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Hypoxic Regulation of VEGF and PAI-1 Expression by HIF-1α and HIF-2α in First Trimester Trophoblasts

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Preeclampsia results from incomplete trophoblast invasion of the spiral arteries during early pregnancy. Vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1) are critical factors involved in angiogenesis, invasion and hemostasis at the maternal-fetal interface. Both factors are transcriptionally regulated by hypoxia inducible factor (HIF), a heterodimeric complex consisting of HIF-1β and either HIF-1α or -2α whose specificity or redundancy in gene regulation is cell-type specific. This study uses siRNA technology to dissect the mechanisms of hypoxia-mediated regulation of PAI-1 and VEGF expression in first trimester trophoblasts. Immortalized first trimester human extravillous trophoblasts (HTR8/SVneo cells) were maintained in serum-free and serum-containing media for 4h (n=3-4), 8h (n=6), 24h (n=5) and 48h (n=5) under normoxic (21% O2) and hypoxic (1-2% O2) conditions to determine a time of maximum induction of both VEGF and PAI-1. Subsequently, cells were maintained for 48h in the presence or absence of siRNA for HIF-1α, HIF-2α, HIF-1α + -2α, a non-targeting (NT) sequence or Cyclophilin B (CB). Media were then removed, cells lysed, and Western blotting used to assess HIF-α knockdown. VEGF and PAI-1 levels in the media were quantified by ELISA and results expressed as pg or ng/μg protein. Results from 3 to 8 independent experiments were analyzed using unpaired t-tests. Under hypoxic conditions treatment of cells with HIF-1α, HIF-2α or HIF -1α + -2α siRNA resulted in >90% HIF-α protein knockdown as determined by Western blotting. 48h of hypoxic treatment caused a statistically significant increase in PAI-1 levels ($p<0.01$) and VEGF levels ($p<0.001$) compared to normoxic controls. Under hypoxic conditions, PAI-1 levels were 4.75 ± 0.46 ng/μg protein and VEGF levels were 7.27 ± 1.08 pg/μg protein. Treatment with siRNA to HIF-1α, HIF-2α and HIF-1α + -2α significantly reduced PAI-1 levels to 3.3 ± 0.35 ($p<0.02$), 3.1 ± 0.38 ($p<0.03$) and 2.4 ± 0.19 ($p<0.003$), respectively. No significant difference in PAI-1 reduction was noted between the three HIF siRNA conditions. Under hypoxic conditions, levels of VEGF in cells treated with siRNA to HIF-1α (5.79 ± 0.55), HIF-2α (5.50 ± 1.24) and HIF-1α + -2α (4.24 ± 0.93) were reduced compared to the hypoxic control (7.27 ± 1.08), yet these effects did not reach statistical significance. However, when compared with

the levels observed in cells treated with NT siRNA (9.90 ± .98), all HIF siRNA treatments promoted a significant reduction in VEGF expression ($p<0.003$, $p<0.02$ and $p<0.003$ for HIF-1α, HIF-2α and HIF-1α+-2α, respectively). In conclusion, these results indicate that hypoxia-mediated changes in PAI-1 and VEGF expression in trophoblasts are regulated similarly by both HIF-1α and HIF-2α. This provides important insight into the molecular mechanisms regulating hemostasis and trophoblast invasion as well as their potential dysfunction in pregnancies complicated by preeclampsia.

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Introduction

Preeclampsia and Abnormal Placentation

Preeclampsia is the leading cause of maternal mortality in the Western world affecting 5-7% of all women (1). It is a disease manifest by hypertension and proteinuria that presents anytime after 20 weeks of gestation, during labor or during the early period after delivery. Without proper management, it can result in maternal ecclampsia, characterized by generalized seizures and fetal intra-uterine growth restriction (IUGR). Women with preeclampsia and eclampsia have a 3- to 25-fold increased risk of severe complications, such as abruptio placentae, thrombocytopenia, disseminated intravascular coagulation, pulmonary edema, and aspiration pneumonia (1). The disease remains one of the most common reasons for a woman to die during pregnancy. Presently incurable, it is managed with screening and labor induction when necessary. Furthermore, it is one of the most common reasons for induced preterm delivery. Risk factors include previous history of preeclampsia, primiparity, obesity, family history of preeclampsia, multiple (twin) pregnancies and chronic medical conditions such as long-term hypertension, renal disease and diabetes (2).

The etiology of preeclampsia is presently unknown. However, it is widely believed that it is a disease of placental dysfunction. Preeclamptic placentas appear histologically abnormal, with evidence of underperfusion and ischemic injury. It is generally thought that focal regions of hypoxia/ischemia stimulate production of various proinflammatory cytokines and other placental factors that are released into the maternal circulation thereby causing endothelial dysfunction and systemic disease (3,4). Reduced uteroplacental perfusion is generally thought to result from incomplete invasion of the maternal spiral arteries by fetally-derived extravillous trophoblast cells (EVTs) (3,5).

During early placentation (weeks 6-9 of pregnancy), trophoblasts exist in a relatively hypoxic environment (6). The trophoblasts are highly proliferative, and uterine invasion is predominantly interstitial. Trophoblast stem cells form two types of chorionic villi: floating and

anchoring (7). The floating villi are primarily responsible for gas and nutrient exchange. They are composed of trophoblasts, which early in placentation, have fused to form the multinucleate synctiotrophoblast layer (8). The cytotrophoblasts of the anchoring villi can fuse to form synctiotrophoblast or break through the synctium to form multi-layered columns, which connect the embryo to the uterine wall (9). The EVTs of the cell columns invade through the interstitium of the first third of the uterine wall and can also form plugs within the uterine spiral arteries to minimize placental perfusion (10). At this time, the fetus is undergoing organogenesis. It requires minimal oxygen and is highly susceptible to teratogenic damage from oxygen radicals (11,12). From weeks 9-12 the uteroplacental arteries recanalize (11,12). There is markedly increased trophoblast growth and differentiation. By the 20th week the EVTs begin to transform from epithelial to endothelial-like cells and migrate along the lumen of the vessels to remodel the decidual and inner third of the myometrial segments of spiral arteries (11,13). As a result the vessels become greatly dilated, and are no longer under maternal vasomotor control. The "pseudoendothelium", composed of endovascular trophoblasts, expresses endothelial markers including angiogenic factors and their receptors (14). The process of "psuedovascularization" enormously expands the vascular capacity of the uteroplacental circulation into the intervillous space, thus guaranteeing maximal placental blood supply regardless of maternal attempts to regulate blood distribution within the body.

In order to invade, trophoblasts must switch expression of adhesion molecules from an epitheilial to a mesenchymal/endothelial profile. Studies have suggested that impaired invasion is due to a failure of trophoblasts to acquire the vascular repertoire of adhesion molecules. Zhou et al. have found that EVTs in normal pregnancies show reduced expression of epithelial cell adhesion markers such as Epithelial-cadherin and up-regulation of endothelial cell markers such as Vascular Endothelial-cadherin, vascular cell adhesion molecule-1, platelet endothelial adhesion molecule 1 and α4-integrins (15,16). They were also found to acquire $\alpha v\beta 3$ integrin, which is present on activated endothelial cells. On the contrary, EVTs in preeclampsia fail to

express these markers. This group hypothesizes that endovascular invasion is dependent on expression of a vascular phenotype. It is worth noting, however, that this model of endovascular mimicry has not held in studies by other groups looking at expression in placental bed biopsies (17,18).

Aberrant Trophoblast Gene Expression in Preeclampsia: VEGF and PAI-1

Presently, it is unclear how incomplete trophoblast invasion with the resulting insufficient uteroplacental circulation translates into the clinical presentation of preeclampsia. There is research to suggest preeclampsia results from an immunologic rejection of the fetal/placental cells by maternal cells with different allogenecity (19,20). As mentioned above, it is also thought that the syndrome could arise from certain factors produced by the dysfunctional placenta, which, once released into the maternal circulation, cause a systemic inflammatory response (21). One such factor is soluble fms-like tyrosine kinase 1 (sFlt-1), which acts as a potent anti-angiogenic molecule by binding to vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). sFlt-1 is produced by trophoblasts as well as other cell types, and levels have been found to be elevated in preeclamptic women compared to normal gestational controls (22,4). Furthermore, treating rats with sFlt-1 results in glomerular endotheliosis, a lesion pathognomonic of preeclampsia (23).

