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Hypoxia is responsible for soluble vascular endothelial growth factor receptor-1 (VEGFR-1) but not for soluble endoglin induction in villous trophoblast

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Abstract

BACKGROUND: Pre-eclampsia is a pregnancy disorder characterized by a maternal endothelial cell dysfunction associated with low levels of circulating placental growth factor (PlGF) and increased levels of total vascular endothelial growth factor (VEGF), soluble VEGF receptor-1 (sVEGFR-1), and soluble endoglin, a transforming growth factor βl and 3 coreceptor. Here, we tested the hypothesis that these altered levels of angiogenic cytokines and of the anti-angiogenic soluble forms of cytokine receptors could be the consequence of hypoxia.
METHODS: Normal human umbilical vein endothelial cells, immortalized first trimester extravillous trophoblast cells (HTR8/SVneo) and first trimester placental villi expiants (8-14 weeks) were used for culture under normoxia (20% O_2) or hypoxia (1% O_2). Culture media were collected for the measurement of cytokines by enzyme-linked immunosorbent assay. Total RNA was extracted for RT-PCR analysis.
RESULTS: Under hypoxia, villous trophoblast expressed higher levels of VEGF, VEGFR-1, sVEGFR-1 and VEGFR-2 mRNAs ($P < 0.001$), and secreted more VEGF and sVEGFR-1 proteins ($P<0.05$). In contrast, P1GΓ mRNA and protein were decreased in 1% O_2 ($P < 0.001$), whereas endoglin (Eng) was not modulated. Additionally, sVEGFR-1 directly abolished VEGF/PlGF-induced angiogenesis in the rat aortic ring assay.
CONCLUSIONS: Our results support the hypotheses that, in pre-eclampsia, (i) overproduction of VEGF family factors by pre-eclamptic placenta is a consequence of induced hypoxia; (ii) overproduction of sVEGFR-1 by hypoxic villous trophoblast accounts for maternal free VEGF depletion; (iii) low circulating level of free P1GΓ is not only related to sVEGFR-1 overproduction, but also to hypoxia-induced mRNA down-regulation; (iv) Eng is not modulated by hypoxia.

Keywords: hypoxic trophoblast; vascular endothelial growth factor receptor-1; soluble endoglin; pre-eclampsia; pregnancy

INTRODUCTION

Pre-eclampsia is a severe disorder unique to human pregnancy that is characterized by onset of proteinuria and hypertension after the 20th week of pregnancy. Etiologic factors are still not fully characterized, although evidence supports the involvement of genetic, immune, angiogenic and other mechanisms (Roberts and Cooper, 2001; Lachmeijer *et al.,* 2002; Bdolah *et al.,* 2004, 2005; Chaouat *et al.,* 2005; Maynard *et al.,* 2005; Roberts and Gammill, 2005).

In normal pregnancy, extravillous cytotrophoblast invades the terminal segments of the uterine arteries transforming these small muscular arterioles to large capacitance vessels of low resistance (Zhou *et al.,* 1993, 1997). This allows increased blood flow (i.e. oxygen, nutrients) to the fetus. Remodeling of the spiral arteries probably begins in the late first trimester and is completed by 18-20 weeks of gestation, although the exact gestational age at which trophoblast invasion of these arteries ceases is unclear. By comparison, in preeclampsia the cytotrophoblast infiltrates the decidual portion of the spiral arteries, but fails to penetrate the myometrial portion (Meekins *et al.,* 1994; Naicker *et al.,* 2003; Guzin *et al.,* 2005). Thus, the large, tortuous vascular channels created by replacement of the musculoelastic wall with fibrinoid material do not develop. Instead, the vessels remain narrow leading to hypoperfusion. This is supposed to impact on the local oxygen pressure leading to placental ischemia/hypoxia, and ultimately to functional anomalies of the maternal vascular endothelium (Granger *et al,* 2002; Challier and Uzan, 2003).

We and others have recently presented evidence that excess secretion of soluble vascular endothelial growth factor receptor-1 (sVEGFR-1 also referred as sFlt1), a naturally occurring circulating VEGF antagonist, could be responsible for the maternal syndrome (Maynard *et al.,* 2003; Tsatsaris *et al.,* 2003; Levine *et al.,* 2004).

Additionally, administration of sVEGFR-1 produces hypertension and proteinuria in pregnant rats (Maynard *et al.,* 2003). sVEGFR-1 arises from an alternative splice variant mRNA that lacks the cytoplasmic and transmembrane domain but contains the ligand-binding domain of VEGFR-1 (Kendall and Thomas, 1993; Kendall *et al.,* 1996). Thus, sVEGFR-1, by forming a complex with VEGF or placental growth factor (PlGF), may antagonize the biological activity of these cytokines (Kendall and Thomas, 1993; Autiero *et al.,* 2003a,b).

Recently, Venkatesha *et al.* (2006) showed that circulating levels of soluble endoglin (sEng), a coreceptor for transforming growth factor β1 and 3 (TGFβ1 and 3), are elevated in women with pre-eclampsia. sEng may impair the binding of TGFβ1 and 3 to endothelial receptors, decreasing endothelial nitric oxide synthase-activated vasodilatation. sEng decreased angio-genesis *in vitro,* but its overexpression in pregnant rats had only minimal effects. However, in pregnant rats the simultaneous introduction of adenoviruses encoding both sVEGFR-1 and sEng induced severe hypertension, heavy proteinuria, elevated liver-enzyme levels, creating a powerful model that simulates most of the manifestations of pre-eclampsia in humans (Venkatesha *et al.,* 2006).

