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The Angiotensin II type 1 receptor mediates the effects of low oxygen on early placental angiogenesis

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ABSTRACT

Introduction: Placental development occurs in a low oxygen environment, which stimulates angiogenesis by upregulating vascular endothelial growth factor A (VEGFA), plasminogen activator inhibitor-1 (SERPINE1) and the angiopoietin-2/-1 ratio (ANGPT2/1). At this time, Angiotensin II type 1 receptor (AT_1R) is highly expressed. We postulated that the early gestation placental oxygen milieu, by stimulating the angiotensin (Ang) II/AT_1R pathway, increases expression of proliferative/angiogenic factors.

Methods: HTR-8/SVneo cells were cultured in 1%, 5% or 20% O_2 with the AT_1R antagonist (losartan) for 48 h. mRNA and protein levels of angiogenic factors were determined by qPCR and ELISA. Angiogenesis and cell viability were assessed by HUVEC tube formation and resazurin assay.

Results: Culture in low oxygen (1%) increased angiogenic *VEGFA, SERPINE1* and placental growth factor (*PGF*) mRNA and VEGFA and SERPINE1 protein levels, and reduced anti-angiogenic *ANGPT1*, endoglin (*ENG*) and soluble fms-like tyrosine kinase-e15a (*sFlt-e15a*) mRNA (all $P = 0.0001$). At 1% oxygen, losartan significantly reduced intracellular *VEGFA* and SERPINE1 levels and secreted VEGF levels ($P = 0.008$, 0.0001 and 0.0001). HUVEC tube formation was increased in cells grown in HTR-8/SVneo conditioned medium from 1 to 5% cultures (all $P = 0.0001$). HUVECs cultured in medium from losartan treated HTR-8/SVneo cells had a reduced number of meshes, branching points and total branching length ($P = 0.004$, 0.003 and 0.0002). At 1% oxygen, losartan partially inhibited the oxygen-induced increase in cell viability ($P = 0.0001$).

Discussion: Thus, AT_1R blockade antagonised the low oxygen induced increase in pro-angiogenic factor expression and cell viability. Our findings highlight a role for an oxygen-sensitive Ang II/AT_1R pathway during placentation.

1. Introduction

Inadequate placental trophoblast invasion of the maternal decidua and uterine spiral arterioles causes placental insufficiency, which contributes to pregnancy complications such as preeclampsia, intrauterine growth restriction (IUGR) and spontaneous abortion [1–3]. During the first trimester, trophoblast cells invade and plug the maternal spiral arterioles and impede maternal blood flow, so that the placenta develops in a low oxygen environment. This low oxygen environment stimulates trophoblast proliferation and placental angiogenesis [4–6] and is critical for successful placentation [7].

The oxygen tension within the intervillous space during the first trimester is 17.9 mmHg (~2.5%), with a range of 5–30 mmHg (~0.7–4.3%), while the oxygen tension in the decidua is higher at 39.6 mmHg (~5.7%), with a range of 25–70 mmHg (~3.5–10%) [8]. At the end of the first trimester, the trophoblast plugs are removed and the oxygen tension within the intervillous space rises steeply to levels similar to those measured in the decidua.

We and others have shown that the placental renin-angiotensin system (RAS) contributes to placental development; moreover, its expression is highest in early gestation and decreases after the first trimester [9–11], when oxygen tension within the placenta are lowest. We have shown that in HTR-8/SVneo cells (a first trimester human placental cell line) a pro-angiogenic RAS pathway is activated when they are cultured in 1% O_2; specifically, expression of angiotensin (Ang) II Type 1 receptor (*AGTR1*) and vascular endothelial growth factor A (*VEGFA*) mRNAs and angiotensin converting enzyme (ACE) and VEGFA protein levels are increased [12]. AGT mRNA and protein levels are significantly increased by hypoxia/reperfusion in term placental explants. Furthermore, both *AGTR1* and *AGTR2* mRNAs were positively

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correlated with hypoxia-inducible factor 1 beta (HIF-1β) in these explants [13]. These studies highlight a potential role for the placental RAS in modulating the pro-angiogenic effects of low oxygen in placental development. However, further studies are required to elucidate the physiological effects of placental RAS activation by low oxygen. *In vitro*, Ang II treatment of first trimester explants mimics the effects of low oxygen (3% O_2) [14]. Extravillous trophoblasts cultured in either low oxygen or Ang II had increased plasminogen activator inhibitor-1 (PAI-1/SERPINE1) expression [14] and hypoxia-inducible factor (HIF)-1α levels [14]. Stabilization of HIF-1α promotes angiogenesis and cell proliferation by stimulating expression of angiogenic factors, including VEGFA and angiopoietins [15,16]. Angiopoietins are critical for vessel homeostasis and angiogenesis. Angiopoietin-1 (Ang-1) promotes vessel maturation by mediating endothelial cell migration, adhesion and survival. Conversely, angiopoietin-2 (Ang-2) promotes cell death and vascular regression. However, in conjunction with VEGFA, Ang-2 promotes neovascularization. The local balance of these factors and the level of other angiogenic factors determine whether blood vessels grow, are maintained or regress. Ang-2 is the dominant angiopoietin in early gestation placenta and is regulated by oxygen tension in placental explants, thus a reduction in the ratio of Ang-2/-1 in early gestation may impair angiogenesis [17].

In this study, we postulated that the Ang II/AT_1R pathway mediated the effects of low oxygen on expression of pro and anti-angiogenic factors in the placenta and on trophoblast proliferation. We studied these interactions between a low oxygen milieu and the placental RAS in HTR-8/SVneo cells by blocking the Ang II/AT_1R pathway.

2. Methods

2.1. Cell culture

HTR-8/SVneo cells are an immortalized first trimester trophoblast cell line (a gift from Prof. Charles Graham, Queens University, Ontario). We have demonstrated previously that the pro-angiogenic/proliferative pathway of the RAS in this cell line is upregulated by culture in low oxygen (1% O_2) [12].

