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Nitric Oxide Generation Affects Pro- and Anti-Angiogenic Growth Factor Expression in Primary Human Trophoblast

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Abstract

Objectives—Preeclampsia is associated with reduced trophoblast placenta growth factor (PGF) expression, elevated soluble fms-like tyrosine kinase-1 (sFlt-1) and decreased bioactivity of nitric oxide (NO). Elevated sFlt-1 reduces bioavailability of PGF and vascular endothelial growth factor (VEGF) leading to maternal endothelial dysfunction. Although NO can regulate gene expression, its ability to regulate trophoblast expression of angiogenic growth factors is not known.

Study Design—Human primary term trophoblast and JEG-3 choriocarcinoma cells were cultured under 21%O_2 or 1%O_2 conditions in the presence or absence of NO donor (SNP) or inhibitor (L-NAME). Effects on PGF, VEGF and Flt-1 isoform mRNA expression were determined by quantitative real time PCR. Changes in expression of soluble protein isoforms of FLT-1 was monitored by ELISA.

Results—Hypoxia decreased PGF mRNA but increased VEGF, sFlt-1 and Flt-1 mRNA expression in the trophoblast. Generation of NO in trophoblast under 1%O_2 culture conditions significantly reversed sFlt-1 mRNA and protein expression, independent of mFlt-1. Conversely NO generation in hypoxic trophoblast increased VEGF and PGF mRNA expression.

Conclusions—NO production in primary human trophoblast cultures had divergent effects on pro-angiogenic (PGF, VEGF) versus anti-angiogenic (sFlt-1) mRNA expression, resulting in an enhanced pro-angiogenic gene expression environment *in-vitro*.

Keywords

placenta growth factor (PGF); soluble fms-like tyrosine kinase-1 (sFlt-1); vascular endothelial growth factor (VEGF); trophoblast; nitric oxide; hypoxia

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Introduction

Preeclampsia remains one of the most common obstetrical complications in humans[1]. The etiology of preeclampsia is not known, but shallow trophoblast invasion of maternal spiral arteries early in gestation is thought to contribute to the development of a relatively hypoxic placenta later in gestation[2]. Preeclampsia results in an imbalance in placental production of pro- versus anti-angiogenic factors[3]. Numerous reports indicate that preeclampsia is associated with reduced maternal systemic levels of placenta growth factor (PGF) protein and elevated presence of a soluble form of its receptor, fms-like tyrosine kinase-1 (sFlt-1) [4-7]. Both of these proteins are prominently expressed by human trophoblast and are differentially regulated by low oxygen tension[8;9]. When elevated, sFlt-1 binds and reduces circulating levels of bioactive PGF and vascular endothelial growth factor (VEGF) which is thought to manifest in peripheral endothelial dysfunction[3;10]. sFlt-1 inhibits endothelial cell tube formation and PGF/VEGF induced vasodilation of renal microvessels *in vitro*[11]. Presence of functional membrane-spanning Flt-1(mFlt-1) receptors on human trophoblast establishes an autocrine loop that that can regulate apoptosis, differentiation and nitric oxide production[12;13]. Numerous reports now confirm that experimentally increased sFlt-1 induces preeclampsia-like symptoms (elevated blood pressure, proteinuria, glomerular endotheliosis) in pregnant animal models[11], [14-16].

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthases (NOS) and is essential for maintaining vascular tone[17]. Although findings have not always been consistent, decreased bioactivity of this potent vasodilator and/or reduced NO serum levels are thought to be common in preeclampsia (see[2;17]) which is substantiated by recent studies[18;19]. Superoxide, a reactive oxygen species, is also produced by NOS [20] and is normally metabolized by superoxide dismutase (SOD). However, the reaction of NO with superoxide to form peroxynitrite out-competes SOD for detoxifying superoxide. Since superoxide can serve as a sink for the bio-availability of NO which results in peroxynitrite production, increasing amounts of superoxide depletes accessible NO within endothelial cells[20]. Elevated levels of superoxide and peroxynitrite are associated with preeclampsia[20] and decreased bioactivity of NO is generally associated with endothelial dysfunction, vasoconstriction and reduced trophoblast invasion[2].

