Upregulation of Urotensin II Receptor in Preeclampsia Causes In Vitro Placental Release of Soluble Vascular Endothelial Growth Factor Receptor 1 in Hypoxia

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Abstract—Preeclampsia is a hypertensive disorder of pregnancy caused by abnormal placental function, partly because of chronic hypoxia at the utero-placental junction. The increase in levels of soluble vascular endothelial growth factor receptor 1, an antiangiogenic agent known to inhibit placental vascularization, is an important cellular factor implicated in the onset of preeclampsia. We investigated the ligand urotensin II (U-II), a potent endogenous vasoconstrictor and proangiogenic agent, for which levels have been reported to increase in patients with preeclampsia. We hypothesized that an increased sensitivity to U-II in preeclampsia might be achieved by upregulation of placental U-II receptors. We further investigated the role of U-II receptor stimulation on soluble vascular endothelial growth factor receptor 1 release in placental explants from diseased and normal patients. Immunohistochemistry, real-time PCR, and Western blotting analysis revealed that U-II receptor expression was significantly upregulated in preeclampsia placentas compared with controls (P<0.01). Cellular models of syncytiotrophoblast and vascular endothelial cells subjected to hypoxic conditions revealed an increase in U-II receptor levels in the syncytiotrophoblast model. This induction is regulated by the transcriptional activator hypoxia-inducible factor 1α. U-II treatment is associated with increased secretion of soluble vascular endothelial growth factor receptor 1 only in preeclamptic placental explants under hypoxia but not in control conditions. Interestingly, normal placental explants did not respond to U-II stimulation. (Hypertension. 2010;56:172-178.)

Key Words: urotensin II ■ preeclampsia ■ placenta ■ sVEGFR-1 ■ HIF-1α

The human placenta develops an extensive vascular network to facilitate exchange of nutrients and waste between maternal and fetal circulations. The placental vasculature is important for the proper functioning of the placenta, and increasing demands from the growing fetus are met by extensive placental vascularization and highly complex mechanisms that regulate vascular tone.1-2 Disorders in these pathways lead to diseases such as preeclampsia. Preeclampsia affects between 3% and 7% of pregnancies worldwide, is a significant cause of maternal mortality and morbidity, and is a major cause of preterm delivery. The disease is known to be placental in origin, although the precise pathogenic pathways remain unclear but is strongly associated with hypoxia,3-4 with the transcriptional regulator hypoxia-inducible factor (HIF) well documented to be elevated in preeclamptic tissues.5-7 Normal placental development uses a combination of angiogenic and antiangiogenic factors including, soluble vascular endothelial growth factor receptor 1 (sVEGFR-1 or sFlt1).8-10 Early in gestation, increased levels of angiogenic factors are produced to facilitate formation of the placental vascular architecture; these are then subsequently antagonized by antiangiogenic factors toward the end of pregnancy. This balance is thought to be altered in preeclampsia, a disease characterized by extensive endothelial damage and dysfunction with clinical sequelae, including rapid-onset hypertension, proteinuria, edema, and fetal growth restriction.11 The roles of antiangiogenic and angiogenic molecules have become more apparent with data suggesting that sVEGFR-1 is increased well before the onset of preeclamptic symptoms.12 Moreover, medium from preeclamptic placentas suppresses angiogenesis, whereas medium from normal placentas promotes angiogenesis.13 Little is known about factors that might regulate sVEGFR-1 release although recently angiotensin II (a potent vasoconstrictor) working through its
receptor, angiotensin II type 1, is known to increase release of this circulating ligand from human placental explants.14