Cross-sectional studies suggest that changes in maternal serum levels of sVEGFR-1 and sEng antedate the onset of clinical symptoms by 5-8 weeks in healthy, nulliparous women and may be useful for screening or early diagnosis of pre-eclampsia (Levine *et al.,* 2004; McKeeman *et al.,* 2004; Levine *et al.,* 2006b; Wathen *et al.,* 2006). More recently, a pilot study, performed to evaluate the clinical utility of sVEGFR-1 and sEng in the differential diagnosis of hypertension in late pregnancy, proved that both cytokines could be useful in differentiating pre-eclampsia from other hypertensive diseases of pregnancy (Salahuddin *et al.,* 2007).

Even though increased soluble factors released in maternal circulation could be the answer to an ischemic placenta, mechanisms of action and the source of these factors are not yet fully understood.

Our study was undertaken to test the hypothesis that placental hypoxia is one of the trigger event leading to the massive placental secretion of these soluble angiogenic cytokines and anti-angiogenic receptors.

MATERIALS AND METHODS

Cell culture and hypoxia treatment

Normal human umbilical vein endothelial cells (HUVEC) were isolated by umbilical vein treatment with 0.1% collagenase (from *Clostridium histolyticum;* Sigma, St Louis, MO, USA) as described previously (Jaffe *et al.,* 1973). HUVEC cells were cultured on gelatin-coated dishes in MCDB 131 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL), 2 mM glutamine (Gibco-BRL), 5 µg/ml endothelial cell growth factor (BD Biosciences, Bedford, USA), 50 µg/ml heparin (Sigma), 25 mM HEPES and 100 IU/ml penicillin-streptomycin (Gibco-BRL).

Immortalized human first trimester extravillous trophoblast cells (HTR8/SVneo), kindly provided by CH Graham, Kingston, Ontario, Canada, were routinely grown in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL), 2 mM glutamine and 100 IU/ml penicillin-streptomycin. Phenotypic characterization has shown that these cells express some markers of extravillous trophoblast cells including cytokeratins, urokinase-type plasminogen activator receptor (uPAR), matrix metalloproteinase-2 and -9 (MMP-2 and -9). As previously described the extravillous trophoblast cells retain the ability to express HLA-G when cultivated on Matrigel (Kilburn *et al,* 2000).

Between 18 and 24 h before undergoing hypoxia, cells were plated at a density of 1.25×10^5 cells per T-flask (12.5 cm^2). The medium was changed 1 h before hypoxic or normoxic treatment to ensure an adequate amount of nutrient and growth factor. Hypoxic treatment was carried out in an incubator supplied with 1% O_2, 5% CO_2 and 94% N_2. Normoxic experiments were performed under 20% O_2, 5% CO_2 and 75% N_2. Oxygen concentration was monitored using an oxygen electrode (OS1000; Oxygen Sensors, Inc, Frazer, PA).

Human trophobMst villous expiant culture

Samples of human placental villi (n = 30) were obtained from first trimester (8-14 weeks) termination of pregnancies with agreement of our local medical ethics committee. Informed consent was obtained from the patients. Placental tissue was placed in ice-cold phosphatebuffered saline (PBS) and processed within 2 h of collection. One part of each tissue sample was fixed in 4% formalin and embedded in paraffin for histological analysis, while the other part was used for expiant culture. The tissue was washed in sterile PBS and aseptically dissected. Small fragments of placental villi (five pieces of 1-2 mm^3) were teased apart and placed on a

transparent Biopore membrane of 12-mm diameter Millicell-CM culture dish inserts with a pore size of 0.4 μm (Millipore Corp., Bedford, MA). Expiants were cultured in Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F12; Gibco-BRL) supplemented with 100 IU/ml penicillin-streptomycin. Hypoxic treatment was carried out in an incubator supplied with 1% O_2, 5% CO_2 and 94% N_2. Normoxic experiments were performed under 20% O_2, 5% CO_2 and 75% N_2. Culture media were collected for measurement of VEGF, PlGF, sEng and sVEGFR-1. Part of the villous expiant culture was fixed in 4% formalin and the other part was snap frozen in liquid N_2 and placed at -80°C for RNA extraction.

Immunoassays for VEGF, sVEGFR-1, PlGF and sEng

Levels of VEGF, PlGF, sVEGFR-1 and sEng were measured in conditioned medium by sandwich enzyme immunoassay technique (R&D Systems® Europe Ltd, Abington, UK for VEGF, PIGF and Eng, or Bender MedSytems®, Vienna, Austria for sVEGFR-1). All samples were measured in duplicate. Sensitivity of assays is 5 pg/ml for VEGF and sEng, 0.1 ng/ml for s VEGFR-1 and 7 pg/ml for PlGF.

RT-PCR analysis

Total RNA was extracted with Rneasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed with a Superscript II reverse transcriptase (Invitrogen) and random hexamers as primer.

Real-time quantitative RT-PCR analyses for VEGF, VEGFR-1 sVEGFR-1, VEGFR-2 (KDR/Flk-1), Eng and PlGF mRNAs and 18S rRNA were performed using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems Inc., Foster City, CA) as previously described (Tsatsaris *et al.* 2003). The sequences of the PCR primer pairs and fluorogenic probes that were used for each gene are shown in Table I.