We have shown that hypoxia significantly reduces human trophoblast PGF gene transcription and mRNA expression[8;21], and increases sFlt-1 expression and VEGF expression *in vitro*[8;22]. Nitric oxide differentially regulates expression of many genes including hypoxia inducible factor-1 (HIF-1), a principle regulatory transcription factor mediating hypoxia gene responses[23]. However, the ability of NO to regulate human trophoblast expression of hypoxia-responsive angiogenesis factors is unknown. We investigated the effects of NO generation in primary human syncytiotrophoblast on PGF, VEGF, mFlt-1 and sFlt-1 gene expression under conditions of varying oxygen tensions *in vitro*. Low oxygen tension ($1\%O_2$) significantly decreased PGF, but increased VEGF, mFlt-1 and especially sFlt-1 mRNA expression. Increased generation of NO within hypoxic primary syncytiotrophoblast tended to increase PGF and VEGF expression and significantly reduced sFlt-1 mRNA and protein expression. NO had little effect on mFlt-1 expression in trophoblast. These results suggest that increasing NO generation at $1\%O_2$ is likely a compensatory response to restore a pro-angiogenic environment in hypoxic trophoblast *in-vitro*.

Materials and Methods

Cell Culture and ELISAs

Primary human term trophoblast from uncomplicated deliveries were isolated [24] and allowed to form syncytium for 48hrs as we have described previously[8]. Collection of placentae was approved by the IRB committees at Southern Illinois University and the University of Alberta. JEG-3 choriocarcinoma cells (ATCC, Manassas, VA) were maintained in DMEM containing 10% FBS (Atlanta Biologicals, Atlanta, GA). Primary trophoblast were cultured in KGM-2 (Lonza, Walkersville, MD) supplemented with the supplied growth media kit (bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, transferrin, epinephrine, gentamicin sulfate) and 10%FBS. Cells were plated at 5×10^5 cells per cm^2 of culture vessel and were treated with 100μM of a spontaneous nitric oxide donor, sodium nitroprusside dihydrate (SNP) (EMD Chemicals, Gibbstown, NJ) or an inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich, St. Louis, MO) under 21% or 1% O_2 for 24hrs. Supernatants were collected and stored at −80°C. Concentration of the stable bi-product of NO, nitrite (NO_2), was used as a surrogate marker for quantitative measurement of nitric oxide production[17]. Nitrite concentration was quantified using Griess reagents (Invitrogen, Carlsbad, CA). Capture ELISAs for human sFlt1 and PGF from cell culture supernatants were performed as instructed (R&D Systems, Minneapolis, MN).

RNA Isolation and Real-Time PCR Analysis

RNA was isolated using TRI Reagent (Sigma-Aldrich) or RNeasy Mini Kit (Qiagen, Valencia, CA) as instructed. 50-100ng of RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and relative changes in PGF, VEGF, mFlt-1 and sFlt-1 mRNA quantified by real-time RT-PCR. Primers for VEGF were: VEGF-A (F): 5′-ATCACGAAGTGGTGAAGTTC-3′ and (R) 5′-TGCTGTAGGAAGCTCATCTC-3′ producing a 265bp product. We verified sFlt-1 mRNA using 2 different sets of primers to confirm that changes in relative expression were identical using both primer sequence: sFlt-1: (F) 5′-GGCCCCAGGGGTGCAAGATG-3′ and (R) 5′-CGCATGAGAGGAGGGAGGGGA-3′ producing a 108bp product and sFlt-1 (F) 5′-TGGCCATCACTAAGGAGCACT-3′ and (R) 5′-TCCGAGCCTGAAAGTTAGCAA-3′ which produced a 314bp amplicon[25]. Both sequences target the most highly conserved and expressed splice variant of sFlt-1 produced in human trophoblast as recently described[25]. All data for sFlt-1 expression were verified with both sFlt-1 primer sets. Primers specific for mFlt-1 were (F)5′-GTCGACACAGTGGCCATCAGCAGTT-3′ and (R)5′-TTCCACAGAGCCCTTCTGGTT-3′ producing a 219bp product as described[25]. Appropriately sized amplicons for all genes of interest were verified by gel electrophoresis and melting curve analyses (data not shown). PGF primers and cycling conditions were utilized as previously described[26]. Ribosomal protein (RPL32) mRNA, which is not altered under hypoxia[26] or TaqMan RNaseP (Applied Biosystems, Carlsbad, CA) were used as internal control genes for normalization. RPL32 was amplified with: (F) 5′-CCCAAGATCGTCAAAAAGA-3′ and (R) 5′-TCAATGCCTCTGGGTTT-3′[21]. Target gene expression was normalized to RPL32 or RNaseP expression within each experiment, and relative changes in expression calculated by the $2^{-\Delta\Delta CT}$ formula [26].

