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Title: 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 ELISA. 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.

RESPONSE TO REVIEWERS

Manuscript # PL-15-100105

Title: 'Effect of oxygen on the expression of renin-angiotensin system (RAS) components in a human trophoblast cell line'
Authors: Sarah J Delforce; Yu Wang; Meg E Van-Aalst; Celine Corbisier de Meaultsart; Brian J Morris; Fiona Broughton-Pipkin; Claire T Roberts; Eugenie R Lumbers; Kirsty G. Pringle

We would like to thank the Editor and reviewers for their generally favorable assessment of our manuscript entitled: 'Effect of oxygen on the expression of renin-angiotensin system (RAS) components in a human trophoblast cell line'. We consider that we have now addressed all of the reviewers' concerns. We have displayed our text corrections in red throughout the manuscript and described them in our response to reviewers (see below). The reviewers' comments are stated first, in italics, followed by our response.

Reviewer #1:
In this study the authors have shown that a low oxygen environment stimulates the expression of AGTR1 and VEGF mRNAs in HTR-8/SVneo cells in vitro. Both AT1R and VEGF are involved in angiogenesis and proliferation, which are key properties of first trimester EVTs. We showed that incubation in a low oxygen environment increased production of ACE and VEGF protein by HTR-8/SVneo cells and increased expression of the Ang II/AT1R pathway. They proposed that the placental RAS is under the influence of many factors including endocrine, molecular and environmental ones.
No response required.

General comment: This is a well-written and designed article by experimented team. I have concerns about the reliability of the use of HTR-8/SVneo cells instead primary trophoblasts or other lines (i.e. BeWo, Jeg-3, JAr …etc). How the authors justify the choice of this line and not another? This justification must be added to the section of discussion.

AUTHORS' RESPONSE: The following passage has now been added to the Materials and methods (page 2, lines 48-55) "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]."

Reviewer #2:

This study by Delforce and colleagues characterize the role of varying oxygen tension on the expression of the renin-angiotensin system in trophoblast cell line HTR-8/SVneo. The main findings of this paper are that the mRNA expression of AGTR1 and VEGF at low oxygen level and the increased VEGF secretion from cells. The methodological approach is sound, although first trimester primary trophoblast cells or explants would have been preferred. The overall conclusion that low oxygen regulates the RAS may be overstated given that only one gene from the RAS is altered (AGTR1) and this was not confirmed at the protein level. This is important since although REN mRNA expression was altered, there were no changes at the protein level. Changes in VEGF expression with oxygen tension are well established and therefore these findings are more confirmatory than novel insights.

AUTHORS' RESPONSE: We agree that the conclusion that low oxygen regulates the RAS was overstated and have revised this section in the Discussion (page 6, lines 164-167): "The study also showed that even small reductions in O_2 have substantial effects on the expression of the AT_1R and pro-angiogenic factors such as VEGF. This was based on our finding that culture in 1% oxygen had a much more powerful effect than culture in 5% oxygen"

Minor comments:
Cell culture: Please provide the passage number(s) of the cell line if possible.

AUTHORS' RESPONSE: Cells were between passages 10-20. This information has now been added to the Materials and methods section of the manuscript (page 2, lines 58-59).

Line 53-54: RNA quality measurements are not appropriate indications of cell viability. Trypan blue exclusion, MTT, apoptosis assays may be required.

AUTHORS' RESPONSE: We have rephrased the text to clarify that RNA quantity, not quality, was used as an indicator of cell viability, page 3 lines 66-69: "RNA quantity was used as an indicator of cell viability 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."

Line 73: Please check if this is supposed to be "one microliter" or "one milliliter"

AUTHORS' RESPONSE: We thank the reviewer for noting this error. This sentence was supposed to say "one millilitre" and has now been corrected (page 3, line 89).

Please provide details for regression analysis. Also please provide the R2 value.

AUTHORS' RESPONSE: The following passage has now been added to the Materials and methods (page 4, lines 115-116): "Pearson correlation coefficients were calculated to determine any relationships between the mRNA expression of genes." R^2 and r values are now present in the figure legends and have been added to the text (page 5, lines 137-138).

Reviewer #3: *In this manuscript, the authors describe in vitro experiments demonstrating that low oxygen influence RAS and VEGF in HTR-8/SVneo cells. The authors have shown by different techniques that culture in low oxygen tension (1%) increased AGTR1 and VEGF mRNAs as well as ACE and VEGF proteins expression in trophoblast cells. Data are interesting and add knowledge to the area, however some points should be elucidate before manuscript publication.*

AUTHORS' RESPONSE: No response required.