Urotensin II (U-II) is a highly conserved cyclic peptide that binds to its cognate receptor urotensin (UT) receptor. The binding of U-II to the receptor elicits an endothelium-dependent vasorelaxation and an endothelium-independent vasoconstriction.15 More recently, U-II has also been reported to be a proangiogenic agent16,17 increasing angiogenesis both in vivo and in vitro in endothelial cells with a potency similar to fibroblast growth factor 2. Given the role that U-II plays in a number of pathogenic cardiovascular diseases, it is a plausible candidate for involvement in the pathogenesis of preeclampsia. The functional U-II peptide in humans is 11 amino acids in length cleaved from a precursor termed prepro–U-II and is one of the most potent vasoconstrictors known.15,18–20 mRNA encoding prepro-U-II has been shown to be expressed in a host of tissues, including whole placental extracts, and the protein has been localized to a host of peripheral vessels, including those in the placenta.21,22 Work on U-II in diseased states has predominantly focused on its upregulation with patients with cardiac complications, portal hypertension, and other hypertensive disorders, as well as reports of upregulation in hypoxic conditions.23,24 Higher circulating levels of U-II have been observed in patients with preeclampsia, inferring a possible role for this ligand in this disease.25 However, no differences in circulating U-II maternal plasma levels were recorded in a different study.26 This study did show a small but significant increase in U-II levels between paired samples of maternal plasma to cord plasma of preeclamptic patients.26 Of particular interest in this respect is that the binding of U-II to a UT receptor is quasi-irreversible.19,27–20 This would then render the key component in the U-II/UT receptor loop to be receptor levels on the cell surface rather than U-II concentration, per se. We, therefore, hypothesized that increased sensitivity to U-II in preeclampsia might be achieved by the upregulation of placental UT receptors and that hypoxia might influence this increase via HIF-1α. Given its role as an angiogenic agent, we also investigated whether it might influence the release of the antiangiogenic agent sVEGFR-1.

Materials and Methods

Experimental Subjects

All of the samples were taken with informed written consent and according to ethically approved protocols (Central Office of Research Ethics Committees 05/Q2802/122). Human tissue samples were collected from consenting patients from the University Hospital Coventry and Warwick. We defined preeclampsia for this study as patients between 37 and 40 weeks’ gestation in their first pregnancy, with a BP >140/100 mm Hg on 2 separate occasions 6 hours apart with >2+ proteinuria on dipstick or >0.3 g/L of proteinuria on 24-hour urine collection. Patients with no underlying medical conditions including existing hypertension or diabetes mellitus were excluded. Controls used in this study were gestationally matched patients showing none of the above clinical signs who were in their first pregnancy without pre-existing medical disease. Blood pressure was recorded by qualified physicians or midwives using an automated brachial cuff-oscillometric sphygmomanometer (Dinamap), with patients lying in a semirecumbent position.

Cell Culture

Human umbilical vein endothelium cells (HUVECs) were isolated from umbilical cords and used between passages 2 and 5.30 U-II was added at a final concentration of 1 μmol/L (Alta Biosciences). For hypoxic challenges, the cells were placed in a hypoxic chamber (1% O2; Billups Rothenberg Inc) for 24 hours at 60% to 70% confluence. BeWo cells, a model of syncytiotrophoblast, were used between passages 10 and 17.31 Forskolin (100 μmol/L, Sigma) was added to BeWo cells that had been subcultured in complete medium for 24 hours to induce syncytialization, where described. After a further 24 hours, these cells were maintained in control conditions (20% O2) or challenged with 24 hours of hypoxia. U-II was added at a final concentration of 1 μmol/L. Treatments described were carried out in triplicate per experiment, and each experiment was carried out ≥3 times. HIF-1α depletion was carried out on syncytialized BeWo cells treated with U-II for 24 hours with a subsequent addition of 10 μmol/L of HIF-1α sense/antisense oligonucleotides for 2 hours before mRNA extraction as described.32 Placental explants were carried out as described previously33 on 7 normal and 7 preeclamptic placentas. These explants were cultivated for 5 days. Syncytiotrophoblast generation was assessed by flow measurement of β-human chorionic gonadotropin). Only explants with rising β-human chorionic gonadotropin were used, and all of the treatments were initiated at day 4 with data shown from ≥7 different patients in each group. Both sVEGFR-1 and β-human chorionic gonadotropin were quantified using ELISA purchased from R&D Systems and IDS Ltd, respectively. U-II was added at a final concentration of 10 μmol/L.

Real-Time PCR

Real-time PCR RNA was extracted (Promega), treated with RQ1 DNase(Promega), and cDNA synthesized (MBI Fermentas). Probes generated for the U-II peptide (Hs00254354_m1) and Hs00246538_m1), UT receptor (Hs00255820_s1), and HIF-1α (Hs00936368m1) were used (Applied Biosciences) with 18s expression used as a standardization control on gestational-matched placentas from normal and preeclamptic patients (n = 7 for each). Samples were cycled on a 7500 Real-Time PCR System (Applied Biosciences).

