PAPPA2 is increased in severe early onset pre-eclampsia and upregulated with hypoxia

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Abstract. Severe early onset pre-eclampsia is a serious pregnancy complication, believed to arise as a result of persistent placental hypoxia due to impaired placentation. Pregnancy-associated plasma protein A2 (*PAPPA2*) is very highly expressed in the placenta relative to all other tissues. There is some evidence that PAPPA2 mRNA and protein are increased in association with pre-eclampsia. The aim of the present study was to characterise the mRNA and protein expression, as well as localisation, of PAPPA2 in an independent cohort of severe early onset pre-eclamptic placentas. We also examined whether exposing placental explants to hypoxia (1% oxygen) changed the expression of PAPPA2. Expression of PAPPA2 mRNA and protein was upregulated in severe early onset pre-eclamptic placentas compared with preterm controls and localised to the syncytiotrophoblast. Interestingly, protein localisation was markedly reduced in term placenta. Syncytialisation of BeWo cells did not change *PAPPA2* expression. However, hypoxia upregulated PAPPA2 mRNA and protein expression in primary placental explants. Together, our data suggest that PAPPA2 may be upregulated in severe pre-eclampsia and, functionally, this may be mediated via increased placental hypoxia known to occur with this pregnancy disorder.

Additional keywords: placenta, pregnancy.

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Introduction

Severe early onset pre-eclampsia (PE) affects 6%–8% of pregnancies and is a leading cause of maternal and perinatal mortality and morbidity (Sibai *et al.* 2005; Powe *et al.* 2011). There are currently no treatments available to halt disease progression, and thus clinicians are often forced to deliver infants regardless of gestation.

During the first trimester of pregnancy, placentation occurs in a tightly controlled series of events, specifically designed to allow adequate blood flow to the fetoplacental unit throughout pregnancy. In PE, it is widely believed that trophoblast invasion is shallow and the spiral arteries are not remodelled adequately to provide the high blood volume required by the developing fetoplacental unit (Redman and Sargent 2005). Failure to establish an adequate blood supply results in chronic and prolonged hypoxia, predisposing the fetus to an increased risk of intrauterine growth restriction and stillbirth (Moffett-King 2002; Redman and Sargent 2005). PE is commonly diagnosed when women present with hypertension and proteinuria, clinical

hallmarks of mounting end-organ damage resulting from widespread maternal endothelial dysfunction, directly attributable to anti-angiogenic factors that are released from the chronically hypoxic placenta (Redman and Sargent 2005).

Pregnancy-associated plasma protein A2 (PAPPA2) is a homologue of the well known first-trimester serum marker of pathological pregnancies, namely PAPPA, sharing 46% sequence identity (Conover *et al.* 2011). Importantly, recent reports demonstrate a link between PAPPA2 and PE (Nishizawa *et al.* 2008; Winn *et al.* 2009; Paiva *et al.* 2011). However, the evidence showing raised PAPPA2 protein in severe preterm PE (responsible for most of the morbidity caused by this disease) is limited to 30 cases described to date (Nishizawa *et al.* 2008; Winn *et al.* 2009). Thus, this association merits further independent validation.

Furthermore, the major mechanism that may potentially differentially regulate PAPPA2 in placenta remains to be robustly described. Given PE placentas are chronically hypoxic, we hypothesised that PAPPA2 would be upregulated in placental

tissues exposed to hypoxia. Although one publication has indeed shown upregulation of *PAPPA2* following hypoxic exposure of BeWo cells, a choriocarcinoma-derived cell line (Wagner *et al.* 2011), it has not been demonstrated in primary placental tissues.

The aim of the present study was to characterise PAPPA2 in an independent cohort of severe early onset PE and gestationally matched preterm control samples at both the mRNA and protein levels. Given that PE is associated with placental hypoxia, we also used functional assays to determine the effects of hypoxia on PAPPA2 mRNA and protein expression in primary placental tissues.