HTR-8/SVneo cells were cultured in RPMI-1640 medium (HyClone), supplemented with 10% fetal bovine serum (SAFC Biosciences, Darmstadt, Germany), 1% antibiotic-antimycotic (Gibco) and 1% L-glutamine in 5% CO_2 in room air at 37 °C (cells were between passages 20–30). Cells were seeded at a density of 2×10^5 cells per well in 6-well plates with 2 ml of complete incubation medium per well and allowed to settle for 24 h, after which time the medium was changed and treatments added. Cells were cultured with either vehicle (PBS) or 10 μM losartan (Sigma, Darmstadt, Germany) and transferred to sealed oxygen chambers containing either 1%, 5% or 20% O_2 and 5% CO_2 in N_2 and cultured for 48 h. Chambers were flushed with the appropriate gas mixture immediately after cells were placed inside and again after 24 h. Cells were harvested at 48 h, and snap frozen in liquid nitrogen and stored at −80 °C for subsequent protein and mRNA analyses. Six experiments were conducted in triplicate.

2.2. Semi-quantitative real-time reverse transcriptase polymerase chain reaction (qPCR)

Total RNA was isolated using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA samples were DNase treated (Qiagen). RNA quantity was used as an indicator of cell viability [18] and was assessed using the Nanodrop spectrophotometer. No differences in RNA quantity were detected between the treatment groups (data not shown). RNA quality was determined by agarose gel electrophoresis.

qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 μl of SYBR Green PCR master mix (Applied Biosystems, California, United States), primers described in Table 1, cDNA reversed transcribed from 10 ng total RNA, and water to 10 μl. Genes examined were angiopoietin-1 (*ANGPT1*), angiopoietin-2 (*ANGPT2*), endoglin (*ENG*), placental growth factor (*PGF*), plasminogen activator inhibitor-1 (*SERPINE1*), soluble fms-like tyrosine kinase-e15a (*sFlt-e15a*), vascular endothelial growth factor A (*VEGFA*) and β-actin (*ACTB*). ACTB mRNA was stably expressed between oxygen tensions and treatment groups and was used as a housekeeper. Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta CT}$ method and expressed relative to *ACTB* mRNA and a calibrator sample (a term placental sample collected at elective Caesarean section) [19].

Table 1
Primers used for PCR.

Gene	GenBank Accession #	Primer Sequence (5' - 3')	Concentration (nM)
ACTB	NM_001101	F: CGCGAGAAGATGACCCAGAT R: GAGTCCATCACGATGCCAGT	1000 nM
ANGPT1	NM_001146	F: TGTAAGTGTCCAAGGTTATGA R: TGCCAACAACTGTCTCTT	100 nM
ANGPT2	NM_001147.2	F: AGCACCAGCAAGCCATAA R: TGACGATTAACATCCTCAGAACT	100 nM
ENG	NM_000118.3	F: ATCTGGACCACTGGAGAATAC R: TGTGTCTGGGAGCTTGAA	100 nM
PGF	NM_002632.5	F: TTACCGTCACACTCTTCA R: CATTCAGCAGGGAAACAG	100 nM
SERPINE1	NM_000602.4	F: TCTGTGTCACCGTATCTCA R: GCTCCGTCACGCTGGATGTC	200 nM
sFlt-e15a	NM_001159920.1	F: ACAATCAGAGGTGAGCACTGCAA R: TCCGAGCCTGAAAGTTAGCAA	100 nM
VEGFA	M32977	F: CTACCTCCACCATGCCAAGT R: GCAGTAGCTGCGCTGATAGA	400 nM

F; Forward, R; Reverse.

2.3. Measurement of VEGFA and PAI-1 proteins by ELISA

Total protein was extracted from cells using a radioimmunoprecipitation assay (RIPA) lysis and extraction buffer. Briefly, 100 μl of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 100 nM sodium orthovanadate and Complete Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and 1 μl of 100 nM PMSF) were added to each sample. Samples were incubated on ice for 30 min with intermittent vortexing, then centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants were collected and stored at −80 °C. Protein was quantified using the Pierce BCA Protein assay kit (Life Technologies, California, United States) according to the manufacturer's instructions. Intracellular and secreted VEGFA and SERPINE1 concentrations or proteins in culture medium were measured using the Human VEGF Duoset ELISA kit (R&D systems, Minnesota, United States) and Human SERPINE1 Duoset ELISA kit (R&D systems) according to the manufacturers' instructions, as described previously [20]. Each experiment was assayed on a single plate. The intra-assay coefficients of variation were 3.3% and 5.2% respectively for VEGFA intracellular and secreted protein and 3.0% and 4.4% for SERPINE1 intracellular and secreted protein; data are presented as a fold change to the respective 1% O_2 vehicle control.

2.4. Primary human umbilical vein endothelial cell isolation and tube formation assay

Primary HUVECS were isolated based on previously described methods [21]. Briefly, term placentas from uncomplicated singleton pregnancies were collected within 30 min of elective caesarean delivery in the absence of labour at the John Hunter Hospital (Newcastle, Australia) following informed consent. Umbilical cords were cut from