Given the radical changes in oxygen tension at the uterine-placental interface that occur throughout early gestation (6), the role of oxygen in trophoblast proliferation, differentiation and invasion is critical to our understanding of normal placental development and potential dysregulation in preeclampsia. Many studies have shown that a hypoxic environment (similar to <10 weeks gestation) promotes trophoblast proliferation and prevents differentiation towards an invasive phenotype. Significant work by Genbacev et al. has found that cytotrophoblasts (10-12 week gestation) *in vitro* appear to enter the preliminary stages of differentiation, but show significantly decreased invasion through an extracellular matrix (ECM) at 2% O_2 (hypoxia)

compared to 20% O_2 (normoxia). Unlike cells at 20% O_2 or 8% O_2, they also failed to up-regulate the integrin $\alpha 1\beta 1$ (a collagen/laminin receptor), normally seen *in vivo* when cells invade the uterus (24). Further work by Caniggia et al. using human villous explants showed that exposure to 3% O_2 resulted in increased cell proliferation and production of biochemical markers characteristic of immature EVTs (25).

However, other studies using first trimester trophoblasts have shown that compared to normoxic conditions, hypoxic treatment stimulated cells to invade through reconstituted basement membrane (26). Furthermore, *in vivo* models of reduced uteroplacental perfusion in non-human primates lead to increased invasion of the uterine wall (27). It is likely that variations in cell-type and experimental models (i.e. primary culture vs. cell-line, *in vitro* vs. *in vivo*) contribute to the differences in outcomes of these experiments. However, it is clear that trophoblast proliferation and invasion are regulated by the oxygen tension.

The processes of interstitial and vascular invasion, angiogenesis and endothelial transformation are very complex and not completely understood. It is known that they involve many different growth factor and cytokine interactions with different cell-types at the maternal-fetal interface. Trophoblasts must undergo regulated changes in the synthesis and degradation of extracellular matrix proteins (ECMs) and their receptors in order to assume an invasive phenotype (similar to tumor cells). Numerous studies have shown that the urokinase-pathway is involved in cellular migration and invasion through the extracellular matrix. Urokinase-type plasminogen activator (uPA) is secreted as a single-chain inactive proenzyme (pro-uPA), which, upon binding to a specific uPA receptor (uPAR) on the cell surface, is cleaved into the active two-chain molecule. Cell bound-uPA converts plasminogen to plasmin. Plasmin degrades various components of the ECM including fibrin, fibronectin, collagen and laminin. For this reason, uPA is essential for ECM degradation, a critical process for cellular invasion. Plasminogen activator inhibitor 1 (PAI-1) inhibits uPA thus blocking uPA conversion of plasminogen to plasmin (28).

The evidence suggesting that PAI-1 may play a role in cellular migration and invasion through the ECM pertains to both normal processes like placentation, as well as certain disease processes such as tumor invasion and metastasis. High levels of PAI-1 indicate poor prognosis from a variety of malignancies including breast (29), gastric (30), lung (31,32) and ovarian (33). Further studies have shown that PAI-1 promotes cellular migration on the ECM protein vitronectin (Vn) by competing for binding with uPAR to the Vn receptor and causing cell dissociation from Vn (34). PAI-1 mediated release of cells from the ECM (in this case, Vn) could potentially help explain why high levels of PAI-1 are indicative of poor prognosis in multiple types of cancer.

Levels of PAI-1 are elevated in the plasma and placenta of preeclamptic women, and are positively correlated to the severity of placental damage (35,36). This finding contrasts what one might expect since high PAI-1 levels seem to correlate with invasiveness, and preeclampsia is a disease of decreased invasion. However, there have been in vitro studies demonstrating a role for PAI-1 in the inhibition of trophoblast migration (37). In addition, PAI-1 also serves an important role in mediating fibrinolyis. PAI-1 has a high affinity for tPA as well as uPA. Whereas uPA binds to plasma membrane receptors, tPA binds to fibrin and other components of the ECM and is a key mediator of fibrinolysis. Therefore, the fibrin deposition and occlusive lesions in the intervillous space and vasculature of preeclamptic placentas could potentially arise from excessive inhibition of the fibrinolytic activity of tPA. As a dynamic molecule with the potential to affect both invasion and hemostasis, PAI-1 is likely to be a key factor in the dysregulation of these processes as seen in preeclmapsia.

PAI-1 expression is stimulated by a number of different growth factors including TGF-β, IL-1 and basic Fibroblast Growth Factor. However studies by Fitzpatrick and Graham using immortalized first trimester EVTs (HTR8/SVneo cells) show that PAI-1 is upregulated by low oxygen levels similar to those of the first trimester placenta, independent of TGF -β regulation

(38). Although it has never been proven, this finding suggests that the increase in PAI-1 levels in preeclampsia might be a consequence of a hypoxic environment.

VEGF, a potent inducer of endothelial cell proliferation and chemotaxis, is a critical factor in vasculogenesis and angiogenesis. The extent of its role in placental development is not fully understood, but it is widely expressed not only by enodothelial cells, but by placental cytotrophoblasts, Hofbauer cells (fetally-derived macrophages), maternal macrophages (39), and first trimester EVTs (40). Lower levels of VEGF mRNA have been found in placental biopsies of women with preeclampsia compared to normal controls (41). Furthermore, in preeclamptic placenta, there is decreased terminal villous volume, and abnormal terminal villous maturation and branching. The capillaries are long, poorly branched and highly coiled (42). It has been suggested that altered VEGF regulation and hence expression at the maternal-fetal interface could contribute to dysregulated angiogenesis and spiral artery remodeling during placentation as seen in preeclampsia (42).

Although VEGF mRNA levels were found to be *lower* in placenta of women with PE, there is conflicting evidence regarding the serum levels of VEGF in preeclamptic women. Numerous studies have found elevated levels of VEGF in the serum of patients with preeclampsia (43,44,45), while other studies have found the opposite (46,47). Resolution of these disparate results may be due to the measurement of free or bound VEGF (i.e. sflt-1 involvement). Despite the abundance of literature linking VEGF to preeclampsia, its actual role in the pathology and clinical presentation is unknown, and under intense investigation.

Role of hypoxia inducible factor (HIF) in gene regulation

Both VEGF and PAI-1 are induced under hypoxic conditions, and are regulated by hypoxia inducible factor (HIF). This transcription factor is a regulator of many genes involved in cell survival, cell proliferation, apoptosis, glucose metabolism and angiogenesis. For this reason, much of the work that has been done on HIF involves its crucial role in tumor growth. HIF

expression and activation has been shown to correlate with tumor progression and resistance to cancer treatments. Hypoxia is the main mechanism by which HIF is activated, although there is increasing evidence that non-hypoxic stimuli are also capable of activating this transcription factor albeit through different mechanisms (48). The regulation of HIF is complex and occurs at the transcriptional, translational and protein levels. The HIF protein is rapidly degraded by the proteasome pathway under normoxic conditions, but under hypoxic conditions it is stabilized and permits activation of genes essential to cell survival under low oxygen conditions.

