction

The pregnancy disorder preeclampsia (PE) has been among the leading causes of maternal, perinatal, and fetal death and morbidity for many years [1]. Despite this fact, the pathophysiology of preeclampsia has yet to be elucidated. The ischemic placenta, however, has been shown to play an important role in the development of this disease [2,3]. In response to hypoxia, the placenta secretes factors into the maternal circulation, leading to endothelial dysfunction, which drives the maternal syndrome [2]. Additionally, the placenta has been shown to alter gene expression [4]. It is possible that the change in gene expression in the placenta are contributing to the secretion of factors in the maternal circulation, thereby driving PE.

One of the hallmark characteristics of PE is a rise in the soluble vascular endothelial growth factor (VEGF) receptor FLT-1 (sFLT-1). Increased sFLT-1 in the maternal circulation has been linked to the angiogenic imbalance and subsequent endothelial dysfunction [5]. As mentioned above, preeclamptic pregnancies are believed to be driven by the under-perfused placenta. In response to hypoxia, placental cells have been shown to increase their release of sFLT-1 [6]. Heparanase, an enzyme which functions to cleave heparan sulfate (HS) chains of the extracellular matrix (ECM), has been shown to be among those proteins which are upregulated in the preeclamptic placenta [7,8]. Not only is there more heparanase in placentas of PE patients, but the activity of heparanase is increased [9]. Because the basement membrane of placentae are largely made up of HS rich proteoglycans [10], and HS chains are able to bind sFLT-1 in its heparin binding domain [11], the increase in heparanase expression and activity could be a source of increased sFLT-1 in the maternal circulation.

While the increase in maternal plasma sFLT-1 is widely known, the link to increased heparanase in preeclamptic placentas has not been examined. Using an immortalized, human placental trophoblast cell line (BeWo), we tested the hypothesis that heparan-bound sFLT-1, rather than newly synthesized protein, accounted for the increased sFLT-1 release in response to hypoxia *in vitro* by increased solubilization by heparanase.

2. Materials and methods

Cell culture: These experiments utilized BeWo choriocarcinoma cells (ATCC; Monassas, VA), an immortalized human placental trophoblast-derived cells. Cells were first cultured at room oxygen with

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https://doi.org/10.1016/j.placenta.2019.07.004
Received 15 April 2019; Received in revised form 5 July 2019; Accepted 8 July 2019

complete media and then exposed to a hypoxic environment with either 8% or 1% oxygen to simulate normal [12] or preeclampsia-like placental oxygen tension [13]. Serum-less media (DMEM F-12 50/50 with L-glutamine; Corning; Monassas, VA), to allow for more accurate ELISA and Western blot analysis, was pre-treated for a minimum of 8 h in 8% or 1% oxygen to ensure calibration. This pre-treated media was added to the cells, which were then placed in their respective oxygen conditions for 6, 12, or 24 h. Additionally, several cell samples were given unfractionated heparin 100U/ml (Frasenius Kabi USA; Lake Zurich, IL) or the heparanase inhibitor O-linked *N*-acetylglucosamine transferase (OGT-2115) at 1 μM (R&D systems; Minneapolis, MN). DMSO was used as the vehicle for OGT-2115, and control samples were treated with vehicle. Experiments involving treatment with heparin or OGT-2115 were carried out for 24 h. After the designated exposure time, the media was collected and stored at −80 °C until further analysis. Cells were collected using Trypsin and stored in RNAlater (Ambion; Carlsbad, CA) for three days at 4 °C until further processing.