Materials and methods

Tissue collection

Women presenting to two tertiary women's hospitals in Melbourne, Australia, between 2008 and 2012 (Monash Medical Centre and Mercy Hospital for Women) provided written informed consent for placental tissue collection. Placentas were obtained from preterm pregnancies (PT) not complicated by PE and those complicated by severe early onset PE. Severe pre-eclamptics were diagnosed in accordance with American Congress of Obstetricians and Gynecologists (ACOG) guidelines (ACOG 2002) and included the presence of hypertension $>160/110$ mmHg on two occasions greater than 6 h apart, proteinuria >5 g day^{-1}, oliguria <500 mL day^{-1}, visual disturbance, pulmonary oedema, right upper quadrant pain, abnormal liver function, thrombocytopenia or fetal growth restriction. In addition, all samples were obtained from cases of early onset preterm PE, defined as requiring delivery <34 weeks gestation. PT control placentas were selected from women presenting with PT rupture of membranes or spontaneous PT labour without evidence of infection (histopathological examination of the placentas), hypertensive disease or maternal comorbidities.

Placental tissue was obtained immediately following delivery. Placental tissue (excluding fetal membranes) was removed and washed briefly in sterile phosphate-buffered saline (PBS). Samples for protein and RNA extraction were frozen within 15 min of delivery and stored at $-80°C$. A portion of each placenta was also fixed in 10% buffered formalin for histology. Human ethics approval was obtained for this study from both the Southern Health Human Research Ethics Committee and the Mercy Health Human Research Ethics Committee.

Western blot analysis

Placental lysate samples (20 μg) were separated on 10% polyacrylamide gels with wet transfer to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked before blotting overnight with antibodies targeting PAPPA2 (1 : 500; Sigma, St Louis, MA, USA) and GAPDH (1 : 5000; Cell Signaling Technology, Danvers, MA, USA). Membranes were then visualised using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ChemiDoc XRS (BioRad, Hercules, CA, USA). GAPDH was used as a loading control. Relative densitometry was determined using Image Laboratory (BioRad).

Immunohistochemistry

PAPPA2 immunohistochemistry was conducted on placental tissue collected from either PE or PT control pregnancies. Paraffin sections (5 μm) of formalin-fixed tissues were dewaxed in xylene and rehydrated through descending grades of ethanol. Sections were then heated for 20 min on defrost in a 700-W microwave oven, followed by cooling to room temperature for 30 min. Sections were then washed for 10 min in PBS (pH 7.6) and immersed in 3% H_2O_2 in methanol for 10 min at room temperature before being washed with PBS. The sections were then immersed in Dako blocking buffer (DAKO, Campbelfield, Vic., Australia) for 10 min and then incubated for 1 h at 37°C with rabbit anti-human PAPPA2 (Sigma) at 10 μg mL^{-1} in 1% bovine serum albumin (BSA)–PBS. For isotype controls, the primary antibody was substituted with mouse IgG. The SuperPicTure kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions to reveal the PAPPA2 staining. Sections were lightly counterstained with Harris haematoxylin (Accustain; Sigma Diagnostics, Castle Hill, NSW, Australia), dehydrated and mounted using Di-N-Butyle Phthalate in Xylene (DPX) mounting medium (BDH Laboratory Supplies, Poole, UK).

Reverse transcription–polymerase chain reaction

RNA was extracted from 50–100 mg frozen placental samples by homogenisation, followed by use of the RNeasy mini-kit (Invitrogen) according to the manufacturer's instructions. The quantity and quality of the RNA were determined using the Nanodrop 2000 (ThermoScientific, Waltham, MA, USA). Only RNA providing a 260/280 ratio of 1.9–2.10 was used in further experiments.

Reverse transcription of RNA was performed using a Super-Script Vilo cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Sybr polymerase chain reaction (PCR) analyses were performed using primers obtained from PrimerBank (a public database of primers that have been extensively tested for PCR specificity and efficiency; http://pga.mgh.harvard.edu/primerbank/) with primers synthesised using SigmaGenosys (Sigma), as previously described (Paiva *et al.* 2011).