HIF is a heterodimer composed of either: HIF-1α or HIF-2α and HIF-1β all of which contain basic helix-loop-helix motifs and Per-ARNT-Sim domains. Either HIF-1α or HIF-2α is the main functional component of the HIF complex and both contain an oxygen degradation domain (ODD). There appears to be overlap with most of the genes that either the HIF-1α or -2α regulate, but recent studies have shown cell-type specific control of certain genes by one or another of the HIF-α subunits (49,50,51). Transcription and translation of HIF-α remain unchanged by the switch from normoxia to hypoxia. However, HIF-α is an extremely labile protein under normoxia, with a half-life of ~ 5 minutes. Under normoxic conditions, HIF-α is hydroxylated on two proline residues, and the HIF herterodimer is ubiquitinated by the Von-Hippel-Lindau complex and rapidly targeted for degradation by the proteasome. Iron and oxygen are necessary for full enzymatic hydroxylation of HIF-α (52) by prolyl hydroxylase. Under low oxygen availability the activity of the prolyl hydroxylase is decreased and HIF-α is stabilized. HIF-α can then translocate to the nucleus to bind with the constitutively expressed HIF-1β. The heterodimer can then bind to hypoxic response elements (HREs) of target genes such as VEGF and PAI-1 to increase their expression.

Hypoxia plays an integral role in the early placental environment, particularly as it effects trophoblast invasion. There has been significant work done by several groups on the expression of HIF in the placenta. Rajakumar et al. have shown that HIF-1α and-2α are highly expressed in

first trimester placenta, and their levels decrease as gestation progresses (53). Caniggia et al. have shown that inhibition of HIF-1α expression in hypoxic villous explants (5-8 weeks gestation) arrested cell proliferation, decreased α5 expression (normally associated with proliferative, non-invasive cells) and triggered biochemical markers of an invasive trophoblast phenotype (such as α_1 integrin and gelatinase B expression) (54). This suggests that in the hypoxic environment of early gestation, HIF expression contributes to the regulation of trophoblasts as proliferative rather than invasive cells. In addition, more studies by Rajakumar et al. determined that HIF-2α protein, unlike HIF-1α or HIF-1β, is over expressed in the placentas of preeclamptic women (55). They have also shown that oxygen dependent down-regulation of HIF-1α and -2α proteins is impaired in placental villous explants from women with preeclampsia (56).

The mechanism of regulation of trophoblast invasion are exceptionally complex, involving numerous cell types and cellular interactions at the maternal-fetal interface that somehow result in degradation of extracellular matrices, invasion, phenotypic changes and angiogenesis. How exactly these processes go awry in preeclampsia, leading to incomplete invasion and to the maternal symptoms of disease, are still largely unknown. Hypoxia has a profound effect on the expression of so many important factors in this complex process that it is likely to play an integral role in development of the disease, which is characterized by ischemic injury and underperfusion of the placenta. HIF, the master regulator of hypoxia, appears to be dysregulated in preeclampsia along with two factors controlled by HIF, VEGF and PAI-1, both of which are critical to the process of angiogenesis and trophoblast invasion. The purpose of this study was to test the hypothesis that HIF regulates VEGF and PAI-1 expression in first trimester trophoblasts using siRNA technology.

Our Specific Aims Are To:

1. Establish a time course of hypoxic induction of VEGF and PAI-1 in first trimester trophoblasts.
2. Perform knock-down of HIF-1α and HIF-2α protein expression in hypoxic cells using siRNA technology.
3. Determine if hypoxic induction of VEGF and PAI-1 is mediated similarly by HIF-1α and HIF-2α.

Methods

Cell culture: Immortalized first trimester extravillous trophoblast cells (HTR8/SVneo), a gift from Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada) were used for all experiments. This cell-line was established following immortalization of a short-lived first trimester extravillous trophoblast cell-line (HTR-8) transformed with SV40 large T-cell antigen (which is known to cause extended life-span in multiple cell-types) (57) and selected for with neomyocin resistance (58). The HTR-8/SVneo cells and the parental cell line are morphologically similar and both express cytokeratin-7 confirming their trophoblastic identity. Both cell lines also express 72kDA type IV collagenase and respond to TGF-β with reduced thymidine incorporation and decreased secretion of PAI-1. In addition, neither cell type was capable of growth in soft agar, and no sign of tumor formation was evident more than 5 months after subcutaneous inocculation of the parental or transfected cells into nude mice. Unlike the parental cells, however, the HTR8/SVneo cells produced human chorionic gonadotropin (hCG) and did not show any decrease in *in vitro* invasion in response to TGF-β (59).

All experiments were conducted in RPMI-1640 (Sigma R8758) supplemented with 5% fetal bovine serum (Gemini bio-products, Woodland CA) and 1% antibiotic/antimicotic (10,000U/ml penicillin G sodium, 10,000ug/ml streptomycin sulfate and 25ug/ml amphotericin

B) (Gibco, Grand Island N.Y.) or RPMI-1640 alone. Cells were maintained at 37°C in a humidified atmosphere of 5%CO_2/95%air.

Hypoxic Induction Studies: All experiments were conducted in 6-well plates (#353046, Becton Dickinson, Franklin Lakes NJ) with $2.5x10^5$ cells (passages 10-30) plated in triplicate wells for each experimental condition. Cells were allowed to grow under normoxic conditions (21% O_2) until 60-70% confluent. At this time, they were washed one time with Dulbecco's phosphate buffered saline (PBS) (Gibco, Grand Island, NY) and then 2ml media were added to each well. Experiments were carried out with serum containing media (5% FBS) or with serum free media consisting of a 1:1 mixture of phenol red-free Hams' F12: Dulbecco's Modified Eagle's medium and ITS^+ (a supplement utilized to obtain a final concentration of insulin of 6.25μg/ml, transferring 6.25μg/ml, selenous acid 6.25ng/ml, bovine serum albumin 1.25mg/ml and linoleic acid 5.35μg/ml) (59). Cells assigned to the normoxic (Nx) group remained in the incubator. Cells assigned to the Hypoxic (Hx) group were incubated in a sealed Plexiglass hypoxia chamber (Belleco Glass Co., Vineland NJ) containing a beaker of water to maintain humidity and a gas oxygen analyzer (Hudson RCI, Temecula CA) (60). The chambers were equilibrated to 0-1% O_2 with CO_2 and balanced N_2 gas flowing into the chamber at a rate of 20 L/min (approximately 10 minutes). The hypoxia chamber was then placed back into the incubator along with the 6-well plates to be maintained under normoxic conditions.

Supernatant and Protein Harvest: After the designated time in the Nx or Hx conditions (4, 8, 24 or 48 hrs) the plates were removed from the incubator and/or hypoxia chamber and rapidly placed on ice to minimize protein degradation. The supernatant was alliquoted into 2.0ml tubes and stored at -20°C until further analysis. The cells were quickly washed with PBS and then lysed with 100ul/well lysis buffer (50mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA,

1mM NaF, 1%Triton X-100, 10% Glycerol) mixed with PMSF (1:100) and protease inhibitor cocktail (1:100). A cell scraper (Fisherbrand, Pittsburgh PA) was used to scrape the lysate from each well, which was then transferred into 1.5ml tubes. Each lysate was then vortexed (30sec), placed on ice (10min), centrifuged (7000 x g, 10min, 4°C) and the supernatant alliquoted into separate tubes and stored at -80°C.

ELISAs: Levels of VEGF and PAI-1 in the culture media were measured by ELISA according to information provided by the manufacturer (R&D Laboratories, Minneapolis, MN for VEGF and American Diagnostica, Stamford, CT for PAI-1). Media for VEGF was diluted 5x (in the provided RDW1 diluent) for

the 24h and 48h samples and media for PAI-1 was diluted 5x (in PBS with 1% BSA) for 4h samples, 20x for 8h samples, 100x for 24h samples and 200x for 48h samples. The concentrations of VEGF and PAI-1 were determined in triplicate wells and normalized to cell protein. The average of the triplicate wells was considered the value for any given experiment. The number (n) of experiments, conducted in the presence of serum and under serum-free conditions, at each time point is included in the following table:

	4h	8h	24h	48h
VEGF	4	6	5	5
PAI-1	3	6	5	5

Protein Assays: Determination of the protein concentration for each sample was done using the D_C Protein Assay from Bio-Rad Laboratories (Hercules, CA). Standards for the protein assay were made with bovine serum albumin (Boerhinger Mannheim, Indianapolis, IN) and lysis buffer (described above).