Table I: Sequence *of primers and TaqMan probes used for RT-PCR studies*

Gene and Accession no	Position	Sequence	Size PCR product
VEGF-FP	748F	5'-TCTACCTCCACCATGCCAAGT-3'	104 bp
VEGF-RP	851R	5'-GCTGCGCTGATAGACATCCA-3'	
VEGF Probe	797	5'-CCACTTCGTGATGATTCTGCCCTCCT-3'	
NM 003376			
VEGFR1-FP	2438F	5'-TCCCTTATGATGCCAGCAAGT-3'	79 bp
VEGFR1-RP	2516R	5'-CCAAAAGCCCCTCTTCCAA-3'	
VEGFR1 Probe	2469	5'-CCGGGAGAGACTTAAACTGGGCAAATCA-3'	
AF063657			
sVEGFR1-FP	2209F	5'-ACAATCAGAGGTGAGCACTGCAA-3'	180 bp
sVEGFR1-RP	2388R	5'-TCCGAGCCTGAAAGTTAGCAA-3'	
sVEGFRl Probe	2257	5'-TCCAAATTTAAAAGCACAAGGAATGATTGTACCAC-3'	
U01 134			
VEGFR2-FP	791F	5'-CTTCGAAGCATCAGCATAAGAAACT-3'	156 bp
VEGFR2-RP	946R	5'-TGGTCATCAGCCCACTGGAT-3'	
VEGFR2 Probe	820	5'-AACCGAGACCTAAAAACCCAGTCTGGGAGT-3'	
AF063658			
PlGF-FP	668F	5'-CCTACGTGGAGCTGACGTTCT-3'	77 bp
PlGF-RP	744R	5'-TCCTTTCCGGCTTCATCTTCT-3'	
PlGF Probe	702	5'-CTGCGAATGCCGGCCTCTGC-3'	
X54 936			
Eng-FP	1597F	5'-TCTACCTCCACCATGCCAAGT-3'	70 bp
Eng-RP	1666R	5'.TTTTTCCGCTGTGGTGATGA-3'	
Eng Probe	1619	5'-TGAGGCGGTGGTCAATATCCTGTCGA-3'	
NM_000118			

F: forward, R: reverse.
VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor-1; sVEGFRl, soluble VEGF receptor-1; VEGFR2, VEGF receptor-2; PlGF, placental growth factor; Eng, endoglin.

Immunohistochemistry (PlGF, VEGFR-1 and Ki67)

Placental villi were fixed in 4% formalin for 1 h and then embedded in paraffin. Paraffin sections (4 μm) were mounted on aminopropyltriethoxysilane (Tespa)-coated glass slides.

Sections were dewaxed in xylene and rehydrated. For Ki67 (a marker of cell proliferation) and VEGFR-1 labeling, antigen retrieval was performed by treatment for 11 min. at 126°C and 1.4 Bar with Target Retrieval Solution (DakoCytomation, Glostrup, Denmark, S1699). For PlGF, a pretreatment with citrate buffer (DakoCytomation, S2031) was performed. Non-specific antibody binding was blocked by incubation for 20 min in a blocking reagent containing 3% H_2O_2 and for 60 min in 10% serum albumin solution. Then, 50 μg/ml of PlGF antibody (mouse monoclonal anti-human PIGF, R&D Systems, MAB264), 5 μg/ml of VEGFR-1 antibody (goat anti-human VEGFR-1, R&D Systems, AF312) or monoclonal mouse anti-human Ki67 antigen (1/100, clone MIB-1 (DakoCytomation, M7240)) were incubated with the sections for 1 h, at room temperature (PlGF and Ki67) or overnight at 4°C (VEGFR-1). Sections were washed in Tris Buffer Saline—0.1% Tween and incubated with a secondary antibody, peroxidase conjugated (goat anti-mouse Envision (DakoCytomation, K4000)) for KI67 and PlGF, or with a rabbit anti-goat biotin conjugated antibody (DakoCytomation, E466) followed by streptavidin/horse-radish peroxidase (DakoCytomation, P397), for VEGFR-1. Staining was detected with the diaminobenzi-dine chromogen after 5 min. Nuclei were counterstained by incubation for 2 min. with hematoxylin. Sections were mounted, examined and photographed under a Leica—DMLS microscope couple to a digital camera (Nikon Coolpix 990). Controls were performed by omitting the primary antibody or by incubating the sections with non-specific immunoglobulin G at the same concentration as the primary antibody.

Aorta ring assay

Rat aortic rings were cultured in 3D collagen gels as previously described (Blacher *et al.,* 2001; Masson *et al.,* 2002). Briefly, thoracic aortas were removed from mice sacrificed by cervical dislocation and immediately transferred to a culture dish containing ice-cold serum-free minimum essential medium (MEM, Life Technologies Ltd, Paisley, Scotland). The peri-aortic fibroadipose tissue was carefully removed. One millimeter long aortic rings (~15 per aorta) were sectioned and extensively rinsed in five consecutive washes of MEM. Ring shaped expiants of mouse aorta were then embedded in a rat tail interstitial collagen gel (1.5 mg/ml) prepared by mixing 7.5 volumes of 2 mg/ml collagen (Collagen R, Serva, Heidelberg, Germany), 1 volume of 10 × MEM, 1.5 volume of NaHC03 (15.6 mg/ml) and ~0.1 volume of 1 M NaOH to adjust the pH to 7.4. The collagen gels containing the aortic rings were polymerized in cylindrical agarose wells and kept in triplicate at 37°C in 60 mm diameter Petri dishes (bacteriological polystyrene, Falcon, Becton Dickinson, Lincoln Park, NJ). Each dish contained 6 ml of MCDB131 (Life technologies Ltd, Paisley, Scotland) supplemented with 25 mM NaHC03, 2.5% mouse serum, 1% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were kept at 37°C in a humidified environment for 9 days and examined with an Olympus microscope at appropriate magnification. Effects of recombinant VEGF (Peprotech, Rocky Hill, NJ), PlGF, sVEGFR-1, endoglin and TGFβ (R&D Systems, Abingdon, UK) on aortic rings were evaluated after 9 days of incubation. Quantification of neoformed micro-vessels number (Vn) was performed by image analysis on a Sun SPARC30 workstation with the software Visilog 5.0 from Noesis according to Blacher *et al.* (2001).

