Human placental renin-angiotensin system in normotensive and pre-eclamptic pregnancies at high altitude and after acute hypoxia-reoxygenation insult.

Lesia O. Kurlak[1*], Hiten D. Mistry[1,2*], Tereza Cindrova-Davies[3], Graham J. Burton[3] & Fiona Broughton Pipkin[1]

[1] *Division of Obstetrics & Gynaecology, School of Medicine, University of Nottingham, City Hospital, UK*

[2] *Division of Hypertension, Department of Nephrology, Hypertension and Clinical Pharmacology and Clinical Research, University of Bern, CH-3010 Berne, Switzerland.*

[3]*Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience University of Cambridge, UK*

** Authors made equal contribution to this study.*

Corresponding author's address: Dr. Lesia O. Kurlak

Division of Obstetrics & Gynaecology,

University of Nottingham,

1st Floor, Maternity Unit,

City Hospital, Hucknall Road,

Nottingham, NG5 1PB, UK

Lesia.kurlak@nottingham.ac.uk

Tel: +44(0)115 82 31955

Fax: +44 (0)115 82 31908

Running Title: The renin angiotensin system responses to hypoxic insults

Key Words: renin angiotensin system; hypoxia, placenta; hypoxia-re-oxygenation; hypoxia-inducible factors

Abstract (250 words)

A functioning placental renin angiotensin system (RAS) appears necessary for uncomplicated pregnancy and is present during placentation, which occurs under low oxygen tensions. Placental RAS is increased in pre-eclampsia (PE), characterised by placental dysfunction and elevated oxidative stress. We investigated the effect of high-altitude hypoxia on the RAS and hypoxia-inducible factors (HIFs) by measuring mRNA and protein expression in term placentae from normotensive (NT) and PE women who delivered at sea level or above 3100m; using an explant model of hypoxia-reoxygenation to assess the impact of acute oxidative stress on the RAS and HIFs. Protein levels of prorenin (P=0.049), prorenin receptor (PRR; P=0.0004), and angiotensin receptors (AT1R, P=0.006) and (AT2R, P=0.002) were all significantly higher in placentae from NT women at altitude, despite mRNA expression being unaffected. However, mRNA expression of all RAS components was significantly lower in PE at altitude than at sea level, yet PRR, angiotensinogen (AGT) and AT1R proteins were all increased. The increase in transcript and protein expression of all the HIFs and NADPH oxidase 4 seen in PE compared to NT at sea-level was blunted at high altitude. Experimentally-induced oxidative stress stimulated AGT mRNA (P=0.04) and protein (P=0.025). AT1R (r=0.77, P<0.001) and AT2R (r=0.81, P<0.001) mRNA both significantly correlated with HIF-1β, whilst AT2R also correlated with HIF-1α (r=0.512, P<0.013).

Our observations suggest that the placental RAS is responsive to changes in tissue oxygenation: this could be important in the interplay between reactive oxygen species as cell-signalling molecules for angiogenesis and hence placental development and function.

Abbreviations: AGT, angiotensinogen; Ang II, angiotensin II; Ang IV, angiotensin IV; Ang (1-7), angiotensin-(1-7); AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; $AT_{2A}R$, adenosine A2A receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, high altitude; HIF, hypoxia-inducible factor; NOX4, NADPH oxidase 4; H/R, hypoxia-reperfusion; NT, normotensive; PE, pre-eclampsia; PRR, prorenin receptor; RAS, renin–angiotensin system; ROS, reactive oxygen species; XO, xanthine oxidase**.**

Introduction

In the early 20th century, Sir Joseph Barcroft (Barcroft, 1946) famously postulated that the fetus may be considered as 'Everest *in utero*' because of its development in an hypoxic environment equivalent to that probably only experienced at the Earth's highest point– the summit of Mount Everest.

The transition from acute hypoxia to hypoxic adaptation may involve the renin angiotensin system (RAS), which has been known as a regulator of systemic blood pressure and vascular function for many decades, reviewed in (Nguyen Dinh Cat & Touyz, 2011; Farag *et al.*, 2015). The systemic RAS in male adults is activated by acute exposure to high altitude (Sun *et al.*, 2013) whereas exposure to chronic hypoxia in rat models of primary hypertension causes the RAS to be suppressed (Cervenka *et al.*, 2015).

The RAS may play a significant role in differing ways throughout pregnancy for example, in the early stages of placentation. Components such as the angiotensin receptors are expressed at very early stages of embryogenesis (Tebbs *et al.*, 1999; Pijacka *et al.*, 2012) and in the placental syncytiotrophoblast and cytotrophoblast (Williams *et al.*, 2010), where they may be responding to maternally-derived angiotensin II (Ang II). Both Ang II and the biologically-active fragment Angiotensin IV (3-8) (Ang IV) promote the invasion and proliferation of trophoblasts (Williams *et al.*, 2010) and angiogenesis in placental tissue, thus supporting the establishment of a placental vasculature. Development of the yolk sac placenta in explanted rat embryos is highly-significantly increased by Ang II (Tebbs *et al.*, 1999). Prorenin and angiotensinogen (AGT) proteins are detected in the syncytiotrophoblast and cytotrophoblasts as early as 6 weeks gestation (Marques *et al.*, 2011; Pringle *et al.*, 2011) with prorenin also being found in the extravillous trophoblasts and AGT in the villous stroma.

Detailed knowledge on the process of implantation in humans is scarce. One fact is unequivocal: trophoblast invasion clearly correlates with oxygenation of the placenta (Huppertz *et al.*, 2014). Initial reorganisation of the syncytiotrophoblast layer and proliferation of the underlying cytotrophoblasts occurs in the first 5 weeks after implantation under oxygen tensions below 20 mmHg (Rodesch *et al.*, 1992; Jauniaux *et al.*, 2000; Jauniaux *et al.*, 2001). These oxygen tensions in other tissues would be considered 'hypoxic' but are physiologically normal for establishment of the placenta (Cindrova-Davies *et al.*, 2015). The oxygen tension in the underlying decidua basalis at this time is ~60 mmHg, and so the extravillous trophoblast invasion occurs along an oxygen gradient. Indirect measurements of oxygen tension , using aspirates in the intervillous space,

indicate that in the second half of normal pregnancy oxygen tensions decline, from 60 mmHg at 18 weeks gestation to 30-40 mmHg at term (Soothill *et al.*, 1986) .

In normal pregnancy, placental growth is stimulated by a number of factors including adenosine, which promotes angiogenesis, proliferation, inflammation and protection against oxidative stress (Hasko & Cronstein, 2004). We have previously established that there is a strong positive association between the RAS and adenosine in first trimester placental tissue (Kurlak *et al.*, 2015) when oxygen tensions are low (Rodesch *et al.*, 1992; Jauniaux *et al.*, 2001). The stimulation of adenosine is a response to hypoxia (Poth *et al.*, 2013) and therefore unsurprisingly placental adenosine receptor $A_{2A}R$ strongly correlates with HIF-1α (Kurlak *et al.*, 2015). Is the RAS acting as a signal transduction regulator for both oxygen sensing and angiogenesis? In other cell systems, such as human fibroblasts, co-operativity between hypoxia- and angiotensin-induced proliferative responses has already been observed (Krick *et al.*, 2005), but a relationship between the RAS and the low oxygen environment in the placenta has not yet been investigated.