Statistical Analyses

The data are presented as mean (±SEM). Analyses were performed using GraphPad InStat 3 statistical software (GraphPad Software, San Diego, CA). To control for inter-assay variations, all data were normalized within individual experiments relative to control and relative changes in expression reported as % of control. Normalized data were analyzed via 2-tailed, 1 sample t-test with the hypothetical mean = 100%. Expression ratios of sFlt1/

mFlt1 were calculated using normalized CT values and analyzed with one-way analysis of variance (ANOVA) and Bonferroni Multiple comparisons post test following $\log_{10}$ transformation of each ratio. A $p \leq 0.05$ was considered statistically significant.

Results

Effects of Hypoxia on Expression of Angiogenic Growth Factors in Trophoblast

Hypoxia (1%O_2) significantly reduced PGF mRNA in primary syncytiotrophoblast to 17.67% (±3.41%) of control cultures ($p<0.0001$) (Figure 1). The effect was specific to PGF, as VEGF expression increased significantly (365.7% ± 47.1%, $p<0.005$) at 1%O_2 (Figure 1). Similar trends in expression were evident in JEG-3 choriocarcinoma cells in that hypoxia reduced PGF expression 44% (±12.7%; $p < 0.05$) and increased VEGF mRNA expression (~50%) at 1%O_2 (data not shown). The relatively lower increase in VEGF expression may be explained by the substantially higher endogenous levels (~4 fold) of VEGF mRNA in the choriocarcinoma cell line compared to primary syncytiotrophoblast. Culturing at 1%O_2 also significantly increased sFlt-1 mRNA expression (256.0% ± 21.7%, $p<0.005$) in syncytiotrophoblast (Figure 1). Baseline sFlt-1 mRNA expression was considerably lower in JEG-3 cells than syncytiotrophoblast (~20 fold), however, a similar trend for increased sFlt-1 mRNA expression occurred in JEG-3 cells (data not shown).

Effects of Nitric Oxide Donor/Inhibitor on PGF, VEGF, mFlt-1 and sFlt-1 mRNA

For both cell types, nitrite levels were at or below the limit of detection (0-1μM) within control supernatants of cells cultured at 21% or 1%O_2. These data suggest that hypoxia does not affect nitric oxide production by syncytiotrophoblast under these culture conditions. In contrast, nitrite levels in cultures treated with SNP increased to 32.9 ± 5.4μM at 21% or 1%O_2.

At 1%O_2, NO generation with SNP significantly increased PGF mRNA expression (183% ± 29%, $p<0.05$) in syncytiotrophoblast (Figure 2A) when compared to the reduced level of PGF at 1%O_2 (set = 100). Similarly, NO generation under 1%O_2 further increased VEGF mRNA expression by ~30% (±12.5, $p= 0.05$). In contrast, SNP treatment significantly reduced sFlt-1 mRNA expression by ~70% (31.4 ± 6.3, $p<0.0001$, Figure 2A) in syncytiotrophoblast. Generation of NO in hypoxic JEG-3 cells had similar affects on PGF,VEGF & sFlt-1 mRNA (data not shown). Inhibition of NO synthesis with L-NAME during hypoxia did not significantly affect expression of PGF, VEGF or sFlt-1 in hypoxic syncytiotrophoblast (Figure 2A) or JEG-3 cells compared to hypoxia alone. This result is not unexpected since 1%O_2 did not detectably increase NO production by these cells (see above). At 21%O_2 culture conditions, neither generation nor inhibition of NO synthesis significantly affected expression of PGF, VEGF or sFlt-1 in either cell type.