Specific comments:
1- How did the authors define oxygen tension (1%, 5% or 20%) for culture? As the effects on gene and protein expression were observed with very low oxygen tension (1%) it is important to clarify whether this oxygen tension reflects the physiological environment of early gestation.

AUTHORS' RESPONSE: We chose 1% and 5% oxygen tensions to closely reflect the environment experienced by the early gestation trophoblasts within the placental villi as well as the extravillous trophoblasts invading into the endometrium. We have now included a section in the introduction outlining the physiological environment in the first trimester (page 1, lines 7-12): "Rodesch *et al.* [2] established that the oxygen tension in the intervillous space at 8 weeks to be 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%). Thus there is an oxygen gradient experienced by the first trimester trophoblasts that can range from ~1% (in the placental villi) to up to 10% in endometrium."

2- Material and Methods- Are VEGF primers also described by Marques et al. (2011)? Did the authors investigate VEGF-A? There are multiple isoforms of VEGF-A. What was the VEGF isoform(s) investigated?

AUTHORS' RESPONSE: The VEGF primers described target VEGF-A and will detect all transcript variants of VEGF-A. VEGF primers were described in Pringle et al. (2011); this reference has been added to the text (page 3, line 81).

3- Material and Methods - Lines 53-54 - There was influence of oxygen tension in cell viability?

AUTHORS' RESPONSE: No influence of oxygen tension on cell viability was observed. This information has now been added to the Materials and methods (page 3, 66-69). Also see response to reviewer 1, above.

4- Material and Methods- Did the authors measure protein content (VEGF, prorenin and ACE) at 24hs of culture?

AUTHORS' RESPONSE: We have measured VEGF protein, as well as prorenin and ACE protein at 24 hours of culture. No effect of oxygen tension on the levels of these proteins was, however, observed. The Materials and methods section has been modified to reflect this (lines 98-99) and these results have now been included in the manuscript:

- Page 5, lines 142-144: "Although measurable levels of *REN* mRNA were found (Fig. 1), prorenin protein was low in both the culture medium and cell lysate of HTR-8/SVneo cells at 24 and 48 h incubation irrespective of the prevailing O_2 (only 48 h data shown; Fig. 3)."
- Page 5, lines 147-148: "No effect of oxygen tension on VEGF protein was observed at 24 h incubation (data not shown)."
- Page 5 lines 153-155: "No effect of oxygen tension on ACE protein in culture medium or cell lysates was observed at 24 h incubation (data not shown)."

5- Did the authors expect to identify ACE2, AGTR2 and MAS1 gene expression in HTR-8/SVneo cells. How to explain the observed data? Could this lack of expression alter expression of other RAS members in HTR-8/SVneo cells.

AUTHORS' RESPONSE: We did not expect to identify *ACE2*, *AGT2R* or *MAS1* gene expression in HTR-8/SVneo cells as we have previously demonstrated that this cell line does not express these mRNAs (Wang et al. 2012). A statement outlining the similarities between these 2 studies has been included in the Results (page 4, lines 126 – 127).

6- Results - Lines 117-118 -What is the physiological meaning of the correlations? What is the molecular correlation between these genes. Manuscript could be improved with more discussion about a hypothesized mechanisms related to the data.

AUTHORS' RESPONSE: The following passage has now been added to the Discussion (page 7, lines 203-209) "We found that *AGT1R* mRNA abundance was highly correlated with levels of both *VEGF* and *ATP6AP2* mRNA. The association between *AGT1R* and *ATP6AP2* mRNA suggests that increased levels of the (pro)renin receptor (ATP6AP2) may stimulate the activation of the placental RAS cascade and in turn increase AT_1R, while the association between *AGT1R* and *VEGF* mRNA suggests that increased AT_1R in first trimester placenta may increase expression of VEGF mRNA and protein, and thereby stimulate angiogenesis within the developing placenta."

7- Result - Lines 123 - How much prorenin was expected in the cell cultures? Why the values are low?

