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Glyceryl Trinitrate Inhibits Hypoxia-Induced Release of Soluble fms-Like Tyrosine Kinase-1 and Endoglin from Placental Tissues

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Preeclampsia is associated with increased circulating levels of proinflammatory molecules, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng). On release by an inadequately perfused placenta into the maternal circulation, these molecules cause systemic endothelial dysfunction and the associated hypertension and proteinuria that characterize preeclampsia. We previously showed that glyceryl trinitrate (GTN) inhibits hypoxia/reoxygenation-induced apoptosis in the syncytiotrophoblast of term chorionic villi explants. Herein, we demonstrate that GTN inhibits the release of sFlt-1 and sEng from term chorionic villi explants exposed to hypoxia. Although transcript levels and secretion of sFlt-1 and sEng increased in explants exposed to hypoxia, low concentrations of GTN significantly inhibited the hypoxia-induced expression of these molecules at the mRNA and protein levels. Treatment of explants with GTN also prevented the hypoxia-induced accumulation of hypoxia-inducible factor-1α, a key mediator of cellular adaptations to hypoxia. Furthermore, knockdown of hypoxia-inducible factor-1α inhibited the hypoxia-induced secretion of sFlt-1 and sEng. This study provides evidence that hypoxia induces the release of sFlt-1 and sEng in the placenta via a mechanism that is inhibited by low concentrations of GTN. Our findings indicate that GTN may have potential applications in the treatment and/or prevention of preeclampsia. ***(Am J Pathol 2011, 178:2888–2896; DOI: 10.1016/j.ajpath.2011.02.013)***

Preeclampsia is a leading cause of perinatal and maternal morbidity and mortality, affecting 3% to 7% of pregnant women worldwide.[1] It is characterized by the development of maternal hypertension, proteinuria, edema, and systemic coagulopathies. Although the etiology of preeclampsia is not well understood, there is evidence that high levels of antiangiogenic molecules [ie, soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng)] in the maternal circulation are linked to the endothelial dysfunction associated with this pregnancy complication.[2–5]

Secreted sFlt-1, usually a splice variant of Flt-1, is the soluble form of vascular endothelial growth factor receptor-1 (VEGFR-1). Soluble Flt-1 binds to angiogenic molecules (ie, placental growth factor and VEGF) and prevents them from interacting with VEGFR-1 and VEGFR-2 on the surface of endothelial cells.[5,6] Consequently, any increase in blood sFlt-1 levels causes a decrease in bioavailable VEGF and placental growth factor, with corresponding inhibition of angiogenesis and vascular function. There is evidence that the placenta is a primary source of sFlt-1 in human pregnancy,[7] and clinical studies have revealed that normal circulating sFlt-1 levels increase during gestation. However, compared with normal pregnancies, blood sFlt-1 levels are substantially higher in preeclamptic pregnancies.[2] Clinical signs of preeclampsia may be a direct outcome of high concentrations of serum sFlt-1.[5,8] Furthermore, animal experimentation has revealed that abnormally high circulating levels of sFlt-1 lead to hypertension, proteinuria, and endothelial damage.[5,9]

Eng is a cell membrane glycoprotein that functions as a coreceptor for transforming growth factor (TGF)-β signaling.[10] sEng competes with the binding of TGF-β1 to its corresponding cell surface receptors and, consequently, interferes with downstream TGF-β signaling in the vascula-

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ture.[4] Similar to signaling by VEGF and placental growth factor, TGF-β signaling plays an important role in the maintenance of vascular function and homeostasis. In preeclampsia, high levels of sEng in the maternal circulation correlate with increased expression of Eng in the placenta.[4,11] Circulating sEng levels were markedly increased 2 to 3 months before the clinical onset of preeclampsia.[12]

Placental hypoxia may play a major role in the pathophysiological characteristics of preeclampsia[9,13,14]; hypoxia increases the secretion of sFlt-1 and sEng from first- and second-trimester chorionic villi explants.[9,15–17] Although hypoxia also increases the secretion of sFlt-1 by third-trimester explants,[18] its effect on sEng secretion by third-trimester explants has not been determined. Because the clinical signs of preeclampsia only appear in the second half of pregnancy, it is possible that a hypoxia-induced increase in sFlt-1 and sEng secretion in the second and third trimesters of gestation contributes to the pathophysiological characteristics of preeclampsia.

The hypoxia response pathway represents a potential therapeutic target for inhibiting placental secretion of sFlt-1 and sEng. A key player that mediates many adaptive responses to hypoxia is the transcriptional activator hypoxia-inducible factor (HIF)-1. This transcription factor is a basic helix-loop-helix-PAS domain protein composed of α and β subunits. Although the HIF-1β subunit is constitutively expressed, the levels of HIF-1α increase in response to hypoxia.[19] The stability of HIF-1α is regulated by hydroxylation of proline residues 402, 564, or both in an oxygen-dependent reaction.[20] Proline hydroxylation of HIF-1α promotes the binding of the von Hippel–Lindau protein, leading to ubiquitination and proteasomal degradation of HIF-1α.[20,21]

There is evidence that relatively low concentrations of nitric oxide (NO) inhibit the accumulation of HIF-1α under hypoxia, thereby interfering with HIF-1–mediated adaptive responses.[22,23] Low concentrations (lower than micromolar) of NO mimetics [eg, glyceryl trinitrate (GTN)] inhibit the hypoxia-induced acquisition of malignant phenotypes in cancer cells, such as invasiveness, metastasis, and drug resistance.[24,25] Although the precise role of endogenous NO in normal and pathological pregnancy is unclear, there is evidence that decreased endogenous NO availability contributes to the pathophysiological characteristics of preeclampsia.[26] A previous study[27] demonstrated that low concentrations of GTN inhibit apoptosis of the syncytiotrophoblast in chorionic villi explants exposed to hypoxia/reoxygenation (H/R). In the present study, we determined the effect of administration of low concentrations of GTN on the secretion of sFlt-1 and sEng from term chorionic villi explants exposed to hypoxia.

