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Effect of oxygen on the expression of renin–angiotensin system components in a human trophoblast cell line

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ABSTRACT

During the first trimester, normal placental development occurs in a low oxygen environment that is known to stimulate angiogenesis via upregulation of vascular endothelial growth factor (VEGF). Expression of the placental renin–angiotensin system (RAS) is highest in early pregnancy. While the RAS and oxygen both stimulate angiogenesis, how they interact within the placenta is unknown. We postulated that low oxygen increases expression of the proangiogenic RAS pathway and that this is associated with increased VEGF in a first trimester human trophoblast cell line (HTR-8/SVneo). HTR-8/SVneo cells were cultured in one of three oxygen tensions (1%, 5% and 20%). RAS and *VEGF* mRNA expression were determined by qPCR. Prorenin, angiotensin converting enzyme (ACE) and VEGF protein levels in the supernatant, as well as prorenin and ACE in cell lysates, were measured using ELISAs. Low oxygen significantly increased the expression of both angiotensin II type 1 receptor (*AGTR1*) and *VEGF* (both $P < 0.05$). There was a positive correlation between *AGTR1* and *VEGF* expression at low oxygen ($r = 0.64$, $P < 0.005$). Corresponding increases in VEGF protein were observed with low oxygen ($P < 0.05$). Despite no change in *ACE1* mRNA expression, ACE levels in the supernatant increased with low oxygen (1% and 5%, $P < 0.05$). Expression of other RAS components did not change. Low oxygen increased *AGTR1* and *VEGF* expression, as well as ACE and VEGF protein levels, suggesting that the proangiogenic RAS pathway is activated. This highlights a potential role for the placental RAS in mediating the proangiogenic effects of low oxygen in placental development.

1. Introduction

A low oxygen environment during the first trimester of pregnancy is required for optimal placental development that is essential for supplying fetal demand in late gestation. This early gestation low oxygen environment occurs as a result of extravillous trophoblast (EVT) cell proliferation and subsequent invasion of the decidua and its vasculature, initially occluding maternal spiral arterioles from about two weeks after implantation [1]. Rodesch et al. [2] established that the oxygen tension in the intervillous space at 8 weeks is 17.9 mmHg (~2.5%) with a range of 5–30 mmHg (~0.7–4.3%) while the oxygen tension in the endometrium is higher at 39.6 mmHg (~5.7%) with a range of 25–70 mmHg (~3.5–10%).

Abbreviations: ACE, angiotensin-converting enzyme; ACE1, angiotensin-converting enzyme 1; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AT1R, angiotensin II type 1 receptor; *AGTR1* mRNA, angiotensin II type 1 receptor mRNA; Ang, angiotensin; ATP6AP2, ATPase, H^+ transporting, lysosomal accessory protein 2 / (pro)renin receptor; BCA, bicinchoninic acid; EVT, extravillous trophoblast; HIFs, hypoxia inducible factors; iNOS, inducible nitric oxide synthase; RAS, renin–angiotensin system; *REN* mRNA, (pro)renin mRNA; VEGF, vascular endothelial growth factor.

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Thus there is an oxygen gradient experienced by first trimester trophoblasts that can range from ~1% (in the placental villi) to up to 10% in the decidua. The low oxygen environment stimulates angiogenesis and vascularization of the placenta. Poor placental development, characterized by insufficient decidual invasion by EVTs, incomplete occlusion of maternal arterioles with inadequate remodeling of maternal spiral arterioles, and early onset of maternal blood flow to the conceptus, ultimately results in poor nutrient and oxygen exchange during the 2nd and 3rd trimesters. These are associated with intrauterine growth restriction and preeclampsia [3,4].

A low oxygen tension stabilizes hypoxia inducible factors (HIFs) which promote angiogenesis and vascularization by activating pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietins, factors involved in regulation of vascular tone such as inducible nitric oxide synthase (iNOS) and proteins involved in nutrient transfer such as transferrin and glycolytic enzymes [5–8].