Quantitative Real-Time PCR: RNA was isolated using the PureLink RNA Mini Kit and the associated protocol was followed (Ambion; Carlsbad, CA). RNA concentration was obtained using the Nanodrop 2000c (Thermo scientific; Rochester, NY). Using the given concentrations, 300 ng of RNA was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific; Rochester, NY) according to instructions. qRT-PCR was performed using the C1000 Touch Thermal Cycler and corresponding CFX96 Optics Module Real-Time system head (Bio-Rad; Hercules, CA). A master mix of Hot Start Taq 2x Master Mix (New England BioLabs; Ipswich, MA), nuclease-free water, and the Taq primer for Heparanase- 1, Heparanase-2, or β-actin (Thermo scientific; Rochester, NY) was made and combined with the cDNA samples. To measure the *FLT1* variants, we designed and validated primers specific to the full length *FLT1* receptor and two of the *sFLT1* variants, the sequences for which are listed below. These two variants, *sFLT1 i13* and *sFLT1 e15a*, are the two most commonly found in the human placenta [14] and referred to here as *sFLT1 V2* and *sFLT1 V3*, respectively. SYBR green master mix (Thermo scientific; Rochester, NY) was utilized for qRT-PCR in samples measuring *FLT1* variants. Each sample was measured in duplicate and normalized to its β-actin expression (dCT). For each gene assessed, the groups were then normalized to the control group (ddCT).

FLT1 (full length)
Forward: GAAATCACCTACGTGCCGGA
Reverse: AGAGCTTTGTACTCGCTGGC
sFLT1 i13 (V2)
Forward: TGGGGAGGGGAGGATGTTAG
Reverse: TAAGGGAGGTGCGTTGAACC
sFLT1 e15a (V3)
Forward: CGAGCCTCAGATCACTTGGTT
Reverse: GTCTTGGCTCTCCAACTAAAGG

ELISA: Released sFLT-1 in media from the cultured cells was measured using a DuoSet ELISA kit (R&D Systems; Minneapolis, MN) specific to human FLT-1. Though this antibody can detect both full length FLT-1 as well as sFLT-1, analyzing media should only detect the soluble forms of the protein. Briefly, a 96-well plate was treated with a Capture antibody for 24 h. The plate was washed with the provided buffer and blocked for one hour with Reagent Diluent. The FLT-1 protein standards and undiluted media samples were plated and incubated for two hours. The plate was washed and FLT-1 specific Detection Antibody was added to the plate for two hours. The plate was washed followed by a 20 min incubation with Streptavidin-HRP. The last wash was performed before addition of the color reagent. After 20 min, the Stop solution was added and the plate was read using the Infinite M200 Pro plate reader and associated Magellan software (Tecan; Grodig, Austria).

Western Blot: Equivalent amounts of serum-less media from cultured cells was loaded with 4x loading dye (BioRad; Hercules, CA). Criterion TGX stain-free gels (BioRad; Hercules, CA) were run at 200 V for approximately 45 min. Wet transfer onto a nitrocellulose membrane was performed at 100 V for approximately 45 min. The membrane was imaged using ChemiDoc MP imager (BioRad; Hercules, CA) to obtain total protein. The membrane was then blocked using Odyssey Blocking Buffer (Li-Cor; Lincoln, NE) for one hour. HPA1 M-45 Primary rabbit antibody (Santa Cruz; Dallas, TX) for heparanase-1 was used at a 1:200 dilution and incubated overnight, followed by three washes of TBS-Tween solution (1% TBS, 0.1% Tween). A donkey anti-rabbit secondary antibody at a 1:15,000 dilution (Li-Cor; Lincoln, NE) was incubated for 45 min before washing the membrane with TBS-T. The membrane was imaged again and analyzed utilizing ImageJ software (NIH).