Materials and Methods

Collection and Culture of Chorionic Villi Explants

Human term placentas (n = 22) were obtained from uncomplicated pregnancies immediately after caesarean deliveries at Kingston General Hospital, Kingston, ON. Placentas were collected with the approval of the Queen's University Research Ethics Board. Explants of chorionic villi were prepared as previously described[27] and incubated in 1.3 mL of serum-free RPMI 1640 medium (Invitrogen, Burlington, ON). For exposures to hypoxia, explants (approximately 60 per experiment) treated with or without GTN (10 nmol/L or 1 μmol/L; Sabex, Boucherville, QC) were incubated at 37°C for 24 hours in a humidified Plexiglas chamber flushed with a gas mixture of 5% CO_2 and 95% N_2. Oxygen concentrations within the chamber were maintained at 0.5% (PO_2 = 3.8 mm Hg) by oxygen regulators (ProOx 110; Biospherix Inc., Lacona, NY). Controls consisted of explants incubated in either 8% O_2 (PO_2 = 60 mm Hg, physiological concentrations) or 20% O_2 (PO_2 = 152 mm Hg) and 5% CO_2 for 24 hours. Explants were then collected or exposed to conditions of reoxygenation (20% O_2) for 3 additional hours. At the end of all incubations, placental tissues were either flash frozen for molecular analysis or fixed in 4% paraformaldehyde for histological analysis. Media of cultured explants were collected, centrifuged for 10 minutes at 8000 $\times$ g, divided into small tubes, and stored at -80°C until further analysis.

Immunohistochemistry

Randomly selected paraffin-embedded sections of chorionic villi were deparaffinized and subjected to antigen retrieval by heating them in a microwave oven for 22 minutes in citrate buffer (0.1 mol/L sodium citrate, pH 6.0). Primary antibodies used were goat polyclonal anti-human Eng or Flt-1 antibody (1 μg/mL; R&D Systems, Minneapolis, MN), mixed with mouse anti–human pan-cytokeratin antibody (1:100; Sigma-Aldrich, St Louis, MO) and incubated at 4°C overnight. Secondary antibodies were a mix of donkey anti-mouse (Alexa Fluor 568) and donkey anti-goat (Alexa Fluor 488) antibodies (1:500 each; Invitrogen, Carlsbad, CA). Slides were mounted with medium (VECTASHIELD Mounting Medium) containing DAPI (Vector Laboratories, Inc., Burlingame, CA). Micrographs were taken using a microscope (Zeiss Imager A1 Microscope; Carl Zeiss Canada Ltd, Toronto, ON) and software (Axiovision v 4.7.1.0; Carl Zeiss Canada Ltd).

Western Blot Analysis of HIF-1α

To assess the ability of chorionic villi to respond to hypoxia, the levels of HIF-1α were determined by Western blot analysis of flash-frozen chorionic villi preexposed to 0.5%, 8%, or 20% O_2 for 24 hours (n = 3 to 6 placentas). Primary antibodies used were mouse monoclonal antibody against HIF-1α (1:250; BD Biosciences, San Diego, CA), goat polyclonal anti–TGF-β1 (1:1000, R&D Systems), and monoclonal anti–β-actin antibody (clone AC-15, 1:8000; Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Goat anti-mouse antibody (1:5000, Vector Laboratories Inc.) labeled with horseradish peroxidase was used as a secondary antibody. The relative intensities of bands were determined by densitometry using software (AlphaErase; α Innotech Corp, San Leandro, CA).

Knockdown of HIF-1α in Chorionic Villi Explants

To down-regulate HIF-1α expression in chorionic villi, *HIF-1α* small-interfering RNA (siRNA) was introduced into explants using a transfection reagent (siPORT NeoFX; Ambion Inc., Austin, TX). This *HIF-1α* siRNA was validated and targets exon 5 of the human *HIF-1α* gene (number 42840; Ambion Inc.). Explants were transfected with 200 nmol/L *HIF-1α* siRNA or negative control siRNA 2 (Silencer; Ambion Inc.) and then incubated in a standard incubator at 5% CO_2 and 20% O_2 (151 mm Hg) for 24 hours. After this incubation, the culture medium was replaced with fresh RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) and explants were further incubated for 24 hours in either 20% or 0.5% O_2, as previously described.

Determination of sFlt-1 and sEng Levels in the Culture Media

To determine the levels of sFlt-1 or sEng in the culture media, kits (DuoSet ELISA Development System kits; R&D Systems) were used according to the manufacturer's instructions.

Real-Time PCR

Total RNA was isolated using a kit (High Pure RNA Isolation Kit; Qiagen, Valencia, CA), according to the manufacturer's protocol. Total RNA, 1 μg, was reverse transcribed with a kit (Omniscript RT Kit; Qiagen) using a random hexamer (Cortex, Kingston). Real-time PCR was performed with a system (LightCycler 480 Real-Time PCR System; Roche, Mississauga, ON) using a mix (SYBR Green PCR Master Mix; Roche) and primers, as follows: *β-actin*, 5′-TGGGACGACATGGAGAAAAT-3′ (sense) and 5′-GAGGCGTACAGGGATAGCAC-3′ (antisense); *Flt-1*, 5′-GCACCTTGGTTGTGGCTGAC-3′ (sense) and 5′-TGGAATTCGTGCTGCTTCCTGGTCC-3′ (antisense); *sFlt-1*, 5′-GCACTGCAACAAAAAGGC-3′ (sense) and 5′-CCAGGAATGTATACACAGG-3′ (antisense); *Eng*, 5′-GCTGGATGAGCCGGGAGCTCCCTGCTG-3′ (sense) and 5′-CACAGGCTGAAGGTCACAATGGACTG-3′ (antisense); and *HIF-1α*, 5′-TGCTTGGTGCTGATTTGTGA-3′ (sense) and 5′-GGTCAGATGATCAGAGTCCA-3′ (antisense). Real-time reaction conditions for all sets of primers were as follows: 94°C for 5 minutes, followed by 60 cycles at 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. The intensity of dye (FastStart SYBR Green) was analyzed using software (LightCycler 480; Roche). Transcript levels were normalized against *β-actin* mRNA levels.

Statistical Analysis

For statistical analysis of enzyme-linked immunosorbent assay and quantitative PCR results, one-way analysis of variance was performed and significant differences between groups were determined using Tukey's multiple comparisons post hoc test. All statistical tests were two sided, and differences were considered significant at $P < 0.05$.