Another system that might regulate placental angiogenesis is the renin–angiotensin system (RAS). Tissue RASs have been shown to be involved in the regulation of angiogenesis, as well as cell proliferation and apoptosis [9]. We have shown that mRNA expression of prorenin (*REN* mRNA), (pro)renin receptor (*ATP6AP2* mRNA), angiotensinogen (*AGT* mRNA), angiotensin (Ang) II type 1 (AT_1R) (*AGTR1* mRNA) and angiotensin converting enzyme 2 (ACE2) (*ACE2* mRNA) are all very high in early gestation placentae compared with term [10]. In addition, we have shown that the mRNA expression of placental *VEGF* is correlated with those of *REN*, *ATP6AP2* and *AGTR1* mRNAs [10]. Thus the placental RAS is most active during the first trimester and therefore could stimulate angiogenesis, as it does in other tissues. The ocular RAS is stimulated by ischemia. The increased activity of the ocular RAS is associated with a potent angiogenic response mediated via the Ang II/AT_1R pathway [11]. In addition, early renal development requires activation of the RAS by a low oxygen milieu [12].

While a low oxygen environment regulates placental development [13], the extent to which the RAS is essential for normal placental development has not been established. To investigate interactions between a low oxygen milieu and the placental RAS, we examined the effects of low oxygen on the expression of the RAS and VEGF in a first trimester human trophoblast cell line, HTR-8/SVneo, which we have previously shown expresses mRNAs encoding those RAS pathway components that stimulate angiogenesis in the eye and kidney [14,15].

2. Materials and methods

2.1. Cell culture

HTR-8/SVneo cells are an immortalized first trimester trophoblast cell line (a kind gift from Prof. Charles Graham, Queens University, Ontario). HTR-8/SVneo trophoblast cells were chosen for the study as they are a transformed first trimester human extravillous trophoblast cell line. Because of this they are an ideal tool for investigating the effect of low oxygen on the proliferative/angiogenic RAS pathways in placental development and are superior to other cell lines such as choriocarcinoma BeWo cells. We have demonstrated previously that the pro-angiogenic/proliferative pathway of the RAS is expressed in the HTR-8/SVneo cell line [14] as occurs in the first trimester placenta *in vivo* [9], but which is not seen in BeWo cells [14].

HTR-8/SVneo cells were cultured in RPMI-1640 medium (HyClone), supplemented with 10% fetal bovine serum (SAFC Biosciences), 1 mg/ml antibiotic-antimycotic (Gibco) and 1% L-glutamine in 5% CO_2 in room air at 37 °C (cells were between passages 10–20). Cells were seeded at a density of 200,000 or 400,000 cells per well for 24 h or 48 h incubation, respectively. They were seeded in 6 well plates with 2 ml of incubation medium per well and allowed to settle for 24 h, after which time the medium was changed. Cells were then transferred to sealed oxygen chambers containing either 1%, 5% or 20% O_2 and 5% CO_2 in N_2 and cultured for 24 or 48 h, with chambers flushed every 24 h. Cells were harvested and the incubation medium was collected at 24 and 48 h, then snap frozen in liquid nitrogen at −80 °C for subsequent protein and mRNA analyses. Three experiments were conducted in triplicate. RNA quantity was used as an indicator of cell viability [16] 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.

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). In addition, we examined the integrity of the total RNA in each sample using gel electrophoresis. RNA samples were DNase treated (Qiagen) and total RNA was spiked with a known amount of Alien RNA (Stratagene), 10^7 copies per μg of total RNA, before the RNA was reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT PCR inhibitor alert system serves as a reference for internal standardization [17]. 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), RAS and VEGF primers that we have described previously [10,18], cDNA reversed transcribed from 10 ng total RNA, and water to 10 μl. Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta CT}$ method and expressed relative to Alien mRNA and a calibrator sample (a term placental sample collected at elective Cesarean section) [10].

2.3. Extraction and quantification of total protein from cells

Protein was extracted from cells using a radioimmunoprecipitation assay (RIPA) lysis and extraction buffer. One milliliter 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 Australia) and 10 μl of 100 nM PMSF were added to each sample. Samples were incubated on ice for 30 min then centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants were collected. Protein was quantified using the Pierce BCA Protein assay kit (Life Technologies) according to the manufacturer's instructions.

2.4. Measurement of prorenin, VEGF and ACE by ELISA

Prorenin, VEGF and ACE concentrations in culture medium and cell lysates (at both 24 and 48 h) were measured using the Human Prorenin ELISA kit (Molecular Innovations), Human VEGF Duoset ELISA kit (R&D systems) and the Human ACE Duoset ELISA kit (R&D Systems), respectively, according to the manufacturers' instructions, as described previously [19]. For prorenin, VEGF and ACE proteins in culture medium and prorenin in cell lysates, all samples were assayed in duplicate on one ELISA plate. Therefore there was no inter-assay variability. For ACE protein in cell lysates, samples were assayed in duplicate over two plates and inter-assay variability was 9.4%. Intra-assay coefficients of variation were