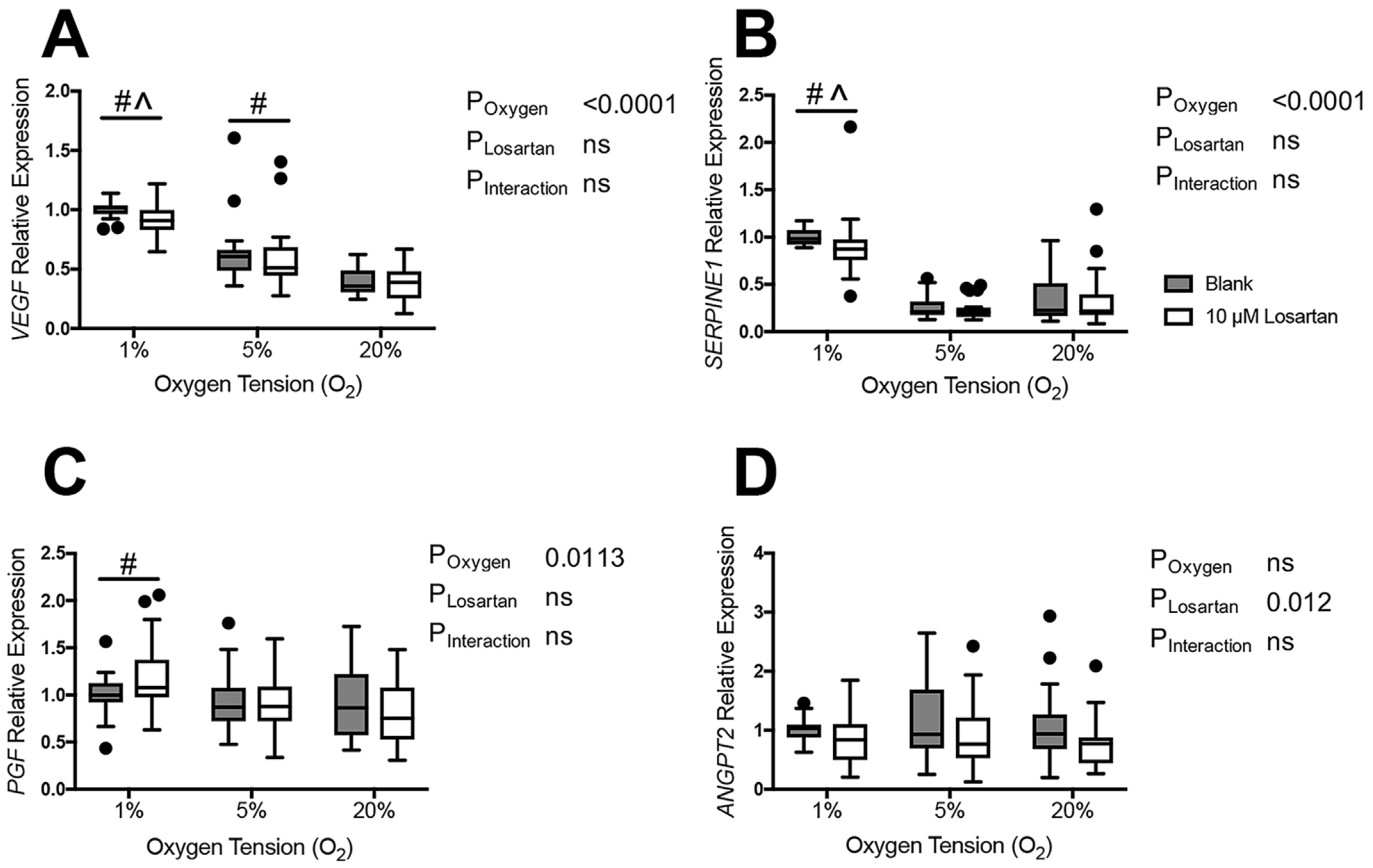

Fig. 1. Effect of oxygen and losartan on the expression of pro-angiogenic downstream targets of AT_1R activation. (A) VEGFA expression was upregulated by culture in 1 and 5% O_2 compared with 20% O_2 culture (#; both P = 0.0001). Culture in 1% oxygen further upregulated VEGFA expression compared with 5% O_2 culture (^; P = 0.0001). (B) SERPINE1 was significantly upregulated by culture in 1% O_2 compared with 5 and 20% O_2 culture (#^; both P = 0.0001). (D) PGF mRNA was significantly upregulated by culture in 1% O_2 compared with culture in 20% O_2 (#; P = 0.008). ANGPT2 mRNA expression was significantly affected by treatment with losartan in 1% O_2 culture however, post-hoc analysis did not reveal any significant effect of losartan within each oxygen tension. Treatment with losartan did not significantly alter VEGF, SERPINE1 and PGF mRNA expression at any oxygen tension. Data expressed as a fold change from the 1% O_2 vehicle control (median and interquartile range). # denotes significance to 20% O_2. ^ denotes significance to 5% O_2. N = 6 experiments in triplicate.

placentas and clamped portions of the cord were removed (University of Newcastle Ethics H-382-0602). The cord was then placed in 1 x Hanks balanced salt solution (HBSS, Gibco) and massaged until all blood was removed. The cord was flushed with 1xHBSS before collagenase (1 mg/ml in DMEM, Worthington, New Jersey, United States) was inserted into the cord until plump. The cord was then incubated at 37 °C for 10 min, after which, the cord was massaged lightly, and contents were placed into a falcon tube with 1–2 mls FCS. The cord was flushed a further 2 times and added to the collagenase solution containing HUVECS. Cells were spun at 300 × *g* for 5 min at RT and re-suspended in 12 ml of media (M − 199 media containing 20% FCS, 0.2 mg/ml endothelial cell growth factor (Sigma), 1% L-glutamine, 1% antibiotic-antimycotic, 10 u/ml Heparin (Sigma)), and then placed into a T75 flask and cultured at in 5% CO_2 in room air at 37 °C.

Ibidi µ-Plate Angiogenesis 96-well plates were pre-coated with growth factor reduced Matrigel (In Vitro Technologies, Victoria, Australia) for 30 min prior to plating. Primary HUVECS (n = 4) were added to each well (1×10^5 cells/ml) in conditioned media. Conditioned media (from HTR-8/SVneo cells as above) was prepared 1:1 with supplemented M199 media. Assay plates were cultured for 6 h in 37 °C, after which Calcein (2 mM final concentration, Merck, Darmstadt, Germany) was added in each well and allowed to culture for a further 20 min. The entire well was captured using the Cytation 3 microscope (Biotek, Vermont, United States) at 2.5× magnification. Image contrast was corrected using Gimp-2.10 software and images were analysed using ImageJ to assess parameters including tube length, number of meshes and branch points.

2.5. Assessment of cell viability

Cell viability was assessed using a Resazurin Assay. At 43 h of culture in various O_2 tensions (as described above), 20 µl of Resazurin Reagent (0.15 mg/ml) was added to each well and plates were returned to their respective O_2 chambers. At 48 h culture, fluorescence was measured at 590 nm emission using the FLUOStar OPTIMA (BMG Labtech, Offenburg, Germany) after 570 nm excitation. Data are expressed as a fold change in relative fluorescence units (RFU) relative to the average of the 1% vehicle control for each experiment.