The RAS has also been well researched in the context of hypertensive disorders of pregnancy such as pre-eclampsia (PE) (Symonds *et al.*, 1975; Irani & Xia, 2011) which is defined as *de novo* hypertension after 20 weeks gestation with significant proteinuria (Brown *et al.*, 2001; Tranquilli *et al.*, 2013). It has previously been suggested that in PE, the placental tissue is more hypoxic than in normotensive pregnancy. Since there are no direct measurements of intervillous oxygen tension beyond early second trimester, these assumptions have largely been based on evidence such as upregulation of global gene expression in PE showing similarities to *in vitro* and *in vivo* models of placental hypoxia (Soleymanlou *et al.*, 2005) and increases in hypoxia-inducible factors (HIFs) (Zamudio *et al.*, 2007; Rajakumar *et al.*, 2008) . These assumptions are being questioned in light of up-to-date evidence (Huppertz *et al.*, 2014), where placental metabolism in PE shows that there is no reduction in energy supplies as would be expected in chronic hypoxia (Bloxam *et al.*, 1987). It has been proposed (Hung & Burton, 2006) that the absolute oxygen concentration in the intervillous space may not be critically important, but rather fluctuations in oxygen tension that may lead to tissue oxidative stress as indicated by rapid degeneration of syncytium (Watson *et al.*, 1998). Sharp rises in oxygen tension within the lobule could induce excessive bursts of production of reactive oxygen species (ROS), which include free radicals such as superoxide anions, nitric oxide and hydroxyl radicals (Hung *et al.*, 2001). Bursts of ROS production that overwhelm local antioxidant defences will result in oxidative stress. PE is characterised by both increased circulatory and placental oxidative stress (reviewed in (Goulopoulou & Davidge, 2015). There are several sources within the placental tissue that could generate ROS bursts, including

mitochondria. These have been proposed as a source of the oxidative stress in PE, as demonstrated by swelling and loss of cristae, increased concentrations of malondialdehyde showing greater potential for lipid peroxidative damage (Wang & Walsh, 1998).

The formation of excessive amounts of free radicals and hence failure of mitochondrial reduction-oxidation balance have been tightly linked to dysregulation in the RAS (Vajapey *et al.*, 2014). One of the main peptides generated from AGT substrate is Ang II, which binds with equal affinity to its type 1 (AT1R) and type 2 (AT2R) receptors (de Gasparo *et al.*, 2000). The binding of Ang II to AT1R activates NADPH oxidase (NOX), which leads to increased generation of cytoplasmic ROS. This Ang II-AT1R-NADPH-ROS signal triggers the opening of mitochondrial potassium (K_{ATP}) channels and mitochondrial ROS production in a positive feedback loop (Vajapey *et al.*, 2014). The maintenance of ROS balance means that the production of ROS needs to be matched with ROS scavenging mechanisms: Ang II has been implicated not only in activating ROS but also in decreasing the activity of scavenging enzymes (decreasing glutathione peroxidase, superoxide dismutase and catalase), thereby contributing even further to detrimental levels of ROS (Vajapey *et al.*, 2014).

Our group has already suggested a role for RAS components in the establishment of the placenta by demonstrating temporal changes in the Ang II receptors - AT1R, AT2R, AT4R (the receptor for bioactive Ang IV peptide) through gestation, and a temporal relationship with the pro-oxidant enzymes – NADPH oxidase isoform (NOX4) and xanthine oxidase (XO) (Williams *et al.*, 2010). Furthermore, the placental RAS is altered in PE with angiotensin receptor type 1 (AT1R) being more highly expressed in the placenta from women with PE than from normotensive women (Mistry *et al.*, 2013). AT1R expression negatively correlates with infant birthweight and placental glutathione peroxidase 3 protein (Mistry *et al.*, 2013).

Pregnancy at high altitude offers a unique opportunity to investigate the effects of chronic hypoxia on the placenta and PE has an increased incidence at high altitude being three-fold greater in areas above 2500 m (Moore *et al.*, 2011).

On the basis of these considerations, we therefore hypothesised that (i) the placental RAS would be activated by high altitude hypoxia; ii) that these changes would be magnified in placentae from pre-eclamptic mothers; iii) that oxidative stress induced by hypoxia/reperfusion (H/R) injury might be the activator of the RAS as opposed to hypoxia *per se*.

We tested this by examining (i) placentae from normotensive women who had delivered at high altitudes compared to those who resided at sea-level before and throughout the pregnancy; (ii) placentae from PE pregnancies at altitude compared to women with PE at sea-level. In addition, (iii) we cultured term placental tissue explants *in vitro* under differing conditions of hypoxia and oxidative stress.

Methods

Ethical approval

All samples were collected following provision of informed, written consent conforming to the standards set by the Declaration of Helsinki, and all procedures were approved by the local ethics committee (Hospital Ethics Committee of the Nottingham University hospitals, University of Colorado Health Sciences Center (Aurora, CO, USA) and University College Hospital London (London, UK), and the Cambridge Local Research Ethics Committee).

Subjects, selection criteria and sample collection

Normotensive control and PE placentae were collected from women at both sea-level and high altitude. PE was stringently defined as per the International Society for the Study of Hypertension in Pregnancy guidelines of a systolic blood pressure of 140 mm Hg or more and diastolic pressure (Korotkoff V) of 90 mm Hg or more on 2 occasions after 20 weeks gestation in a previously normotensive woman and proteinuria ≥300 mg/L, ≥500 mg/day or ≥2+ on dipstick analysis of midstream urine if 24-hour collection result was not available (Brown *et al.*, 2001). Exclusion criteria included smoking, renal disease, cardiac disease, diabetes, chronic hypertension, pregnancy-induced hypertension, or any other complications of pregnancy, and known risk factors for these conditions. All placentae were from term deliveries, either by elective Caesarean section or vaginal delivery. The high altitude placentae were collected at St. Vincent's General Hospital (Leadville, CO, USA; 3100 m) from stable, but recently migrant, populations of European descent. The sea-level placentae were collected at the Rosie Hospital (Cambridge, UK) and, Nottingham University Hospitals (Nottingham, UK) and University College Hospital (London, UK). By the very nature of the collections, the subject numbers differed in the various parts of the study and will be specified with the results.

Samples were obtained using a systematic random system by which the placenta was divided into 5 areas (Hung *et al.*, 2001). Two full-thickness samples were taken from each area, washed in phosphate buffered saline (PBS) to remove blood contamination, snap-frozen in liquid nitrogen within 10 min of delivery, and maintained at −80°C until further processed. Samples for immunohistochemistry were immediately submersed in formalin to be fixed for 12–24 h. After being treated with 70% ethanol for 24 h, placental samples were further dissected before being embedded in paraffin wax.

Acute hypoxia experiments

Villous samples were taken midway between the chorionic and basal plates, from the periphery of lobules free of visible infarction, calcification, haematoma or tears. After a brief rinse in cold PBS, samples were placed into ice-cold transport medium (TCS large vessel endothelial cell basal medium (TCS CellWorks, Milton Keynes, UK), containing 2% fetal bovine serum, heparin, hydrocortisone, human epidermal growth factor, human basic fibroblast growth factor, 25 μg/ml gentamicin and 50 ng/ml amphotericin B, 1 mM vitamin C and 1 mM Trolox) that had been equilibrated with 5% O_2/90% N_2/5% CO_2, as described before (Cindrova-Davies *et al.*, 2007).