Immunoblotting and Localization Studies

Standard slide preparations were used with UT receptor antibody (Novus Biologicals) added at 1:200 dilution overnight at 4°C using an ABC kit (Pierce). Western blots were carried out using standard protocols with placentals proteins harvested in 0.3 mol/L of NaOH neutralized with Tris HCl (pH 6.8). Membranes were blocked using 5% PBS in Tris-buffered saline-Tween (0.1%) buffer. UT receptor antibody was used at 1/1000 (CalTag Medsystems) and β-actin at 1/5000 (Abcam). Appropriate secondary antibodies (BioRad) were used at a dilution of 1/150 000 or 1/15 000 and developed with enhanced chemiluminescence advanced (UT receptor) or enhanced chemiluminescence (HIF-1α). The treatments were initiated at day 4 with data shown from ≥7 different patients in each group. Both sVEGFR-1 and β-human chorionic gonadotropin were quantified using ELISA purchased from R&D Systems and IDS Ltd, respectively. U-II was added at a final concentration of 10 μmol/L.

Statistical Analysis

Statistical analysis was performed using 2-way repeated ANOVA.

Results

U-II Receptor Is Significantly Upregulated in Preeclamptic Placentas

UT receptor expression was seen in human placenta. This by definition is essential for the transmission of the signal into a cellular response. We confirmed the presence of the UT
confirmed this increase in the UT receptor at the protein level (Figure 2B and 2C).

Expression of UT Receptor in BeWo and HUVECS: Effects of Hypoxia
To try to delineate which cell type in a placenta might be responsible for the increase in UT receptors seen in preeclampsia, we investigated the expression of UT receptors in BeWo cells (a recognized model of the syncytiotrophoblast, which syncytializes in response to forskolin) and HUVECs (vascular endothelial model). We confirmed that the UT receptor is expressed in both cell types under standard growth conditions (20% oxygen) via a detectable signal in extracted mRNA (Figure 3). Preeclampsia is caused by defective trophoblast invasion leading to relative hypoxia at the materno-fetal interface, and UT-II is reported to also be upregulated in hypoxia in other organ systems. We, therefore, attempted to mimic these conditions by challenging HUVECs and BeWo cells with 24 hours of hypoxia (1% O2). UT treatment of HUVECs, in both control and hypoxia, showed no change in UT receptor expression (Figure 3i) and a significant \( (P<0.01) \) 3-fold induction of UT receptor expression in syncytialized BeWo cells (Figure 3ii). Interestingly, opposing effects of hypoxia were seen in nonsyncytialized BeWo cells with hypoxia causing a 50% reduction in UT receptor expression. BeWo cells showed decreased expression of the UT receptor in both nonsyncytialized and syncytialized cells with the addition of U-II. However, a similar 3-fold induction of the UT receptor was demonstrated in hypoxic conditions with U-II treatment in both nonsyncytialized and syncytialized BeWo cells.

HIF-1α is a transcriptional activator well documented to be elevated in preeclampsia, which binds to hypoxia response elements to activate transcription. Analysis of the upstream sequence (1500 nucleotides from the start codon) revealed 4 putative hypoxia response elements (–163, –885, –1020, and –1301), which were perfect matches for the consensus sequence (RCGTG). The site at –163 had been identified previously as a potential HIF-1α cMyc binding site. We, therefore, investigated the role of HIF-1α in UT receptor upregulation. We confirmed that HIF-1α is expressed in our BeWo cell model and, further, that its expression was upregulated in these cells when subjected to 1% oxygen (Figure 4i). Pretreatment of BeWo cells with HIF-1α antisense oligonucleotides completely inhibited the induction of HIF-1α seen under hypoxic conditions. In the same samples, UT receptor induction followed the same pattern with the induction of mRNA expression in hypoxia as described above. This induction was not seen in the presence of HIF-1α antisense treatment, suggesting that the induction of the UT receptor in hypoxia depends on HIF-1α (Figure 4ii). Downregulation of HIF-1α or UT receptor was not seen when BeWo cells were transfected with HIF-1α sense sequence oligonucleotides (data not shown).

