The placental renin-angiotensin system and oxidative stress in pre-eclampsia

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Short Tital: Placental RAS in Pre-eclampsia

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Abstract

There is an inverse correlation between human birthweight and umbilical venous angiotensin II (AngII) concentrations. Oxidative stress and increased prorenin receptor (PRR) both enhance the cleavage of angiotensin I from angiotensinogen (AGT). Pre-eclampsia, a hypertensive disorder of pregnancy, manifests as high blood pressure and proteinuria, and is a state of increased oxidative stress.

Objectives, study design and main outcome measures: Hypothesis: Pre-eclampsia will be associated with increased placental expression of components of the renin-angiotensin system, which could result in reduced infant birthweight. Biopsies were taken 1cm from the placental edge from 27 normotensive controls and 23 pre-eclamptic White European women. Immunohistochemistry was performed for AGT, PRR, glutathione peroxidase 3 (GPx3) and the AT1R and AT2R AngII receptors. Protein expression was semi-quantitatively assessed (H-score).

Results: AT1R expression was significantly increased in pre-eclamptic placentae, and negatively correlated with birthweight (r = - 0.529, P = 0.009). AT1R expression was also negatively correlated with GPx3 expression overall (r = -0.647; P = 0.005). AT2R expression positively correlated with AGT (r = 0.615, P = 0.002) in the pre-eclamptic placentae only.

Conclusions: The raised AT1R expression in pre-eclampsia, together with inadequate antioxidant protection, possibly through lower GPx activity, might enhance the vasoconstrictor effect of locally-generated AngII, contributing to the restricted fetal growth characteristic of pre-eclampsia. Conversely, the AT2R:AGT association within the pre-eclamptic placenta may provide a compensatory mechanism.

Keywords: Pre-eclampsia, Renin-angiotensin-system, angiotensin receptors, angiotensinogen, pro-renin receptor, placenta.

Introduction

Pre-eclampsia is a hypertensive disorder of pregnancy that occurs in ~3% of all pregnancies. This disease, which manifests as high blood pressure and proteinuria, is probably mediated by endothelial damage and may affect multiple systems of the body and contribute to adverse pregnancy outcomes including maternal death, preterm birth, intrauterine growth restriction (IUGR) and fetal death [1]. Together with other hypertensive disorders of pregnancy, pre-eclampsia is responsible for at least 60,000 maternal deaths each year [2] and increases perinatal mortality 5-fold [3]. Placental and maternal systemic oxidative stress are components of the syndrome [4, 5] and contribute to a generalised maternal systemic inflammatory activation [6]. Placental ischemia-reperfusion injury has been implicated in excessive production of reactive oxygen species (ROS), which could cause release of placental factors that mediate the inflammatory responses [7]. We have recently shown that placental antioxidant glutathione peroxidise (GPx) activity and expression are reduced in pre-eclampsia [8].

The renin–angiotensin system (RAS) is known to be an important regulator of blood pressure, sodium and fluid homeostasis. In non-pregnant models, enhanced RAS activity causes hypertension [9], salt retention [10], and hyperaldosteronism [11]. Although the exact mechanism of pre-eclampsia remains unclear, it appears that angiotensinogen (AGT) could play critical roles in its development [12]. AGT is the only renin substrate, and is thus a major molecule in the RAS. Prorenin (PR) is the biosynthetic precursor of renin, the structure of which includes a peptide which obstructs the access of AGT to the active site of renin, which binds to its receptor, prorenin receptor (PRR or ATPase H^+ transporting lysosomal accessory protein 2; ATP6AP2) [13]. Oxidative stress and increased PRR expression both enhance the cleavage of angiotensin I (Ang I) from AGT. Hypoxaemia in the chronically-cannulated fetal lamb was associated with a doubling of circulating angiotensin II (AngII)

concentrations [14], and umbilical venous Ang II concentrations are higher in smaller babies [15, 16]. AngII exerts part of its vasoconstrictor effect through the generation of ROS [17].