siRNA Experiments: It has been well established that small interfering RNA (19-21bp RNA fragments) upon binding to the complementary mRNA within the cell, leads to degradation of the mRNA (61). siRNA to HIF-1α has been successfully used in the past to knock-down HIF-1α at both the RNA and protein levels (62,53,51). For these studies, $2.5x10^5$ cells/well were plated in triplicate in 6-well dishes with RPMI (5% FBS, 1% antibiotic/antimicotic) and allowed to grow until 60-70% confluence (48-72 hrs). Cells were then washed once with PBS and transfected with siRNA duplexes (Dharmacon Incorportated, Lafayette, CO) to HIF-1α (100nM) (sense: GGA CAC AGA UUU AGA CUU GUU, antisense: CAA GUC UAA AUC UGU GUC CUU), HIF-2α (sense: GCA AAU GUA CCC AAU GAU AUU, antisense: UAU CAU UGG GUA CAU UUG CUU) (100nM), HIF-1α+ -2α (200nM), non-targeting siRNA (NT) (sequence not released, cat # D-001210-01-05) (100nM) as a negative control, cyclophilin B siRNA(CB) (sequence not released, cat # D-001136-0105) (100nM) as a positive control or transfection reagent alone for mock transfection (M) (DharmaFECT 1 Transfection Reagent) (4μl transfection reagent/100nM siRNA) in serum-free RPMI. The cells were transfected according to Dharmacon Incorporated protocol for 6-well plates. For any given experiment each transfection/siRNA condition was done in triplicate wells.

Once treated with siRNA, all cells were placed at 37°C, 21% O_2. After 30 minutes, cells being treated under hypoxic conditions were placed into a hypoxic chamber following the same protocol as for the *hypoxic induction experiments*. After 48 hrs, the chambers were opened and the supernatant was removed to be stored at -20°C until further analysis by ELISA. The adherent cells were harvested for protein analysis. The protein harvest was conducted on ice, within 1-2 minutes of opening the hypoxia chamber in order to minimize HIF protein degradation due to re-oxygenation. Each well was lysed with 100μl lysis buffer (50mMHepes pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 1% Triton-X 100%, 10% Glycerol) supplemented with 1% protease inhibitor cocktail, 1%PMSF and 0.1%SDS. The lysate was vortexed (30sec) and

placed on ice (10 min) before being centrifuged (7000 x g, 10 min, 4°C). Supernatant was subsequently alliquoted to 1.5ml tubes and stored at -80°C until further analysis. The number (n) of each siRNA experiment is as follows:

	Untreated	Mock	NT	CB	HIF-1α	HIF-2α	HIF-1α + -2α
Normoxia	8	--	3	--	--	--	--
Hypoxia	8	6	8	6	8	5	5

Western blotting: Western blotting was used to assess protein expression and knock-down of HIF-1α, HIF-2α and Cyclophilin B. 25μg of cell lysate diluted with Laemelli sample buffer (Bio-Rad, Hercules, CA) was loaded per well. Prior to loading samples they were heated to 37°C (5 min). Samples along with 10μl Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA) were electrophoresed (100V for 1.5 hrs) on a 4-15% Tris-HCL Ready-Gel (Bio-Rad, Hercules, CA) and transferred to a Hybond™ ECL™ Nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) at 4°C (100V for 1.0 hr). The membranes were then blocked with 5% Carnation non-fat dry milk in PBS-2%Tween. Proteins were detected using a monoclonal antibody to HIF-1α (BD Biosciences, Palo Alto, CA), HIF-2α (Novus Biologicals, Littleton, CO) and cyclophilin B (Abcam Incorporated, Cambridge, MA) at 1:250, 1:1000 and 1:2000 respectively. Cyclophilin B served as a loading control as well as a positive control for siRNA knock-down. Overnight primary antibody incubation (4°C) was followed by incubation with goat anti-mouse (1:5000) or goat anti-rabbit (1:15,000) horseradish peroxidase (1 hr, RT) secondary antibodies (Bio-Rad, Hercules, CA) followed by signal detection using enhanced chemiluminescence developing reagents (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL). Blots were exposed to film (Denville Scientific, Metuchen, NJ) for between 1sec and 5min. Blots were washed 3 times (10 min each) with PBS-2% tween

between each incubation step. Prior to reprobing any blots, they were stripped with Blot Restore (Chemicon Intenational, Temecula, CA).

Statistics. Results are expressed as a mean ± s.e. Statistical comparison of VEGF and PAI-1 expression between normoxic and hypoxic conditions for each time point was done using paired t-tests performed by Graphpad Prism software (San Diego, CA). Comparisons of VEGF and PAI-1 expression between any two siRNA groups were also carried out using unpaired t-tests performed by Graphpad. A P value <0.05 was considered significant.

All experiments described above were done by the author.

Results

Hypoxic effects on expression of VEGF and PAI-1 in first trimester trophoblasts

Placental development within the first 10 weeks of pregnancy occurs in a relatively hypoxic environment (6). Preeclamptic placentas demonstrate regions of ischemic injury and *in vitro* studies have shown that hypoxia affects trophoblast invasion (16). Two factors in the process of placentation, VEGF and PAI-1, are critical to angiogenesis and trophoblast invasion and are regulated by hypoxia. In order to better understand how these two factors are regulated by hypoxic treatment in first trimester trophoblast cells, this study investigated the protein expression of VEGF and PAI- 1 in an immortalized first trimester trophoblast cell-line (HTR8/SVneo cells). Fitzpatrick el. al (39) have evaluated the effects of hypoxia on PAI-1 mRNA expression, however the mechanism of this regulation and the direct role of HIF remain unelucidated. In addition, no studies on hypoxia-mediated changes on PAI-1 protein expression have been carried out. Furthermore, hypoxia-mediated expression of VEGF in these cells has not been examined. We were also interested in determining if there was a difference in the time

course of VEGF and PAI-1 induction, since there could be other mechanisms involved in their hypoxic induction, aside from HIF.

HTR8/SVneo cells were incubated for 4, 8, 24 and 48 hours under normoxic (21%O_2) or hypoxic conditions (0-2%O_2). Levels of PAI-1 and VEGF in culture media were determined by ELISA following normalization to total cellular protein in order to control for potential changes in overall rates of protein synthesis. Experiments were preliminarily conducted in serum-supplemented media and then repeated in serum-free media to be sure that endogenous levels of VEGF and PAI-1, or other serum factors, did not affect ELISA results. We observed similar hypoxia-dependent changes in PAI-1 and VEGF levels in both the absence and presence of serum. For cells in serum-containing media, hypoxic conditions promoted a 1.5 fold induction of VEGF at 4h (*$p<0.05$) compared to normoxia (0.90 ± 0.15 pg/μg protein vs. 0.59 ± 0.18 pg/μg protein), a 2.4 fold induction at 8h (**$p<0.004$) (1.90 ± 0.33 pg/μg protein vs. 0.80 ± 0.18 pg/μg protein), a 3.0 fold induction at 24h (**$p<0.006$) (4.15 ± 0.86 pg/μg protein vs. 1.37 ± 0.38 pg/μg protein) and a 3.2 fold induction at 48h (**$p<0.001$) (7.84 ± 0.98 pg/μg protein vs. 2.42 ± 0.54 pg/μg protein) (fig. 3A). Cells cultured in serum-free media expressed levels of VEGF comparable to cells cultured in serum-supplemented media, and there was a statistically significant induction in VEGF at all four time points (fig. 3B). These results demonstrate that hypoxic induction of VEGF occurs as early as 4h, and the induction continues for at least 48hrs. Time points beyond 48h were not investigated because of concerns about cell survival under extensively long hypoxic conditions. Preliminary studies showed decreased protein levels in cells under hypoxic conditions at 96 hrs (data not shown).