2.6. Statistical analysis

Each mRNA, protein (intracellular or supernatant), tube formation experiment and cell viability assay (n = 6, 3, 4 and 6 respectively) was conducted in technical triplicates. A two-way non-parametric ANOVA was used to determine the effects of differing oxygen tensions and drug treatments on the relative abundance of mRNA and protein expression as well as cell viability. A Kruskal Wallis test with Dunnett's multiple comparisons test was used to determine effect of prevailing oxygen tension on vehicle control cells. Dunnett's multiple comparisons tests were used to determine effect of treatment with losartan compared with vehicle at each oxygen tension. GraphPad Prism (Prism version 6.0) was used for all graphs and statistical analyses. Significance was set at

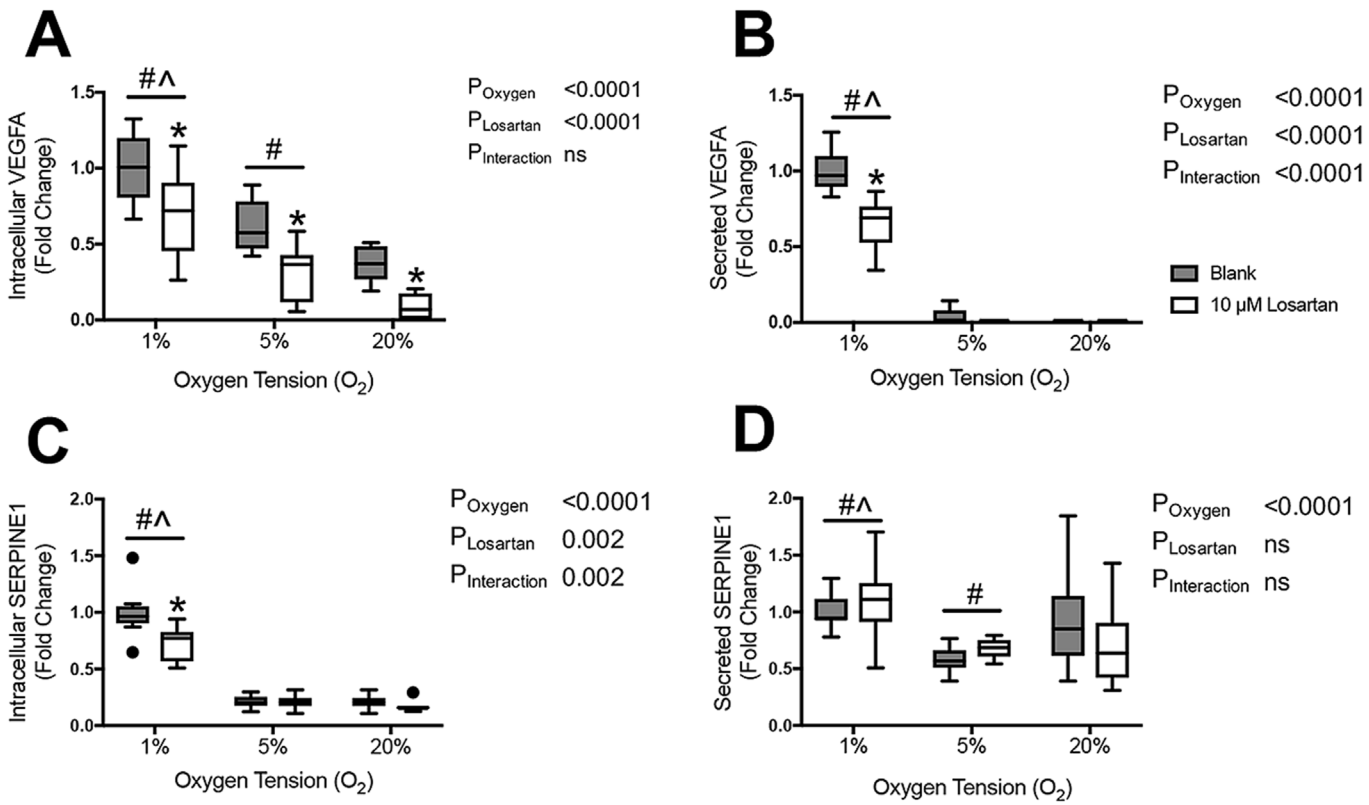

Fig. 2. Effect of oxygen tension and treatment with losartan on VEGFA and SERPINE1 protein levels in HTR-8/SVneo cells. (A) Intracellular VEGFA protein levels were upregulated by culture in 1% O_2 compared with 5 and 20% O_2 culture (#; P = 0.0001). Culture in 5% O_2 further enhanced intracellular VEGFA protein compared with 20% O_2 (^; P = 0.001). (B) VEGF protein secretion was enhanced by culture in low oxygen (1% O_2) compared with culture in 5 and 20% O_2 (#; P = 0.0001). (C) Intracellular SERPINE1 was enhanced by culture in 1% O_2 compared with 5 and 20% O_2 (#; both P = 0.0001). (C) Secreted SERPINE1 was enhanced by culture in 1% compared with 5 and 20% O_2 (P = 0.0001 and 0.013 respectively). Secreted SERPINE1 was significantly lower in culture at 5% O_2 compared with 20% O_2 (P = 0.042). Intracellular VEGFA protein was significantly reduced by treatment with 10 μM losartan after culture in 1, 5 and 20% O_2 tensions compared with the vehicle control (*; P = 0.008, 0.003 and 0.009 respectively). VEGFA protein secretion was significantly inhibited by losartan treatment when cultured in 1% O_2 at treatment with 10 μM compared with the vehicle control (*; P = 0.0001). Treatment with losartan at 10 μM also significantly inhibited SERPINE1 intracellular protein in 1% O_2 compared with the vehicle control (*; P = 0.0001). Secreted SERPINE1 was unaffected by treatment with losartan at all oxygen tensions. Data expressed as a fold change from the 1% O_2 vehicle control (median and interquartile range). ^ denotes significance to 5% O_2. # denotes significance to 20% O_2. * denotes significance to vehicle control. N = 3.

$P < 0.05$.