Comparisons between treatment groups normalized to 21% O_2 culture conditions highlights the differential effects NO had on each gene product (Figure 2B). At 1%O_2 NO generation increased PGF mRNA expression, although not to levels comparable to 21%O_2. NO generation further augmented VEGF expression in the hypoxic trophoblast. In contrast, SNP treatment at 1%O_2 inhibited sFlt-1 mRNA expression by >3.0 fold to levels not significantly different from 21%O_2 culture conditions ($p=0.22$). Thus, under 1% O_2 culture conditions, NO production had divergent effects on pro-angiogenic (PGF, VEGF) versus anti-angiogenic (sFlt-1) mRNA expression in primary syncytiotrophoblast.

Nitric oxide selectively alters sFlt-1 mRNA expression in hypoxic trophoblast

It was possible that NO was influencing sFlt-1 expression during hypoxia by regulating *flt1* gene transcription. We then re-analyzed the cDNA samples using primers that differentiated

membrane (m)Flt-1 from sFlt-1[25]. Hypoxia tended to increase mFlt-1 mRNA expression (167% ± 34%) compared to 21%O_2, although this increase was not statistically significant (p=0.09) (Figure 3A). However, in sharp contrast to the effects on sFlt-1, increased NO generation during hypoxia had no effect on mFlt-1 mRNA expression (166%± 39%, p=0.82). Analyses of normalized CT ratios (Figure 3B) showed that under standard culture conditions, primary trophoblast express relatively more sFlt-1 (~3.7 fold) than mFlt-1 mRNA. Low oxygen tension greatly increased expression of sFlt-1 mRNA which significantly increased the sFlt1/mFlt1 ratio ($p<0.01$). SNP generation of NO at 1%O_2 preferentially reduced sFlt-1 mRNA but had little effect on mFlt-1 resulting in a sFlt-1/mFlt-1 ratio significantly ($p< 0.001$) lower than at 1%O_2 alone and similar to the ratio at 21%O_2 ($p>0.05$).

Effects of Nitric Oxide Donor/Inhibitor on sFlt-1 protein expression

Presence of sFlt-1 proteins in syncytiotrophoblast supernatants were analyzed to confirm that protein concentrations reflected the changes in mRNA expression. Similarly to mRNA expression, SNP treatment significantly reduced sFlt-1 protein to 38.1% ±1.4% ($p<0.005$) of hypoxic cultures (Figure 4). In contrast, L-NAME treatment did not significantly ($p= 0.8$) alter sFlt-1 protein levels. These protein findings corroborate sFlt-1 mRNA findings and confirm that NO can significantly decrease sFlt-1 production in hypoxic primary human trophoblast. PGF protein was also measured in these same cell culture supernatants. Despite the modest (~2 fold) increase of PGF mRNA following SNP treatment of hypoxic trophoblast (Figure 2B), we did not detect a significant change in *free* PGF protein in the supernatants (data not shown). However, these results may be confounded by the presence of sFlt-1 protein in the supernatants.

Discussion

Aside from hypoxia, factors that regulate trophoblast expression of PGF, VEGF and sFlt-1 are not well understood. We are the first to report that NO generation mediated a significant reduction in sFlt-1 mRNA and protein expression in primary human syncytiotrophoblast cultured at 1%O_2, but had little effect on mFlt-1 expression. Furthermore, generation of NO tended to increase PGF mRNA and further augment VEGF mRNA expression in syncytiotrophoblast cultured at 1%O_2. To our knowledge, this is the first report of NO-mediated regulation of these pro- and anti-angiogenic genes in primary human trophoblast. Assuming similar results occur with hypoxic preeclamptic trophoblast *in vivo*, these results suggest that increased trophoblast NO production could enhance the bio-availability of PGF and VEGF angiogenic factors which may help ameliorate the anti-angiogenic environment commonly associated with this complication.