Immunofluorescence: BeWo cells were grown and treated as stated above, with the exception of growing the cells on a two-chamber glass slide, with one chamber containing OGT-2115 treated cells, and the other containing control cells. Two chamber slides were used, one at 8% and the other at 1% oxygen. After 24 h of these exposures, the media was aspirated and the slides were washed with iced PBS (GE Healthcare Life Sciences; Logan, UT). Cooled methanol was added to the chamber before incubating for five minutes at 37 °C. The methanol was aspirated and the chamber walls were removed from the slide. The slide was washed in iced PBS and blocked with a 1:10 dilution of normal goat serum in PBS-T (PBS and Tween) (Fisher Scientific; Fair Lawn, NJ) solution at 37 °C for 30 min. After aspirating the blocking solution, primary antibody for sFLT-1/FLT-1 was combined with the blocking solution and incubated for one hour at 37 °C. The primary antibody solution was aspirated and the slide was washed three times for five minutes in iced PBS. The secondary antibody was also combined with the serum/PBS-T blocking solution and added to the slide for one hour at 37 °C. The slide was aspirated and washed, followed by the addition of 1:120,000 Hoechst in PBS, which was added for five minutes. After aspirating and three washes in iced PBS, the slide was dried, ProLong Gold AntiFade (Life Technologies; Eugene, OR) was applied and a coverslip placed. The EVOS FL (Life Technologies; Eugene, OR) was used to image the slides and detect the level of fluorescence.

Cell Proliferation Determination: Cell proliferation was determined by performing an MTS assay. Cells were cultured and treated with OGT-2115 as described above in a 96-well plate for 24 h before addition of CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega; Madison, WI) solution. After three hours of incubation, the plate reader above was used to determine the proliferation in the samples, and total absorbance signal was normalized to control levels as a percentage of proliferation.

Statistical Analysis: All statistical analysis was performed using the Prism 7 software (GraphPad). A student's T-test (for two sample groups) or a Two-way ANOVA (four sample groups) was performed with the Tukey post-hoc test and statistical significance was determined by a $p < 0.05$ between the groups.

3. Results

Media sFLT-1 is increased in hypoxia: We first wished to demonstrate sFLT-1 increase that occurs during hypoxia. To accomplish this, we utilized BeWo placental trophoblasts and cultured them in 8% or 1% oxygen for 24 h in order to mimic the oxygen tension in healthy and preeclamptic placentas, respectively. Upon collection of the media, we saw a significant increase in sFLT-1 of the samples cultured in 1% oxygen (6120 ± 415.1 pg/mL at 8% compared with 8492 ± 568.7 pg/mL at 1%; $p < 0.05$) (Fig. 1).

sFLT1 mRNA increases in acute hypoxia but not sustained exposure: In order to see if increased message was responsible for the increased media sFLT-1, qRT-PCR was performed. As shown in Fig. 2, a trend for increased *sFLT1 V2* is observed after 6 h of hypoxia exposure (1.06 ± 0.21 in 8% vs 2.55 ± 0.90 fold change in 1%; $p = 0.12$), but is not different from normoxia expression at 12 or 24 h exposure (2B). In contrast, *sFLT1 V3* mRNA expression remains unchanged in hypoxia

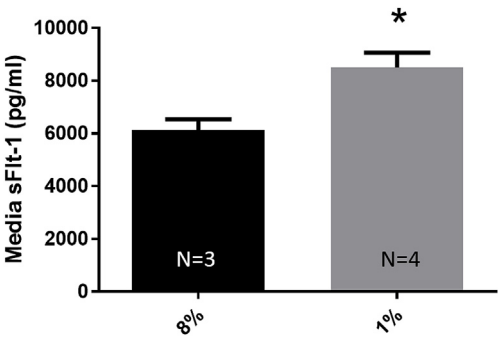

Fig. 1. Media sFlt-1: sFlt-1 in the media collected from cells cultured in 8% and 1% oxygen as measured by ELISA demonstrated a significant increase in the concentration of sFlt-1 in cells exposed to 1% oxygen. *P < 0.05.