Following transport to the laboratory on ice, placental samples were further dissected into small pieces (about 5 mm in diameter) in ice-cold culture medium, in a glove box under 10% O_2/85% N_2/5% CO_2. Samples were cultured on individual Costar Netwell (24 mm diameter, 500 μm mesh) supports in 4 ml culture medium per well in 6-well plates. Approximately 6-10 pieces were added to each well, depending on the experimental requirements. Placental explants were incubated in pre-gassed medium under: i) normoxic conditions (controls) (10% O_2/85% N_2/5% CO_2), ii) treatment with 1mM H_2O_2 under normoxia, or iii) conditions of repetitive hypoxia±reoxygenation (HR - repetitive cycles of 0.5% O_2/94.5% N_2/5% CO_2 for 6 hr and 10 % O_2/85% N_2/5% CO_2 for 6 hr) in the ExVivo system (BioSpherix, NY, USA) for 24 hr. The Biospherix incubator was calibrated before each experiment and set to maintain the normoxic cultures under 10%O_2, 5% CO_2 in order to mimic the oxygen concentration within the term placenta (Soothill *et al.*, 1986).

RNA extraction and cDNA synthesis

Total RNA was extracted from a known amount of placental tissue (~100 mg) using QIAzol lysis reagent (Qiagen, Crawley, UK) as previously described (Mistry *et al.*, 2011). RNA concentration and purity were verified spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Labtech, Ringmer, UK); all of the samples had an A260/A280 ratio >1.96. RNA (1 μg) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Crawley, UK) in a Primus96 thermocycler (Peqlab Ltd, Fareham, UK).

Quantitative real time Polymerase Chain Reaction (PCR)

Real-time PCR was carried out using SYBR Green chemistry (2x QuantiFast SYBR Green, Qiagen, Crawley, UK) on a ABI7500 Fast (Life Technologies,UK) using the primers detailed in

Table 1. Melt-curve analysis was performed to confirm the presence of one single product and non-template controls were run to assess contamination. The standard curve method was used to quantify gene expression. Abundance data for the genes of interest were expressed following normalisation using GeNORM (http://medgen.ugent.be/~jvdesomp/genorm/), with 3 stably expressed housekeeping genes, suitable for human placental samples: GAPDH, β-2-microglobulin and β-actin (Murthi *et al.*, 2008; Kurlak *et al.*, 2013) and expressed normalised to copy number.

Immunohistochemical staining of RAS components and markers of hypoxia/oxidative stress

Placental protein expression was analysed by immunohistochemistry. Serial sections of placental tissue were cut (5 μm) in the same orientation from paraffin-embedded tissue blocks (Sledge Microtome, Anglia Scientific, Norwich, UK) and mounted onto Superfrost Plus glass microscope slides (Menzel-Glaser, Braunschweig, Germany). Before use, sections were dewaxed by immersion in xylene followed by rehydration in descending concentrations of alcohol.

Immunohistochemical staining was performed using the Dako Envision staining kits (Dako Ltd Germany). Eight rabbit and 2 mouse antibodies were used for immunostaining of paraffin-embedded placental sections. The optimal dilutions and details of all antibodies used are shown in Table 2. Heat-induced epitope retrieval was achieved by heating in a citrate buffer (pH 6.0) using a microwave oven for 15 minutes (800 W), followed by incubation for 30 minutes in normal goat serum (Sigma-Aldrich, UK) to block nonspecific binding; slides were then incubated with primary antibodies overnight at 4°C. A negative control was performed for each test section by incubation with mouse or rabbit IgG as appropriate. Specificity of antibodies was verified by Western blot analysis with identification of the target protein confirmed by specific bands of correct molecular weight (data not shown). Sections were dehydrated and cleared in ascending concentrations of alcohol and xylene before mounting in DPX (BDH, Poole, United Kingdom).

For analysis of placental sections, digital images of 5 randomly selected, high-power fields (Nikon Eclipse II microscope,×400 magnification) were captured using NIS-Elements F2.20 (Nikon UK Ltd, UK). Protein expression was semi-quantitatively assessed (H-score) by two blinded observers (LOK & HDM), as described previously (Mistry *et al.*, 2013). Between-observer agreement for H-scoring was excellent (kappa 0.97). A visual check was also performed to ensure accurate discrimination of immunolabelled regions.

Statistical analysis

All tests were performed using SPSS for Windows version 22 and GraphPad PRISM, version 6. Summary data are presented as means ± standard deviation (SD) or median and interquartile range (IQR) as appropriate. One-way ANOVA or Kruskal-Wallis tests followed by Gabriel's or Mann-Whitney U *post hoc* tests were used depending on the distribution of the data, after testing using the Kolmogorov-Smirnov test. Correlations between the parameters were tested with a Spearman's Rank test. The null hypothesis was rejected where $P<0.05$. The data from the *in vitro* culture experiments were evaluated using repeated measures analysis.

Results

A. *Ex vivo* data

(i). *Placental RAS in normotensive women at high altitude*

mRNA measurements were available from 9 placentae collected at sea-level (spontaneous vaginal delivery (SVD, n=4); elective Caesarean section (CS, n=5) and 8 at high altitude (CS, n=4; SVD, n=4). For the protein expression study, 16 placentae were collected at sea level n=16, (SVD, n=9; CS, n=7), and 8 at high altitude (SVD, n=4; CS, n=4).

The mRNA expression of all RAS components was not significantly affected by altitude in normotensive women, although variation was higher for all components at high altitude (Fig.1A). In contrast, the protein levels of prorenin ($P = 0.049$), prorenin receptor (PRR; $P = 0.0004$) and both AT1R ($P = 0.006$) and AT2R ($P = 0.002$) were all significantly higher in the high-altitude placentae (Fig. 1B), although AGT was not different ($P > 0.1$). All proteins were confined to the outer layer of the villous syncytiotrophoblast. The ratio between prorenin and its receptor, (PRR/prorenin) also rose significantly ($P = 0.008$): sea level median 0.34 [IQR 0.1-0.6]; high altitude median 0.86 [0.75-0.93] whilst that between the AT1R and AT2R was unchanged ($P > 0.8$): sea level median 0.97 [IQR 0.79-1.4]; high altitude median 0.96 [0.82-1.30]. The mode of delivery did not influence protein expression of any of the RAS components ($P > 0.2$ to > 0.7) and therefore we did not consider this as a confounder in further analyses.

(ii). *Placental RAS in women with PE at high altitude*

mRNA measurements were available from 19 placentae collected at sea-level (n=11) and at high altitude (n=8). Samples analysed for proteins included 6 collected at sea-level and 5 at high altitude.

The mRNA expression was significantly lower in placentae from pre-eclamptic women at high altitude for all RAS components (Fig. 1A), except the *AT1R*. In contrast with samples from normotensive women , the variability in mRNA expression was also markedly less. However, the protein levels of PRR, AGT and AT1R were all increased in PE at altitude (Fig. 1B) although this only reached statistical significance for AGT and AT1R.

Placental HIFs and NOX4 in normotensive and PE pregnancies at high altitude

In the normotensive pregnancies, placental mRNA expression of all HIFs and NOX4 was unaffected by altitude (Fig. 2A). However, the protein level of all the HIFs: HIF-1α (P< 0.001); HIF-1β (P < 0.001); HIF-2α (P = 0.04) as well as NOX4 (P = 0.001) was significantly raised in high altitude pregnancies (Fig. 2B). Immunohistochemical staining localised the proteins to the syncytiotrophoblast and around the fetal vessels of the placental villi. The enzyme XO, was unaffected by altitude (data not shown).