AUTHORS' RESPONSE: The values observed in the study were expected and were similar to those obtained in previous studies in which increases in prorenin protein were only observed when HTR-8/SVneo cells were stimulated with cAMP (see Wang *et al.* 2012). This was addressed in the Discussion (page 6, lines 168-176) where we suggest that the expression of prorenin (mRNA and protein) may be influenced by factors other than low oxygen including hormonal activation and/or activation by cAMP: "A low oxygen environment was not associated with increased expression of prorenin mRNA or

its protein. This suggests that a low oxygen tension alone is not responsible for the high levels of *REN* mRNA found in first trimester placentae [10]. It is more likely that increased *REN* mRNA expression in the placenta early in pregnancy is the result of hormonal activation and/or activation by cAMP of *REN* mRNA expression. Human chorionic gonadotropin (hCG) has been recognized as a stimulus for prorenin production by villous placenta [19], as have β-adrenoceptor agonists [19]. We have also shown previously that cAMP increases *REN* mRNA expression and prorenin protein secretion by HTR-8/SVneo cells [14]."

8- Discussion - Please check this statement in lines 141-145.

AUTHORS' RESPONSE: We have adjusted the text because we agree that the effect of 1% O_2 was only seen for *AGT1R* mRNA and not the whole RAS cascade. Please also see our response to Reviewer 1, above.

Conflict of Interest

The authors have no competing interests to declare.

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 ELISA. 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.

Keywords: renin-angiotensin system; pregnancy; placenta; hypoxia; gene expression

Abbreviations

ACE, angiotensin-converting enzyme; ACE1, angiotensin-converting enzyme 1; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AT_1R, 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.

Highlights

- The human placenta develops in a low oxygen environment
- The placental renin-angiotensin system (RAS) is highly expressed in early pregnancy
- Culture in 1% O_2 increased *AGTR1* mRNA and ACE protein in HTR-8/SVneo cells
- The latter was associated with increased *VEGF* mRNA and VEGF protein
- Stimulation of the placental RAS by low oxygen may be proliferative and angiogenic

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%). 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 Ang 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].

Materials and methods

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 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.

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 [16]. 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, 17], 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].

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.

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 [18]. 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 13.1% and 3.2% for prorenin culture medium and cell lysate, 6.8% for VEGF culture medium and 14.2% and 2.9% for ACE culture medium and cell lysate, respectively.

Data analysis

Two-way ANOVA with Tukey's multiple comparisons test was used to determine the effects of differing oxygen tensions on the relative abundance of RAS mRNAs, prorenin, ACE and VEGF protein levels. A Kruskal-Wallis non-parametric test was used to determine the effects of differing oxygen tensions on prorenin concentration in both the culture medium and cell lysate at 48 h. Pearson correlation coefficients were calculated to determine any relationships between the various mRNA levels. GraphPad Prism (Prism version 6.0) was used for all graphs and correlation analyses and the SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for all other analyses. Significance was set at $P < 0.05$.

Results

Effect of O_2 on RAS and VEGF mRNA expression

At all oxygen tensions, HTR-8/SVneo cells expressed genes of the pro-angiogenic pathway of the RAS, i.e., *AGT*, *REN*, *ATP6AP2*, *ACE1* and *AGTR1*, as well as *VEGF*, but expression of genes of the anti-angiogenic RAS pathway, i.e., *ACE2*, *AGT2R*, *MAS1* was not detected (Figure 1); this is consistent with previously published data on the expression of RAS genes in this cell line [14].

Expression of *AGT*, *REN*, *ATP6AP2* and *ACE1* mRNAs was not affected by the prevailing oxygen tension, but *AGTR1* and *VEGF* mRNA expression were significantly higher in HTR-8/SVneo cells cultured in low oxygen (1% O_2) compared with 5% or 20% O_2 at 48 h (all $P<0.01$). A similar trend was seen in 24 h incubates. This did not, however, reach statistical significance (Fig. 1).

There was a significant effect of incubation time on the expression of *AGT*, *REN*, *ATP6AP2*, *ACE1* and *AGTR1* mRNAs, in that expression of these mRNAs was significantly higher at 48 h compared with 24 h (all $P<0.05$; Fig. 1).

A significant correlation was found between *AGTR1* and *VEGF* mRNA and between *AGTR1* and *ATP6AP2* mRNA (r=0.6378 and 0.6500 respectively, R^2= 0.4067 and 0.4733 respectively, both $P<0.01$; Fig. 2).

Effect of O_2 on the concentration on prorenin and VEGF protein concentration in the culture medium

Although measurable levels of *REN* mRNA were found (Fig. 1), prorenin protein was low in both the culture medium and cell lysate of HTR-8/SVneo cells at 24 and 48 h incubation irrespective of the prevailing O_2 (only 48 h data shown; Fig. 3).