Results

Immunolocalization of Flt-1 and Eng in Chorionic Villi Explants

To determine the potential source of secreted Flt-1 and Eng, immunofluorescence using polyclonal antibodies against Flt-1 and Eng was performed on sections of chorionic villi explants incubated in 20% O_2 (see Supplemental Figure S1, A and E, at *http://ajp.amjpathol.org*). Cytokeratin was used as a marker for trophoblast (see Supplemental Figure S1, B and F, at *http://ajp.amjpathol.org*). Both Flt-1 and Eng colocalized with cytokeratin primarily to the syncytiotrophoblast layer (see Supplemental Figure S1, D and H, at *http://ajp.amjpathol.org*), indicating that this tissue is a potential source of the secreted molecules. We also observed that Flt-1, but not Eng, is expressed in the fetal vessels within the villous stroma, indicating that fetal vessels are another potential source of sFlt-1 but not of sEng (see Supplemental Figure S1, D versus H, respectively, at *http://ajp.amjpathol.org*).

Effect of Hypoxia on the Levels of sFlt-1 and Eng mRNA and Secreted Protein

Previous studies[14,15,17,28] reported hypoxia-induced sFlt-1 and sEng secretion by trophoblast cell lines and first-trimester chorionic villi explants. Given that preeclampsia is mostly a disease of the second half of pregnancy, we examined the effect of hypoxia on the mRNA levels of *Flt-1* variants and *Eng* by term chorionic villi explants. The secreted form of Flt-1 either can be encoded by a spliced variant of the *Flt-1* (*spliced Flt-1*) gene or, less frequently, is the product of the cleaved ectodomain of the Flt-1 receptor (*receptor Flt-1*).[29,30] To determine whether a specific variant of Flt-1 is responsive to hypoxia in term villous explants, we examined both species of *Flt-1* mRNA using real-time PCR. Exposure to 0.5% O_2 increased the expression of both the *spliced Flt-1* and *receptor Flt-1* mRNA species compared with exposure to either 20% or 8% O_2 (Figure 1A). However, although the expression of the *spliced Flt-1* variant increased several thousandfold, the levels of transcript encoding the *receptor Flt-1* variant increased by only approximately twofold. Consequently, the ratio of *spliced Flt-1*/*receptor Flt-1* significantly increased in chorionic villi exposed to hypoxia compared with chorionic villi exposed to either 20% or 8% O_2 (Figure 1A: $P < 0.001$ for both). This ratio is an indication of increased bioavailability of the spliced (secreted) Flt-1 variant compared with nonspliced Flt-1 receptor in chorionic villi exposed to hypoxia. Exposure of chorionic villi to hypoxia (0.5% O_2) resulted in a fivefold and a threefold increase in Eng mRNA levels compared with exposure to 20% and 8% O_2, respectively (Figure 1B: $P < 0.001$ for both). Compared with exposure to 20% O_2, exposure to 8% O_2 did not result in statistically significant increases in the levels of sFlt-1 and sEng mRNA.

To further characterize the effect of hypoxia on the secretion of sFlt-1 and sEng, we performed an enzyme-

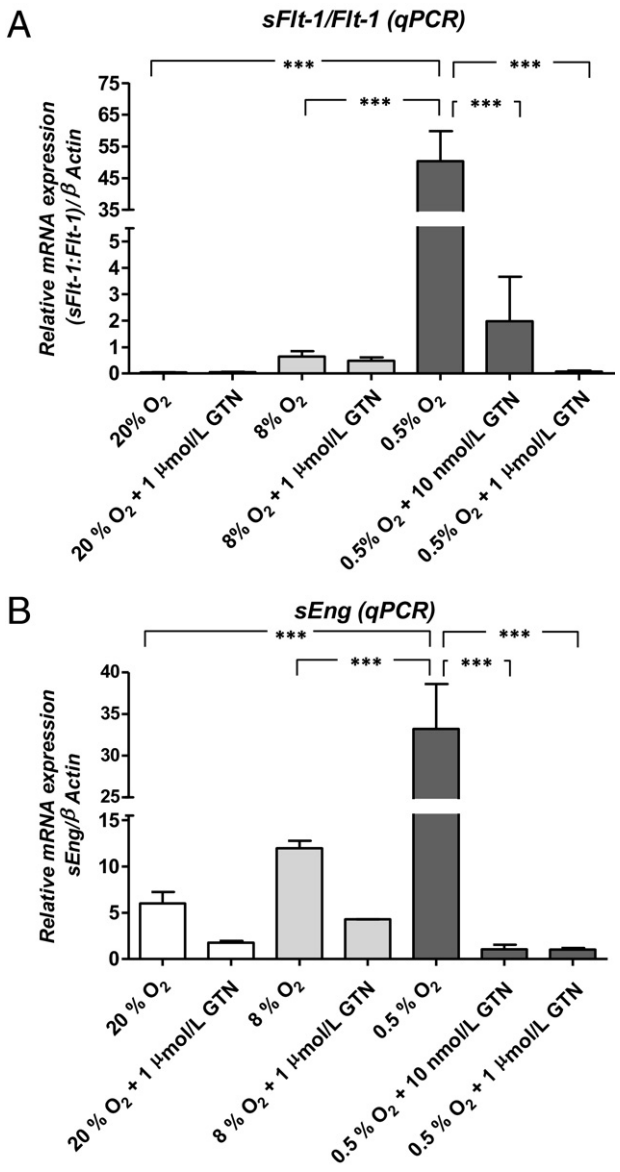

Figure 1. Effect of GTN on hypoxia-induced increases in *sFlt-1* and *sEng* mRNA levels in chorionic villi explants. (minimum of three placentas per treatment) **A:** Quantitative PCR (qPCR) analysis of *Flt-1* mRNA levels (expressed as the ratio of *spliced Flt-1/receptor Flt-1* mRNA forms). Transcript levels were normalized against β-actin mRNA levels. **B:** qPCR analysis of sEng; mRNA levels were quantified relative to β-actin transcript levels. Error bars represent SEM. ***$P < 0.001$. Transverse lines link two groups to indicate statistical significance.