Statistical analyses

Mann-Whitney test was used for statistical analysis. Results are shown as mean $\pm$ SEM. In the aortic ring assay Student-Newman-Keuls or Kruskal-Wallis Anova tests were used. A value of $P < 0.05$ were considered statistically significant.

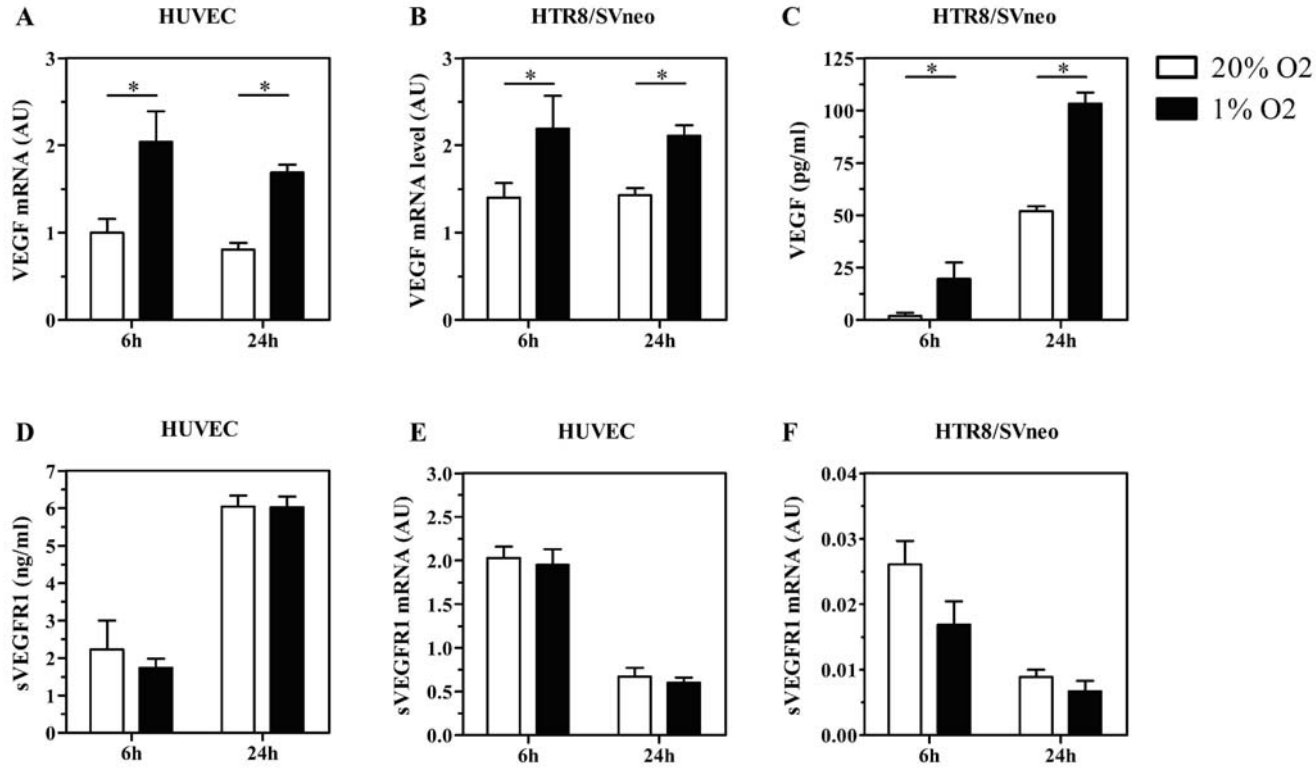
RESULTS

Impact of hypoxia on angiogenic cytokines and anti-angiogenic soluble receptors in cell culture

HUVEC and HTR8/SVneo cells cultured in the presence of 20% O_2 contained similar amounts of VEGF mRNA levels, as measured by quantitative real-time PCR. After 6 and 24 h of hypoxia (1% O_2) VEGF mRNA levels were doubled in HUVEC cells (Fig. 1A) and 1.5-fold higher in HTR8/SVneo (Fig. 1B). VEGF secretion measured by enzyme-linked immunosorbent assay (ELISA) in medium conditioned by HUVEC cells remained undetectable even after 24 h hypoxia, whereas HTR8/SVneo cells doubled their secretion of VEGF in hypoxic conditions (Fig. 1C).

sVEGFR-1 protein secreted by HUVEC cells was not affected by hypoxia (Fig. 1D). No sVEGFR-1 was detected in conditioned medium from HTR8/SVneo cells. Quantitative RT-PCR confirmed that sVEGFR-1 mRNA was not modulated under hypoxia in HUVEC cells (Fig. 1E) and that HTR8/SVneo cells contained minimal amounts of sVEGFR-1 mRNA, ~100 times less than in HUVEC cells (Fig. 1F). In contrast to the anticipated rise, sVEGFR-1 mRNA level in HTR8/SVneo cells decreased, but not significantly, upon hypoxic treatment.

Figure 1: *Effect of hypoxia on vascular endothelial growth factor (VEGF) and soluble VEGF receptor-1 (sVEGFR-1) mRNA and secretion. Cells were incubated for 6 or 24 h under normoxia (20% O_2) or hypoxia (1% O_2). mRNA levels of VEGF and sVEGFR-1 in HUVEC cells (A, E) and HTR8/SVneo (B, F) were quantified by real-time RT-PCR, normalized by 18S rRNA and expressed as arbitrary units (AU) (mean ± SD), n = 4. Levels of VEGF (pg/ml) in HTR8/SVneo (C) and sVEGFR-1 (ng/ml) in HUVEC cells (D) were assessed by enzyme-linked immunosorbent assay (ELISA) (mean ± SE), n = 4.*P < 0.05.*



Hypoxia in placental villi culture expiants

Placental villi expiants cultured under hypoxia were used to study the impact of low oxygen on placental physiology.