Despite the significant role of sFlt-1 in preeclampsia, there is limited information concerning the regulation of sFlt-1 expression in human trophoblast. Under 1%O_2, trophoblast increase Flt-1 expression[27], and this may be mediated at least in part by HIF-1[28]. NO may regulate HIF-1 stability[29] but our studies show that hypoxic regulation of VEGF, a classic HIF-1-mediated response, was not inhibited by NO generation in trophoblast at 1%O_2. In addition, we found that NO, reported to increase HIF-1 activity at 21%O_2[29], increased neither sFlt-1 nor VEGF expression at this oxygen level (data not shown). Thus, it is not likely that HIF-1 is involved in NO regulation of sFlt-1 production. An alternative explanation is that NO may regulate trophoblast sFlt-1 accumulation under hypoxia independently of transcription. Production of sFlt-1 arises via alternative splicing of mature Flt-1 mRNA, our data (Figure 3B) confirms that human trophoblast favor sFlt-1 production, especially under 1%O_2[25]. Our data further implies that generation of NO alters post-transcriptional processing of mature Flt-1 mRNA to produce less of the sFlt-1 isoform and/or increase sFlt-1 mRNA degradation. We propose that one posttranscriptional mechanism

involves Jumonji domain-containing protein 6 (Jmjd6), an oxygen sensitive lysyl hydroxylase, recently shown to regulate alternative splicing of Flt-1 mRNA in HUVECs[30]. Potential sFlt-1 transcriptional and post-transcriptional control mechanisms regulated by NO in human trophoblast warrants future investigation.

Although NO generation did not alter trophoblast VEGF expression at 21%O_2, the combination of hypoxia plus NO generation tended to further elevate VEGF mRNA expression in trophoblast. This effect may also be independent of HIF-1α in trophoblast as NO is generally thought to destabilize this subunit under hypoxia[29]. However, the effect of NO on hypoxia-induced HIF-1α accumulation is highly variable and depends upon the level of NO generated and the O_2 tension[29]. Most importantly, there are cell type specific differences in the ability of cells to sense hypoxia[29] as exemplified by the unique trophoblast responses with PGF production[21].

NO generation at 1%O_2 significantly increased PGF mRNA expression when compared to PGF levels at 1%O_2. However, the mechanism involved appears independent of GCM-1, one principle regulator of PGF expression in trophoblast [26]. NO generation at 1%O_2 did not effect GCM-1 expression from 1%O_2 alone. Perhaps increased PGF mRNA was mediated via NO through metal responsive transcription factor-1 (MTF-1) interaction with its response elements within the PGF promoter[31]. MTF-1 is activated in cultured sheep pulmonary artery endothelial cells following treatment with a NO donor[31]. Interestingly, MTF-1 expression is decreased under hypoxic conditions in trophoblast and has been implicated in the hypoxic regulation of PGF[32]. The potential effects of NO modulation on MTF-1 expression and/or function in trophoblast are unknown.

Our results provide mechanistic insights into the association between hypoxia, NO and preeclampsia. Systemic inhibition of NO synthesis with L-NAME in pregnant rats selectively increased sFlt-1 levels and maternal hypoxia selectively decreased PGF while the combination resulted in severe fetal growth restriction[33]. Our studies suggest that one of the biological mechanisms by which NO inhibition affects fetal outcome in this model may involve trophoblast. Conceptually, during hypoxic stress in vivo trophoblast increase sFlt-1 production and concomitant L-NAME treatment to inhibit NO would further increase sFlt-1 mRNA and protein production. Indeed, there is an inverse relationship between high levels of sFlt-1 and lower levels of NO production in preeclamptic patients[34]. Both VEGF and PGF act through mFlt-1 receptors to induce NO production in endothelial cells[35] and trophoblast [36]. Treatment of trophoblast cells with PGF augmented NO production and PGF binding to mFlt-1 receptors [36], which we hypothesize would result in a selective decrease in sFlt-1 production via alternative splicing of *flt1* by trophoblast at 1%O_2. The effects of PGF treatment on NO production at varying oxygen concentrations has not been examined to our knowledge. While low oxygen tension does not affect NO production quantified in the supernatants of trophoblast, it has recently been shown that hypoxia reduces eNOS protein expression [37]. Thus, decreases in trophoblast PGF production concomitant with increased sFlt-1 production, of which both are apparent in a hypoxic trophoblast environment, could act in paracrine and autocrine fashions to limit NO production and trophoblast function. Regulating angiogenesis and vasodilation at the maternal-fetal interface is complex, and many mechanisms act through the NO pathway [38]. In the context of trophoblast, we suggest that decreasing NO production in trophoblast perpetuates a further prominence of an anti-angiogenic state by increased sFlt-1 production.