U-II Treatment of Regenerated Placental Explants Alters sVEGFR-1 Release in Preeclamptic Tissue Only
Having demonstrated that UT receptor upregulation in hypoxic conditions occurred via an HIF-1α-mediated pathway,
in BeWo cells and given the documented role for HIF-1α-mediated control of sVEGFR1 release in the preeclamptic placenta,\textsuperscript{38} we hypothesized a role for involvement of the UT receptor in sVEGFR1 release. Because BeWo cells do not produce sVEGFR-1\textsuperscript{39} and to explore the role of UT receptor induction on sVEGFR-1 release, we confined further experiments to regenerated syncytiotrophoblast explants using normal and preeclampsia placentas. These explants were challenged on day 4 with U-II in 20% or 1% oxygen, and the medium was assayed for sVEGFR-1 after 24 hours. In normal explants, the addition of U-II in either 20% or 1% oxygen elicited no change in sVEGFR-1 release into the medium in p= <0.01.

**Figure 2.** A, Quantitative UT receptor expression in the placenta of normal and preeclamptic patients. B, Representative Western blot using antibody raised against UT receptor; lanes 1 to 5, normal placenta; lane 6, HEK 293 cells; lane 7, HEK 293 cells overexpressing UT receptor; lanes 8 to 12, preeclamptic placenta. C, Quantification of protein expression in normal and preeclamptic placentas (based on 3 repeats).

**Figure 3.** Quantitative real-time PCR of UT receptor expression in the following: (i) HUVECs; (ii) syncytialized BeWo (+F); and (iii) nonsyncytialized BeWo cells grown under control (20% O\textsubscript{2}) or hypoxic conditions (1% O\textsubscript{2}) treated with 1 μmol/L of U-II where stated. Samples that show statistical differences against the untreated sample control for each cell type are shown (P<0.05 and ***P<0.001).

**Figure 4.** Relative fold induction of HIF-1α (i) and UT receptor (ii) grown in 20% or 1% O\textsubscript{2} with 1 μmol/L of U-II in the presence or absence of HIF-1α antisense oligonucleotides measured against control (20% O\textsubscript{2}). *P>0.05.
caused by liver cirrhosis. Moreover, U-II is also reported in diabetes mellitus, renal failure, and portal hypertension disease, ischemic cardiomyopathy, congestive heart failure, levels occur in essential hypertension, severe coronary artery potential role in preeclampsia, because significantly elevated between normal and preeclampsia. It is possible that the preeclampsia, whereas Cowley et al reported no differences reported a significant increase in circulating levels of U-II in comparison with identical sample not treated with U-II (Figure 5A). In contrast, preeclampsia explants showed marked differences in U-II-mediated sVEGFR-1 release depending on ambient oxygen concentration. The addition of U-II to preeclamptic explants in 20% oxygen markedly reduced the release of sVEGFR-1 into the medium, whereas the same placentas in 1% oxygen showed an increase in sVEGFR-1 release on U-II stimulation (Figure 5B).

### Discussion

Preeclampsia is a disease characterized by rapid onset of hypertension, proteinuria, and edema. Moreover, associations between increased circulating vasoactive peptides and preeclampsia are established (eg, neurokinin B and angiotensin II). U-II is a potent hypertensive agent, and we investigated its potential role in preeclampsia, because significantly elevated levels occur in essential hypertension, severe coronary artery disease, ischemic cardiomyopathy, congestive heart failure, diabetes mellitus, renal failure, and portal hypertension caused by liver cirrhosis. Moreover, U-II is also reported to be a proangiogenic agent and, thus, a potential candidate in disease pathogenesis. Interestingly, 2 studies have looked at circulating levels in preeclampsia. Balat et al reported a significant increase in circulating levels of U-II in preeclampsia, whereas Cowley et al reported no differences between normal and preeclampsia. It is possible that the increased levels of U-II seen may represent changes in expression of another protein, urotensin related peptide, which has the same epitope. We show a significant upregulation of the UT receptor in preeclampsia (Figure 2). Because the binding of U-II to its receptor is quasi-irreversible, such an increase in receptor levels is far more likely to affect the sensitivity of the U-II/UT receptor system than changes in U-II levels, per se. Surprisingly, we were unable to identify the U-II ligand within placental tissue despite previous reports, suggesting that either U-II is synthesized at levels beyond the limit of detection by our Taqman probes or that U-II is being synthesized remotely from an unknown source, of which there are a number of candidates, including heart, kidney, and adipose tissue. Therefore, it is not clear at this stage where the source of U-II is in these patients, and further work is required to clarify this.