Tissue integrity in normoxic or hypoxic conditions was assessed by histological analysis after hematoxylin-eosin staining and Ki67 labeling. Hypoxia did not alter cell and tissue morphology even after 48 h. Ki67 labeling was not significantly different in specimens cultured under 20% O_2 or 1 % O_2 (Fig. 2A-C'). Immunodetection of sVEGFR-1 was particularly evident after culture in 1 % 0_2, whereas immunodetection of PlGF became faint in such specimens (Fig. 2D-I').

Hypoxia increased 2- to 4-fold the amount of secreted VEGF after 48 h of culture (14.01 ± 4.10 versus 33.69 ± 10.91 pg/ml, $P = 0.0479$) (Fig. 3A). sVEGFR-1 protein was also increased 2-fold in the conditioned medium of culture upon hypoxic treatment (7.507 ± 0.519 versus 12.54 ± 2.215 ng/ml, $P = 0.0086$) (Fig. 3B). In contrast, hypoxia caused a 4-fold decrease in secreted PlGF (226.87 ± 39.16 versus 51.44 ± 8.654 pg/ml, $P < 0.0001$) (Fig. 3C). Hypoxia did not modulate sEng level after 48 h of culture (Fig. 3D). Since an imbalance exists between the proangiogenic PlGF and the anti-angiogenic sVEGFR-1 in preeclampsia, we calculated PlGF/sVEGFR-1 ratio. The ratio was significantly decreased in villous expiants cultured under hypoxia (Fig. 3E, 39.40 ± 8.393 in normoxia versus 6.794 44 ± 1.507 in hypoxia, $P < 0.0001$).

In cultured expiants, mean levels of VEGF mRNAs significantly increased 5-fold under hypoxia ($P < 0.0001$) (Fig. 4A). In contrast, PlGF mRNA levels decreased 2-fold after 48 h under hypoxia ($P = 0.0002$) (Fig. 4C). Quantification of sVEGFR-1 mRNA levels showed a 5-fold up-regulation under hypoxia ($P = 0.0002$) (Fig. 4B), although the increase of VEGFR-1 mRNA was only 2-fold ($P = 0.0004$) (Fig. 4E). In our experimental conditions, Eng mRNA levels did not show any modulation under hypoxia as compared to normoxia (Fig. 4D). VEGFR-2 mRNA exhibited an up-regulation of 10-fold ($P = 0.0002$) after 48 h of hypoxia (Fig. 4F).

Collectively these data indicate that oxygen level directly affects the mRNA levels of these cytokines and their receptors, but does not modulate endoglin.

Figure 2: *Immunolocalization of Ki67 (A, A', B, B', C and C), VEGFR-1 (D, D', E, E', F and F') and placental growth factor (PlGF, G, G', H, H', I and I') in villous expiants before culture (A, A', D,D', G and G'), after 48 h under normoxia (20% O_2) (B, B', E, E', H and H') or hypoxia (1% O_2) (C, C, F, F', I and I').*