Ang II exerts a majority of its effects through two major angiotensin receptors: AT1R and AT2R. These highly conserved seven-transmembrane G-protein–coupled receptors share a 34% sequence identity and have comparable affinities for Ang II [18]. Most of the actions of Ang II, including vasoconstriction and stimulation of aldosterone synthesis, angiogenesis and cell growth are mediated by the AT1R [17]. The AT2R is implicated in apoptosis, reduction in endothelial cell growth and migration, and vasodilation in the adult, the reduction in neointima formation after vascular injury [19-23], although it is usually expressed at low density. However, expression is much higher during fetal life, where it may counterbalance the effects of the AT1Rs during fetal development. Endothelial cell growth, migration, angiogenesis and apoptosis allowing spiral artery remodelling are all central to placentation.

In the placenta, an intrinsic angiotensin-generating system has been well documented based on the presence of major RAS components including renin, AGT, angiotensin-converting enzyme (ACE), and Ang II as well as its receptor subtypes [24-26]. The conversion of Ang I to Ang II by ACE in the feto-placental bed leads to a potent vasoconstrictor activity in the feto-placental circulation [27, 28]. Placental trophoblasts are particularly AT1R rich [29]. A functioning placental RAS appears necessary for an uncomplicated pregnancy [30]. There is an inverse correlation between human birthweight and umbilical venous AngII concentrations [31]. An elevated fetal plasma renin activity [32] and fetal Ang II concentration [16] have also been reported in IUGR. However, there is controversy about the expression of placental angiotensin receptors in pre-eclampsia.

These early studies did not examine the placental RAS in detail. A recent paper has reported on placental expression of PRR and AGT in normal term pregnancy [33], but these have not been described in relation to pre-eclampsia. We thus hypothesised that pre-eclampsia, a state of increased oxidative stress, will be associated with increased placental expression of components of the RAS, which could result in reduced infant birthweight.

Methods

Subjects:

The study population consisted of White European women who had either a normotensive or pre-eclamptic pregnancy as previously described [4]. The investigations were approved by the Nottingham Hospital Ethics Committee and written, informed consent was obtained from each subject. Cases were defined on admission with a clinical diagnosis of pre-eclampsia, defined using the International Study for the Study of Hypertension in Pregnancy guidelines [34]. Medical and obstetric histories, including delivery data, were obtained for each woman. The birthweight centile for each baby was computed, correcting for gestation age, sex, maternal parity and body mass index (BMI) [35].

Sample collection:

Full depth placental biopsies were taken 1cm from the placental edge from 27 normotensive and 23 pre-eclamptic women, avoiding placental infarcts. Tissues were taken within 10 min of delivery, membranes removed and tissue washed in ice cold 1× PBS to remove maternal blood contamination. Biopsies were formalin fixed and wax-embedded for immunohistochemical analysis.

Immunohistochemistry:

Immunohistochemical staining was performed on serial sections as previously described [8] using the Dako Envision™ visualization system (Dako, Ely, UK). Table 1 provides further details on antibody dilutions used, which were optimised by performing a dilution series for each antibody. The heat-induced epitope retrieval was achieved by heating in a citrate buffer (pH 6.0) using a microwave oven for 15 min. A negative control was performed for each test section by incubation with mouse or rabbit IgG as appropriate. Sections were dehydrated and

cleaned in ascending concentrations of alcohol and xylene before coverslips were mounted (DPX mountant, BDH). Protein expression was semi-quantitatively assessed (H-score) at x200 magnification (Nikon Eclipse II microscope) by two blinded observers as described previously [36]. Between-observer agreement for H-scoring was excellent (kappa 0.97).

Maternal plasma thiobarbituric acid reactive substances (TBARS) concentrations were measured as a global measure of oxidative stress; plasma total GPx activity and placental GPx3 protein expression were measured as indices of specific antioxidant activity. These data have been previously reported [4].

Statistical analysis

All tests were performed using SPSS for Windows version 19.0. Summary data are presented as median (interquartile range) as appropriate for their distribution after testing for normality using the Kolmogorov-Smirnov test. Mann‑Whitney *U*-tests were used to test differences between normotensive and pre-eclampsia groups. Spearman's Rho correlation and Kendall's Tau ranking tests were also conducted as appropriate. The null hypothesis was rejected where $P < 0.05$.