linked immunosorbent assay on the media of explant cultures. Explants exposed to 0.5% O_2 for 24 hours secreted significantly higher levels of sFlt-1 and sEng than explants incubated in 20% or 8% O_2 (Figure 2, A and B). Although the data shown in Figure 2 are expressed as pictograms of sFlt-1 or sEng per microgram of protein in the media, differences in the reported concentrations of these molecules were not the result of differences in the total amount of protein secreted by chorionic villi; the latter was similar across the different treatment groups (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Compared with exposure to 20% O_2 alone, exposure of chorionic villi to H/R (24 hours in 0.5% O_2, followed by 3 hours in 20% O_2) did not significantly increase the detectable levels of sFlt-1 and sEng in the culture medium (see Supplemental Figure S3, A and B, at *http://ajp.amjpathol.org*). Accordingly, compared with exposure to hypoxia for 24 hours, subsequent reoxygenation of chorionic villi explants (3 hours in 20% O_2) resulted in a significant *decrease* in the levels of detectable sFlt-1 and sEng (see Supplemental Figure S4, A and B, at *http://ajp.amjpathol.org*; $P < 0.05$ for both).

Effect of GTN on the Hypoxia-Induced Increases in sFlt-1 and sEng mRNA Levels and Secretion

The hypoxia-mediated increases in sFlt-1 and sEng mRNA accumulation and protein secretion were significantly inhibited by GTN at concentrations of 10 nmol/L or 1 μmol/L (Figure 1, A and B, and Figure 2, A and B). The inhibitory effect of GTN at 1 μmol/L was confirmed by immunofluorescence microscopy of explants using polyclonal antibodies against Flt-1 or Eng. Compared with exposure to hypoxia alone, Flt-1 and Eng immunofluorescence intensity under hypoxic conditions was substantially decreased after treatment with 1 μmol/L GTN (Figure 2, C and D).

Effect of Hypoxia and GTN on HIF-1α Accumulation

To elucidate the mechanism by which GTN inhibits hypoxia-induced secretion of sFlt-1 and sEng, we examined the effect of GTN on the accumulation of HIF-1α in explants incubated in 0.5% O_2. HIF-1α levels were significantly increased in explants exposed to 0.5% O_2 compared with HIF-1α levels in explants exposed to 20% O_2 (Figure 3, A and B: $P < 0.01$) or 8% O_2 (Figure 3, A and B: $P < 0.05$). Treatment with GTN at a concentration of 1 μmol/L or 10 nmol/L significantly inhibited the accumulation of HIF-1α in explants exposed to 0.5% O_2 (Figure 3, A and B: $P < 0.001$ and $P < 0.01$, respectively). Consistent with the inhibitory effects of GTN on sFlt-1 and sEng secretion, the most pronounced suppression of HIF-1α accumulation was achieved with GTN at a concentration of 1 μmol/L.

Effect of HIF-1α Knockdown on the Secretion of sFlt-1 and sEng

To determine whether HIF-1 mediates the hypoxia-induced secretion of sFlt-1 and sEng, we knocked down *HIF-1α* mRNA in chorionic villi explants. Villi exposed to 0.5% O_2 significantly expressed higher levels of *HIF-1α* mRNA compared with villi exposed to 20% O_2 or villi exposed to 0.5% O_2 and transfected with *HIF-1α* siRNA (Figure 4A: $P < 0.001$ for both). Chorionic villi with higher *HIF-1α* expression levels (ie, exposed to 0.5% O_2) secreted more sFlt-1 and sEng than those with lower *HIF-1α* mRNA levels (ie, exposed to 20% O_2; Figure 4, B and C: $P < 0.05$ and $P < 0.01$, respectively). Knockdown of *HIF-1α* with siRNA inhibited the hypoxia-induced secretion of sFlt-1 and sEng (Figure 4, B and C: $P < 0.001$ for