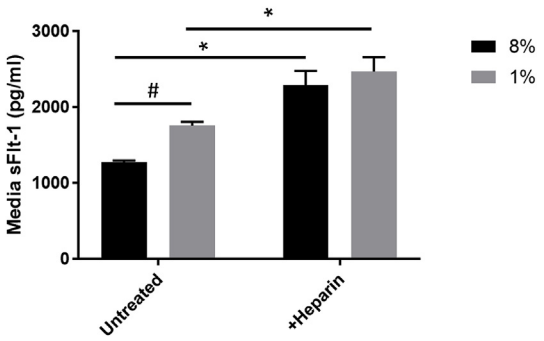

Fig. 3. Heparin treatment increases sFlt-1 release: A significant increase in sFlt-1 release into the media occurs when cells are cultured in 1% oxygen compared to 8%. Additionally, treatment with heparin displaces the sFlt-1 bound to the heparan strands, increasing the media sFlt-1 regardless of oxygen tension. N = 6 in all groups; #P < 0.05; *P < 0.01.

at the 6 h time point, but is significantly increased after 12 h of hypoxia exposure (1.04 ± 0.15 in 8% vs 2.47 ± 0.42 fold change in 1%; p < 0.05), and returns to normal again at the 24 h time point (2C). Because full length *FLT1* was undetectable at the 6 h and 12 h time points, and expression was unchanged in hypoxia compared to normoxia after 24 h, it appears unlikely that expression of the transmembrane receptor is changed in hypoxic conditions (2A).

Treatment with heparin displaces bound sFLT-1: It has been reported that sFLT-1 contains a heparin binding site, and sFLT-1 is able to bind HS chains on extracellular proteins [11]. To examine this relationship, we exposed cultured BeWo placental trophoblastic cells to unfractionated heparin to compete for heparan binding and solubilize membrane-bound sFLT-1. As indicated by ELISA, media sFLT-1 in the heparin treated cells was significantly higher compared to controls in both 8% oxygen exposure (1274 ± 25.19 pg/mL untreated vs 2294 ± 184.9 pg/mL heparin treated; p < 0.01) and 1% oxygen (1758 ± 49.72 pg/mL untreated vs 2472 ± 189.4 pg/mL heparin; p < 0.01) (Fig. 3). This indicated that in both normal and hypoxic conditions, significant amounts of trophoblastic sFLT-1 are bound to HS moieties in the extracellular matrix.

Heparanase acts as a modulator for the release of sFLT-1 in hypoxia: Having found that longer term hypoxia had little effect on sFLT-1 mRNA production and that significant amounts of sFLT-1 were retained in the extracellular matrix of trophoblasts, we hypothesized that extracellular matrix remodeling enzymes could be partially responsible for hypoxia-dependent sFLT-1 release in trophoblasts. One potential candidate for this activity is heparanase, which cleaves the HS chains that bind sFLT-1. To that end, we performed qRT-PCR on BeWo cells cultured in 8% and 1% oxygen to determine levels of HPSE expression. Two forms of heparanase, HPSE1 and HPSE2, were found to be significantly upregulated in cells grown in hypoxia, with greater than three-fold increase in both HPSE1 (1.363 ± 0.55 in 8% vs. 4.298 ± 1.09 fold change in 1%, p < 0.01) and HPSE2 (1.083 ± 0.19 at 8% vs 3.563 ± 0.30 fold change at 1%, p < 0.01) (Fig. 4). Additionally, Western blot analysis of the media confirmed that protein expression of heparanase was increased compared to total protein, with an increase of 56% in 1% cultured media compared to 8% oxygen (7671 ± 582.4 AU in 8% vs 11994 ± 1047 AU in 1%, p < 0.05). This suggested that placental trophoblast heparanase was directly upregulated by hypoxia *in vitro*.