175 **Results**

176 All women conceived naturally and carried singleton pregnancies. The demographic,
177 obstetric and pregnancy data of the 50 women (27 normotensive, 23 pre-eclampsia) who
178 participated in the study are shown in Table 2. The complete and detailed data have been
179 previously published [4]. In summary, women in the normotensive group gave birth without
180 developing hypertension or proteinuria, to infants weighing >2500 g, delivered at 37 weeks
181 or later. Overall, the pre-eclamptic women all had moderate to severe disease and had lower
182 gestational ages at delivery than the control group ($P < 0.05$; Table 2). No pre-eclamptic
183 woman had Haemolysis Elevated Liver enzymes, and Low Platelet count (HELLP). All
184 neonates from both pregnancy groups survived.

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186 All components of the RAS that were measured in this study were found to be expressed in
187 placental tissue, mainly localised to the villous syncytiotrophoblast, with some stromal
188 staining (Figure 1). Table 3 summarises the staining intensity of AGT, PRR, AT1R, AT2R
189 and GPx3 in placentae from normotensive and pre-eclamptic women. AT1R expression was
190 significantly increased in pre-eclamptic compared to normotensive placentae ($P = 0.032$;
191 Table 3); all other RAS components were similarly expressed in both groups. Placental GPx3
192 expression (Table 3) and maternal plasma total GPx activity [4, 8] were significantly reduced
193 in pre-eclampsia while TBARS were highly significantly increased (1.2 [0.6, 1.6] compared
194 to 0.45 [0.2, 0.8] μmol/l ($P < 0.001$)[4].

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196 A negative association was observed between AT1R and birthweight in the pre-eclamptic
197 group (Figure 2; r = - 0.529, P=0.009) but not in the normotensive group ($P > 0.5$). A
198 significant inverse correlation was also found overall between placental AT1R and GPx3
199 protein expression (Figure 3; r = 0.634; $P = 0.006$). AT2R expression was found to be

positively related to AGT in pre-eclampsia (r = 0.615, $P = 0.002$), but not control, placentae (r = 0.064, $P > 0.05$; Figure 4).

Although staining density was diffuse throughout the villous syncytiotrophoblasts, there was also dense staining localised specifically around fetal vessels in 18 (PRR) and 8 (AGT) of the 50 placenta (Figure 1). The presence of perivascular staining for PRR and AGT was found to be associated with increased maternal TBARS (Chi-squared $P = 0.001$ and $P = 0.033$).

Discussion

It has been known for nearly 40 years [37] that there is a tissue-based RAS in the uteroplacental unit, suggesting a locally acting RAS. Our localised staining of the RAS components to the syncytiotrophoblast is consistent with other very recent results [38]. Our study demonstrates that the placental RAS may be involved in pre-eclampsia, with raised AT1R expression, together with the raised AngII concentrations previously reported [15, 16], contributing to the restricted fetal growth characteristically observed in this syndrome. The pressor response to AngII is well known to be enhanced before pre-eclampsia is clinically detectable [39] and is associated with an increased density of AT1Rs [40]. Agonist autoantibodies to the AT1R are also increased in pre-eclampsia [41].

The inverse correlation between placental AT1R and GPx3 expression (Figure 3) is particularly interesting in light of the stimulatory effect of AngII on ROS generation [17]. The absence of adequate antioxidant protection in pre-eclampsia might enhance the vasoconstrictor effect of locally-generated AngII. The specific clustering of both the PRR and AGT around the vessels, in conjunction with elevated measures of oxidative stress (TBARS), suggests that the RAS may be contributing to the heightened state of oxidative stress in placental tissue of pre-eclamptic pregnancies. ROS superoxide anion (O_2^-) has been implicated in Ang II-mediated hypertension [42] and experimental data in the non-pregnant state support the concept that Ang II-mediated hypertension in pregnancy may also be due, in part, to effects on the oxidative state in vascular-endothelial tissue [43].