Real-time quantitative PCR was performed on the CFX 384 (Bio-Rad, Hercules, CA, USA) using Fast Sybr Green Master Mix (Applied Biosystems) with the following run conditions: for Sybr PCR, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 30 s, finishing off with 95°C for 15 s, 60°C for 15 s and 95°C for 15 s to obtain a dissociation curve. The specificity of the PCR products obtained using these primers has been confirmed previously (Paiva *et al.* 2011).

All data were normalised against an appropriate housekeeping gene (*GAPDH* or *YWHAZ*) used as an internal control and calibrated against the average cycle threshold (Ct) of the control samples. GAPDH was used as the control for all experiments except in the hypoxia experiments, because it has been suggested previously that GAPDH levels vary with hypoxia (Zhong and Simons 1999). The results are expressed as fold changes relative to control. Samples were run in triplicate. Negative
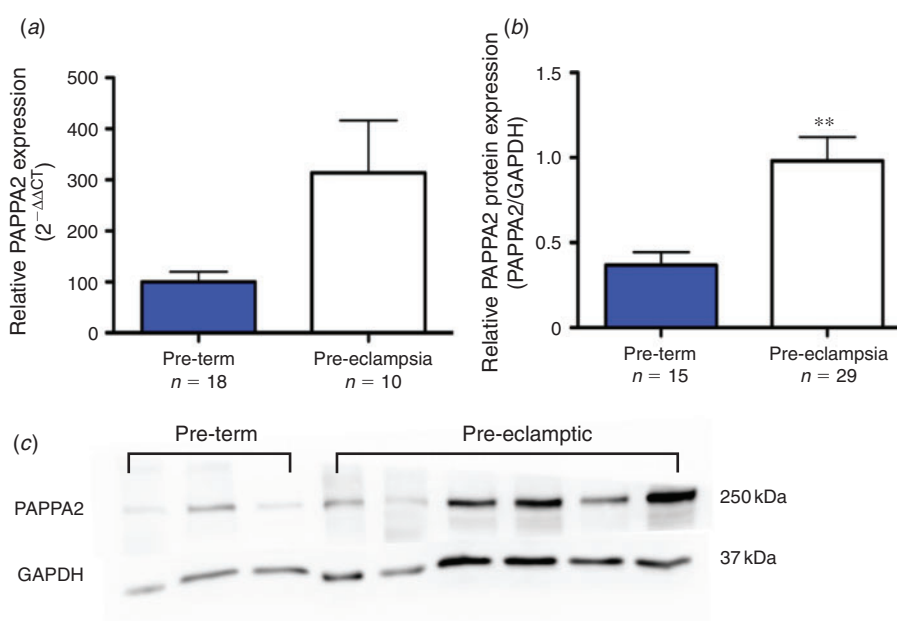
Fig. 1. Pregnancy-associated plasma protein A2 (PAPPA2) mRNA and protein levels are increased in severe early onset pre-eclamptic placentas compared with gestationally matched preterm controls. (*a*) At the mRNA level, PAPPA2 expression was elevated in the pre-eclamptic cohort ($P = 0.06$). (*b*, *c*) Western blot analysis with densitometry indicated a significant ($P < 0.01$) increase in PAPPA2 protein expression. (Representative western blot is shown.) Data are the mean $\pm$ s.e.m. **$P < 0.01$ compared with control.

controls lacking template and containing RNA that was not reverse transcribed were included in all runs.

Syncytialisation of BeWo cells

Cells were plated at 50 000 cells per well of a six-well plate and allowed to plate down for 24 h. Cells to be syncytialised were treated with 20 μM forskolin, whereas control cells received an equivalent volume of vehicle control (dimethylsulfoxide; DMSO). Cells were incubated for 48 h before monolayers were processed for RNA or protein extraction.

Hypoxic culture of term placental explants

Fresh placental specimens were dissected into 5–10 mm cubes and allowed to equilibrate at 37°C for 1 h before being transferred to fresh culture medium and cultured under either hypoxic (1% oxygen, 5% CO_2) or normal (20% oxygen, 5% CO_2) culture conditions for 24 h ($n = 6$ placentas).