Heparanase inhibition blocks hypoxia-induced sFLT-1 release from trophoblasts: To confirm the role of heparanase in the release of sFLT-1, we utilized the heparanase inhibitor OGT-2115. OGT-2115 has been shown to exhibit heparanase inhibition at levels of 0.4 μM and angiogenesis inhibition was observed at a 1 μM exposure [15]. Therefore, BeWo trophoblasts were cultured in both 8% and 1% oxygen in the presence and absence of 1 μM concentration of this inhibitor. As indicated by ELISA, there was a significant decrease in the released sFLT-1 from cells given the heparanase inhibitor compared to vehicle controls. As shown in Fig. 5, treatment with OGT-2115 ameliorated the hypoxic response to increase sFLT-1 secretion (47.5 ± 4.07 pg/mL untreated vs 21.17 ± 4.36 pg/mL OGT-2115 treated, p < 0.05). While media sFLT-1 in 8% oxygen with the OGT-2115 treatment was not significantly decreased compared to 8% oxygen vehicle controls, sFLT-1 in the media of the treated cells was below the detection limit. To verify that this was not due to changes in cell proliferation, thereby accounting for the decreased sFLT-1 release into the media with OGT-2115 treatment, an MTS assay was performed. While there was a significant decrease in cell proliferation of the OGT-2115 treated samples, this decrease was a mere 7% (100% ± 0.658 control vs 93.5% ± 0.715 OGT-2115 treated, p < 0.01), which is unlikely to account for the observed difference.

Proposed mechanism of sFLT-1 release in hypoxia: In order to complete the picture of our results, we created a diagram to describe the

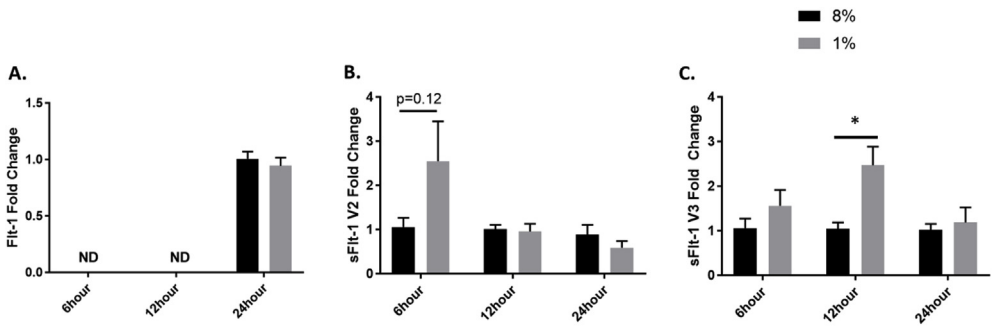

Fig. 2. Expression of FLT variants: Real time PCR was used to examine the expression of full length Flt-1 as well as common sFlt-1 variants. BeWo cells express Flt-1 at low levels, such that it was undetectable in 6 h and 12 h time points, and expression was not changed under hypoxic conditions for 24 h (A). Acute hypoxia exposure lead to a non-significant trend to increase sFlt-1 V2 expression, but prolonged exposure did not alter expression of sFlt-1 V2 (B). Expression of sFlt-1 V3 was not changed in hypoxia exposure of 6 h or 24 h, but was significantly elevated in 12 h hypoxia exposure (C). For 6 and 12 h samples N = 6; for 24 h samples N = 4. The 8% control for each time point was used to calculate the fold change for each FLT variant. *P < 0.05.