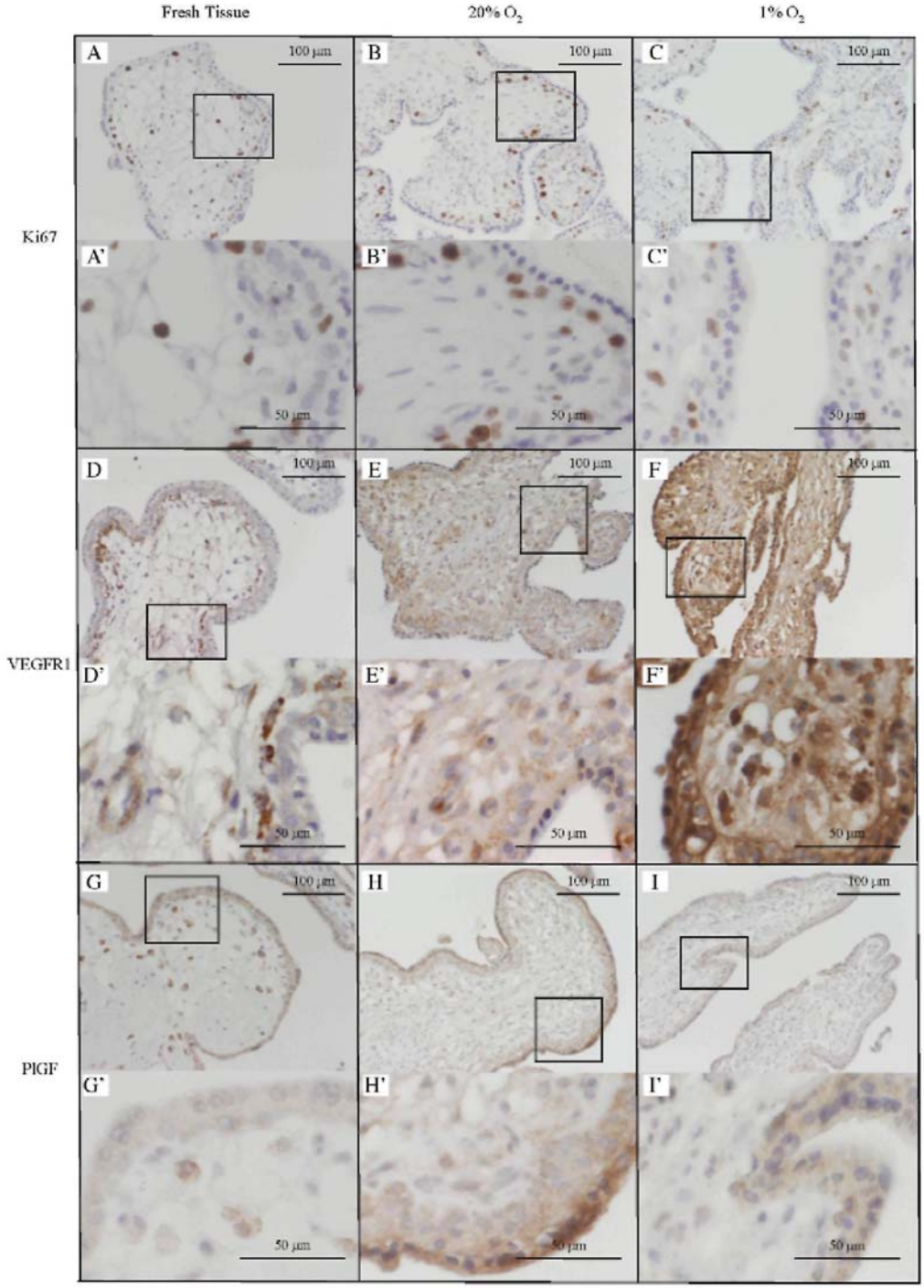
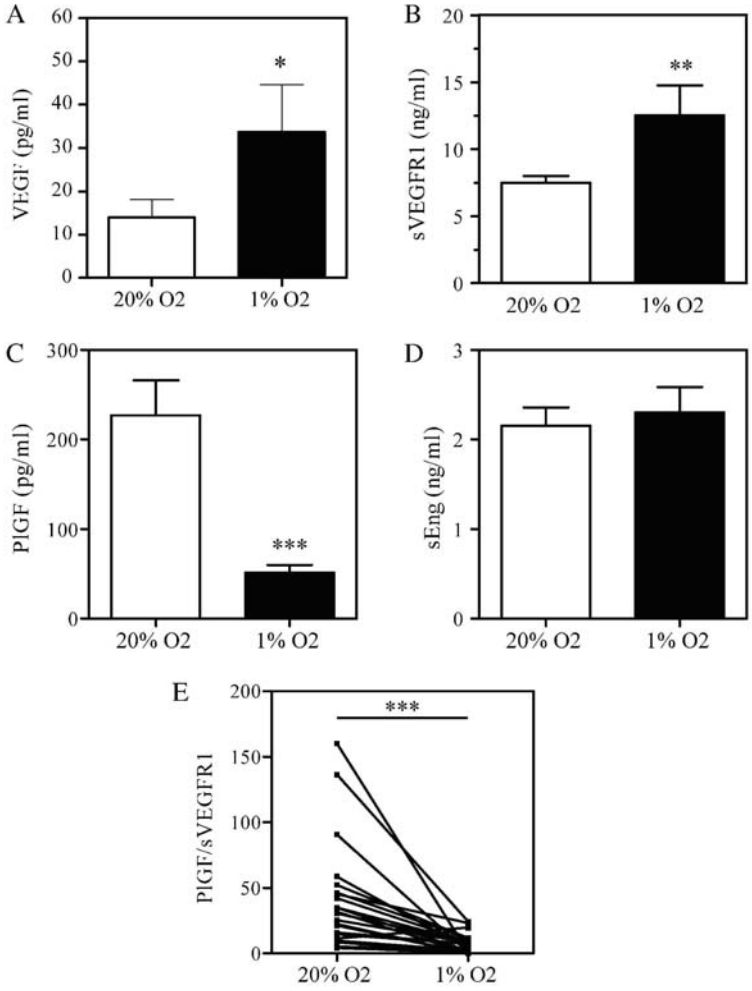


Figure 3: *VEGF, sVEGFR-1, PlGF and soluble endoglin (sEng) secretion in conditioned medium of villous expiants after 48 h of culture under normoxia (20% O_2) or hypoxia (1% O_2). Levels of VEGF (A), sVEGFR-1 (B), PlGF (C) and sEng (D) were assessed by ELISA and expressed as pg/ml or ng/ml (mean ± SE), n = 30. Levels of PlGF and sVEGFR-1 released from placental villous expiants were expressed as ratio (E), n = 30. *P < 0.05, **P < 0.01 and ***P < 0.001.*