3. Results

3.1. Regulation of angiogenic mediators by oxygen tension in HTR-8/SVneo cells

Expression of the pro-angiogenic factor *VEGFA* was greatest in cells cultured in 1% oxygen compared with those cultured in 5 and 20% O_2 (median [IQR]; 1%-0.98 [0.89, 1.03], 5%-0.57 [0.46, 0.68] and 20%-0.36 [0.29, 0.67], both $P = 0.0001$; Fig. 1A). Culture in 5% O_2 also resulted in higher levels of expression of *VEGFA* mRNA compared with levels in HTR-8/SVneo cells incubated at 20% O_2 ($P = 0.0001$; Fig. 1A). *SERPINE1* mRNA expression was significantly greater in 1% O_2 culture compared with 5 and 20% (median [IQR]; 1%-0.95 [0.88, 1.10], 5%-0.21 [0.17, 0.26] and 20%- 0.22 [0.17, 0.51], both $P = 0.0001$; Fig. 1B). *PGF* mRNA was upregulated in 1% O_2 compared with culture in 20% O_2 (median [IQR]; 1%-1.03 [0.92, 1.23] and 20%- 0.84 [0.56, 1.12], $P = 0.008$; Fig. 1C). *ANGPT2* mRNA expression was not affected by oxygen (Fig. 1D).

Similar to *VEGFA* mRNA levels (Fig. 1A), intracellular and secreted VEGFA protein levels were highest in cells cultured in 1% O_2 compared with 5 and 20% (median [IQR]; 1%-0.85 [0.68, 0.98], 5%-0.008 [0.003, 0.03] and 20%-0.003 [0.003, 0.03], all $P = 0.0001$; Fig. 2A and B). Culture in 5% O_2 was also associated with increased intracellular VEGFA protein levels compared with cells cultured in 20% O_2 (median [IQR]; 1%-0.90 [0.68, 1.06], 5%-0.47 [0.34, 0.59] and 20%-0.19 [0.05, 0.37], $P = 0.001$, Fig. 2A). Cells cultured in 1% O_2 also had significantly higher intracellular and secreted levels of SERPINE1 protein compared with levels in cells cultured in 5 and 20% O_2 (median [IQR]; intracellular SERPINE1 1%-0.87 [0.70, 1.00], 5%-0.21 [0.17, 0.24] and 20%-0.17 [0.15, 0.21], secreted SERPINE1 1%-1.02 [0.93, 1.13], 5%-0.62 [0.54, 0.71] and 20%-0.77 [0.54, 0.96], $P = 0.0001$ and 0.0001 and 0.013, Fig. 2C and D). Low oxygen culture suppressed SERPINE1 secretion in 5% O_2 when compared with 20% O_2 ($P = 0.042$, Fig. 2D).

Culture in low oxygen inhibited the expression of anti-angiogenic factors. *ANGPT1* mRNA expression was less in cells cultured in 1% O_2 compared with cells cultured in 5 and 20% O_2 (median [IQR]; 1%-0.99 [0.58, 1.46], 5%-3.37 [2.79, 5.05] and 20%-4.19 [1.72, 6.41], both $P = 0.0001$; Fig. 3A), and expression of *sFlt-e15a* and *ENG* mRNA were also reduced after culture in 1 and 5% O_2 compared with 20% O_2 (median [IQR]; *sFlt-e15a* 1%-0.94 [0.83, 1.11], 5%-1.09 [0.90, 1.22] and 20%-1.46 [1.23, 1.78], *ENG* 1%-1.01 [0.90, 1.08], 5%-1.11 [0.99, 1.34] and 20%-1.35 [1.12, 1.74], both $P = 0.0001$ and 0.0001 and 0.003 respectively, Fig. 3B and C).

Conditioned medium from HTR-8/SVneo cells cultured in 1 and 5% O_2, was associated with an increase in HUVEC tube formation, as measured by the number of meshes, total branching points and total branching length (median [IQR]; no. meshes 1%-0.85 [0.68, 1.01], 5%-0.89 [0.82, 1.24] and 20%-0.38 [0.25, 0.59], no. branching points 1%-