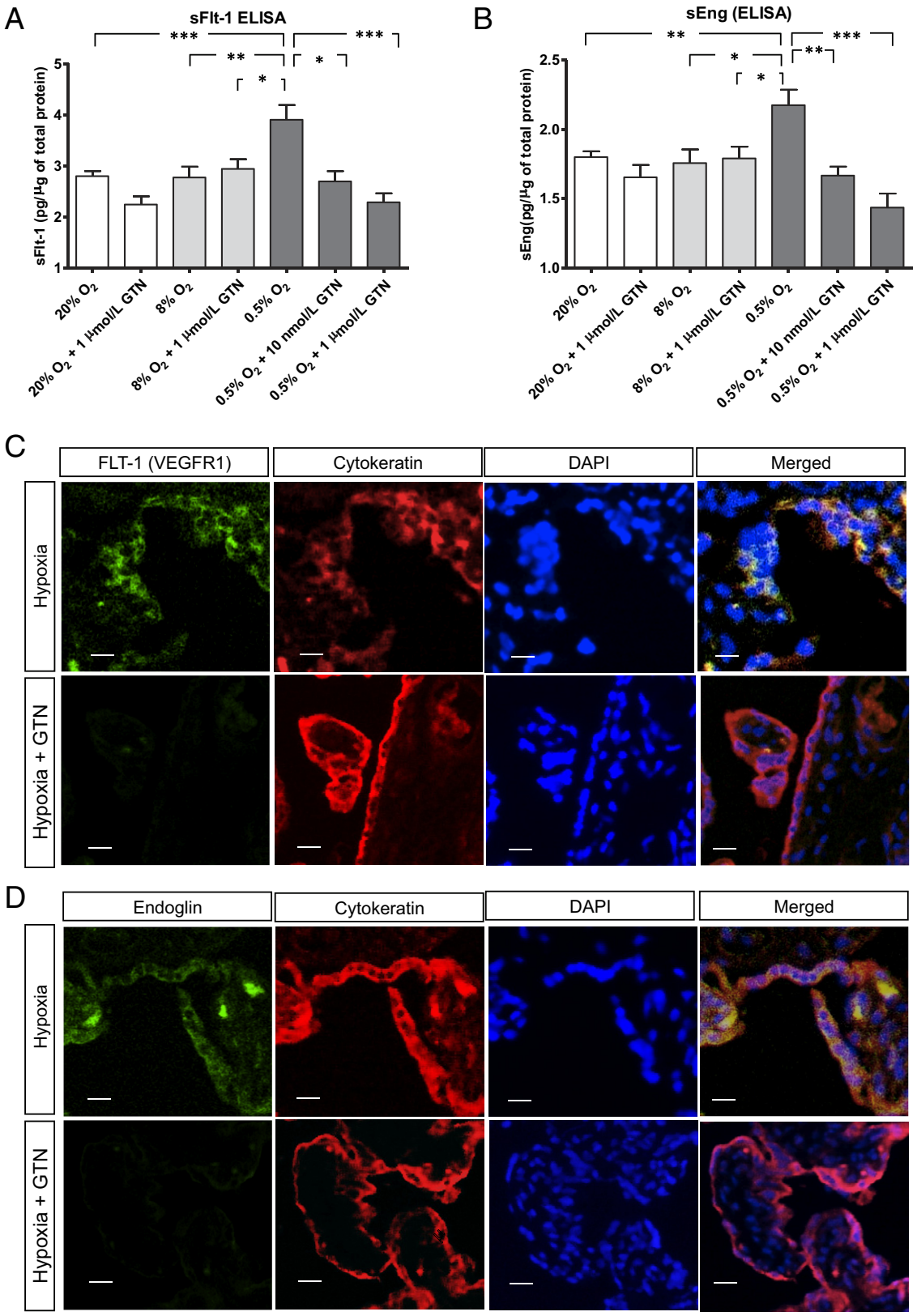

Figure 2. Effect of GTN on the secretion and expression of sFlt-1 and sEng by chorionic villi explants exposed to hypoxia. **A** and **B:** Relative sFlt-1 and sEng levels, respectively, in the medium of explant cultures (six explants per placenta, with a minimum of three placentas per treatment). Concentrations (in ng/mL) were divided by the total amount of protein (in μg/mL) in the media. Error bars represent SEM. The lines in **A** and **B** identify the comparison groups by the vertical lines pointing to the corresponding bars. **C** and **D:** Localization of Flt-1, Eng, cytokeratin (trophoblast marker), and nuclei (DAPI) by fluorescence microscopy. **Far right:** The merged images with all three markers combined, with yellow representing areas of colocalization of sFlt-1 or Eng with cytokeratin, are shown. **Top:** Explants exposed to hypoxia (0.5% O_2). **Bottom:** Explants exposed to hypoxia in the presence of 1 μmol/L GTN. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Transverse lines link two groups to indicate statistical significance. Scale bars = 10 μm. ELISA indicates enzyme-linked immunosorbent assay.

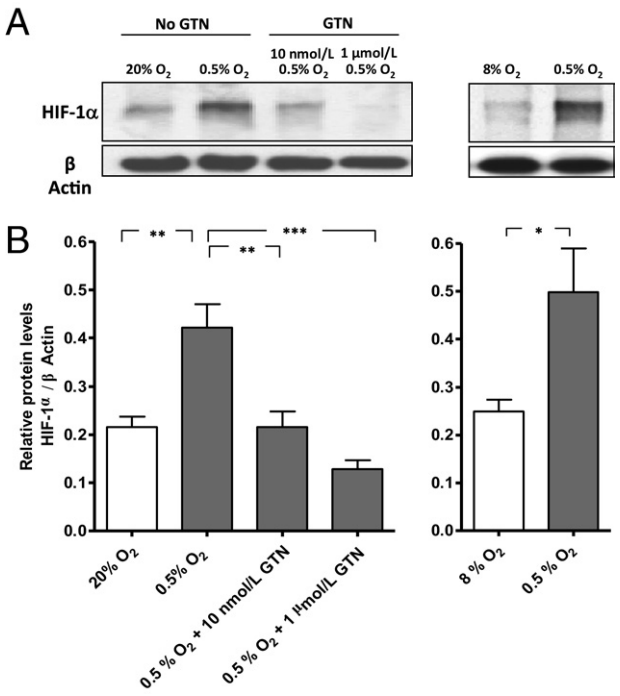

Figure 3. Effect of GTN on the hypoxia-induced accumulation of HIF-1α in chorionic villi explants. **A:** Western blot analysis of HIF-1α protein in explants cultured in 20%, 8%, or 0.5% O_2. The administration of GTN (1 μmol/L or 10 nmol/L) inhibited the accumulation of HIF-1α in explants incubated in 0.5% O_2. Incubation of explants in 8% O_2 did not lead to accumulation of HIF-1α. **B:** Densitometric quantification of pooled Western blots from three independent experiments. The lines in **A** and **B** identify the comparison groups by the vertical lines pointing to the corresponding bars. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (Minimum of three placentas per treatment).

both). Furthermore, explants transfected with *HIF-1α* siRNA secreted significantly lower levels of sFlt-1 and sEng than explants transfected with scrambled negative control siRNA (Figure 4, B and C: $P < 0.05$ and $P < 0.01$, respectively). These results indicate that HIF-1α is a key mediator of the hypoxia-induced secretion of sFlt-1 and sEng. These findings also indicate a hypoxia-induced up-regulation of HIF-1α at the mRNA level, which was previously reported.[31] However, our findings do not exclude the possibility that hypoxia may also increase HIF-1α levels by increasing protein stability.[23,32]

Discussion

The main novel finding that we report herein is that hypoxia-induced secretion of sFlt-1 and sEng by chorionic villi explants can be inhibited by low concentrations of GTN. Given that hypoxia and increased circulating levels of sFlt-1 and sEng contribute to the pathophysiological characteristics of preeclampsia, the results of this study indicate that administration of low doses of NO mimetics to women with preeclampsia or at a high risk of developing this complication may be a useful therapeutic or preventative strategy.

Other studies[14,17,28] have focused primarily on determining the effect of hypoxia on placental sFlt-1 secretion; increased sFlt-1 secretion during preeclampsia may be part of a placental response to hypoxia, whereby sFlt-1 increases maternal blood pressure to improve placental perfusion. Hypoxia-mediated stimulation of sFlt-1 and sEng secretion by *first-trimester* chorionic villi explants was previously reported.[15,17,28] However, the role of hypoxia in the regulation of sEng secretion is controversial. Although some studies[28,33] have described a lack of effect of hypoxia on sEng secretion, others[15,34] have found a stimulatory effect in first-trimester explants and preeclamptic term placentas but not in normal-term placentas. In light of our results, it is possible that sEng secretion is dependent on gestational age and length of exposure to hypoxia.