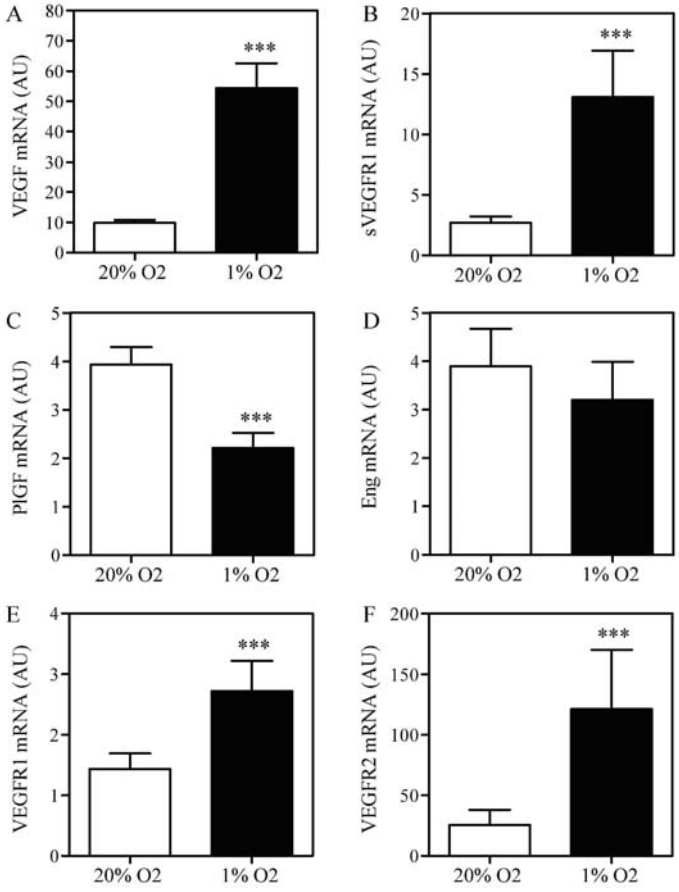


sVEGFR-1 and sEng effect on angiogenesis

In order to further evaluate the consequences of an increased production of sVEGFR-1 or endoglin upon endothelial cells and angiogenesis, we next incubated rat aortic rings with angiogenic cytokines VEGF (10ng/ml)±PlGF (10ng/ml) mixed with increasing doses of sVEGFR-1 (100 and 250 ng/ ml) or endoglin (100 or 250 ng/ml). In this model, sVEGFR-1 was able to totally abolish the angiostimulation induced by VEGF+PlGF, thus demonstrating the anti-angiogenic effect of excess of sVEGFR-1. Endoglin (100 ng/ml) was not able to modulate the neoangiogenesis in this experimental setting whereas a higher dose (250 ng/ml) had some additional effect on VEGF/PlGF-driven angiogenesis (Fig. 5A and B).

Since endoglin has been described as a coreceptor for TGFβ, we performed the same assay with TGFβ present. In the presence of TGFβ (15 ng/ml), neoangiogenesis was inhibited and increasing amounts of endoglin was not able to reverse this effect (Fig. 5A and C). Endoglin alone did not modulate neoangiogenesis (data not shown) in our experimental conditions.

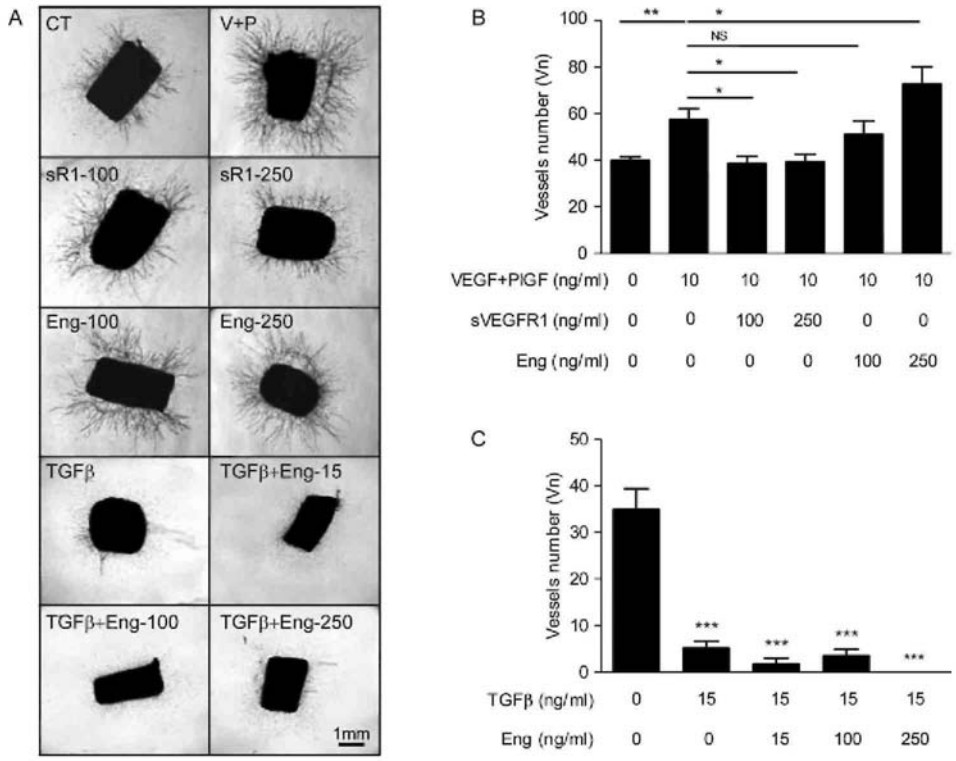
Figure 4: *VEGF, sVEGFR-1, PlGF, Eng, VEGFR-1 and VEGFR-2 mRNA in villous expiants after 48 h of culture under normoxia (20% O_2) or hypoxia (1% O_2). mRNA levels of VEGF (A), sVEGFR-1 (B), PlGF (C), Eng (D), VEGFR-1 (E) and VEGFR-2 (F) were quantified by real-time RT-PCR, normalized by 18S rRNA and expressed as arbitrary units (AU) (mean ± SE), n = 30. ***P < 0.001.*