Reducing systemic sFlt-1 protein by increasing ligand availability reverses PE-like conditions in animal models[11;39]. Conceptually, our results suggest that it might be possible to decrease the systemic effects of inverted pro- to anti-angiogenic gene products expression via manipulation of NO levels in preeclampsia. Indeed, a recent large scale study

found that L-arginine supplementation during pregnancy significantly decreases the incidence of preeclampsia in a high risk population [40]. However, benefits of NO donors in pregnancy have not been uniform and additional clinical studies are needed (see [41]). Given the clinical heterogeneity of preeclampsia and the potential for patient-specific responses to NO-altering approaches (see[42]), the variability in results is not surprising. Studies assessing the effects of NO augmentation on serum levels of PGF/VEGF and sFlt-1 during human preeclampsia are lacking.

Clinical management approaches such as anti-hypertensive drugs to control blood pressure in preeclampsia have been shown to reduce maternal serum and placental production of sFlt-1[43]. However, many anti-hypertensive agents have no effect on lowering sFlt-1 production from human placenta explants in vitro[44]. Non-pharmacological approaches, such as regular exercise during pregnancy, has been shown to increase serum PGF while simultaneously decrease sFlt-1 and soluble endoglin during the third trimester and reduces the risk of developing preeclampsia[45]. The molecular explanations behind these observations are not known but continued investigation into mechanisms to re-establish expression of pro-angiogenic while limiting anti-angiogenic gene expression in trophoblast may provide novel treatment avenues for obstetrical complications like preeclampsia and/or fetal growth restriction.

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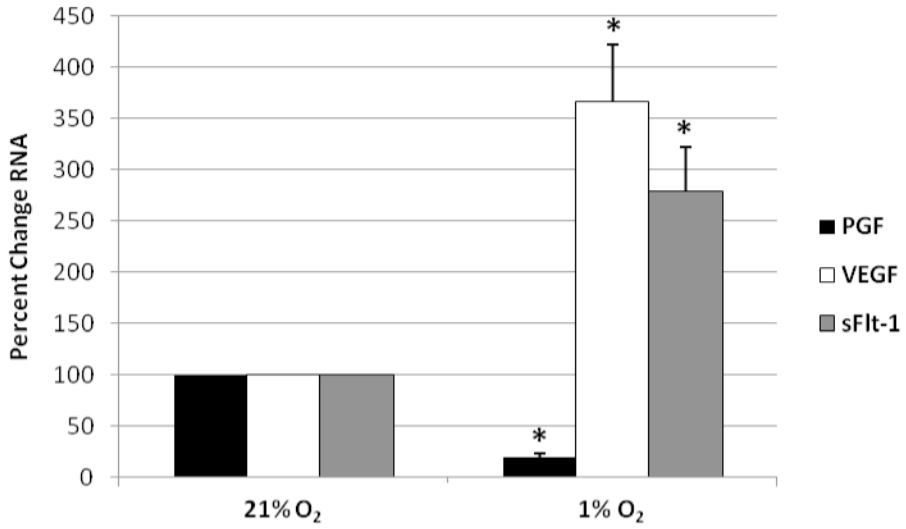

Figure 1.

Effects of hypoxia on PGF, VEGF and sFlt-1 mRNA expression in syncytiotrophoblast. Cells were cultured under 21%O_2 or 1%O_2 for 24 hrs, RNA was isolated and relative changes in gene expression determined by qRT-PCR. Data were normalized to controls at 21%O_2; set to 100% and plotted; n = 7 cultures, *=p< 0.01.