After 48 h VEGF levels in the culture medium of HTR-8/SVneo cells grown in 1% O_2 were much higher than levels in medium from samples cultured in 5% and 20% O_2 ($P<0.05$ and $P<0.01$, respectively; Fig. 4). No effect of oxygen tension on VEGF protein was observed at 24 h incubation (data not shown).

Effect of O_2 on the concentration of ACE in the culture medium

After 48 h culture, HTR-8/SVneo cells cultured in 1% and 5% O_2 had significantly higher levels of ACE protein in the supernatant than cells cultured in 20% O_2 ($P<0.05$; Fig. 5). ACE levels in the cell lysate were not affected by O_2. No effect of oxygen tension on ACE protein in culture medium or cell lysates was observed at 24 h incubation (data not shown).

Discussion

Incubation of primary HTR-8/SVneo trophoblast cells at a low (1%) oxygen tension increased the expression of *AGTR1* and *VEGF* mRNA and stimulated the production of

ACE and VEGF proteins. We propose, therefore, that a low oxygen milieu activates the pro-angiogenic pathway of the RAS by increasing AT_1R and ACE protein levels and that these changes are associated with increases in VEGF, an important angiogenic factor.

The study also showed that even small reductions in O_2 have substantial effects on the expression of the AT_1R and pro-angiogenic factors such as VEGF. This was based on our finding that culture in 1% oxygen had a much more powerful effect than culture in 5% oxygen. ~~Thus, increased *AGTR1* and *VEGF* mRNA expression and VEGF protein only occurred at 1% O_2.~~ A low oxygen environment was not associated with increased expression of prorenin mRNA or its protein. This suggests that a low oxygen tension alone is not responsible for the high levels of *REN* mRNA found in first trimester placentae [10]. It is more likely that increased *REN* mRNA expression in the placenta early in pregnancy is the result of hormonal activation and/or activation by cAMP of *REN* mRNA expression. Human chorionic gonadotropin (hCG) has been recognized as a stimulus for prorenin production by villous placenta [19], as have β-adrenoceptor agonists [19]. We have shown previously that cAMP increases *REN* mRNA expression and prorenin protein secretion by HTR-8/SVneo cells [14]. Expression of *AGT* and *ATP6AP2* mRNAs were not affected by oxygen. This suggests that their high expression in early gestation placentae [10] is not due to the low oxygen milieu.

Although the expression of *ACE1* was not stimulated by culturing cells in a low oxygen environment, we did observe increased ACE protein in medium from incubations carried out in low oxygen. Goyal *et al.* have observed a similar effect [20]. In animal studies they showed that maternal hypoxia was associated with upregulation of placental ACE protein without a corresponding increase in *ACE1* mRNA levels [20]. They concluded that this was because hypoxia-regulated miR-27, which regulates ACE translation, was down-regulated [20]. It is possible that a hypoxia-regulated miRNA in the HTR-8/SVneo cell cultures could be similarly affected by oxygen.

In a previous publication we reviewed evidence suggesting that the low oxygen milieu of early placental development activated the placental RAS, stimulating angiogenesis and cell proliferation [15]. The RAS pathway most likely to be involved in inducing these effects is the prorenin/AGT/ACE/Ang II/AT_1R pathway because the Ang II/AT_1R blocking drug, candesartan, blocks Ang II-induced endothelial proliferation

[21]. Hypoxia inducible factor (HIF-1α) is essential for normal trophoblast differentiation, since both HIF-1α and AT_1R gene ablation in mice impairs placental vascularization [22, 23]. Ang II acting via the AT_1R causes cell proliferation [24].

We were surprised that a low oxygen milieu had no effect on prorenin expression and secretion. There may, however, be a number of factors acting on the placental renin/AGT/ACE/Ang II/AT_1R pathway that upregulate different components, so ensuring increased placental Ang II production. As mentioned above, hCG and cAMP are potent stimuli for placental prorenin release. cAMP also stimulates *AGT* mRNA expression [25]. MicroRNAs (miRNAs) may affect the stability or translation of *ACE1* mRNA as has been observed in animal experiments [20]. *AGTR1* mRNA expression in HTR8/SVneo cells is, moreover, increased by low oxygen.

We found that *AGTR1* mRNA abundance was highly correlated with levels of both *VEGF* and *ATP6AP2* mRNA. The association between *AGTR1* and *ATP6AP2* mRNA suggests that increased levels of the (pro)renin receptor (ATP6AP2) may stimulate the activation of the placental RAS cascade and in turn increase AT_1R, while the association between *AGTR1* and *VEGF* mRNA suggests that increased AT_1R in first trimester placenta may increase expression of VEGF mRNA and protein, and thereby stimulate angiogenesis within the developing placenta.