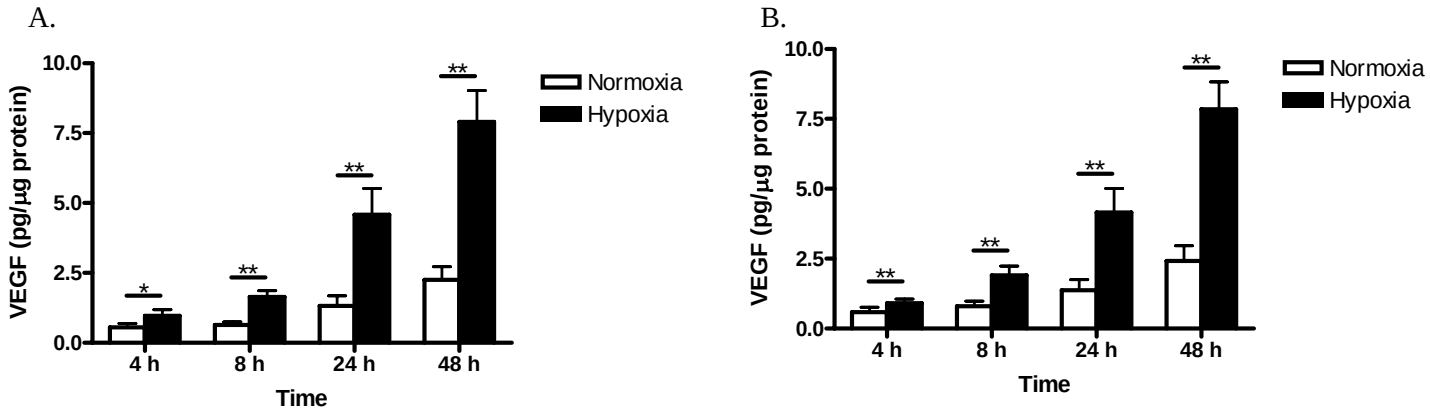

Figure 3. Hypoxia induces VEGF expression by first trimester trophoblast cells cultured in either serum free or serum supplemented media. HTR8/SVneo cells were cultured in either serum free media (A) or media supplemented with 5% FCS (B). These cells were exposed to ambient oxygen (normoxia) or 0-2% oxygen (hypoxia) and at the indicated times supernatant was harvested and analyzed by ELISA for VEGF protein expression. Results were normalized to total protein content.

The same time course was evaluated for PAI-1 expression. For cells in serum-supplemented media, hypoxic conditions promoted a 3.0 fold induction of PAI-1 at 24h (*$p<0.03$) compared to normoxic conditions (3.42 ± 0.82 ng/µg protein vs. 1.14 ± 0.28 ng/µg protein) and a 5.0 fold induction at 48h (*$p<0.02$) compared to normoxic conditions (4.77 ± 0.51 ng/µg protein vs. 0.96 ± 0.09 ng/µg protein) (fig. 4A). PAI-1 levels were not significantly induced at 4h (0.08 ± 0.0 ng/µg protein vs. 0.14 ± 0.04 ng/µg protein) or 8h (0.86 ± 0.20 ng/µg protein vs. 0.58 ± 0.09 ng/µg protein). Similar results were found with experiments performed in serum-free conditions (fig. 4B). These results indicate that PAI-1 is also induced by hypoxia, although significant induction occurs later than VEGF induction. For this reason it was determined that 48h would be an appropriate time point to conduct experiments aimed at examining the role of HIFs in hypoxia-dependent changes in PAI-1 and VEGF expression.

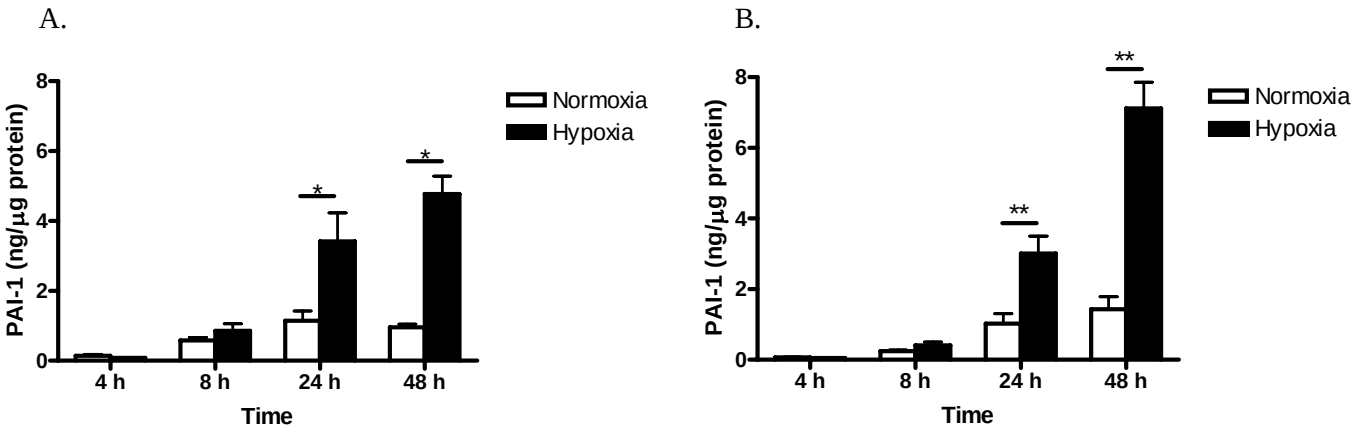

Figure 4. Hypoxia induces PAI-1 expression by first trimester trophoblast cells cultured in either serum free or serum supplemented media. HTR8/SVneo cells were cultured in either serum free media (A) or media supplemented with 5% FCS (B). These cells were exposed to ambient oxygen (normoxia) or 0-2% oxygen (hypoxia) and at the indicated times supernatant was harvested and analyzed by ELISA for PAI-1 protein expression. Results were normalized to total protein content.

Protein Expression of HIF-1α and HIF-2α in HTR8/SVneo after siRNA treatment

HIF is a heterodimeric transcription factor composed of a constitutively expressed β subunit and a hypoxically regulated α subunit (-1α/-2α). Under normoxic conditions, the α subunit is hydroxylated, ubiquitinated and targeted for proteasomal degradation. In hypoxic conditions, the α subunit is stabilized, and it is able to translocate to the nucleus and bind to the β subunit. The complex is then able to bind to the promoter region of certain genes (ie VEGF and PAI-1), through hypoxia response elements, to induce transcription.

siRNA technology has been used previously to knock-down HIF expression at the RNA and protein level (63,53,51) in various cancer cell lines and villous explants, but not in HTR8/SVneo cells. Before evaluating VEGF and PAI-1 levels after treatment with HIF-1α and HIF-2α siRNA, it was necessary to determine the efficacy and specificity of HIF knock-down after siRNA treatment. HTR8/SVneo cells were transfected with siRNA to HIF-1α, HIF-2α, HIF-1α + -2α, a non-targeting sequence (NT), cyclophilin B (CB) or transfection reagent alone (mock). The NT siRNA was used as a negative control to determine if there were any non-specific effects of siRNA transfection on HIF -1α (-2α) expression. Cyclophilin B siRNA served

as a positive control for siRNA knock-down of a known protein, as well as a loading control because cyclophilin B is a constitutively expressed protein. Thirty minutes after siRNA transfection, cells were placed under normoxic or hypoxic conditions. After 48 hrs, cells were lysed and cellular protein was extracted and analyzed by Western blotting for HIF-1α, -2α and cyclophilin B knock-down. Figure 5 is a representative of 5-8 independent experiments.