Analysis of the PAPPA2 promoter

Promoter analysis of human *PAPPA2* (gene ID 60676) was performed using a 2000-nucleotide region upstream of the *PAPPA2* mRNA (NM_020318.2), together with the first exon of *PAPPA2*, covering a 2248-base region defined by the coordinates chr1 : 176430307–176432554 (hg19 assembly), using MatInspector (Genomatix Software, Munich, Germany). Predictions of hypoxia responsive element (HRE) and nuclear factor (NF)-κB binding sites were confirmed using independent software relying on TRANSFAC (BioBase, Wolfenbuettel, Germany) matrices (Messeguer *et al.* 2002).

Statistical analysis

Continuous variables were compared using either an unpaired *t*-test (parametric data) or a Mann–Whitney *U*-test (nonparametric data). $P \leq 0.05$ was considered significant. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Increased PAPPA2 mRNA and protein expression in severe early onset PE

Expression of *PAPPA2* mRNA was assessed in a cohort of severe early onset PE ($n = 18$) and PT control ($n = 10$) placental samples and was found to be increased in PE placentas (Fig. 1*a*; $P = 0.06$). A larger cohort of samples (PE, $n = 29$; PT, $n = 15$) was assessed by western blot analysis, which verified a significant ($P < 0.01$) increase in PAPPA2 protein production with severe early onset PE (Fig. 1*b*, *c*).

Localisation of PAPPA2 to the syncytiotrophoblast of PE and PT placentas does not change with syncytialisation