The effect of altitude on the pattern of expression differed in the placentae from women with PE. Whereas there was no effect of altitude in the normotensive women, the placental mRNA expression of *HIF-1α, HIF-1β* and *NOX4* mRNA expression were all lowered at high altitude compared to the sea-level PE women (P < 0.001 for all; Fig. 2A). When comparing placentae from normotensive and PE women at sea-level, *HIF-1α* and *NOX4* mRNA expression was markedly higher in the PE compared to normotensive controls (P = 0.01 and P = 0.001 respectively). At high altitude, *HIF-2α* mRNA expression was lower in PE than the normotensive group (P = 0.003; Fig. 2A).

Placental protein levels were equally affected by altitude in the PE women: HIF-1α (P < 0.01); HIF-1β (P = 0.03); HIF-2α (P = 0.017) as well as NOX4 (P = 0.004) were significantly lower in high altitude pregnancies in women with PE (Fig. 2B). XO protein was not altered (data not shown).

In high-altitude samples, protein levels of HIF-1α (P = 0.001); HIF-1β (P = 0.001); HIF-2α (P = 0.029) and NOX4 (P = 0.019) were significantly lower in women with PE as compared to normotensive women (Fig. 2B).

B. *In vitro* data

(i) In vitro explant culture under experimental oxidative stress conditions

Due to the nature of the explant methodology, sample numbers in each of the treatment groups were 4-6 depending on availability of sufficient quality tissue. Oxidative stress was induced by

subjecting explants to repetitive cycles of hypoxia-reoxygenation for 24 hr, or by treating explants with hydrogen peroxide under normoxia.

Experimental hypoxia/reperfusion treatment significantly increased the mRNA expression of only *AGT* ($P = 0.04$) compared to normoxic conditions. Protein expression followed the same pattern (AGT, $P = 0.025$; Fig. 3). Treatment with hydrogen peroxide under normoxic oxygen concentrations had no effect on *AGT* mRNA, although both hydrogen peroxide and hypoxia/reperfusion were associated with significantly-increased AGT protein (Fig. 3). No other components of the RAS were significantly altered by any of the treatments.

The HIFs and antioxidant enzymes were also measured in these experiments, to establish whether hypoxia was being sensed within this model. There was no observable effect of these experimental conditions on either mRNA expression or protein of any of the HIFs, NOX4, or XO. Interestingly, we identified strong positive correlations in gene expression between *HIF-1β* and both angiotensin receptors *AT1R* ($r = 0.77$, $P < 0.001$) and *AT2R* ($r = 0.81$, $P < 0.001$), but only *AT2R* also correlated with *HIF-1α* ($r = 0.512$, $P < 0.013$; Fig. 4).

Discussion

Our aim was to investigate the effects of various forms of oxidative stress on the RAS in the human placenta, including exposure to the lower pO_2 of high altitude chronic hypoxia, the elevated tissue and circulatory chronic oxidative stress which characterises PE, and the acute experimental oxidative stress induced *in vitro* by hypoxia/reperfusion or hydrogen peroxide insults. Our results demonstrate, for the first time, that components of the RAS are activated under these conditions. Furthermore, we show that the type of component activated and the degree of activation are dependent on the nature of the insult.

At high altitude, there is a generalised, significant increase in activity of the placental RAS, which, to our knowledge, has not been reported previously. The rise in PRR protein is statistically highly significant. Binding of (pro) renin to this receptor induces a 4-fold increase in its catalytic efficiency in the generation of angiotensin I (Ang I) from AGT (Nguyen *et al.*, 2002). Thus, changes in receptor expression can have a substantial effect on the generation rate of Ang I, and hence Ang II, even without changes in AGT. Furthermore, our results from the *in vitro* hypoxia-reperfusion model show a significant increase in both mRNA and protein expression of AGT, supporting our premise that this element of the renin-angiotensin axis may be instrumental in 'tuning' the regulation of the placental RAS in a redox environment. When oxidised AGT is reacted with renin in the presence of the PRR, there is a 4-fold increase in the generation of Ang I (in addition to the 4-fold increase induced by the binding of prorenin to the receptor), which does not occur if reduced AGT is reacted (Zhou *et al.*, 2010). The hypobaric hypoxia of high altitude might be sufficient to increase the ratio of oxidised: reduced AGT and further stimulate the system. This appears to be corroborated by the rise in AGT observed after an acute hypoxic insult in the experimental model. Our observations suggest that this increase in AGT is not simply a reaction to lower oxygen tension *per se*, but potentially relates to the increased oxidative stress induced by this model (Cindrova-Davies *et al.*, 2007). We could only examine total AGT expression, as it is not yet possible to distinguish the oxidised and reduced forms immunohistochemically; total expression was not different at the two altitudes.

Low concentrations of endogenous ROS are important in the activation of signalling pathways which relate to adaptations to hypoxia, such as genes involved in angiogenesis (e.g. *VEGF*), where the promoter region has a binding site for hydrogen peroxide (Oshikawa *et al.*, 2010), which is essential for full activation of *VEGF receptor 2* (*VEGFR2*). Accumulating evidence points towards the existence of direct interactions between Ang II and VEGF; a recent study has

demonstrated that AT2R mediates the synergistic effect of a combination of Ang II and VEGF-A to promote differentiation of bone marrow-derived mesenchymal stem cells into endothelial cells (Ikhapoh *et al.*, 2015). These authors suggest that AT2R could be critical to repair and regenerate damaged vascular endothelium. In other cell systems, such as mouse Lewis lung carcinoma cells, hypoxia appears to upregulate ACE, Ang II, and AT1R. Most intriguingly, this regulation is diphasic: in the early phase, after 6 hr hypoxia, AT1R protein is decreased, whilst AT2R protein is increased; yet if hypoxia is continued up to 24 hr this effect is reversed, with AT1R protein significantly increased and AT2R decreased (Fan *et al.*, 2014). Employing specific pharmacological inhibitors, these authors concluded that this diphasic response may be due to a bi-directional control mechanism: hypoxia enhances the expression of AT1R and AT2R via increased Ang II, but elevated AT2R inhibits AT1R during the early stage whilst AT1R inhibits AT2R expression as hypoxia continues. This may explain why neither the AT1R nor the AT2R were significantly altered in our hypoxia/reperfusion model. Similarly, Cindrova-Davies *et al* reported increased HIF-1α protein in placental explants when challenged with hypoxia/reperfusion for only 7h (Cindrova-Davies, 2009) but these changes were no longer evident after a longer, 24h period.

HIF-1β knockout mice die in midgestation having reduced levels of VEGF protein and increased numbers of apoptotic hematopoietic cells (Ramirez-Bergeron *et al.*, 2006) : HIF coordinates early endothelial cell emergence and vessel development by promoting hematopoietic cell survival and paracrine growth factor production. Thus the strong association, observed in this study (Fig.4), between the angiotensin receptors and oxygen-sensing proteins, HIF-1β and HIF-1α, adds credence to the idea that these receptors are involved in the oxygen sensing mechanism of the syncytiotrophoblast. As expression of AT2R is highest early in pregnancy, the association we have found with both HIF-1 α and HIF-1β oxygen-sensing units, supports a possible role for RAS in normal placental development.