There is some controversy surrounding the regulation of placental AT1R in pre-eclampsia. The increase in placental AT1R expression in pre-eclampsia we observed is in agreement with previous studies in placental [44] and decidual [17] tissue. However, two earlier studies

reported that the capacity and affinity of AT1R were significantly lower in placentae from pregnancies complicated by pre-eclampsia and IUGR [45] and in a previous study we also found no significant increase in placental AT1R expression [26]. It is now well established from several studies that gradients in gene expression [46] or enzyme activity [8] exist across the placenta. In our original paper [26], results were reported from biopsies taken within 1cm of the cord insertion. We have subsequently demonstrated an increase in placental ACE activity at the periphery [47], where sensitivity of chorionic plate arteries to Ang II is greatest [48]. In the current investigation, we therefore studied samples collected from 1cm from the periphery of the placenta. This adds further weight to the requirement for structured, rather than random, sampling across the placental disc, with clear reporting of sampling site.

We have, for the first time, identified a clear relationship between placental AT2R and AGT expression in pre-eclamptic, but not normotensive pregnancy (Figure 4). The AT2R appears to function as an 'antagonist' of AT1R [49], and is predominantly expressed in fetal tissue [50]. Ang II binding to AT2Rs increases apoptosis, causes vasodilation and is thought to be involved in fetal tissue development [51, 52]. Thus, the strong positive associations observed in the pre-eclamptic placentae between AT2R and AGT may indicate a potential compensatory mechanism, not activated in normotensive pregnancy.

The human placenta possesses an autonomous RAS, allowing the local generation of Ang II and its fragments. Our data suggest that, at term, increased activity in this local system, particularly evident in the syncytiotrophoblast, may contribute to both pre-eclampsia itself and the IUGR which frequently accompanies it. We have not addressed the question of distinguishing between the effects of placentally-generated AngII and fetal systemic AngII.

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Figure Legends

Figure 1: Immunohistochemical staining of AGT (A1 to A3), PRR (B1 to B3), AT1R (C1 to C3) and AT2R (D1 to D3) at x200 magnification. Positive staining shown in brown, black arrows indicate syncytiotrophoblast cells and red arrows show fetal vessels. ATIR expression was increased in the pre-eclamptic compared to the normotensive control placentae ($P < 0.05$).

Figure 2: Scatter plot illustrating the inverse association between AT1R expression in the placenta, and the birthweight in the pre-eclamptic group (r = -0.529; $P = 0.009$); not observed in the controls (r = -0.117; $P > 0.05$).

Figure 3: Inverse association between the expression of GPx3 and AT1R in the placenta (r = -0.634; $P = 0.006$). Placental GPx3 activity was only measured in 10 pre-eclampsia and 10 normotensive control samples.

Figure 4: Scatter plots showing the positive association between the placental AGT and AT2R expression in pre-eclamptic (r = 0.615; $P = 0.002$) but not normotensive controls (r = 0.064; $P > 0.05$).

460 **Table 1.** Details of antibody sources and dilutions.

Antigen	**Suppler information**	**Concentration (mg/mL)**
PRR	Sigma Prestige, rabbit polyclonal: HPA003156	1.8
AGT	Sigma Prestige, rabbit polyclonal: HPA0031557	0.26
AT1R	Abcam, mouse momoclonal: ab9391	80
AT2R	Abcam, rabbit polyclonal: ab19134	5.2

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Table 2: Obstetric data of subject groups. Data are presented as mean ±SD or median [IQR] as appropriate. *P <0.05, **P < 0.001; more detailed data has been previously published (Mistry et al 2008).

Parameter	**Normotensive control (n = 27)**	**Pre-eclampsia (n = 23)**
Gestational age at delivery (weeks)	40 ± 1.1	36.4 ± 3.8*
Birthweight (kg)	3.55 [3.25, 3.86]	2.92 [1.92, 3.51]*
Birthweight centiles (%)	45 [23, 67]	35 [2, 87]

488 **Table 3:** Placental protein expression (H-Score) of AGT, PRR, AT1R, ATT2R and GPx3. *P
489 < 0.05; #previously published (Mistry et al 2008) and placental GPx3 activity was only
490 measured in 10 pre-eclampsia and 10 normotensive control samples.

Protein expression	**H-Score (Median [interquartile range])**	
	Normotensive control	Pre-eclampsia
AGT	34 [10,95]	30 [9,60]
PRR	117 [16,144]	139 [72,187]
AT1R	40 [10,70]	68 [30,112]*
AT2R	152 [120,180]	184 [74,220]
Placental GPx3 expression (AU) #	197 [124, 254]	110 [82, 124]*

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