Discussion

Pre-eclampsia is associated with defect of cytotrophoblasts to invade the spiral arterioles (Zhou *etal.,* 1998). Events, that normally take place during the first 20 weeks of pregnancy, which convert the maternal-fetal interface from a relatively hypoxic environment to one that is well oxygenated, fail to occur. Consequently, villous cytotrophoblast cells remain in a relatively hypoxic environment. In this study, we evaluated if hypoxia could be the trigger event at the origin of the abnormal angiogenic protein secretion pattern characterizing pre-eclampsia. In that view, we compared protein secretion and mRNA expression of these angiogenic proteins in first trimester villous expiants cultured under normoxia and hypoxia. Because of the difficulty in obtaining tissue, no work was carried out on tissue from pre-eclamptic pregnancies. Low oxygen induces some of the classical features of pre-eclampsia such as the up-regulation of sVEGFR-1 and VEGF and an imbalance of PlGF to sVEGFR-1 ratio. Interestingly, we demonstrated that the decreased level of circulating PlGF observed in pre-eclampsia could not only be a consequence of the trapping by sVEGFR-1 overproduced, but could also be due to hypoxia-induced PlGF mRNA down-regulation. By comparing mRNA expression of sVEGFR-1 and VEGFR-1, we observed that hypoxia increases 2.5-fold more the sVEGFR-1 mRNA transcription. These data strongly suggest that hypoxia promotes the alternative splicing of VEGFR-1 pre-mRNA in placenta. However, sEng was not shown to be modulated in the same experimental setting. Our data highlight that distinct placental pathogenic mechanisms are involved in the differential expression of sVEGFR-1 and sEng.

Figure 5: *Effect of sVEGR1 and/or endoglin in the aortic ring assay. Rat aortic rings were cultured in a collagen gel for 9 days with vehicle (CT), with VEGF+ PlGF (10ng/ml) (V+P), with a combination of sVEGFR-1 (100 or 250 ng/ml) and VEGF+PlGF (10 ng/ml) (sR1-100 and sR1-250), with a combination of endoglin (100 or 250 ng/ml) and VEGF+PlGF (10 ng/ml), with transforming growth factor β (TGFβ) (15 ng/ml) or with a combination of endoglin (15, 100 or 250 ng/ml) and TGFβ (15 ng/ml). (A) Representative photomicrographs (scale 25 ×) of aortic rings after 9 days of incubation (B) and (C) Quantification of microvessel outgrowth by computerized image analysis of aortic ring assay. Vn, vessels number; n = 4, mean ± SEM *P < 0.05, **P < 0.01 and ***P < 0.001.*



Reduced placental perfusion with attendant placental hypoxia is considered to be an important early feature of pregnancies complicated by pre-eclampsia or by intrauterine fetal growth restriction (Khong *et al.,* 1986; Brosens *et al.,* 2002). Hypoxia has been shown previously to increase sVEGFR-1 but not VEGF production by primary cytotrophoblasts culture (Lash *et al.,* 2002; Nagamatsu *et al.,* 2004). In their primary cytotrophoblasts cultures, they also showed a significant increase in the level of total VEGF and a decrease of free PlGF under reduced oxygen condition (2,5 and 8% O_2). We show here that the extravillous trophoblast cells HTR-8/SVneo respond to hypoxia by an increase of VEGF mRNA and protein secretion without any sVEGFR-1 modulation. Soluble VEGFR-1 mRNA detected in this extravillous cell lineage was not modulated by hypoxia. Our results are in agreement with those of Koklanaris *et al.* (2006). By microarray analysis, they indeed identified, in HTR8/SVneo cells cultured in hypoxic conditions, several hypoxia inducible factor 1 alpha regulated genes, like VEGF, tissue inhibitor of metalloproteinases-3 and plasminogen activator inhibitor-1, but sVEGFR-1 was not detected. Collectively these data indicate a differential modulation of villous versus extra villous trophoblast by hypoxia.

In HUVEC cells, VEGF mRNA was shown also to be upre-gulated by hypoxia even though VEGF secretion in the conditioned medium cells was under the sensitivity threshold of the ELISA. In contrast, sVEGFR-1 in HUVEC cells was not modulated by hypoxia. Similar results were described earlier by Nagamatsu *et al.* (2004).

As demonstrated by the *ex vivo* angiogenesis model of aorta ring assay, s VEGFR-1 counteracted angiogenesis induced by VEGF + PlGF. These results agree with the hypothesis that placental sVEGFR-1 secretion could contribute to maternal endothelial dysfunction.

sEng is an anti-angiogenic protein that inhibits TGF-β signaling in vasculature (Toporsian *et al.,* 2005; Venkatesha *et al.,* 2006). Endoglin is highly expressed on cell membranes of vascular endothelium and syncytiotrophoblasts (Cheifetz *et al.,* 1992; Gougos *et al.,* 1992). Recently, sEng has been implicated in the pathogenesis and prediction of pre-eclampsia, but high levels of sEng alone do not seem to be sufficient for the development of pre-eclampsia (Levine *et al.,* 2006a). Recent data indicate that hypoxia activates the endoglin promoter and significantly induces endoglin gene expression in human microvascular endothelial cells (Li *et al.,* 2003). Our data are in agreement with those of Venkatescha *et al.* (2006) who suggested that sEng is probably the result of a proteolytic cleavage rather than the presence of an alternate splice variant in placenta. Our data indicate that the enhanced levels of sEng observed in pre-eclampsia are not directly linked to villous trophoblast exposure to hypoxia.

In conclusion, our results support the hypothesis that, in preeclampsia, (i) overproduction of VEGF family factors by preeclamptic placenta is a consequence of induced hypoxia; (ii) overproduction of sVEGFR-1 by hypoxic villous trophoblast account for maternal free VEGF depletion; (iii) low circulating level of free PlGF is not only related to sVEGFR-1 overproduction, but also to hypoxia-induced mRNA down-regulation; (iv) sEng overproduction is probably due to an enhanced proteolytic cleavage independent of hypoxia.

We have demonstrated the suitability of an *in vitro* first trimester villous expiant model for studying the effects of hypoxia in placental tissues and more interestingly, we demonstrated that the increase in sVEGFR-1 found in maternal circulation of pre-eclamptic women is probably of placental origin and that hypoxia could be the trigger event.

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