AT2R may also have a protective role in the repair of oxygenation-induced damage (Namsolleck *et al.*, 2014), as seen in ischaemic stroke (McCarthy *et al.*, 2009), myocardial ischaemic injury (Parlakpinar *et al.*, 2011; Park *et al.*, 2013) and neurological pathologies (Sumners *et al.*, 2013).

By the same token, prorenin has been shown to stimulate proliferation of retinal endothelial cells to an equivalent degree as that induced by VEGF, and independently of Ang II (Zhu *et al.*, 2015). In pancreatic cancer cells, Ang II induces VEGF, through AT1R and ERK1/2 signalling (Anandanadesan *et al.*, 2008). Studies in AGT-knockout mice report blunted hypoxia-induced

endothelial progenitor cell function, indicating that AGT is required for hypoxia-induced vasculogenesis (Choi *et al.*, 2014).

Since hypoxia stimulates the release of ROS, we postulated that the low oxygen partial pressure at altitude (hypobaric hypoxia) would amplify the oxidative stress of PE through increased production of ROS, and thus augment the stimulation of the RAS already observed in PE at sea-level. However, this stimulation did not occur. Whereas mRNA expression of the RAS was unaffected in normotensive women at high altitude (Fig. 1A), it was reduced in PE placentae at high altitude (Fig. 1A). However, translation followed a very different pattern, showing increased translation (protein expression) in a majority of components of the RAS, in both normotensive and PE women at high altitude.

Concurrent analysis of the mRNA expression of the HIFs (Fig. 2) revealed that there were no detectable differences between sea-level and high altitude placentae in the normotensive women; this agrees with previously published data (Tissot van Patot *et al.*, 2004). In contrast, the increases in HIF-1α, and NOX4 observed in PE compared to normotensive women at sea-level were not observed in PE at altitude; indeed, the mRNAs encoding HIF-1α, HIF-1β and NOX4 were significantly lower in placentas from PE women at high altitude. Our protein data (Fig. 2) expand the study of Zamudio et al (Zamudio *et al.*, 2007), which reported increases in the HIF-1α protein expression at high altitude in normotensive women. However, the observation that placental responses to oxygen deprivation are completely blunted at high altitude in women with PE, as demonstrated by the lower mRNA and protein parameters of all the HIFs, is completely novel. Despite the low sample numbers, these observed differences are striking. This certainly begs the question – do pregnant women who develop PE at altitude have an adaptive response to hypoxia that differs to that of normotensive women? In healthy pregnancies at altitude, placental HIF activity is reduced as seen by lower HIF-1/DNA binding despite unchanged HIF protein levels (Tissot van Patot *et al.*, 2004); placentae at high altitude display little evidence of oxidative stress following labour normally seen in laboured placentae at low altitude (Tissot van Patot *et al.*, 2010). Rajakumar et al. (Rajakumar *et al.*, 2008) suggest that impairment of protein degradation, rather than increased synthesis causes inadequate oxygen-dependent reduction of HIF-1α protein in PE and is supported by the fact that, in early-onset PE placentae at least, there is decreased expression of the prolyl hydroxylases, PHD1 and PHD2 (Rolfo *et al.*, 2010). Hence, molecular events leading to the formation of the HIF-1α:VHL-ubiquitin ligase complex (Ivan *et al.*, 2001), as well as proteasomal trypsin, chymotrypsin, and peptidyl glutamyl-like activities may be the focus of adaptation. In well-oxygenated cells, PHDs hydroxylate the HIF-1α

subunits, thereby targeting them for proteosomal degradation and resulting in a half-life of less than 5 minutes (Wang *et al.*, 1995). PHDs are the key enzymes triggering HIF-1 α desensitization, a feedback mechanism required to protect cells against necrotic cell death and thus to adapt them to chronic hypoxia (Ginouves *et al.*, 2008). Whereas humans living at low altitudes ordinarily maintain low levels of HIF-1α and HIF-2α through constitutive hydroxylation and degradation, upon ascent to high altitude attenuated PHD2 hydroxylase activity due to hypoxia leads to increased levels of HIF-1α and HIF-2α. Even so, the Tibetan population shows the strongest support for the hypothesis that multiple genes in the HIF pathway can be reconfigured in response to chronic hypoxia (Bigham & Lee, 2014); (Lorenzo *et al.*, 2014): the most convincing candidates for this reconfiguration being PHD2 and HIF-2α. Tibetans are the first humans in whom a hypo-responsive HIF system has been demonstrated, and may represent an evolutionary resetting of the HIF system to operate within a hypoxic environment (Petousi *et al.*, 2014). However, this may differentiate populations long-acclimatised to life at high altitude from those relatively-recently arrived, such as the inhabitants of Colorado included in this study.

As noted in the Introduction, deficient spiral artery remodelling may lead to a hypoxia/reperfusion insult. In order to explore the possibility that oxidative stress and not hypoxia *per se,* is the driving force behind the activation of the RAS at high altitude and in PE, we used the fully-validated experimental model of hypoxia/reperfusion. In this model, increased oxidative stress is demonstrated by the increase in heat shock proteins Hsp27 and Hsp90, lipid peroxidation and formation of peroxynitrite (Cindrova-Davies *et al.*, 2007) localised mainly to the syncytiotrophoblast. Under these acute conditions of oxidative stress, our study reveals a more specific stimulation of the RAS, as seen by increased mRNA and protein expression of AGT only (Fig. 3). The fact that other RAS components were not increased by the acute oxidative stress of these experimental conditions implies that their generalised stimulation at high altitude may be a response to the chronically-reduced oxygen tensions. This is supported by the fact that in a mouse model of alveolar hypoxia, such as may occur in pulmonary diseases (e.g. chronic obstructive pulmonary disease), the RAS is stimulated by the low pO_2 (Gonzalez & Wood, 2010) and it is the activation of NOX by the RAS that leads to an inflammatory response. However, if the animals remain under hypoxic conditions for several days, the inflammation resolves and exposure to lower pO_2 does not elicit further inflammation, suggesting that the vascular endothelium has "acclimatised" to hypoxia (Gonzalez & Wood, 2010). Furthermore, in human lung fibroblast cells,

hypoxia directly activates the RAS to induce collagen type I expression, mediated by NF-κB signalling, which in turn activates AT1R expression (Liu *et al.*, 2013).

Previously, using the different methodology of Western blotting to measure protein in term villi, and a shorter time period of 7 hr, hypoxia/reperfusion was found to stimulate HIF-1 α in this model (Cindrova-Davies, 2009). Using the semi-quantitative method of immunohistochemical scoring and a longer time period of 24hr, we did not detect this stimulation. Likewise, use of hydrogen peroxide to mimic the effect of oxygen in acutely lowering HIF-1 α, over the longer 24hr period, did not have the same effect as previously.

Hypoxia cannot be merely defined by just the prevailing oxygen tension (Cindrova-Davies *et al.*, 2015), but must be related to the metabolic demands of the tissue. As Barcroft himself said "oxygen –is- but one of the proximate principles of nourishment" (Young, 1992). The placenta is an extremely metabolically active tissue (Carter, 2000; Illsley *et al.*, 2010). Oxygen sensing is of fundamental importance to all cells as oxygen consumption is required for ATP generation (Guzy & Schumacker, 2006). In tissue such as the placenta, which is exposed not only to altered oxygen concentrations and hypoxia/reperfusion insults during development, but also within its own lobular structures, oxygen sensing is critical in order to stimulate gene expression and transcription of genes relating to growth processes, including angiogenesis. An ability to control oxygen sensing mechanisms using molecules which do not disrupt oxygen consumption would have a profound impact on a diverse range of pathologies. We have here described such a potential system.