In conclusion, we have shown that a low oxygen environment stimulates the expression of *AGTR1* and *VEGF* mRNAs in HTR-8/SVneo cells *in vitro*. Both AT_1R and VEGF are involved in angiogenesis and proliferation, which are key properties of first trimester EVTs. We showed that incubation in a low oxygen environment increased production of ACE and VEGF protein by HTR-8/SVneo cells and increased expression of the Ang II/AT_1R pathway. We propose that the placental RAS is under the influence of a number of endocrine, molecular and environmental factors.

Author Contributions

E.R. Lumbers and K.G. Pringle were responsible for designing the experiments. C.T. Roberts, B.J. Morris and F. Broughton-Pipkin assisted in developing the experimental concepts that were the basis of the experiments.

Y. Wang, S.J. Delforce, M.E. Van-Aalst and C. Corbisier de Meaultsart carried out the experiments. Wang and Delforce analysed the data.

Delforce, Wang, Lumbers, Morris and Pringle interpreted the data and drafted the manuscript.

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LEGENDS TO FIGURES

Fig. 1. Effect of O_2 on expression of RAS components in HTR-8/SVneo cells at 24 and 48 h. (A) *AGTR1* and (B) *VEGF* mRNA were significantly upregulated by low oxygen (1% O_2) at 48 h when compared with 5% and 20% O_2 (*, both $P<0.01$). (B) *VEGF* mRNA was significantly increased at 48 h compared with 24 h by 1% O_2 (#, $P<0.05$). (A) *AGTR1*, (C) *ATP6AP2*, (D) *REN*, (E) *ACE1* and (F) *AGT* mRNA expression significantly increased with incubation time (•, all $P<0.05$). Data are presented as mean ± SEM.

Fig. 2. The correlations between *VEGF* and *AGTR1* mRNA abundance and between *AGTR1* and *ATP6AP2* mRNA abundance in HTR-8/SVneo cells cultured at 1% O_2. (A) *VEGF* and *AGTR1* (r=0.638, R^2=0.407 and P=0.0044); (B) *AGTR1 and ATP6AP2* (r=0.650, R^2=0.473 and P=0.0035).

Fig. 3. Levels of prorenin protein in culture medium and cell lysates of HTR-8/SVneo cells cultured in varying oxygen tensions for 48 h. Neither culture medium (A) nor cell lysate (B) prorenin protein levels were affected by oxygen tension. Data are presented as mean ± SEM.

Figure 4. The effect of O_2 on VEGF protein concentration in the culture medium of HTR-8/SVneo cells at 48 h. Culture in 1% O_2 for 48 h significantly increased the concentration of VEGF compared with culture in either 5% or 20% O_2 (*, $P< 0.05$ and $P<0.01$ respectively). Data are presented as mean ± SEM.

Fig. 5. The effect of O_2 on ACE protein concentration in the culture medium and cell lysate of HTR-8/SVneo cells at 48 h. (A) The concentration of ACE in the culture medium of HTR-8/SVneo cells was significantly increased after 48 h of culture in both 1% and 5% O_2 when compared with the concentration measured in medium cultured in 20% O_2 (*, both $P<0.05$). (B) ACE protein levels in cell lysates were unaffected by oxygen. Data are presented as mean ± SEM.