Immunohistochemistry was used to investigate the localisation of PAPPA2 in placental tissue (Fig. 2). Strong syncytiotrophoblast staining was observed in both the PT (Fig. 2*a*) and PE (Fig. 2*b*) cohort. Interestingly, little to no staining was observed in term placental sections (Fig. 2*c, d*). Negative controls, in which the primary antibody was substituted with an IgG control, were clean (Fig. 2*e*). Given PAPPA2 was localised to the syncytiotrophoblast, we assessed *PAPPA2* mRNA levels in BeWo
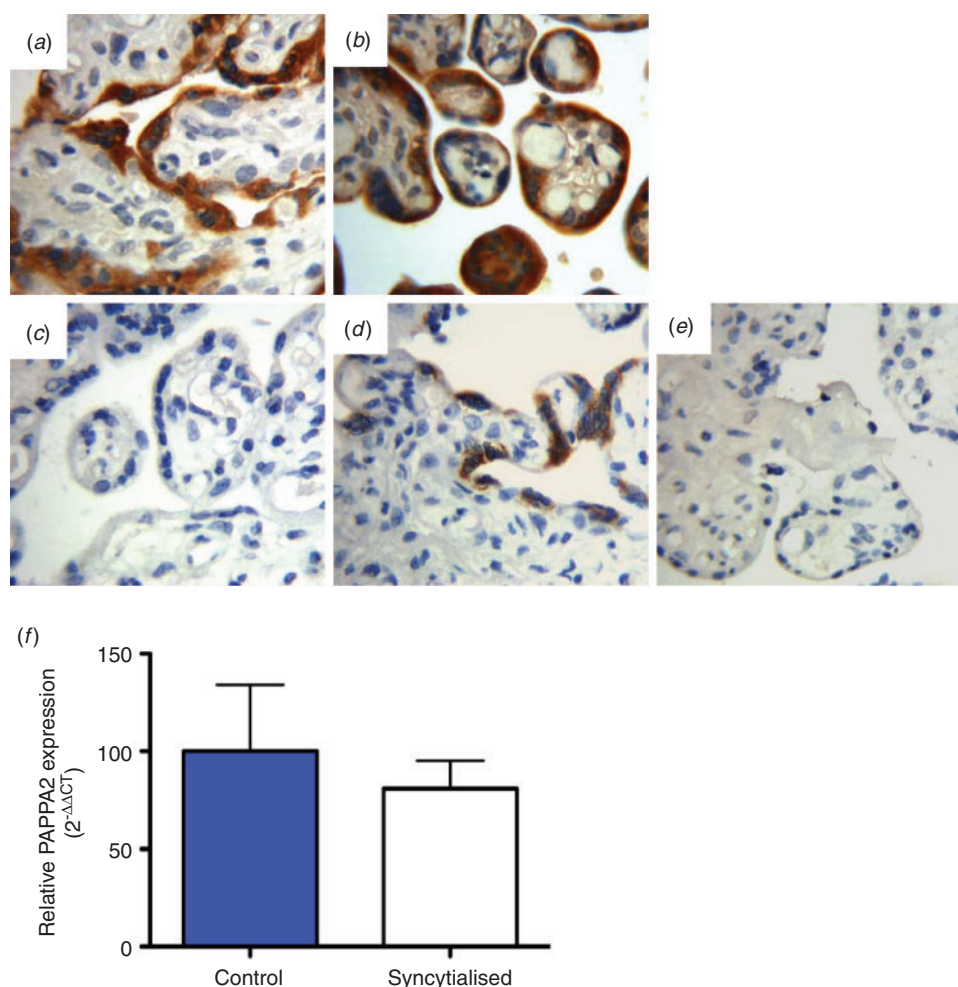
Fig. 2.   Pregnancy-associated plasma protein A2 (PAPPA2) protein is localised to the syncytiotrophoblast, but expression does not alter when BeWo cells are syncytialised. PAPPA2 protein was localised to the syncytiotrophoblast layer in both (*a*) preterm and (*b*) pre-eclamptic placental sections. (*c, d*) Interestingly, in term placental sections, PAPPA2 protein was observed as absent (*c*) or scarcely expressed (*d*). (*e*) Negative controls were clean. The images shown are representative of six samples per group (original magnification ×400). (*f*) To assess changes in *PAPPA2* mRNA expression with syncytialisation, BeWo cells were induced to syncytialise by exposure to 20 µM forskolin for 48 h. Analysis of *PAPPA2* mRNA expression indicated no significant change with syncytialisation compared with the vehicle control. Data are the mean ± s.e.m.