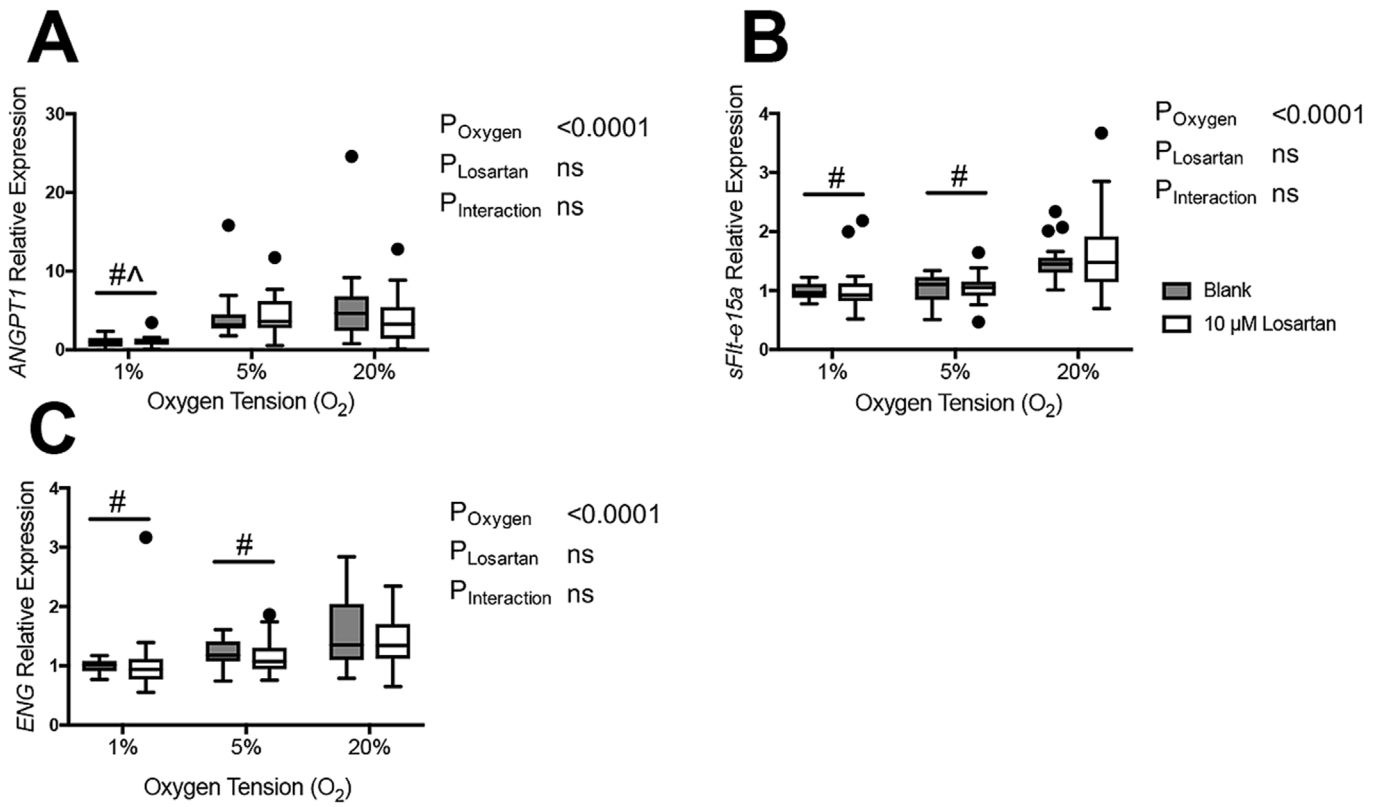

Fig. 3. Regulation of anti-angiogenic factor expression by oxygen and losartan in HTR-8/SVneo cells. (A) ANGPT1 expression was decreased by culture in 1% O_2 compared to 5 and 20% O_2 (#,^; both P = 0.0001). Both (B) sFlt-e15a and (C) ENG were significantly downregulated by culture in 1 and 5% O_2 compared to 20% (#; both P = 0.0001 and 0.0001 and 0.003 respectively). Expression of ANGPT1, sFlt-e15a and ENG were unaffected by treatment with losartan. Data expressed as a fold change from the 1% O_2 vehicle control (median and interquartile range). # denotes significance to 20% O_2. ^ denotes significance to 5% O_2. N = 6.

0.82 [0.75, 1.06], 5%-0.88 [0.70, 1.05] and 20%-0.50 [0.43, 0.60] and total branching length 1%-0.92 [0.82, 1.01], 5%- 0.88 [0.79, 0.94] and 20%-0.61 [0.51, 0.66], all $P = 0.0001$; Fig. 4 A, B and C), compared with HUVECS grown in conditioned medium from HTR-8/SVneo cells cultured at 20% O_2.

HTR-8/SVneo cells cultured at low O_2 tensions produced oxygen-dependent increases in cell viability. Cell viability was significantly greater in 1% O2 compared with culture in 5 and 20% O_2 (median [IQR]; 1%-0.98 [0.83, 1.00], 5%-0.72 [0.66, 0.81] and 20%-0.48 [0.44, 0.55], both P = 0.0001; Fig. 5) and the viability of cells in 5% O_2 was greater compared with those cultured in 20% O_2 (P = 0.0001; Fig. 5).

3.2. Ang II/AT_1R partially mediates upregulation of pro-angiogenic factors

Inhibition of Ang II/AT_1R signalling with 10 μM of the specific AT_1R antagonist, losartan, had no effect on the expression of any downstream pro-angiogenic target of Ang II/AT_1R signalling (Fig. 1). Whilst treatment with losartan tended to reduce mRNA expression of *ANGPT2* (P_{ANOVA} = 0.012; Fig. 1D), post-hoc analysis did not reveal any significant effect of losartan within each oxygen tension.

Treatment with losartan significantly reduced both intracellular and secreted levels of VEGFA protein (Fig. 2A and B) in cells cultured in 1% O_2 and reduced VEGFA intracellular protein levels in cells cultured at all three oxygen tensions (median [IQR]; 1% Vehicle-1.01 [0.66, 1.32], 1% Losartan-0.72 [0.26, 1.15], 5% Vehicle-0.58 [0.42, 0.89], 5% Losartan-0.37 [0.06, 0.58], 20% Vehicle-0.37 [0.19, 0.51] and 20% Losartan-0.07 [0.003, 0.21], $P = 0.008$, 0.003 and 0.009 respectively; Fig. 2A). Furthermore, VEGFA protein was significantly reduced by treatment with losartan when cells were cultured in 1% O_2 only (median [IQR]; 1% Vehicle-0.97 [0.83, 1.26] and 1% Losartan-0.69 [0.34, 0.87], $P = 0.0001$; Fig. 2B) because little to no VEGFA was secreted by cells cultured in 5 or 20% O_2.

In addition, intracellular SERPINE1 protein was significantly reduced when cells were treated with 10 μM losartan (median [IQR]; 1% Vehicle-0.96 [0.65, 1.48] and 1% Losartan-0.77 [0.51, 0.94], $P = 0.0001$; Fig. 2C), but this was only seen in cells cultured in 1% O_2 because levels of SERPINE1 were very low in 5 and 20% O_2. The secretion of SERPINE1 was not affected by treatment with losartan at any oxygen tension (Fig. 2D).

Treatment with losartan had no effect on the expression of any of the anti-angiogenic factors at any oxygen tension (Fig. 3).

3.3. Effect of HTR-8/SVneo losartan treated conditioned media on tube formation in human umbilical vein endothelial cells (HUVECs)

Overall, conditioned media from HTR-8/SVneo cells treated with losartan significantly affected HUVEC tube formation in all parameters measured (all P_{ANOVA} = 0.004, 0.003 and 0.0002; Fig. 4). The total number of meshes was significantly reduced by the presence of losartan treated conditioned media only from HTR-8/SVneo cells cultured in 20% O_2 (median [IQR]; 20% Vehicle-0.46 [0.22, 1.44] and 20% Losartan-0.30 [0.06, 0.54], $P = 0.031$; Fig. 4A). Total branching length was also significantly reduced by the presence of losartan treated conditioned media from HTR-8/SVneo cells cultured in 1 and 5% O_2 (median [IQR]; 1% Vehicle-1.01 [0.79, 1.37], 1% Losartan-0.83 [0.78, 0.96], 5% Vehicle-0.93 [0.79, 1.26] and 5% Losartan-0.80 [0.64, 1.16], $P = 0.022$ and 0.050 respectively; Fig. 4C). The total number of branching points tended to be reduced by culture with losartan treated conditioned media from HTR-8/SVneo cells cultured in 1% O_2, however this failed to reach statistical significance ($P = 0.086$; Fig. 4B).