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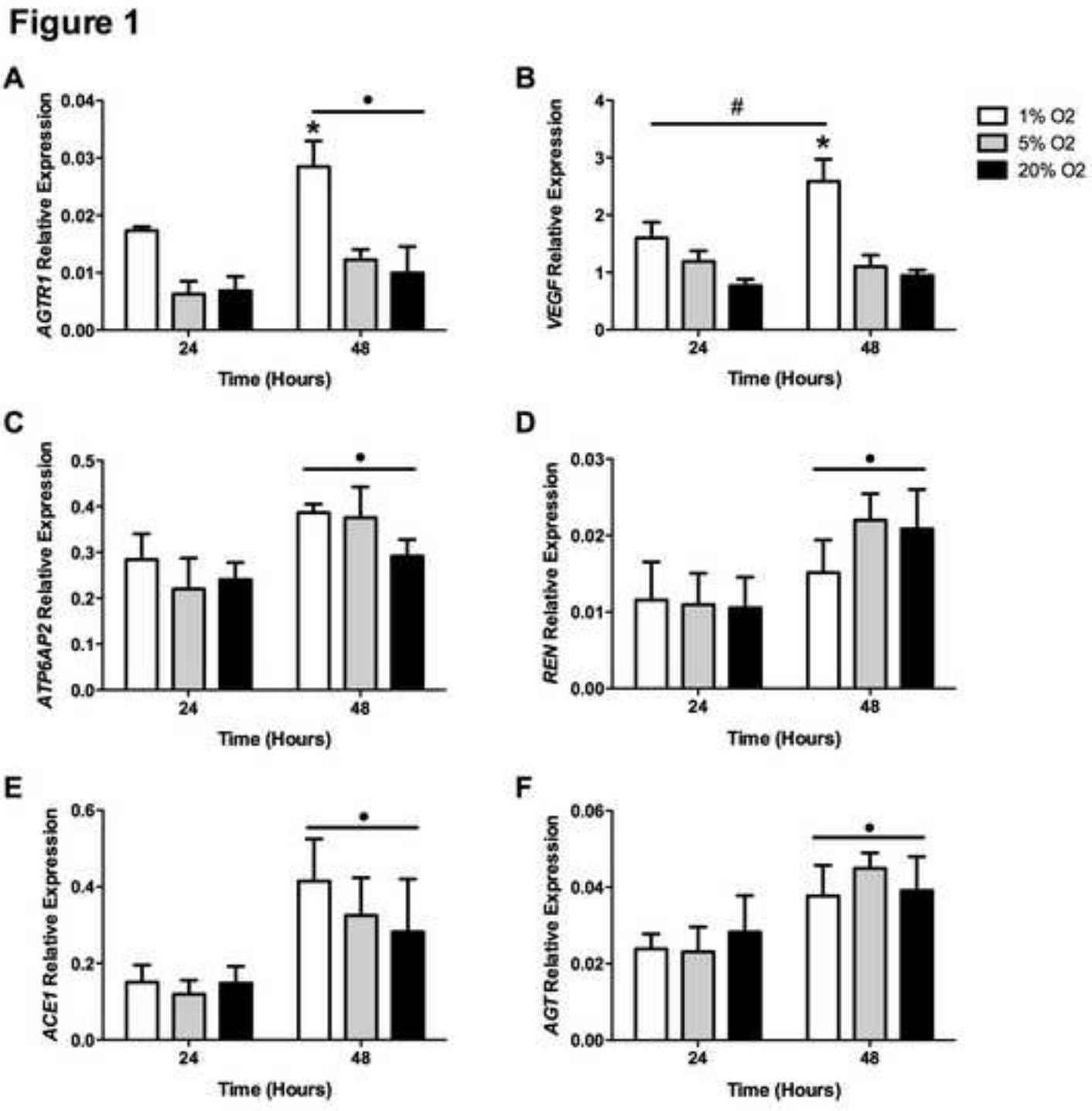

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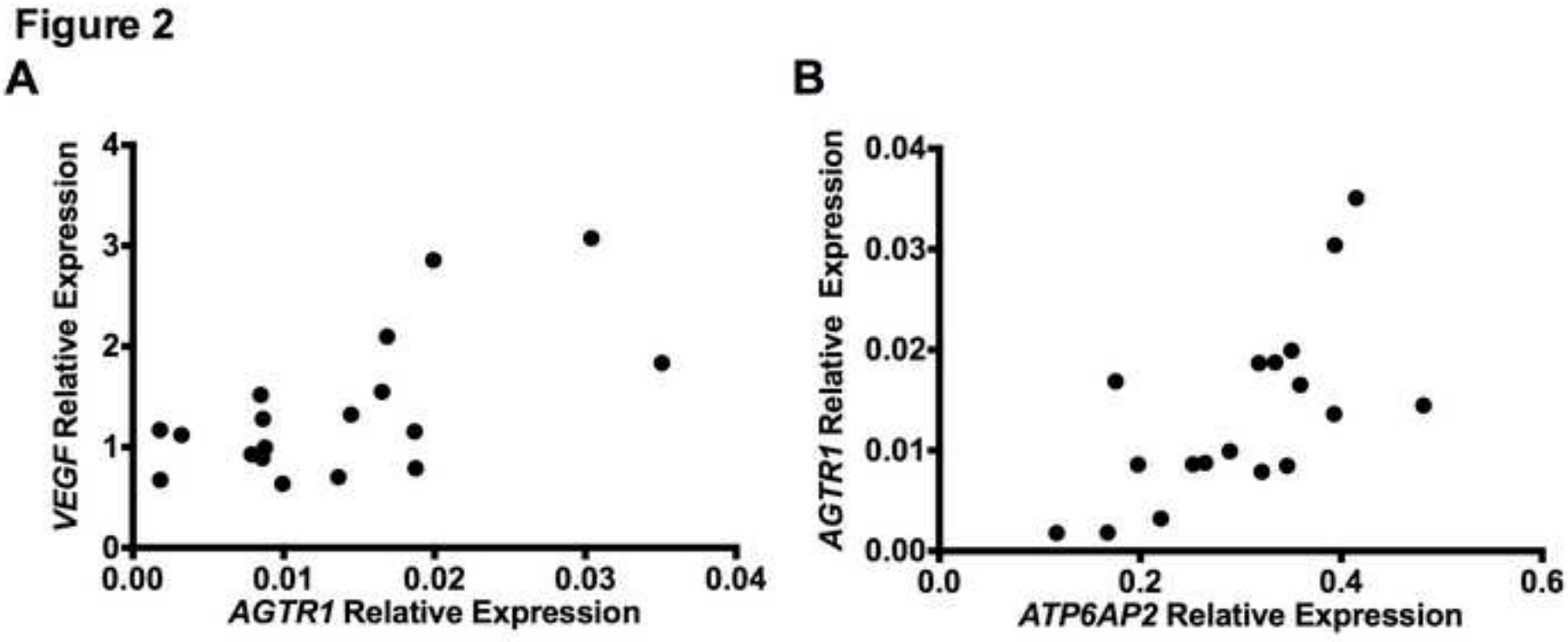