cells that were induced to syncytialise, but observed no significant changes in *PAPPA2* expression (Fig. 2*f*).

*Increased PAPPA2 mRNA and protein expression in primary term placental explants exposed to hypoxia*

In silico analysis identified the presence of an HRE and two NF-κB target sites within the PAPPA2 promoter region (Fig. 3*a*). To examine whether placental PAPPA2 expression changed with exposure to hypoxia, we exposed primary term placental explants to hypoxia (1% oxygen) for 24 h. There was a significant ($P < 0.001$) increase in *PAPPA2* mRNA expression (Fig. 3*b*) following exposure to hypoxia. There was also a tendency for increased protein expression in the same samples on densitometric analysis, but the differences did not reach statistical significance (Fig. 3*c, d*).

Discussion

In the present study we demonstrated a significant increase in PAPPA2 mRNA and protein expression in an independent cohort of severe early onset PE placentas. We demonstrated protein localisation to the syncytiotrophoblast layer in PE and PT placentas but, surprisingly, observed a marked decrease and/or absent staining in term placenta. We also demonstrated no change in *PAPPA2* expression with syncytialisation, but a significant increase in *PAPPA2* mRNA expression in term placental explants exposed to hypoxia.
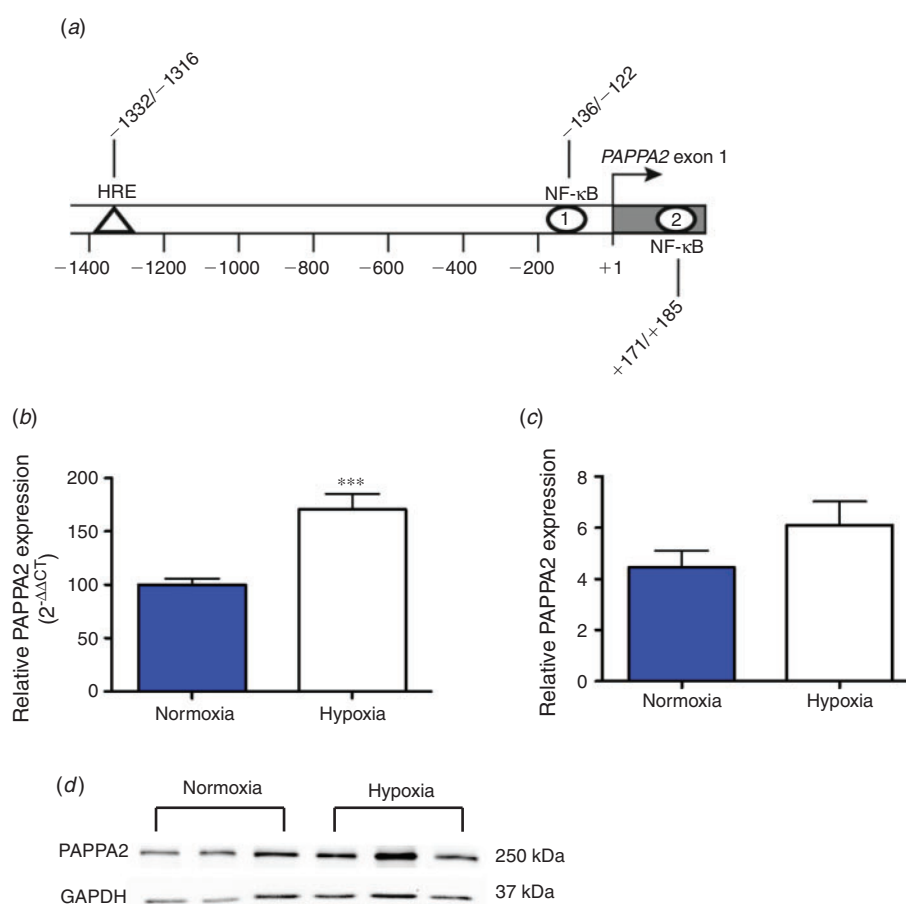
Fig. 3. Pregnancy-associated plasma protein A2 (PAPPA2) is upregulated following exposure to hypoxia. (*a*) Schematic representation of the promoter region of human *PAPPA2*: +1 denotes the beginning of *PAPPA2* mRNA (NM_020318.2). For each transcription factor binding site, the relative position of the beginning and end of the regulatory region is given. The predicted nuclear factor (NF)-κB target sites are (1 – c-Rel) tttggggtgTTCCcgg and (2 – NF-κB1) tgGGGAcctccccaa, whereas the predicted hypoxia responsive element (HRE) site is tgggagaaCGTGctcaa (note, uppercase letters denote the core sequence of the binding site). To assess changes in PAPPA2 expression following exposure to hypoxia, primary term explants were exposed to 1% oxygen for 24 h. (*b*) Exposure to hypoxia induced a significant increase in PAPPA2 mRNA expression. (*c, d*) A similar trend was observed at the protein level using western blot and densitometric analysis, although it did not reach statistical significance. (A representative western blot is shown.) Data are the mean ± s.e.m. ($n = 6$ primary placental explants).

Several previous studies have published data indicating that PAPPA2 is increased in PE (Buimer *et al.* 2008; Sitras *et al.* 2009; Várkonyi *et al.* 2010; Wagner and Christians 2010). In addition, PAPPA2 has also been localised to the syncytiotrophoblast previously (Nishizawa *et al.* 2008; Wagner and Christians 2010) in both PE and PT placentas.

Severe preterm PE represents the most serious spectrum of this disease and is responsible for most of the serious morbidity and mortality. The collective cohort size in all reports demonstrating PAPPA2 protein is increased in severe preterm PE has thus far been limited to only 30 (Nishizawa *et al.* 2008; Winn *et al.* 2009). Therefore, we thought it was important to provide independent confirmation of this finding. Thus, in the present study, we demonstrated increased PAPPA2 in a further large cohort of 29 severe PE placentas. In light of our findings, we

now believe there is collectively very strong evidence showing PAPPA2 protein is increased in severe preterm PE.