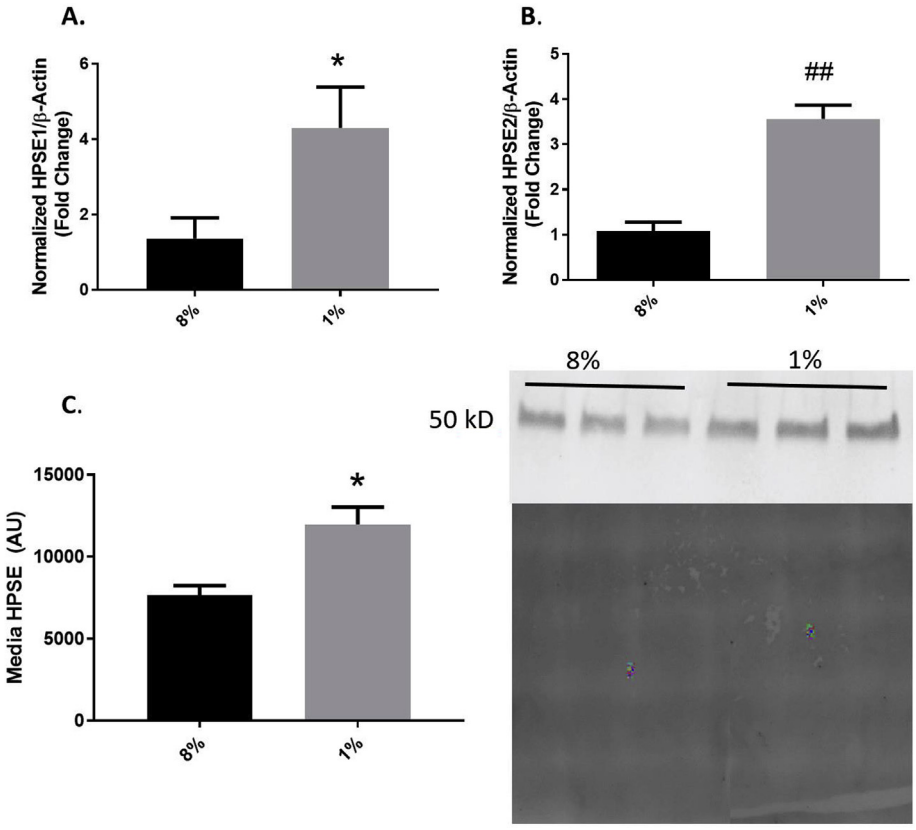

Fig. 4. Media HPSE increases in hypoxia: mRNA expression of HPSE 1 (N = 5) (**A**) and HPSE 2 (N = 6) (**B**) are both increased under hypoxic conditions when normalized to the beta actin expression. Additionally, protein expression of HPSE 1 is increased under hypoxic conditions when media samples are measured by Western blot (N = 3). Transfer from a stain-free gel in order to show total protein in the samples was similar is also pictured with the blot (**C**). *P < 0.05; ##P < 0.001.

mechanism in which increased sFLT-1 is released in hypoxic conditions (Fig. 6). In the panel labeled 8% oxygen, there is relatively little heparanase, and therefore decreased sFLT-1 released from the HS chains on the syndecan-1. The right panel labeled 1% oxygen shows that increased heparanase expression leads to increased cleavage of the HS chains, accounting for increased sFLT-1 release into the surrounding media.