Figure 2A.

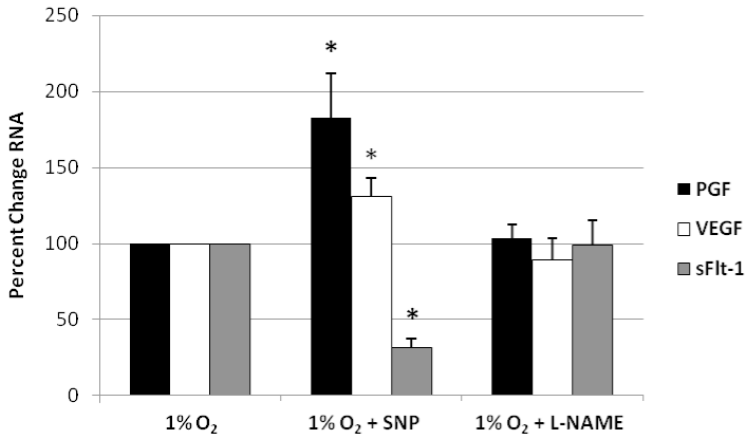

Figure 2B.

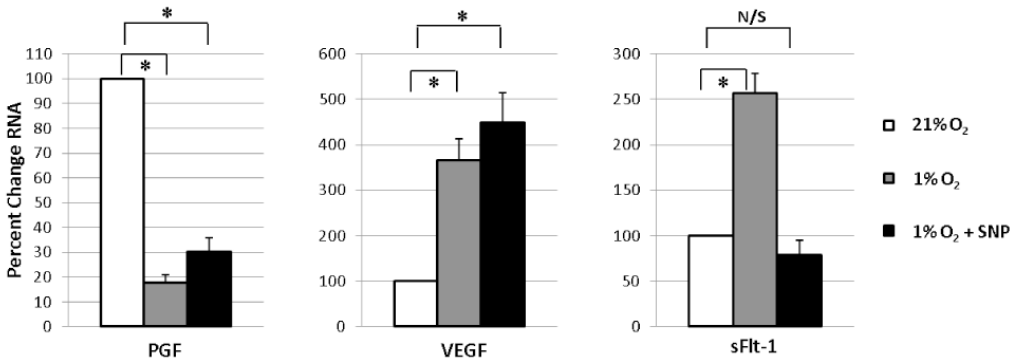

Figure 2.

Nitric oxide generation significantly increases PGF and VEGF mRNA, but decreases sFlt-1 mRNA in hypoxic syncytiotrophoblast. (A.) Primary syncytiotrophoblast were treated with SNP or L-NAME and subjected to 1%O_2 for 24hrs. Relative changes in RNA expression was determined by qRT-PCR, normalized to values at 1%O_2; set to 100% and plotted; n=7, *=$p<0.05$. (B.) Divergent effects of NO generation under hypoxia on PGF, VEGF and sFlt-1 mRNA in primary syncytiotrophoblast. Relative changes in RNA for each treatment were determined by qRT-PCR, normalized to controls at 21%O_2; set to 100% and plotted, n=7, *=$p<0.005$.

Figure 3A.

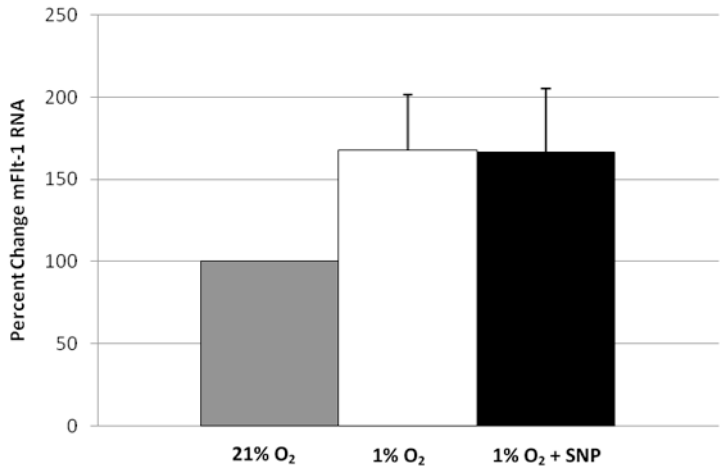

Figure 3B.