Interestingly, we observed little to no PAPPA2 staining within term placentas. Nishizawa *et al.* (2008) previously assessed PAPPA2 immunohistochemistry in term control (but not preterm) and severe early onset PE placentas and also suggested a decrease in PAPPA2 in control samples compared with PE. However, the present study appears to be the first to comprehensively compare PAPPA2 immunohistochemistry between PT, PE and term samples and to observe a lack of staining in term samples. Although variable, placental *PAPPA2* mRNA levels have also been reported to decline with gestation (Winn *et al.* 2009) and PAPPA2 has been shown to be a cleavage protease for insulin-like growth factor (IGF) binding protein 5 (Wang *et al.* 2009), suggesting that it contributes to IGF

bioavailability. Thus, the decline in placental PAPPA2 protein observed at term in our immunohistochemical samples is possibly a result of the decreased placental growth occurring during this phase of pregnancy.

It is now well accepted that the pre-eclamptic placenta is exposed to chronic hypoxia throughout gestation as a consequence of the failure to establish an adequate blood supply during implantation (Redman and Sargent 2005; Sibai *et al.* 2005). Recent studies have also suggested that reactive oxygen species triggered by hypoxia produce oxysterols that may contribute to the pathogenesis of PE (Valbuena-Diez *et al.* 2012) and that oxysterols inhibit differentiation and fusion of primary trophoblasts (Aye *et al.* 2011) For this reason, we examined PAPPA2 mRNA and protein expression in term placental explants exposed to hypoxia for 24 h. Previous data indicate a 47-fold increase in *PAPPA2* mRNA when BeWo cells are exposed to hypoxia (2% oxygen; Wagner *et al.* 2011). However, primary tissue has not been examined previously. Given that we identified a putative HRE within the PAPPA2 promoter, it was not surprising to observe a highly significant increase in *PAPPA2* mRNA levels when placental explants were exposed to hypoxia. Although the increase in protein levels from these same samples did not reach statistical significance, there was the same trend for increased levels with hypoxia. It is possible that the lack of statistically significant increases was related to the timing of the experiment (only 24 h exposure), and that protein levels would have been more definitively elevated if given further time in culture. Interestingly, we also identified two NF-κB binding sites within the promoter region, which may also contribute to the decrease in *PAPPA2* observed with hypoxia. Certainly there is good evidence to suggest a strong overlap between hypoxia and NF-κB activity, whereby hypoxia induces NF-κB activation and NF-κB regulates hypoxia-inducible factor-1 expression (Rius *et al.* 2008; Culver *et al.* 2010). Thus, the presence of NF-κB binding sites in PAPPA2 may also contribute to the effect observed.

Currently soluble FMS-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), anti-angiogenic factors produced by the failing placenta in PE, are considered the best biomarkers of PE, with levels of both rising before the clinical hallmarks of PE are apparent (Levine *et al.* 2004; Venkatesha *et al.* 2006). Interestingly, PAPPA, which is used clinically to screen for the presence of Downs syndrome, has been reported to be decreased in all trimesters of patients with PE (for a review, see Grill *et al.* 2009). Given the present data confirming that PAPPA2 is increased in PE and previous data indicating that it is significantly increased in the blood of PE patients (Paiva *et al.* 2011), it is possible that PAPPA2 may also represent a potential biomarker for this disease. It is interesting that PAPPA2 appears to trend in the opposite direction of PAPPA, suggesting that despite their homology, they may be regulated very differently and probably perform very different cellular functions.

In conclusion, the findings in the present study confirm and extend previous evidence to indicate that, in severe PE, there is a marked increase in PAPPA2. We also demonstrate that the upregulation of PAPPA2 in PE may be attributable to placental hypoxic exposure *in utero*.

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