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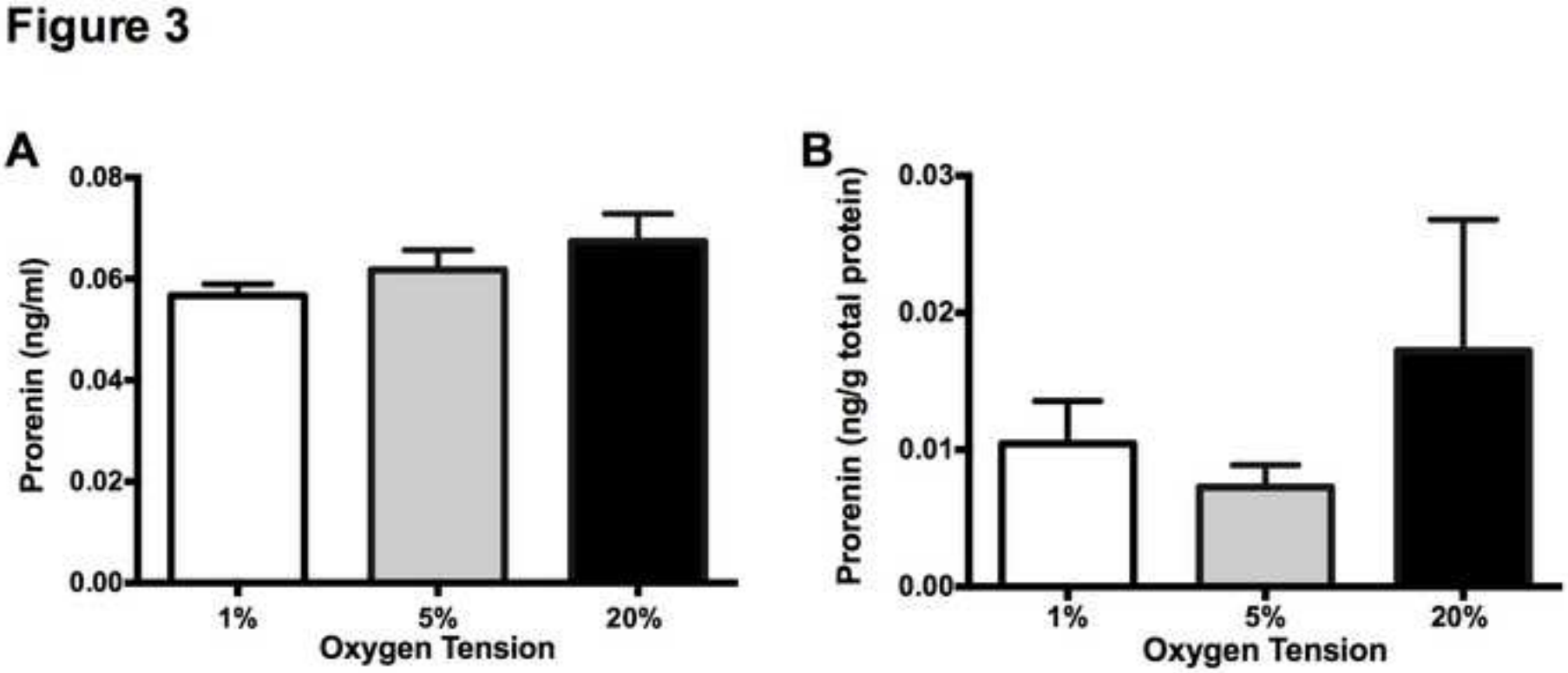