Competing interests: No conflict of interest for any authors.

Acknowledgements

We thank Dr. Martha Tissot van Patot for supplying high altitude samples from Colorado

and Mr.Yosef Mansour for technical assistance with qPCR .

Author contributions: Conception and design of the experiments (FBP & GJB); Collection, assembly, analysis and interpretation of data (HDM, LOK & TC-D); Drafting the article or revising it critically for important intellectual content (LOK, HDM, TC-D, FBP & GJB)

Sources of Funding: HDM is supported by an ERA-EDTA Fellowship (ERA LTF 137-2013).

References

Anandanadesan R, Gong Q, Chipitsyna G, Witkiewicz A, Yeo CJ & Arafat HA. (2008). Angiotensin II induces vascular endothelial growth factor in pancreatic cancer cells through an angiotensin II type 1 receptor and ERK1/2 signaling. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract* **12,** 57-66.

Barcroft J. (1946). *Researches in Pre-natal Life*. Blackwell, Oxford.

Bigham AW & Lee FS. (2014). Human high-altitude adaptation: forward genetics meets the HIF pathway. *Genes & development* **28,** 2189-2204.

Bloxam DL, Bullen BE, Walters BN & Lao TT. (1987). Placental glycolysis and energy metabolism in preeclampsia. *Am J Obstet Gynecol* **157,** 97-101.

Brown MA, Lindheimer MD, de Swiet M, Van Assche A & Moutquin JM. (2001). The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* **20,** IX-XIV.

Carter AM. (2000). Placental oxygen consumption. Part I: in vivo studies--a review. *Placenta* **21 Suppl A,** S31-37.

Cervenka L, Bibova J, Huskova Z, Vanourkova Z, Kramer HJ, Herget J, Jichova S, Sadowski J & Hampl V. (2015). Combined suppression of the intrarenal and circulating vasoconstrictor renin-ACE-ANG II axis and augmentation of the vasodilator ACE2-ANG 1-7-Mas axis attenuates the systemic hypertension in Ren-2 transgenic rats exposed to chronic hypoxia. *Physiological research / Academia Scientiarum Bohemoslovaca* **64,** 11-24.

Choi JH, Nguyen MP, Lee D, Oh GT & Lee YM. (2014). Hypoxia-induced endothelial progenitor cell function is blunted in angiotensinogen knockout mice. *Molecules and cells* **37,** 487-496.

Cindrova-Davies T. (2009). Gabor Than Award Lecture 2008: pre-eclampsia - from placental oxidative stress to maternal endothelial dysfunction. *Placenta* **30 Suppl A,** S55-65.

Cindrova-Davies T, Spasic-Boskovic O, Jauniaux E, Charnock-Jones DS & Burton GJ. (2007). Nuclear Factor-κB, p38, and Stress-Activated Protein Kinase Mitogen-Activated Protein Kinase Signaling Pathways Regulate Proinflammatory Cytokines and Apoptosis in Human Placental Explants in Response to Oxidative Stress: Effects of Antioxidant Vitamins. *The American journal of pathology* **170,** 1511-1520.

Cindrova-Davies T, van Patot MT, Gardner L, Jauniaux E, Burton GJ & Charnock-Jones DS. (2015). Energy status and HIF signalling in chorionic villi show no evidence of hypoxic stress during human early placental development. *Mol Hum Reprod* **21,** 296-308.

de Gasparo M, Catt KJ, Inagami T, Wright JW & Unger T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacological reviews* **52,** 415-472.

Fan L, Feng Y, Wan HY, Ni L, Qian YR, Guo Y, Xiang Y & Li QY. (2014). Hypoxia induces dysregulation of local renin-angiotensin system in mouse Lewis lung carcinoma cells. *Genetics and molecular research : GMR* **13,** 10562-10573.

Farag E, Maheshwari K, Morgan J, Sakr Esa WA & Doyle DJ. (2015). An update of the role of renin angiotensin in cardiovascular homeostasis. *Anesthesia and analgesia* **120,** 275-292.

Ginouves A, Ilc K, Macias N, Pouyssegur J & Berra E. (2008). PHDs overactivation during chronic hypoxia "desensitizes" HIFalpha and protects cells from necrosis. *Proc Natl Acad Sci U S A* **105,** 4745-4750.

Gonzalez NC & Wood JG. (2010). Alveolar hypoxia-induced systemic inflammation: what low PO(2) does and does not do. *Adv Exp Med Biol* **662,** 27-32.

Goulopoulou S & Davidge ST. (2015). Molecular mechanisms of maternal vascular dysfunction in preeclampsia. *Trends in Molecular Medicine* **21,** 88-97.

Guzy RD & Schumacker PT. (2006). Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol* **91,** 807-819.

Hasko G & Cronstein BN. (2004). Adenosine: an endogenous regulator of innate immunity. *Trends in immunology* **25,** 33-39.

Hung TH & Burton GJ. (2006). Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia. *Taiwan J Obstet Gynecol* **45,** 189-200.

Hung TH, Skepper JN & Burton GJ. (2001). In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. *The American journal of pathology* **159,** 1031-1043.

Huppertz B, Weiss G & Moser G. (2014). Trophoblast invasion and oxygenation of the placenta: measurements versus presumptions. *J Reprod Immunol* **101-102,** 74-79.

Ikhapoh IA, Pelham CJ & Agrawal DK. (2015). Synergistic effect of angiotensin II on vascular endothelial growth factor-A-mediated differentiation of bone marrow-derived mesenchymal stem cells into endothelial cells. *Stem Cell Res Ther* **6**.

Illsley NP, Caniggia I & Zamudio S. (2010). Placental metabolic reprogramming: do changes in the mix of energy-generating substrates modulate fetal growth? *Int J Dev Biol* **54,** 409-419.

Irani RA & Xia Y. (2011). Renin angiotensin signaling in normal pregnancy and preeclampsia. *Semin Nephrol* **31,** 47-58.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS & Kaelin Jr. WG. (2001). HIFα Targeted for VHL-Mediated Destruction by Proline Hydroxylation: Implications for O2 Sensing. *Science* **292,** 464-468.

Jauniaux E, Watson A & Burton G. (2001). Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. *Am J Obstet Gynecol* **184,** 998-1003.

Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN & Burton GJ. (2000). Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *The American journal of pathology* **157,** 2111-2122.

Krick S, Hanze J, Eul B, Savai R, Seay U, Grimminger F, Lohmeyer J, Klepetko W, Seeger W & Rose F. (2005). Hypoxia-driven proliferation of human pulmonary artery fibroblasts:

cross-talk between HIF-1alpha and an autocrine angiotensin system. *FASEB J* **19,** 857-859.

Kurlak LO, Mistry HD, Kaptein E, Visser TJ & Broughton Pipkin F. (2013). Thyroid hormones and their placental deiodination in normal and pre-eclamptic pregnancy. *Placenta* **34,** 395-400.