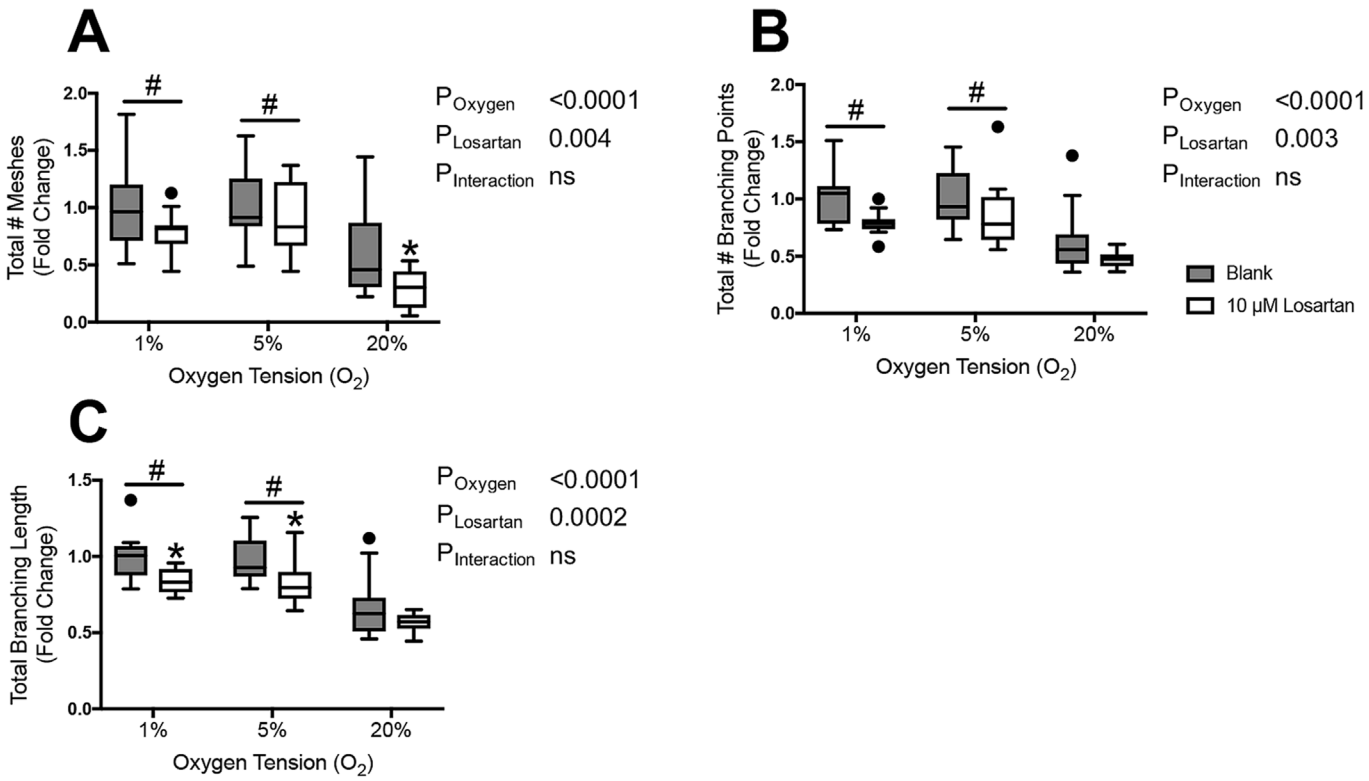

Fig. 4. Effect of HTR-8/SVneo conditioned media on HUVEC tube formation capacity. (A) Total number of meshes, (B) number of branching points and (C) branching length were significantly higher in conditioned media from 1% O_2 and 5% O_2 culture compared with 20% O_2 (#; all P = 0.0001). While conditioned media from HTR-8/SVneo cells treated with losartan affected all parameters measured (ANOVA), post-hoc analyses did not reveal any significant effects of losartan within each oxygen tension with (B) total number of branching points despite tending to show a reduction in 1% O_2 medium treated with losartan (P = 0.086). (A) Total number of meshes was significantly reduced by treatment with losartan only in 20% O_2 (*; P = 0.031). (C) Total branching length was significantly reduced in 1 and 5% O_2 losartan treated medium (*; P = 0.022 and 0.050 respectively). Data expressed as a fold change from the 1% O_2 vehicle control (median and interquartile range). ^ denotes significance to 5% O_2. # denotes significance to 20% O_2. * denotes significance to vehicle control. N = 4.

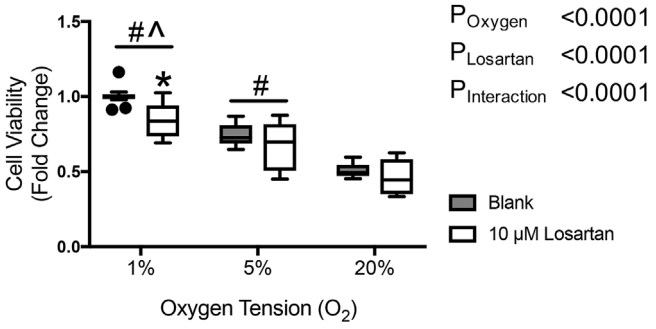

Fig. 5. Effect of oxygen tension and AT_1R inhibition on HTR-8/SVneo cell viability. Cell viability was enhanced by culture in 1% O_2 when compared with culture in 5 and 20% O_2 (^,#; all P = 0.0001). Furthermore, culture in 5% O_2 significantly enhanced cell viability compared with culture in 20% O_2 (#; P = 0.0001). Treatment with losartan (10 μM) significantly reduced the viability of cells cultured in 1% O_2 (*; P = 0.0001). Treatment with losartan had no effect on cell viability in culture at either 5 or 20% O_2. Data expressed as a fold change from the 1% O_2 vehicle control (median and interquartile range). ^ denotes significance to 5% O_2. # denotes significance to 20% O_2. * denotes significance to vehicle control. N = 6.

3.4. Cell viability induced by culturing HTR-8/SVneo cells in 1% O_2 is partially mediated via the AT_1 receptor

Treatment with losartan was associated with a significant reduction in the number of viable cells when cultured in 1% O_2 (median [IQR]; 1% Vehicle-1.00 [0.91, 1.16], 1% Losartan-0.84 [0.69, 1.03], $P = 0.0001$; Fig. 5). Treatment with losartan did not significantly affect cell viability at either 5 nor 20% O_2.