The expression of the UT receptor was localized to both syncytiotrophoblast and fetal vascular tissue (Figure 1A). Although it is difficult to ascertain an increase in UT receptor expression from the immunohistochemistry (a 1.9-fold induction would not be easy to visualize), both Western blotting and real-time PCR data show a significant upregulation in preeclampsia.

Given both the anatomic proximity of these tissues and their different physiologic functions, identification of the respective contribution to the noted increase in UT receptors in preeclampsia required the use of established cellular models of these compartments. BeWo cells are widely used as a model of trophoblast function. HUVECs similarly are a well accepted model of the vascular endothelium. UT receptor upregulation was only noted in syncytiotialized BeWo cells (Figure 3iii) when subjected to hypoxia but not in HUVECs (Figure 3i) or nonsyncytiotialized BeWo cells (Figure 3iii). Interestingly, U-II treatment in nonsyncytiotialized BeWo cells (Figure 3iii) led to a reduction in the relative UT receptor expression in control conditions, but hypoxia again resulted in a 3-fold induction of the UT receptor. This suggests that hypoxia might be an important stimulus for UT receptor expression. The hypoxic transcriptional regulator HIF-1α appears to have a role in this induction, because antisense oligonucleotides that downregulate HIF-1α also downregulate UT receptor induction under hypoxic conditions. Analysis of the upstream sequence of the UT receptor revealed 4 putative hypoxia response elements, and these data suggest that UT receptor expression is, in hypoxic conditions, regulated at least partly by HIF-1α.

Given that our data suggest that UT receptor upregulation is mediated via HIF-1α, and HIF-1α has a documented role in regulating sVEGFR1 release, we hypothesized that UT receptor stimulation might regulated sVEGFR1. sVEGFR-1 is known to be significantly increased in preeclampsia and acts as an inhibitor of angiogenesis. There has been a paucity of work examining the processes that regulate the production of sVEGFR-1. Recent work has implicated both angiotensin II and heme oxygenase as playing roles in controlling sVEGFR-1 release in placental explants. We used explants from normal pregnancies and pregnancies affected by preeclampsia to explore the relationship between U-II and sVEGFR-1 in the diseased state (BeWo cells do not produce

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**Figure 5.** sVEGFR-1 measured from conditioned medium standardized against untreated explants on addition of U-II (A) normal and (B) preeclampsic patients taken on day 5.
sVEGFR-1[^39]). Preeclampsia placental explants revealed marked differences in U-II–mediated sVEGFR-1 release depending on ambient oxygen concentration. In 20% oxygen, the addition of U-II markedly reduced the release of sVEGFR-1 into the media in preeclampsia explants, but there was no effect in nondiseased explants. However, the preeclampsia placental explants placed in hypoxic conditions showed an increase in sVEGFR-1 release on U-II stimulation, whereas the normal placental explants again showed no difference. Twenty-percent oxygen concentration might be perceived as hyperoxic stress and be the cause of the decrease in sVEGFR-1 in the preeclampsia placental explants; however, this decrease in sVEGFR-1 is still only seen in diseased tissues and not in normal tissues subjected to the same conditions. This variance in behavior between control and diseased states may suggest a significant pathogenic role for the UT receptor in preeclampsia given that the receptor is upregulated in the disease, and U-II further stimulates sVEGFR-1 under hypoxic conditions only in placentas from patients with preeclampsia, a disease known to involve chronic hypoxia.

**Perspectives**

These novel data show that the UT receptor is significantly upregulated in preeclampsia principally within the syncytiotrophoblast. This upregulation depends on HIF-1α. More importantly, stimulation of UT receptors with U-II alters the release of sVEGFR-1 from regenerated syncytiotrophoblasts in the preeclamptic placenta only. This release shows a significant variation depending on ambient oxygen concentrations with hypoxia provoking a significant stimulation of U-II–mediated sVEGFR-1 release. These data indicate that these tissues are fundamentally different in their response to U-II stimulation and suggest a role for the UT receptor in the pathogenic process. It is also reassuring that high levels of HIF-1α expression have also been described in preeclampsia tissues. Dissection of the pathway(s) by which this varying effect of ambient oxygen concentration on U-II mediated release of sVEGFR-1 in placental explants is beyond the scope of this study but represents an interesting avenue for further research.

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**Disclosures**

None.

**References**

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