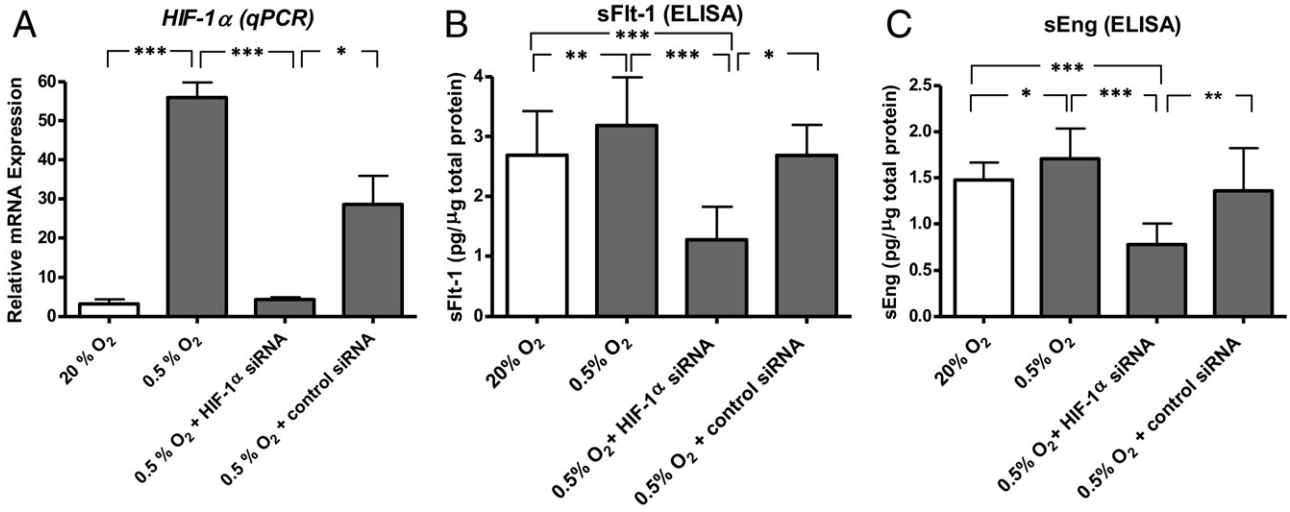

Figure 4. Effect of HIF-1α knockdown on the hypoxia-induced increase in mRNA levels and secretion of sFlt-1 and sEng by third-trimester chorionic villi explants. (minimum of three placentas per treatment). **A:** Expression of HIF-1α transcript levels relative to β-actin transcript levels as determined by real-time quantitative PCR (qPCR). **B** and **C:** Relative levels of sFlt-1 and sEng protein in the tissue culture media. Values (in ng/mL) were divided by the total amount of protein (in μg/mL) in the media. The lines in **A** and **B** identify the comparison groups by the vertical lines pointing to the corresponding bars. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Lines with **asterisks** denote the comparison groups. ELISA indicates enzyme-linked immunosorbent assay.

In women who later develop preeclampsia, increased circulating sFlt-1 and sEng levels are first detected in the late second and early third trimesters, respectively, before the clinical onset of disease.[12,35] Thus, cultures of third-trimester explants may represent a more accurate model to study the effects of hypoxia on placental sFlt-1 and sEng secretion.

In our study, only hypoxia, and not H/R, resulted in increased detection of sFlt-1 and sEng in the culture medium. Although the effect of H/R on the secretion of sFlt-1 and sEng by chorionic villi was not previously examined, Cindrova-Davies[36] reported a significant increase in tissue-associated sFlt-1 levels in chorionic villi after exposure to H/R. In our study, compared with exposure to hypoxia alone, exposure to conditions of reoxygenation resulted in decreased detectable levels of sFlt-1 and sEng. We cannot exclude that H/R increased the secretion of sFlt-1 and sEng by chorionic villi explants. It is possible that the decreased detection of these molecules after H/R was the result of sequestration by high levels of secreted VEGF and TGF-β, which can potentially interfere with the detection assay. In support of this concept, Western blot analysis revealed substantial increases in the levels of TGF-β1 in the culture medium of explants exposed to H/R compared with media from explants incubated in 20% or 0.5% O_2 alone (see Supplemental Figure S4C at *http://ajp.amjpathol.org*).

Although exposure of chorionic villi to hypoxia alone can lead to apoptosis,[27,37] thereby resulting in the release of vasoactive molecules, we showed herein that the hypoxia-induced release of sFlt-1 and sEng can also be transcriptionally regulated. This conclusion is based on the observation that the hypoxia-induced expression and release of these molecules was mediated by HIF-1α. In our study, both HIF-1α protein and mRNA levels increased in chorionic villi exposed to hypoxia. Although HIF-1α levels are mostly regulated at the level of protein stabilization, there is evidence that hypoxia can increase the levels of HIF-1α mRNA in chorionic villi exposed to hypoxia.[31]

Our results also revealed that the increased ratio of *spliced Flt-1*/*receptor Flt-1* mRNA and the increase in *Eng* mRNA levels were much higher than the secreted protein levels. To our knowledge, this is the first report on the ratio of *spliced Flt-1*/receptor *Flt-1* mRNA in the placenta and may, in part, explain the observed large increase in *sFlt-1* mRNA levels. Differences between secreted protein and mRNA levels may be explained at the level of RNA stability and efficiency of the translational machinery.

Our findings support the results of previous studies[34,38] showing that the syncytiotrophoblast is a major source of Flt-1 and Eng. We have demonstrated that the expression and secretion of Flt-1 and Eng from syncytiotrophoblast exposed to hypoxia can be inhibited by GTN. The rationale for testing the effect of GTN on the expression and release of these molecules was based on a previous study[27] in which H/R-mediated apoptosis of term chorionic villi was significantly attenuated by low concentrations of GTN. It is possible that the effects of GTN, as an NO mimetic, reflect the well-known actions of NO. At lower concentrations, such as those used in our study, NO activates soluble guanylyl cyclase to generate cGMP. Under certain conditions involving low oxygenation, NO can also inhibit mitochondrial respiration so that oxygen becomes available for the activation of prolyl hydroxylases and the degradation of HIF-1α.[23,32] Herein, we showed that GTN was able to inhibit HIF-1α protein accumulation. The precise mechanism by which GTN inhibited HIF-1α accumulation requires further investigation. However, based on this observation, together with the finding that siRNA-mediated knockdown of HIF-1α prevented the hypoxia-induced release of sFlt-1 and sEng, we conclude that the inhibitory effect of GTN on the hypoxia-induced secretion of sFlt-1 and sEng was likely the result of inhibition of HIF-1 activity.