Kurlak LO, Williams PJ, Bulmer JN, Pipkin FB & Mistry HD. (2015). Placental expression of adenosine A2A receptor and hypoxia inducible factor-1 alpha in early pregnancy, term and pre-eclamptic pregnancies; interactions with placental renin-angiotensin system. *Placenta*.

Liu SS, Wang HY, Tang JM & Zhou XM. (2013). Hypoxia-induced collagen synthesis of human lung fibroblasts by activating the angiotensin system. *International journal of molecular sciences* **14,** 24029-24045.

Lorenzo FR, Huff C, Myllymaki M, Olenchock B, Swierczek S, Tashi T, Gordeuk V, Wuren T, Ri-Li G, McClain DA, Khan TM, Koul PA, Guchhait P, Salama ME, Xing J, Semenza GL, Liberzon E, Wilson A, Simonson TS, Jorde LB, Kaelin WG, Jr., Koivunen P & Prchal JT. (2014). A genetic mechanism for Tibetan high-altitude adaptation. *Nature genetics* **46,** 951-956.

Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, Zakar T, Morris BJ & Lumbers ER. (2011). Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section. *Placenta* **32,** 214-221.

McCarthy CA, Vinh A, Callaway JK & Widdop RE. (2009). Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke. *Stroke; a journal of cerebral circulation* **40,** 1482-1489.

Mistry HD, Kurlak LO & Broughton Pipkin F. (2013). The placental renin-angiotensin system and oxidative stress in pre-eclampsia. *Placenta* **34,** 182-186.

Mistry HD, McCallum LA, Kurlak LO, Greenwood IA, Broughton Pipkin F & Tribe RM. (2011). Novel expression and regulation of voltage-dependent potassium channels in placentas from women with preeclampsia. *Hypertension* **58,** 497-504.

Moore LG, Charles SM & Julian CG. (2011). Humans at high altitude: hypoxia and fetal growth. *Respiratory physiology & neurobiology* **178,** 181-190.

Murthi P, Fitzpatrick E, Borg AJ, Donath S, Brennecke SP & Kalionis B. (2008). GAPDH, 18S rRNA and YWHAZ are suitable endogenous reference genes for relative gene expression studies in placental tissues from human idiopathic fetal growth restriction. *Placenta* **29,** 798-801.

Namsolleck P, Recarti C, Foulquier S, Steckelings UM & Unger T. (2014). AT(2) receptor and tissue injury: therapeutic implications. *Current hypertension reports* **16,** 416.

Nguyen Dinh Cat A & Touyz RM. (2011). A new look at the renin–angiotensin system—Focusing on the vascular system. *Peptides* **32,** 2141-2150.

Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T & Sraer JD. (2002). Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *The Journal of clinical investigation* **109,** 1417-1427.

Oshikawa J, Urao N, Kim HW, Kaplan N, Razvi M, McKinney R, Poole LB, Fukai T & Ushio-Fukai M. (2010). Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PloS one* **5,** e10189.

Park BM, Gao S, Cha SA, Park BH & Kim SH. (2013). Cardioprotective effects of angiotensin III against ischemic injury via the AT2 receptor and KATP channels. *Physiological reports* **1,** e00151.

Parlakpinar H, Ozer MK & Acet A. (2011). Effects of captopril and angiotensin II receptor blockers (AT1, AT2) on myocardial ischemia-reperfusion induced infarct size. *Cytokine* **56,** 688-694.

Petousi N, Croft QP, Cavalleri GL, Cheng HY, Formenti F, Ishida K, Lunn D, McCormack M, Shianna KV, Talbot NP, Ratcliffe PJ & Robbins PA. (2014). Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *Journal of applied physiology (Bethesda, Md : 1985)* **116,** 893-904.

Pijacka W, Hunter MG, Broughton Pipkin F & Luck MR. (2012). Expression of renin-angiotensin system components in the early bovine embryo. *Endocrine connections* **1,** 22-30.

Poth JM, Brodsky K, Ehrentraut H, Grenz A & Eltzschig HK. (2013). Transcriptional control of adenosine signaling by hypoxia-inducible transcription factors during ischemic or inflammatory disease. *Journal of molecular medicine* **91,** 183-193.

Pringle KG, Tadros MA, Callister RJ & Lumbers ER. (2011). The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis? *Placenta* **32,** 956-962.

Rajakumar A, Michael HM, Daftary A, Jeyabalan A, Gilmour C & Conrad KP. (2008). Proteasomal activity in placentas from women with preeclampsia and intrauterine growth restriction: implications for expression of HIF-alpha proteins. *Placenta* **29,** 290-299.

Ramirez-Bergeron DL, Runge A, Adelman DM, Gohil M & Simon MC. (2006). HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. *Dev Cell* **11,** 81-92.

Rodesch F, Simon P, Donner C & Jauniaux E. (1992). Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* **80,** 283-285.

Rolfo A, Many A, Racano A, Tal R, Tagliaferro A, Ietta F, Wang J, Post M & Caniggia I. (2010). Abnormalities in oxygen sensing define early and late onset preeclampsia as distinct pathologies. *PloS one* **5,** e13288.

Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S, Post M & Caniggia I. (2005). Molecular evidence of placental hypoxia in preeclampsia. *J Clin Endocrinol Metab* **90,** 4299-4308.

Soothill PW, Nicolaides KH, Rodeck CH & Campbell S. (1986). Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy. *Fetal therapy* **1,** 168-175.

Sumners C, Horiuchi M, Widdop RE, McCarthy C, Unger T & Steckelings UM. (2013). Protective arms of the renin-angiotensin-system in neurological disease. *Clinical and experimental pharmacology & physiology* **40,** 580-588.

Sun NL, Wang LY, Xi Y & Hu QZ. (2013). Effect of renin-angiotensin system on arterial function in persons with acute mountain sickness. *International journal of cardiology* **167,** 1641-1642.

Symonds EM, Broughton Pipkin F & Craven DJ. (1975). Changes in the renin-angiotensin system in primigravidae with hypertensive disease of pregnancy. *Br J Obstet Gynaecol* **82,** 643-650.

Tebbs C, Pratten MK & Broughton Pipkin F. (1999). Angiotensin II is a growth factor in the peri-implantation rat embryo. *J Anat* **195 (Pt 1),** 75-86.

Tissot van Patot MC, Bendrick-Peart J, Beckey VE, Serkova N & Zwerdlinger L. (2004). Greater vascularity, lowered HIF-1/DNA binding, and elevated GSH as markers of adaptation to in vivo chronic hypoxia. *American journal of physiology Lung cellular and molecular physiology* **287,** L525-532.

Tissot van Patot MC, Murray AJ, Beckey V, Cindrova-Davies T, Johns J, Zwerdlinger L, Jauniaux E, Burton GJ & Serkova NJ. (2010). Human placental metabolic adaptation to chronic hypoxia, high altitude: hypoxic preconditioning. *Am J Physiol Regul Integr Comp Physiol* **298,** R166-172.

Tranquilli AL, Brown MA, Zeeman GG, Dekker G & Sibai BM. (2013). The definition of severe and early-onset preeclampsia. Statements from the Interantion Society for the Study of Hypertension in Pregnancy (ISSHP). *Pregnancy Hypertension: An Interantional Journal of Women's Cardiovascular Health* **3,** 44-47.

Vajapey R, Rini D, Walston J & Abadir P. (2014). The impact of age-related dysregulation of the angiotensin system on mitochondrial redox balance. *Front Physiol* **5,** 439.