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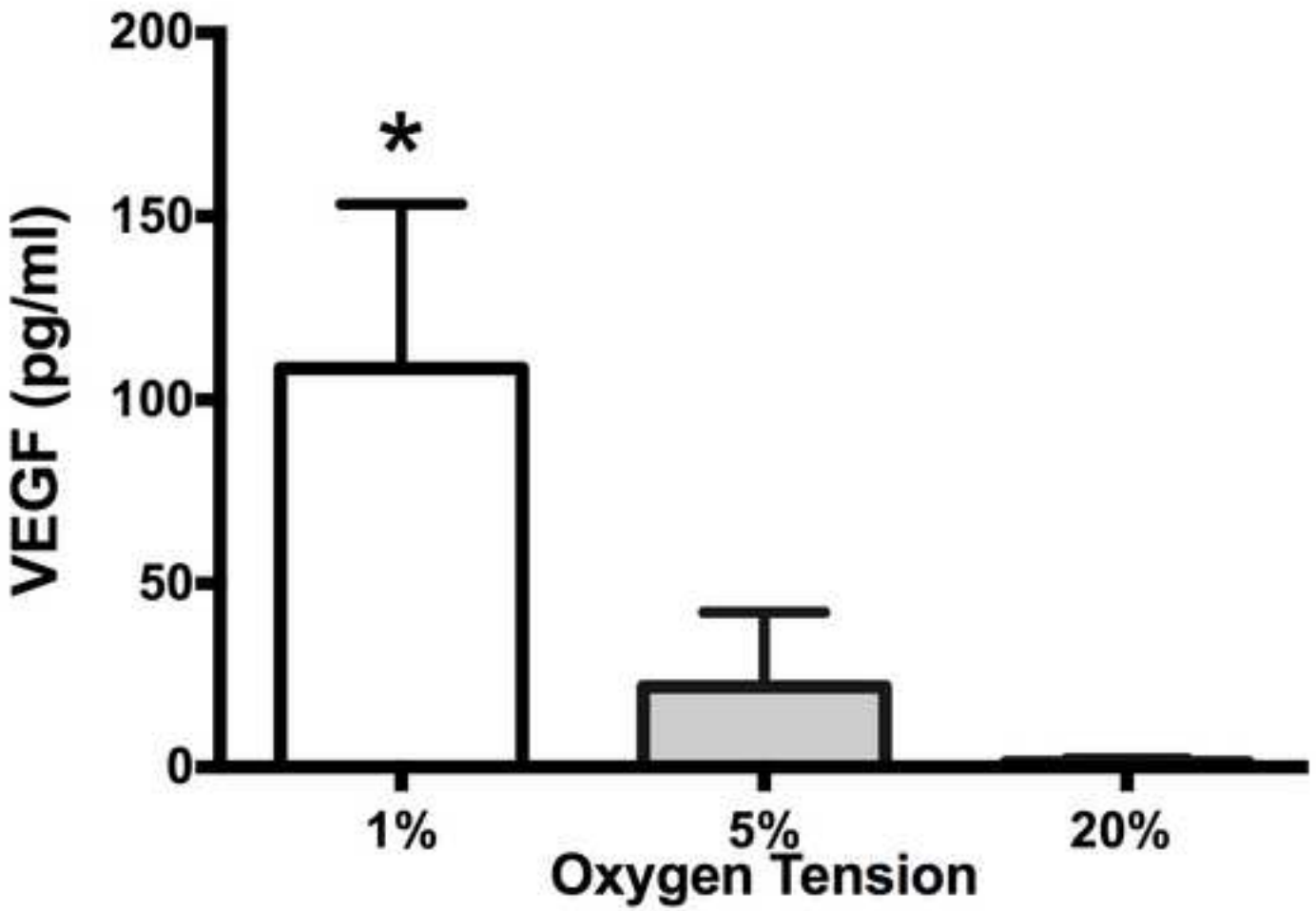

Figure 5

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