4. Discussion

This study shows that a low oxygen milieu promoted a proliferative and angiogenic phenotype in HTR-8/SVneo cells similar to that seen in first trimester extravillous trophoblasts. This effect was more marked in trophoblasts cultured in 1% O_2 compared with 5% O_2 [22–24]. Furthermore, this study shows that the induction of a proliferative and angiogenic trophoblast phenotype by a low oxygen milieu is influenced by the pro-proliferative/pro-angiogenic pathway of the RAS, namely the Ang II/AT_1R pathway, which is upregulated in HTR-8/SVneo cells incubated in 1% O_2 [12]. Inhibition of the Ang II/AT_1R pathway with the specific receptor antagonist, losartan, significantly reduced the angiogenic responses of HTR-8/SVneo cells caused by 1% O_2. That is, treatment with losartan reduced the low oxygen mediated increase in VEGFA, and SERPINE1 proteins (Fig. 2).

AT_1R inhibition with losartan was also associated with a reduction in *ANGPT2* mRNA expression in cells cultured in all O_2 tensions (Fig. 1). This, combined with the inhibition of *ANGPT1* mRNA expression in 1% oxygen and reduction in the ratio of *ANGPT1:ANGPT2* (allowing ANGPT2 to be the dominant angiopoietin), is pro-angiogenic. Treatment of cells with losartan in any O_2 tension tends to reverse this angiogenic profile because it inhibits expression of *ANGPT2* but has no effect on expression of *ANGPT1*. The *ANGPT1:ANGPT2* ratio and VEGFA influences the capacity of extravillous trophoblasts to promote elongation and vascular network growth [25,26]. Treatment with losartan did not alter the expression of the other anti-angiogenic factors (*sFlt-e15a* and *ENG*) studied (Fig. 2).

Thus, our data support the hypothesis that low oxygen-induced

increases in the expression of pro-angiogenic factors are partly mediated via Ang II/AT_1R signalling. Low oxygen is a known regulator of cell growth and *SERPINE1* mRNA expression in the trophoblast through stabilization of HIF-1α [27–29]. This is particularly relevant, because HIF-1α and AT_1R gene ablation in mice impair placental vascularization, suggesting that low oxygen driven AT_1R mediated actions are key regulators of early placental angiogenesis [30,31].

mRNA and protein levels of angiogenic factors affected by antagonising the interaction of Ang II with AT_1R also reduced HUVEC tube formation and cell viability of HTR-8/SV neo cells. These findings further strengthen the link between Ang II/AT_1R and placental angiogenesis.

The role of Ang II/AT_1R in angiogenesis has been extensively reviewed [32]. Both Ang II applied directly to endothelial cells and exposing endothelial cells to conditioned media from immortalized mouse myocyte cells can induce tube formation, and this can be attenuated with either AT_1R inhibition or the ACE inhibitor, captopril, respectively [33,34]. This indicates that Ang II may similarly mediate the production of angiogenic factors in HTR-8/SVneo cells. Interestingly, the role of Ang II in placental angiogenesis is poorly characterised. We suggest that dysregulation in Ang II/AT_1R signalling may disrupt placental angiogenesis, potentially contributing to the impaired placental vasculature seen in some pregnancy complications [35].

We have shown that the viability of the extravillous trophoblasts was affected by oxygen as has been shown by others [36,37] highlighting the role of O_2 promoted growth of chorionic villi. Since losartan antagonised the positive effects of 1% O_2 on HTR-8/SVneo cell viability, upregulation of AT_1R by low oxygen [7] must be required to promote this low oxygen-induced increase in cell viability.

Importantly, there were several differences in the response of the HTR-8/SVneo cells to culture in 1 and 5% O_2. As mentioned, the oxygen tension within the intervillous space during the first trimester is approximately 1–3% O_2, whereas the oxygen tension towards the decidual bed is approximately 5–8% O_2 [8]. Whilst there is conjecture, it is thought that trophoblast differentiation is regulated by oxygen tension [7,23,36]. It has been shown that only trophoblasts within the chorionic villi and those EVTs closest to the villi proliferate [38]. As EVTs invade into the maternal decidua, that is towards a higher oxygen tension, they develop a more invasive phenotype [36,37].

Regulation of the angiogenic capacity by low oxygen is much more poorly characterised. VEGFA mRNA and intracellular protein was incrementally increased by decreasing oxygen tensions, whilst secreted VEGFA and other angiogenic factors such as SERPINE1, were only induced under 1% O_2 culture. *ANGPT1* mRNA was also only reduced by culture in 1% O_2 indicating it would be the dominant angiopoietin in oxygen conditions above 1% O_2. Although there were no differences between 1 and 5% O_2 conditioned media in the ability of HUVECs to form tubes in this study, further titration and/or altered incubation time of HUVECs may reveal these subtler differences. Thus, clinically this data suggests that the low oxygen environment (1% O_2) that promotes a proliferative trophoblast phenotype may also enhance angiogenic capacity and that impaired oxygen regulation within the first trimester may inhibit angiogenesis within the chorionic villi.

As this study was conducted in an immortalized first trimester cell line, future work should be extended to examine the role of oxygen in regulating angiogenic capacity in more physiological models, such as first trimester chorionic villi or trophoblasts isolated from first trimester placentae, to strengthen these findings. However, the relationship between oxygen and the placental RAS both *in vivo* and *in vitro,* particularly evidenced by AT_1R, has been recognised. As mentioned above, *AGTR1* mRNA is positively correlated with HIF-1β in a model of placental hypoxia/reoxygenation [13]. AT_1R protein is also more highly abundant in placentae from high altitude (the most common cause of maternofetal hypoxia [39]) compared to sea level controls [13].

In conclusion, culture of HTR-8/SVneo cells in a low oxygen environment similar to that seen in chorionic villi (1% O_2) in the first trimester of gestation promotes an angiogenic/proliferative trophoblast phenotype mediated in part by the increased Ang II/AT_1R expression as the angiotensin converting enzyme (ACE) and the Ang II/AT_1R are both increased when HTR-8/SVneo cells are cultured in 1% O_2 [40].

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