The pathophysiological characteristics of preeclampsia and other complications of pregnancy have been linked to a maternal immune imbalance with greater secretion of proinflammatory cytokines and a relative decrease in the levels of anti-inflammatory molecules, such as IL-10.[39,40] Although the administration of IL-10 to chorionic villi explants did not affect the secretion of sFlt-1,[41] treatment of explants with exogenous sFlt-1 increased IL-10 and tumor necrosis factor-α secretion.[42] It is possible that, by increasing the secretion of tumor necrosis factor-α in the placenta, sFlt-1 contributes to the maternal inflammatory state characteristic of severe preeclampsia.

Women who have had preeclampsia are at increased risk of recurrence in a subsequent pregnancy.[43,44] Despite the fact that the cause of preeclampsia is largely unknown, the increase in sFlt-1 and sEng levels in the maternal circulation is tightly linked to the pathophysiological characteristics of preeclampsia. Current therapeutic strategies have relied primarily on managing symptoms, with little evidence that any intervention alters the underlying pathophysiological characteristics.[43] For example, antihypertensive and antiplatelet drugs have been used to treat severe hypertension and coagulopathies associated with preeclampsia.[43] However, it is unclear that such approaches have an impact on other maternal and fetal comorbidities. Furthermore, a recent study[45] revealed that antihypertensive drugs had no effect on placental secretion of sFlt-1 and sEng. There are few published studies on the use of GTN for the treatment of preeclampsia. Some studies[46–48] have reported beneficial effects of GTN in preeclamptic patients with deficient uteroplacental blood flow. Other studies[49] did not reveal the beneficial effects of this drug. In those studies, it is possible that patients developed tolerance to GTN because of continuous use of relatively high doses of this drug. It is also possible that only a subset of patients with preeclampsia benefit from GTN therapy (specifically, patients with hypoxic placentas and elevated serum levels of sFlt-1 and/or sEng).

In summary, our current study reveals a novel strategy to inhibit the secretion of sFlt-1 and sEng from placental tissues exposed to hypoxia and, therefore, has the potential of leading to novel modalities for the management and prevention of preeclampsia.

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References

1. Redman CW, Sargent IL: Latest advances in understanding pre-eclampsia. Science 2005, 308:1592–1594
2. Koga K, Osuga Y, Yoshino O, Hirota Y, Ruimeng X, Hirata T, Takeda S, Yano T, Tsutsumi O, Taketani Y: Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. J Clin Endocrinol Metab 2003, 88:2348–2351
3. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA: Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004, 350:672–683
4. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA: Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 2006, 12:642–649
5. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003, 111:649–658
6. Ahmad S, Ahmed A: Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circ Res 2004, 95:884–891
7. Banks RE, Forbes MA, Searles J, Pappin D, Canas B, Rahman D, Kaufmann S, Walters CE, Jackson A, Eves P, Linton G, Keen J, Walker JJ, Selby PJ: Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor: Flt-1. Mol Human Reprod 1998, 4:377–386
8. Tsatsaris V, Goffin F, Munaut C, Brichant J-F, Pignon M-R, Noel A, Schaaps J-P, Cabrol D, Frankenne F, Foidart J-M: Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. J Clin Endocrinol Metab 2003, 88:5555–5563
9. Makris A, Thornton C, Thompson J, Thomson S, Martin R, Ogle R, Waugh R, McKenzie P, Kirwan P, Hennessy A: Uteroplacental ischemia results in proteinuric hypertension and elevated sFlt-1. Kidney Int 2007, 71:977–984
10. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M: Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells. J Biol Chem 1992, 267:19027
11. Masuyama H, Nakatsukasa H, Takamoto N, Hiramatsu Y: Correlation between soluble endoglin, vascular endothelial growth factor receptor-1, and adipocytokines in preeclampsia. J Clin Endocrinol Metab 2007, 92:2672–2679
12. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA: Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006, 355:992–1005
13. Sandau KB, Zhou J, Kietzmann T, Brune B: Regulation of the hypoxia-inducible factor 1alpha by the inflammatory mediators nitric oxide and tumor necrosis factor-alpha in contrast to desferroxamine and phenylarsine oxide. J Biol Chem 2001, 276:39805–39811
14. Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S, Post M, Caniggia I: Molecular evidence of placental hypoxia in preeclampsia. J Clin Endocrinol Metab 2005, 90:4299–4308
15. Yinon Y, Nevo O, Xu J, Many A, Rolfo A, Todros T, Post M, Caniggia I: Severe intrauterine growth restriction pregnancies have increased placental endoglin levels: hypoxic regulation via transforming growth factor-beta 3. Am J Pathol 2008, 172:77–85
16. Zamudio S, Wu Y, Ietta F, Rolfo A, Cross A, Wheeler T, Post M, Illsley NP, Caniggia I: Human placental hypoxia-inducible factor-1α expression correlates with clinical outcomes in chronic hypoxia in vivo. Am J Pathol 2007, 170:2171–2179
17. Nevo O, Soleymanlou N, Wu Y: Increased expression of sFlt-1 in in vivo and in vitro models of human placental hypoxia is mediated by HIF-1. Am J Physiol Regul Integr Comp Physiol 2006, 291:R1085
18. Rajakumar A, Powers R, Hubel C, Shibata E, von Versen-Hoynck F, Plymire D, Jeyabalan A: Novel soluble Flt-1 isoforms in plasma and cultured placental explants from normotensive pregnant and pre-eclamptic women. Placenta 2009, 30:25–34
19. Wang GL, Jiang BH, Rue EA, Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995, 92:5510–5514
20. Semenza GL: Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE 2007, 2007:cm8
21. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001, 292:468–472
22. Brune B, Zhou J: Nitric oxide and superoxide: interference with hypoxic signaling. Cardiovasc Res 2007, 75:275–282
23. Hagen T, Taylor CT, Lam F, Moncada S: Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science 2003, 302:1975–1978
24. Frederiksen LJ, Sullivan R, Maxwell LR, Macdonald-Goodfellow SK, Adams MA, Bennett BM, Siemens DR, Graham CH: Chemosensitization of cancer in vitro and in vivo by nitric oxide signaling. Clin Cancer Res 2007, 13:2199–2206
25. Frederiksen LJ, Siemens DR, Heaton JP, Maxwell LR, Adams MA, Graham CH: Hypoxia induced resistance to doxorubicin in prostate cancer cells is inhibited by low concentrations of glyceryl trinitrate. J Urol 2003, 170:1003–1007
26. Lowe DT: Nitric oxide dysfunction in the pathophysiology of pre-eclampsia. Nitric Oxide 2000, 4:441–458
27. Belkacemi L, Bainbridge SA, Dickinson MA, Smith GN, Graham CH: Glyceryl trinitrate inhibits hypoxia/reoxygenation-induced apoptosis in the syncytiotrophoblast of the human placenta: therapeutic implications for preeclampsia. Am J Pathol 2007, 170: 909–920
28. Munaut C, Lorquet S, Pequeux C, Blacher S, Berndt S, Frankenne F, Foidart J-M: Hypoxia is responsible for soluble vascular endothelial growth factor receptor-1 (VEGFR-1) but not for soluble endoglin induction in villous trophoblast. Hum Reprod 2008, 23: 1407–1415
29. Kishuku M, Nishioka Y, Abe S, Kishi J, Ogino H, Aono Y, Azuma M, Kinoshita K, Rentsenhand B, Makino H, Ranjan P, Minakuchi K, Sone S: Expression of soluble vascular endothelial growth factor receptor-1 in human monocyte-derived mature dendritic cells contributes to their antiangiogenic property. J Immunol 2009, 183:8176–8185
30. Kendall RL, Thomas KA: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A 1993, 90:10705–10709
31. Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, Post M: Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3), J Clin Inves 2000, 105:577–587
32. Palacios-Callender M, Quintero M, Hollis VS, Springett RJ, Moncada S: Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome c oxidase, Proc Natl Acad Sci U S A 2004, 101:7630–7635
33. Jeyabalan A, McGonigal S, Gilmour C, Hubel C, Rajakumar A: Circulating and placental endoglin concentrations in pregnancies complicated by intrauterine growth restriction and preeclampsia, Placenta 2008, 29:555–563
34. Gu Y, Lewis DF, Wang Y: Placental productions and expressions of soluble endoglin, soluble fms-like tyrosine kinase receptor-1, and placental growth factor in normal and preeclamptic pregnancies, J Clin Endocrinol Metab 2008, 93:260–266
35. De Vivo A, Baviera G, Giordano D, Todarello G, Corrado F, D'Anna R: PlGF and sFlt-1 as markers for predicting pre-eclampsia. Acta Obstet Gynecol Scand 2008, 87:837–842
36. Cindrova-Davies T: Gabor Than Award Lecture 2008: Pre-eclampsia – from placental oxidative stress to maternal endothelial dysfunction, Placenta 2009, 30:55–65
37. Burton GJ, Jauniaux E: Placental oxidative stress: from miscarriage to preeclampsia, J Soc Gynecol Investig 2004, 11:342–352