Wang GL, Jiang BH, Rue EA & Semenza GL. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proceedings of the National Academy of Sciences* **92,** 5510-5514.

Wang Y & Walsh SW. (1998). Placental mitochondria as a source of oxidative stress in pre-eclampsia. *Placenta* **19,** 581-586.

Watson AL, Skepper JN, Jauniaux E & Burton GJ. (1998). Susceptibility of human placental syncytiotrophoblastic mitochondria to oxygen-mediated damage in relation to gestational age. *J Clin Endocrinol Metab* **83,** 1697-1705.

Williams PJ, Mistry HD, Innes BA, Bulmer JN & Broughton Pipkin F. (2010). Expression of AT1R, AT2R and AT4R and their roles in extravillous trophoblast invasion in the human. *Placenta* **31,** 448-455.

Young M. (1992). Classics revisited: researches on pre-natal life by Sir Joseph Barcroft. *Placenta* **13,** 607-612.

Zamudio S, Wu Y, Ietta F, Rolfo A, Cross A, Wheeler T, Post M, Illsley NP & Caniggia I. (2007). Human placental hypoxia-inducible factor-1alpha expression correlates with clinical outcomes in chronic hypoxia in vivo. *The American journal of pathology* **170,** 2171-2179.

Zhou A, Carrell RW, Murphy MP, Wei Z, Yan Y, Stanley PL, Stein PE, Broughton Pipkin F & Read RJ. (2010). A redox switch in angiotensinogen modulates angiotensin release. *Nature* **468,** 108-111.

Zhu T, Miller AG, Deliyanti D, Berka DR, Agrotis A, Campbell DJ & Wilkinson-Berka JL. (2015). Prorenin stimulates a pro-angiogenic and pro-inflammatory response in retinal endothelial cells and an m1 phenotype in retinal microglia. *Clinical and experimental pharmacology & physiology*.

Figure Legends

Figure 1: A) Normalised mRNA expression (copy number) of all RAS components in placentae from: normotensive controls residing and delivering at sea level (n = 9) or at high altitude (n = 8); pre-eclamptic women residing and delivering at sea level (n = 11) or at high altitude (n = 8).
B) Placental protein expression and localisation of RAS components from: normotensive controls residing and delivering at sea level (n = 16) or at high altitude (n = 8); pre-eclamptic women residing and delivering at sea level (n = 6) or at high altitude (n = 5).
Boxplots represent median [interquartile range]; **P*<0.05; ***P*<0.001; ****P*<0.0001. Positive staining (brown) was localised mainly in the syncytiotrophoblast (black arrows). All photomicrographs taken at x400 magnification, scale bars=100μm. PRR: prorenin receptor; AGT: angiotensinogen; AT1R/2R: angiotensin receptors type 1 and 2.

Figure 2: A) Normalised mRNA expression (copy number) of all hypoxia-inducible factor (HIF) components and NADPH oxidase (NOX4) in placentae from normotensive controls (NT) and pre-eclampsia (PE) residing and delivering at sea level or at high altitude. B) Placental protein expression and localisation of HIF components and NOX4 from NT and PE women residing and delivering at sea level or at high altitude. Boxplots represent median [interquartile range]; **P*<0.05; ***P*<0.001; ****P*<0.0001. Positive staining (brown) was localised mainly in the syncytiotrophoblast (black arrows), with some staining around fetal vessels (red arrows). All photomicrographs taken at x400 magnification, scale bars=100μm.

Figure 3: Placental angiotensinogen A) mRNA and B) protein expression of term placental explants (n = 4-6) following incubation in normoxic conditions; following hydrogen peroxide (H_2O_2); after hypoxia/reperfusion (H/R). Data analysed using repeated measures analysis and are represented at mean ± standard deviations; ***P*<0.001. Positive staining (brown) was localised mainly in the syncytiotrophoblast (black arrows). All photomicrographs taken at x400 magnification, scale bars=100μm. AGT: angiotensinogen.

Figure 4: Scatter plots demonstrating associations in placental mRNA expression of AT1R, AT2R, HIF-1β and HIF-α. HIF-1β strongly correlated with both AT1R (r=0.77; *P*<0.001) and AT2R (r=0.81; *P*<0.001) under all oxygenation conditions. AT2R also correlated with HIF-1α (r=0.512; *P*<0.01).

Table 1. Details of primers used in qPCR.

Gene	Accession number	Primers	Length (bp)
PRR	NM	5’ cctcattaggaagacaaggactatcc 3’ 5’ gggttcttcgcttgttttgc 3’	50
Prorenin	NM	5’ ccacctcctccgtgatcct 3’ 5’ gcggatagtactgggtgtccat 3’	46
AGT	NM_000029	5` ccccagtctgagatggctc 3` 5` gacgaggtggaaggggtgta 3`	117
AT1R	NM_004835	5’ ggctattgttcacccatgaagt 3’ 5’ tgggactcataatggaaagcac 3’	177
AT2R	NM_000686	5’ tatggcctgtttgtcctcattg 3’ 5’ ccattgggcatatttctcaggt 3’	115
HIF1α	NM_ 181054	5’ atccatgtgaccatgaggaaatg 3’ 5’ tcggctagttagggtacacttc 3’	125
HIF1β	NM_ 001197325	5’ tgactcctgttttgaaccagc 3’ 5’ ctgctcacgaagtttatccacat 3’	95
HIF2α	NM_ 001430	5’ ggacttacacaggtggagcta 3’ 5’ tctcacgaatctcctcatggt 3’	79
NOX4	NM_016931.3	5’ tgaactatgaggtcagcctctg 3’ 5’ aagcttgtatggtttccagtcat 3’	107
GAPDH	NM_002046.3	5’ ggaagcttgtcatcaatggaa 3’ 5’ tggactccacgacgtactca 3’	102
B-Actin	NM_001101.2	5’ ccaaccgcgagaagatga 3’ 5’ ccagaggcgtacaggatag 3’	97
B2M	NM_004048.2	5’ cttatgcacgcttaactatcttaacaa 3’ 5’ taggagggctggcaacttag 3’	127

Table 2. Product descriptions and concentrations for immunohistochemistry

	Product name/Description	**Concentration (mg/mL)**
PRR	Anti- ATP6AP2 - *Sigma Prestige*, HPA003156. Rabbit polyclonal	1.8
Prorenin	Anti-prorenin –*Abcam*, ab82450. Mouse monoclonal	0.042
AGT	Anti-AGT – *Sigma Prestige*, HPA0031557. Rabbit polyclonal	0.26
AT1R	Anti-AT1R – *Abcam*, ab9391. Mouse monoclonal	80
AT2R	Anti-AT2R - *Abcam*, ab19134 Rabbit ploYclonal	5.2
HIF1α	Anti- HIF1α – *Sigma Prestige*, HPA0031557 Rabbit polyclonal	0.001
HIF1β	Anti- Arnt – *Aviva Systems Biology*, ARP30979. Rabbit polyclonal	0.001
HIF2α	Anti-EPAS1 - *Aviva Systems Biology*, ARP32253. Rabbit polyclonal	0.005
NOX4	Anti-NOX4 - *Abcam*, ab133303. Rabbit polyclonal	1:500
XDH	Anti-XDH - *Aviva systems biology* OAAB05869. Rabbit polyclonal	0.5µg/ml