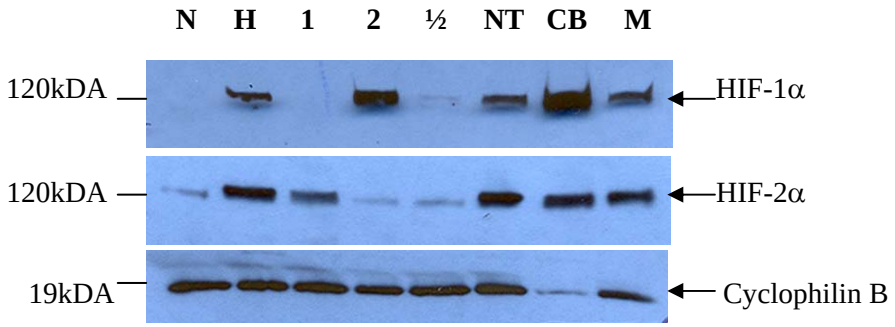

Figure 5. Western Blot of specific siRNA knockdown. HTR8/SVneo cells were transfected with the indicated siRNA constructs. Normoxia: untreated (**N**), Hypoxia: untreated (**H**), Hypoxia: HIF-1α siRNA (**1**), Hypoxia: HIF-2α siRNA (**2**), Hypoxia: HIF-1α + -2α siRNA (**1/2**), Hypoxia: Non-targeting siRNA (**NT**), Hypoxia: Cyclophilin B siRNA (**CB**) and Hypoxia: transfection alone -mock (**M**). Equal amounts of protein were loaded per well. Membranes were blotted with antibodies to HIF-1α (top blot), HIF-2α (middle blot) and Cyclophilin B (bottom blot).

Comparison of normoxia (**N**) and hypoxia (**H**) show that, as expected, there is induction of both HIF-1α (top blot) and HIF-2α (middle blot) under hypoxia compared to normoxia. Furthermore, cyclophilin B (bottom blot) is not induced, a finding that is expected since it is not a hypoxically-regulated protein. Blot 1 demonstrates that treatment with siRNA to HIF-1α leads to significant knock-down of HIF-1α protein (**1**) compared to hypoxic treatment alone (**H**). Similarly, treatment with siRNA to both HIF-1α + -2α (**1/2**) also leads to significant knock-down of HIF-1α protein. Blot 2 demonstrates that treatment with siRNA to HIF-2α (**2**) leads to significant knock-down of HIF-2α protein compared to hypoxic treatment alone (**H**). Furthermore, similar HIF-2α knock-down was seen when cells were treated with siRNA to both HIF-2α + -1α (**1/2**). Non-targeting siRNA (**NT**), cyclophilin B siRNA (**CB**) and transfection

reagent alone-mock (**M**) did not appear to have significant effects on protein expression of HIF-1α (top blot) or HIF-2α (middle blot). Cyclophilin B siRNA (**CB**) resulted in significant knock-down of CB protein (bottom blot) indicating that the siRNA treatment protocol was effective. Furthermore cyclophilin B (bottom blot) levels were similar for all other lanes, serving as an appropriate loading control.

Non-specific effects of siRNA treatment on VEGF and PAI-1 expression in HTR8/SVneo cells

In addition to treating the HTR8/SVneo cells with siRNA to HIF-1α and HIF-2α, cells were transfected with transfection reagent alone-mock (M), a non-targeting siRNA (NT) and cyclophilin B (CB) siRNA. These reagents were used to determine if the transfection protocol or siRNA treatment per se had any non-specific effects on VEGF and PAI-1 expression. After 48h of treatment with siRNA, the media from the HTR8/SVneo cells was harvested and ELISAs were performed to quantitate levels of VEGF and PAI-1 in culture media. Results were then normalized to cellular protein. Figure 6A demonstrates that there was no significant difference in VEGF levels in untreated cells under hypoxic conditions (Hx) (7.27 ± 1.08 pg/μg protein) compared to transfection reagent alone (M) under hypoxia (7.43 ± 1.09 pg/μg protein). Compared with Hx, there was a significant increase in VEGF levels under hypoxia after siRNA transfection both with NT siRNA (9.90 ± 0.98 pg/μg protein) ($p<0.02$) and CB siRNA (9.20 ± 1.36 pg/μg protein) ($p<0.02$). This suggests siRNA per se non-specifically induces VEGF levels under hypoxic conditions. Although there was a non-specific increase in VEGF levels under normoxic conditions between untreated cells (Nx) and cells treated with NT siRNA, this effect was not statistically significant.

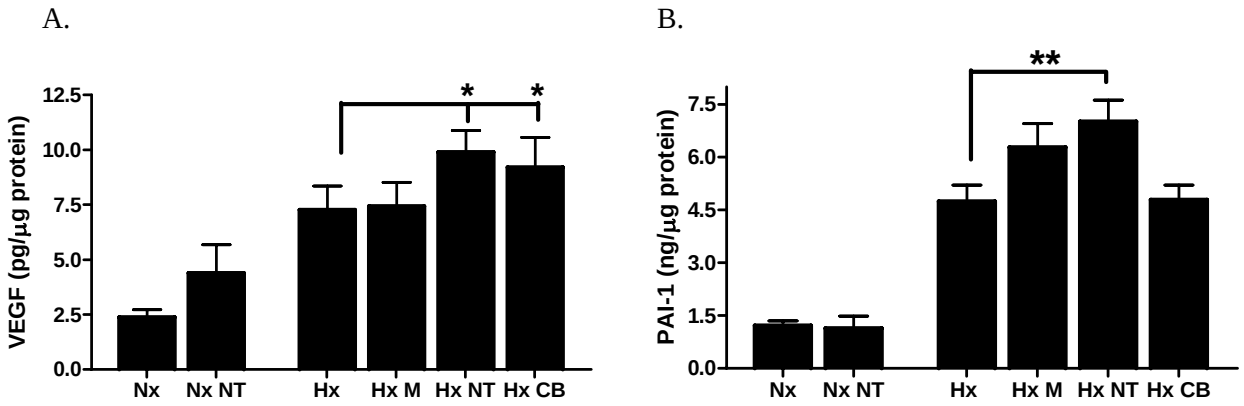

Figure 6. VEGF and PAI-1 expression after treatment with siRNA controls. HTR8/SVneo cells were exposed to either normoxic (Nx) or hypoxic (Hx) conditions for 48 hrs. Cells were then transfected with the indicated siRNA controls; non-targeting siRNA (NT), transfection reagent alone - mock (M), cyclophilin B siRNA (CB). Serum was analyzed for VEGF (A) and PAI-1 (B) protein expression by ELISA.

Figure 6B also demonstrates the non-specific effects of siRNA treatment on expression of PAI-1 levels in media of HTR8/SVneo cells after treatment for 48 hrs. Similar to the VEGF results, there was no significant difference in PAI-1 levels in untreated cells under hypoxia (Hx) (4.75 ± 0.46 ng/μg protein) compared to M under hypoxia (6.28 ± 0.68 ng/μg protein). Furthermore, compared to Hx, there was a significant increase in PAI-1 levels with NT siRNA (7.02 ± 0.61 ng/μg protein) ($p<0.003$). However, unlike the results of VEGF expression, treatment with CB siRNA did not affect PAI-1 levels under hypoxic conditions (4.79 ± 0.42 ng/μg protein) compared to hypoxia alone (Hx). Under normoxic conditions, there was not a significant difference between levels of PAI-1 in cells treated with NT siRNA (1.11 ± 0.34 ng/μg protein) compared to Nx alone (Nx) (1.21 ± 0.13 ng/μg protein).

The overall results from these experiments suggest that transfection itself does not affect VEGF and PAI-1 expression. The siRNA, however, appears to cause an induction of both VEGF and PAI-1 expression under hypoxic conditions. VEGF levels were induced after treatment with NT and CB siRNAs whereas PAI-1 levels were induced only after treatment with NT siRNA. This result suggests that individual siRNA sequences have different effects on protein expression

depending on the siRNA sequence as well as the specific protein under analysis. Thus, we must test the effects of NT siRNA on levels of PAI-1 and VEGF expression in studies of HIF knock-down under hypoxic conditions.

Effects of HIF-1α and HIF-2α siRNA on VEGF and PAI-1 expression in HTR8/SVneo cells

Current evidence suggests that HIF-1α and HIF-2α are dysregulated in preeclamptic placentas (58,57). As stated above, VEGF and PAI-1 are two soluble factors that are also dysregulated during preeclampsia and many studies suggest that they have a role in the etiology of this disease. It is known in cancer cells and villous tissue explants that HIF regulates VEGF and PAI-1 expression, but how this complex regulates expression of these soluble factors early in placentation is unclear. Furthermore, HIF-1α and HIF-2α have previously been shown to have different effects on the regulation of certain genes depending on cell–type (52). Therefore, in this study we wanted to characterize the effect of genetic knock-down of various elements of the HIF complex on the expression of VEGF and PAI-1 during hypoxia in early first trimester trophoblast cells. Figure 7A demonstrates the effects of siRNA for HIF-1α, HIF-2α, HIF-1α + HIF-2α and non-targeting (NT) on VEGF expression under hypoxia compared to untreated cells under hypoxia (Hx) or normoxia (Nx).