38. Helske S, Vuorela P, Carpen O, Hornig C, Weich H, Halmesmaki E: Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. Mol Human Reprod 2001, 7:205–210
39. Saito S, Sakai M: Th1/Th2 balance in preeclampsia. J Reprod Immunol 2003, 59:161–173
40. Raghupathy R, Makhseed M, Azizieh F, Hassan N, Al-Azemi M, Al-Shamali E: Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cell Immunol 1999, 196:122–130
41. Royle C, Lim S, Xu B, Tooher J, Ogle R, Hennessy A: Effect of hypoxia and exogenous IL-10 on the pro-inflammatory cytokine TNF-alpha and the anti-angiogenic molecule soluble Flt-1 in placental villous explants. Cytokine 2009, 47:56–60
42. Xu B, Thornton C, Tooher J, Hennessy A: Exogenous soluble VEGF receptor-1 (sFlt-1) regulates Th1/Th2 cytokine production from normal placental explants via intracellular calcium. Hypertens Pregnancy 2009, 28:448–456
43. Duley L, Meher S, Abalos E: Management of pre-eclampsia. BMJ 2006, 332:463–468
44. Roberts DJ, Post MD: The placenta in pre-eclampsia and intrauterine growth restriction. J Clin Pathol 2008, 61:1254–1260
45. Xu B, Thornton C, Tooher J, Ogle R, Lim S, Makris A, Hennessy A: Effects of anti-hypertensive drugs on production of soluble fms-like tyrosine kinase 1 and soluble endoglin from human normal and pre-eclamptic placentas in vitro. Clin Exp Pharmacol Physiol 2009, 36:839–842
46. Cacciatore B, Halmesmaki E, Kaaja R, Teramo K, Ylikorkala O: Effects of transdermal nitroglycerin on impedance to flow in the uterine, umbilical, and fetal middle cerebral arteries in pregnancies complicated by preeclampsia and intrauterine growth retardation. Am J Obstet Gynecol 1998, 179:140–145
47. Grunewald C, Kublickas M, Carlstrom K, Lunell N, Nisell H: Effects of nitroglycerin on the uterine and umbilical circulation in severe preeclampsia. Obstet Gynecol 1995, 86:600–604
48. Cetin A, Yurtcu N, Guvenal T, Imir A, Duran B, Cetin M: The effect of glyceryl trinitrate on hypertension in women with severe preeclampsia. HELLP syndrome, and eclampsia. Hypertens Pregnancy 2004, 23:37–46
49. Lees C, Valensise H, Black R, Harrington K, Byiers S, Romanini C, Campbell S: The efficacy and fetal-maternal cardiovascular effects of transdermal glyceryl trinitrate in the prophylaxis of pre-eclampsia and its complications: a randomized double-blind placebo-controlled trial. Ultrasound Obstet Gynecol 1998, 12:334–338