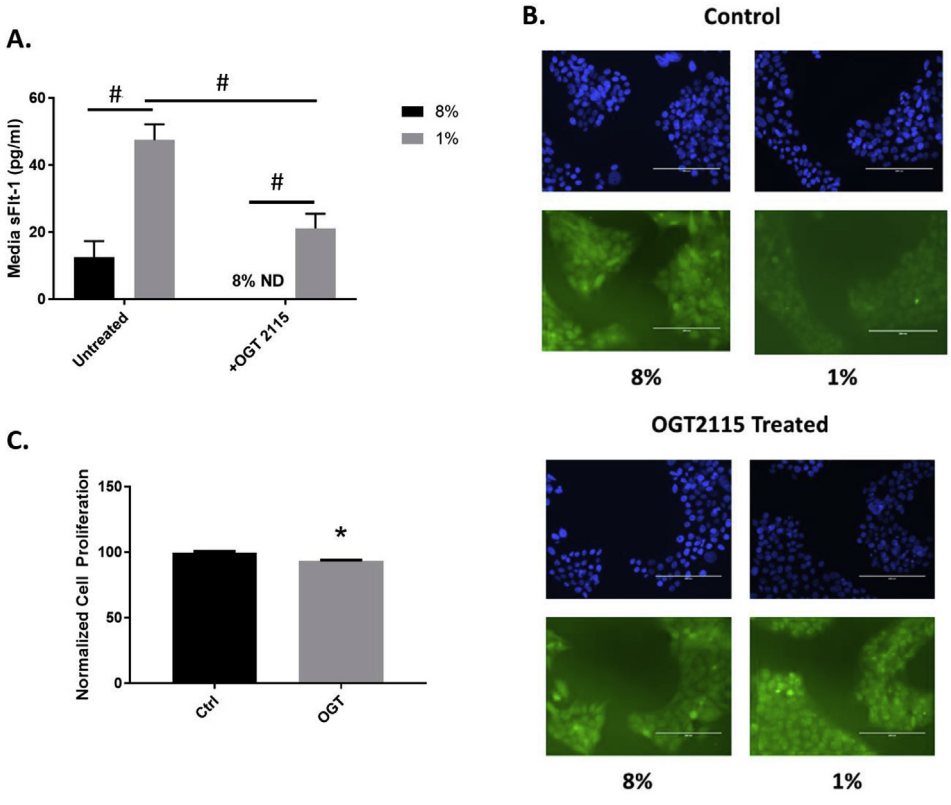

Figure 5. sFlt-1 in response to HPSE inhibition: Under hypoxic conditions, sFlt-1 release into the media is significantly increased. Inhibition of HPSE by OGT results in an attenuation of sFlt-1 release (N = 6) (**A**). This is further shown in levels of sFlt-1 bound to the cell surface. Cells cultured in 1% oxygen have less sFlt-1 bound compared to 8% oxygen. Treatment with OGT restores sFlt-1 binding to the trophoblast membrane (**B**). MTS assay showed that there was a slight, but significant, decrease in cell proliferation with OGT treatment (N = 18) (**C**). *P < 0.05; #P < 0.01.

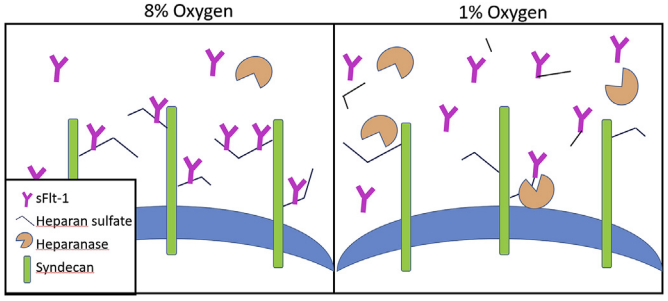

Fig. 6. Schematic of hypothesized sFlt-1 release in hypoxia: Under normal placental oxygen conditions (8% oxygen in the left panel), there is relatively low heparanase expression. This allows the sFlt-1 present to remain bound to the syndecan-1 heparan sulfate chains. In hypoxia (1% oxygen in right panel), increased heparanase expression leads to increased cleavage of heparan sulfate, allowing release of sFlt-1.

4. Discussion

While the underlying cause of preeclampsia is uncertain, it is generally accepted that placental hypoxia is an important factor [16]. Increased maternal circulating sFLT-1 has also been widely established, having been linked to placental hypoxia [6]. Several lines of evidence implicate sFLT-1 in the maternal syndrome. sFLT-1 infusion in late gestation causes a preeclampsia-like phenotype in rodents [17]. Furthermore, removal of sFLT-1 from the circulation by apheresis helps to prolong gestation and reduce proteinuria [18]. Understanding the mechanisms by which sFLT-1 secretion occurs in preeclampsia is, therefore, of great importance.

Hoffman-Kiefer et al. demonstrated that HS chain fragments, a major component of the placental glycocalyx, were increased in the circulation of women with HELLP syndrome (Hemolysis, Elevated Liver enzymes, and Low Platelet count), often considered an acute stage and extremely severe relative of preeclampsia [19,20]. Additional studies have demonstrated the importance of the HS chains in normal pregnancy to regulate sFLT-1 localization in the placenta [11]. Heparin treatment has been shown to elevate the levels of circulating sFLT-1 in pregnant women [21,22]. Therefore, increases in heparanase could lead to increased sFLT-1 release.