Culture media levels of VEGF under Hx (7.27 ± 1.08 ng/μg protein) were reduced after treatment with either HIF-1α (5.79 ± 0.55 pg/μg protein), HIF-2α (5.50 ± 1.24 pg/μg protein) or HIF-1α + HIF-2α (4.24 ± 0.93 pg/μg protein) under Hx, although these effects did not reach statistical significance. However, compared to cells treated with NT siRNA under Hx (9.90 ± 0.98 pg/μg protein) treatment with HIF specific siRNA caused a statistically significant decrease in VEGF with HIF-1α siRNA (**$p<0.003$), with HIF-2α siRNA (*$p<0.02$) and with both HIF-1α + HIF-2α siRNA (**$p<0.003$). VEGF levels in cells treated with both HIF-1α + HIF-2α siRNA

were not statistically different from levels noted in cells treated with HIF-1α or HIF-2α siRNA alone.

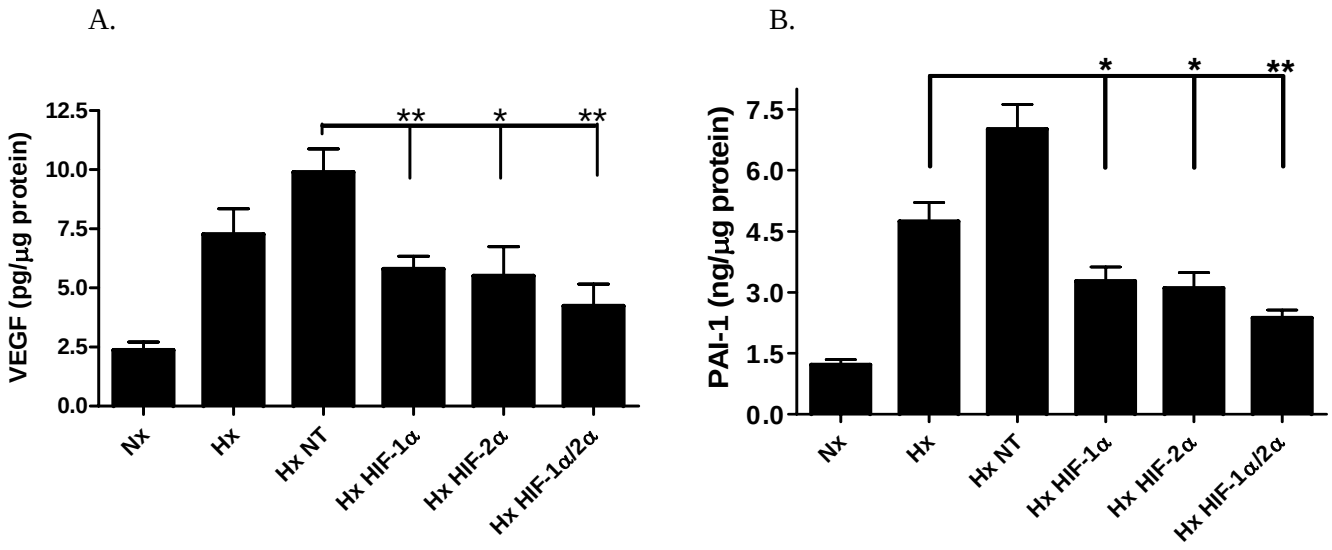

Figure 7. VEGF and PAI-1 expression under hypoxia are reduced by HIF-1α and HIF-2α siRNA knock-down. HTR8/SVneo cells were exposed to either normoxic (Nx) or hypoxic (Hx) conditions for 48 hrs. Cells were transfected with the indicated siRNA specific for HIF-1α, HIF-2α, both HIF-1α and HIF-2α, or non-targeting (NT). Serum was analyzed for VEGF (A) and PAI-1 (B) protein expression by ELISA.

Figure 7B demonstrates the effects of siRNA for HIF-1α, HIF-2α, HIF-1α + HIF-2α and NT on PAI-1 expression under hypoxic conditions along with the same controls as discussed for Figure 7A. PAI-1 levels in untreated cells under Hx (4.75 ± 0.46 ng/μg protein) were significantly reduced after treatment with HIF-1α siRNA (3.27 ± 0.35 ng/μg protein) (*$p<0.02$), with HIF-2α siRNA (3.10 ± 0.38 ng/μg protein) (*$p<0.03$) and with both HIF-1α + HIF-2α siRNA (2.37 ± 0.19 ng/μg protein) (**$p<0.003$). Since PAI-1 expression from cells treated under Hx with the NT siRNA were significantly higher than from cells under Hx alone, it follows that levels of PAI-1 in HIF-α siRNA treated cells were significantly less than that noted for cells treated with NT siRNA. The results from these experiments suggest that both HIF-1α and HIF-2α regulate VEGF and PAI-1 expression in early first trimester trophoblast cells. Furthermore,

HIF-1α and HIF-2α are each necessary but independently insufficient to promote maximal changes in PAI-1 and VEGF expression under hypoxic conditions.

Discussion

Preeclampsia, a disease manifest by hypertension and proteinuria during pregnancy, remains a leading global cause of maternal and fetal illness and death. Although the etiology remains unknown, it is a disease of placental dysfunction, with incomplete trophoblast invasion of the maternal spiral arteries during the first trimester (3). Early placental development occurs in a hypoxic environment, and there is evidence to suggest that hypoxia regulates trophoblast invasion of the extracellular matrix and transformation from epithelial-like to endothelial-like cells (25,56). Vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1), two critical factors in the process of trophoblast invasion, hemostasis and angiogenesis, are induced under hypoxia and are dysregulated in preeclampsia (39,37,38,46). Hypoxia Inducible Factor (HIF) transcriptionally regulates the expression of PAI-1 and VEGF along with many other genes that are necessary for cellular adaptation to a hypoxic environment. HIF consists of a constitutively expressed 1β subunit, also known as the arylhydrocarbon receptor nuclear translocator (ARNT), and a hypoxia-regulated α subunit (1α or 2α). These two α subunits have regulatory effects which are gene- and cell-type specific (51). It has been shown that HIF-2α is upregulated in preeclamptic placentas (57) and that both HIF-1α and HIF-2α in preeclamptic villous explants remain up-regulated inappropriately in response to re-oxygenation (58). This study examined the regulation of VEGF and PAI-1 by HIF-1α and -2α in first trimester trophoblast cells in order to better understand the potential mechanism of dysregulation of these factors in preeeclampsia.

Placental trophoblasts differentiate throughout gestation to perform numerous roles, including nutrient/waste exchange at the maternal-fetal interface, placental anchoring to the

uterus and invasion of the endometrium and myometrium. The first trimester extravillous trophoblasts are responsible for uterine invasion and are thus critical to the invasion deficiency seen in preeclamptic placentas. It is extremely difficult to study this population of cells, however, for several reasons. First and foremost, it is exceedingly challenging to examine *in vivo* the pathogenesis of a disease process in the first trimester when the disease does not manifest clinically until the third trimester. Furthermore, there is no animal model of preeclampsia presently in existence. For this reason we felt it was important to study an isolated population of extravillous trophoblasts in order to better characterize their inherent regulatory mechanisms, and how they might be disrupted. By electing to study the regulation of VEGF and PAI-1 by HIF in an immortalized cell line, there is a compromise of physiologic relevance compared to using primary cultures. However, establishing optimal transfection conditions for primary cultures can be exceptionally difficult and the HTR8/SVneo cell-line, although immortalized, maintains many of the same characteristics as the non-immortalized HTR-8 parental cells (39,60). Both cell types share the same phenotype (both express cytokeratin 7 and other epithelial-cell markers), similar functions (both are responsive to anti-proliferative, anti-migratory and anti-invasive effects of TGF-β) and neither cell type showed any sign of tumor formation more than 5 months after subcutaneous inoculation into nude mice (59).