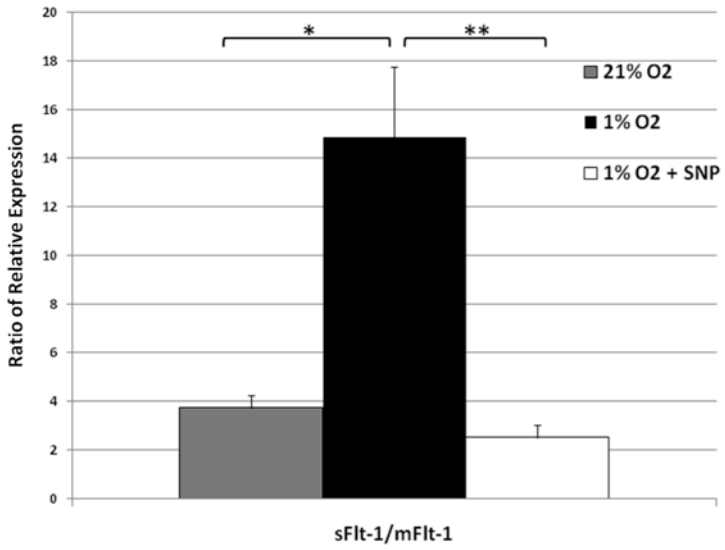

Figure 3.

NO generation decreases sFlt-1/mFlt-1 mRNA expression ratios in hypoxic syncytiotrophoblast. (A.) Relative changes in mFlt-1 mRNA for each culture condition were determined by qRT-PCR and normalized data plotted relative to values at 21% O_2; set =100%, n=7. (B.) Relative sFlt-1/mFlt-1 expression ratios in syncytiotrophoblast under hypoxia and hypoxia with SNP-induced NO-production shows preferential decrease in sFlt-1 mRNA expression with SNP treatment during hypoxia. ANOVA $p<0.0001$; *=$p<0.01$; **=$p<0.001$, n=7 each.

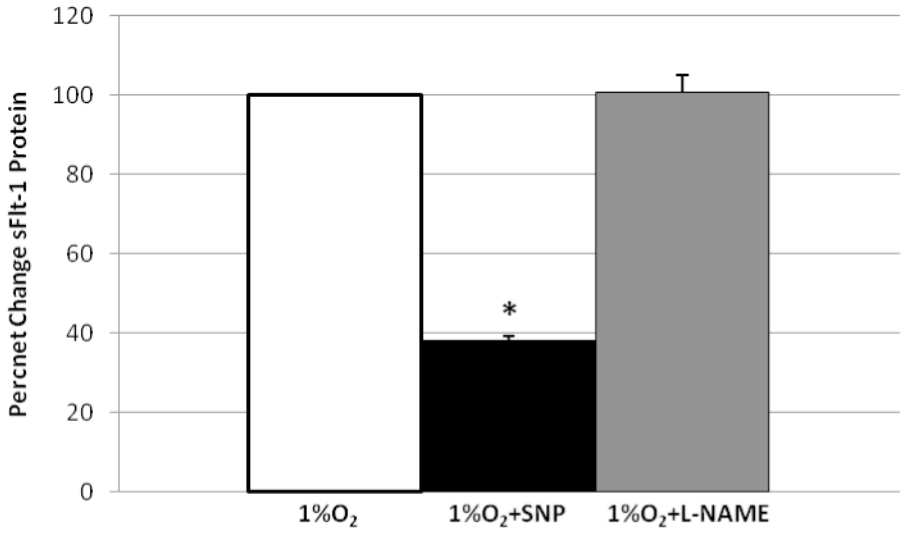

Figure 4.

Nitric oxide generation significantly inhibits sFlt-1 protein release in hypoxic syncytiotrophoblast. Relative changes in sFlt-1 protein were determined by capture ELISA and data were normalized to control values from trophoblast cultured at 1% O_2; set = 100% and plotted, n=3 each, *=p< 0.005.