Our results offer a potential explanation for the increase in sFLT-1 release from the preeclamptic placenta *in vivo*. The specialized trophoblasts of the placenta create an extracellular matrix which include heparin sulfate chains. As our *in vitro* culture of BeWo placental trophoblasts demonstrated, heparan chains of the extracellular matrix are able to bind and act as a reservoir for sFLT-1. Though we did not specifically identify heparan as the site for sFLT-1 binding, previous studies [11], and the fact that treatment with heparin displaced sFLT-1 into the media, lead us to believe that we have confirmed this site for sFLT-1 storage.

We examined heparanase as potential modulator of sFLT-1 shedding due to its function as an extracellular matrix enzyme cleaving heparan. Not only did we find that HPSE is upregulated in hypoxic trophoblasts, but blockade of this enzyme with OGT-2115 lead to a significant decrease in sFLT-1 shedding. As a complementary experiment, we looked at combined membrane-bound soluble/full length FLT-1 by immunofluorescence in both control and OGT-2115 treated samples. As hypothesized, BeWo cells treated with the heparanase inhibitor had significantly increased FLT-1/sFLT-1 signal compared to the untreated cells (data not shown).Though we only examined one concentration of heparanase inhibition by OGT-2115, this concentration has been shown previously to exhibit both hepearanase inhibition as well as anti-angiogenic potential (breakdown of the ECM is required for angiogenesis), while higher concentrations can have off-target effects in cytochrome P450 enzymes [15]. Zhao et al. examined the role of chymotrypsin/chymase in sFLT-1 shedding by proteolytic cleavage of full length FLT-1. Though they observed increasing sFLT-1 release with increasing concentrations of chymotrypsin, application of an inhibitor did not alter the FLT-1 expression on the placental trophoblasts studied [23]. Considering their results in conjunction with our own, we believe that chymotrypsin may be acting on other extracellular matrix enzymes contributing to the release of sFLT-1 bound to HS.

While this data links placental hypoxia, heparanase, and sFLT-1 secretion, there is still a great deal to that is unknown. Though BeWo cells can offer us an idea of potential interactions in the placenta, we cannot assume that the observations *in vitro* will translate to *in vivo*. Though these cells are derived from placental choriocarcinoma, they closely resemble the characteristics of placental syncytiotrophoblasts, which are specialized trophoblasts that form the placental glycocalyx *in vivo* [24]. While it is clear that sFLT-1 secretion is increased by hypoxia, the mechanism remains unclear. Measuring the most common *sFLT1* variants in the placenta [14], we did see increases in *sFLT1* mRNA under acute exposure. The preeclamptic hypoxic placenta is a chronic condition, leading us to believe that the 24 h sustained hypoxia exposure is a more accurate representation of protein expression in preeclampsia. However, Maynard et al. showed that both *sFLT1* and *FLT1* transcripts were increased in placentas from preeclamptic patients [5]. Therefore, the potential importance of increased splicing of the *FLT1* transcript must also be examined further in extended hypoxia exposures in order to better understand the transient changes in mRNA expression.

Though these *in vitro* data are suggestive, confirmation of these mechanisms in relevant *in vivo* models and further elucidation of the underlying mechanisms should be revealing.

5. Perspectives and significance

Here, for the first time, we demonstrate that hypoxia induces heparanase-dependent sFLT-1 release from trophoblast cells *in vitro*. We believe this data may aid in the understanding of one potential mechanism of increased sFLT-1 release in the preeclamptic placenta. Should future studies utilizing primary cultured placental trophoblasts and preeclampsia animal models additionally suggest that heparanase plays a significant role in sFLT-1 release, heparanase inhibitors may be an important investigative avenue for the treatment of preeclampsia.

Conflicts of interest

There are no conflicts of interest to report for any of the authors.

Acknowledgements

This work was supported by the following grants NIH grants P01HL51971, P20GM104357, R00HL116774, R01HL 137791, and AHA GRNT 31460000 and 19PRE34430044.

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