Using the HTR8/SVneo cells, we have shown a temporal sequence of induction of VEGF and PAI-1 under hypoxic conditions. Specifically, VEGF expression is significantly induced under hypoxic conditions as early as 4 h whereas PAI-1 expression is not significantly up-regulated until 24 h under hypoxic conditions. There are multiple explanations for why VEGF induction occurred before PAI-1 induction. Aside from HIF, other factors are known to regulate both VEGF and PAI-1 levels. VEGF expression in HTR8/SVneo cells is induced by TNF-α and TGF-β (63). Furthermore, it has been shown that the VEGF secretion is synergistically enhanced by the combination of hypoxia and TGF-β (64). Similarly, PAI-1 expression in HTR8/SVneo

cells is also up-regulated by TGF-β through a transcriptional mechanism (60) independent of hypoxic induction (39). Therefore, it is likely that other factors are contributing to the induction of these factors. In addition, studies have shown that VEGF can stimulate the activity of PAI-1(65), and perhaps might also affect induction. It is also possible that there are differences in post-transcriptional, post-translational or secretory processing of these two factors which could impact on the time course of protein induction. Whatever the causes might be, recognizing that there is a temporal sequence of activation of these two factors could be of utility in understanding the mechanism of hypoxia-mediated changes in trophoblast gene expression. After successfully knocking-down HIF-1α and -2α protein expression with siRNA technology, we found that both the -1α and -2α subunits were necessary, although independently insufficient, for maximum hypoxic induction of both VEGF and PAI-1 expression at 48h. Demonstrating that both α subunits are necessary for maximum hypoxic induction of both VEGF and PAI-1 is of critical importance to understanding how these factors are regulated and potentially dysregulated in preeclampsia. This is of particular interest because of overwhelming evidence that HIF-1α and HIF-2α are expressed differentially in a variety of cell types and show remarkable target gene specificity. HIF-1α is ubiquitously expressed, and it was previously thought that HIF-2α was expressed only in vascular endothelial cells (66). It is now known that HIF-2α is expressed in a variety of cell types including kidney fibroblasts, hepatocytes, intestinal epithelial cells, heart myocytes and lung type II pneumocytes (67). Gene array analysis in HEK293T human embryonic kidney cells has shown that 21 genes were found to be up-regulated by both HIF-1α and HIF-2α (including VEGF), 14 preferentially activated by HIF-1α, including several involved in glycolysis, and 10 genes were preferentially activated by HIF-2α (68). PAI-1 was not investigated in this particular study. It has been shown that in human osteoblast-like cells, hypoxia-induced VEGF gene transcription is controlled by HIF-2α and not HIF-1α (69). Interestingly, Sowter et al. have shown, using siRNA to knock-down HIF-1α and -2α, that in

breast carcinoma and endothelial cell lines, HIF-2α cannot substitute for HIF-1α in regulating VEGF or uPAR (53). The differential regulation of VEGF and PAI-1 by HIF-1α and HIF-2α in trophoblast cells has not been reported. Our results indicate that dysregulation of either HIF-1α or HIF-2α in trophoblasts affects expression of both VEGF and PAI-1.

Rajakumar et al. have studied expression patterns of HIF-1α and HIF-2α in placentas of preeclamptic women. They have demonstrated that HIF-2α, but not HIF-1α or HIF-1β, is selectively over expressed in preeclamptic placentas (57). Further work by this group have shown that isolated cells from villous explants of preeclamptic placentas fail to adequately down regulate both HIF-1α and -2α protein expression upon re-oxygenation (58). Although such work characterizes HIF expression in tissue from whole placenta and villous trophoblasts rather than the extravillous trophoblasts which are responsible for uterine invasion, it is still of note that alternations in villous HIF expression and its regulation are associated with preeclampsia.

Increased HIF activity may be responsible for elevated placental PAI-1 expression noted in preeclamptic placentas (37). The pathological lesions of preeclamptic placentas characteristically contain areas of intervillous fibrin deposition and thrombosis which result from increased anti-fibrinolytic activity due to an excess of PAI-1. However, the syncytiotrophoblasts were specifically implicated in the elevated PAI-1 levels (37) found in preeclamptic placentas, a finding that makes sense since these cells, being in contact with maternal blood, are well-situated to regulate fibrinolytic activity at the maternal-fetal interface (60). Conversely, it is more difficult to explain decreased invasion by extravillous trophoblasts to elevated HIF and PAI-1 expression. PAI-1 levels are elevated in a variety of tumors and increased levels correlate with increased mortality, presumably due to increased invasion (33,34). However, attempting to dissect a process as complex as cellular invasion by differences in expression of a single factor is likely to be flawed. Invasion involves cell interactions with many different receptors, numerous proteases

and extracellular matrix proteins which are all subject to regulatory mechanisms, many of which are also independently regulated by HIF (70).

The role of HIF in the regulation of VEGF in preeclampsia is a complex issue, because HIF regulates sFlt-1, the soluble anti-angiogenic VEGF receptor that is also up-regulated in preeclampsia (71). *In vitro* studies have shown that free VEGF is not detectable in the culture media of primary cultures of cytotrophoblasts despite an apparent increase in total VEGF concentrations under hypoxic conditions. This suggests that sFlt-1 production, concurrently increased under hypoxia, may bind VEGF thereby negating its biological effects (72). This finding is likely to account for the ostensibly disparate results concerning whether VEGF levels increase in maternal serum in preeclampsia (73). Low or high levels are likely a function of whether measurements were of "free" or "total" VEGF respectively; compared to normal controls, total VEGF levels in the serum of women with preeclampsia are high, but the levels of functionally active, unbound VEGF are low. Concomitant elevations of VEGF and sFlt-1 levels in preeclamptic placenta do not explain why lower levels of VEGF mRNA were seen in placental biopsies of women with preeclampsia (38). However, perhaps sFlt-1 itself or the high VEGF levels in the maternal circulation are affecting cellular production of VEGF in the placenta in ways yet to be determined.

Presently, it appears that the elevated placental levels of sFlt-1 could play a significant role in the pathogenesis of preeclampsia. Adenoviral gene transfer of sFlt-1 into rats resulted in a preeclampsia-like phenotype including hypertension, proteinuria and glomerular endotheliosis (24,4). As a hypoxia-induced gene, it is important to also determine the regulation of sFlt-1 by HIF. There are currently no studies that have evaluated the specificity of HIF-1α versus -2α in the regulation of sFlt-1. We attempted to investigate the regulation of sFlt-1 in HTR8/SVneo cells, but expression levels even under hypoxic conditions were too low to be studied further (data not shown).

Early placental development, involving trophoblast phenotype-switching, trophoblast invasion and angiogenesis is an extremely complex process dependent on a variety of different growth factors, cytokines and cell-types, all communicating at the maternal-fetal interface. It follows, that the dysregulation of this process, as seen in preeclampsia is also quite complex and difficult to study. Since the human trophoblast is by far the most highly invasive of all animal species, and human placentation is unique in several respects, one must be cautious of using animal models in studies of trophoblast biology. Hypoxia is certain to play a critical role in successful placentation given the low oxygen milieu that is known to exist through the first 10-12 weeks of gestation. HIF, the predominant mediator of hypoxia-induced gene regulation, likely has a central role in orchestrating the complex interplay of cells and soluble mediators that characterize early placentation. Our results have demonstrated that in human extravillous trophoblasts the HIF complex regulates the expression of PAI-1 and VEGF using a unique mechanism that requires both HIF-1α and HIF-2α. Given their well established role in directing invasion and placentation, determining the fine details of cellular regulation of VEGF and PAI-1 expression will likely contribute to a better understanding of normal placental biology and the pathogenesis of preeclampsia.

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