Impaired G-Protein Coupled Receptor 30-endothelial NOS signaling axis in pre-eclampsia is mediated by PI3K/Akt down-regulation.

Running title: Impaired GPR30-NO signalling axis in pre-eclampsia.

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None of the authors have any conflict of interest in this study.

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Word count: **6711** including references

Number of table: 2

Number of figure: 7

Abstract:

Introduction: Pre-eclampsia (PE) is associated with lowered circulating levels of oestrogen. The effects of oestrogen are normally mediated by the classical oestrogen receptors (ERs). Intriguingly, a new oestrogen receptor, G-protein coupled receptor 30 (GPR30), has been recently found to play

an important role in many oestrogenic effects. However, the potential role of GPR30 in PE remains poorly understood.

M**ethods**: Placentas, decidua and umbilical cord tissues were collected in 21 PE cases and following 20 uncomplicated pregnancies. Immunohistochemistry was used to detect the expression of GPR30 in the placental tissues. An *in vitro* cellular hypoxia/reoxygenation (H/R) model was established by using the immortalized human umbilical vein endothelial cell (HUVEC) line. Immunofluorescence was used to detect the expression of GPR30 and p-eNOS (Ser^{1177}) protein. The expression of GPR30, PI3K/Akt pathway and p-eNOS (Ser^{1177})/eNOS proteins in HUVECs were assessed using Western blotting techniques. An *in vitro* tube formation assay and a migration assay were used to detect angiogenesis of HUVECs. The effect of GPR agonists and inhibitors was determined.

R**esults**: The expression of GPR30 in vascular endothelial cells of placentas, decidua and umbilical cord tissues was lower in women with pre-eclampsia compared to normal pregnant women. The selective GPR30 agonist G1 and a general oestrogen receptors agonist 17-β-estradiol (E2) both exerted a protective effect in hypoxia/reoxygenation (H/R) exposed HUVECs by up regulating NO production though the phosphoinositide 3-Kinase/Akt (PI3K/Akt) pathway. This effect was abolished by the selective GPR30 inhibitor G15.

C**onclusion**: This study suggests that GPR30, as an upstream signaling molecule of PI3K/Akt/eNOS pathway, playing a critical role in the pathogenesis of PE. GPR30 also may be a potential therapeutic target for PE and other pregnancy complications that result from placental endothelial dysfunction.

Condensed abstract:

The expression of G-Protein Coupled Receptor 30 (GPR30), a recently identified oestrogen receptor, in vascular endothelial cells of placentas, decidua and umbilical cord tissues was lower in

women with pre-eclampsia compared to normal pregnant women . Impaired expression of GPR30 leads to Nitric Oxide (NO) decrease through down-regulation of the PI3K/Akt pathway in pre-eclampsia. These findings suggest that GPR30 regulates NO production in the placenta, decidua and umbilical cord tissues, and may be a potential therapeutic target for PE and other pregnancy complications that result from placental endothelial dysfunction.

Keywords: Pre-eclampsia; GPR30; eNOS; Endothelium.

Introduction

Pre-eclampsia (PE) is a severe pregnancy-associated disorder, characterised by the development of hypertension and proteinuria after 20 weeks of gestation. It complicates 2–7% of pregnancies globally and contributes to maternal and fetal mortality [1]. Although its aetiology and pathogenesis have not yet been fully elucidated, many *in vitro* and animal studies link the development of PE to impaired placental perfusion and ischemia, with some evidence linking ischemia to endothelial dysfunction [2, 3].

Previous studies have reported significantly reduced plasma levels of oestrogens and oestrogen metabolites in patients with PE compared with normotensive pregnant women[4, 5]. The effects of oestrogen in PE have been attributed to the classical oestrogen receptors (ERs), namely, ERα and ERβ [6, 7]. The role of the novel oestrogen receptor, G-Protein Coupled Receptor 30 (GPR30) in PE remains largely unknown.

GPR30 was firstly defined as a novel oestrogen receptor in 2005 [8]; since then its roles in cardiovascular diseases have been intensively studied [9-11]. Accumulated evidence reveals that GPR30 is associated with the pathogenesis of hypertension [9, 11-15]. Since hypertension is one of the defining symptoms of PE we hypothesised that GPR30 might be involved in the pathogenesis of PE.

It is known that oestrogen regulates rapid vascular effects including contraction, relaxation, and self-regulatory function of blood vessels through its receptors [16]. Specifically, these rapid vascular effects of oestrogen are potentially mediated through GPR30; in contrast, the classic oestrogen receptors, ERα and ERβ are thought to only play minor roles in these effects [16]. Other groups have confirmed that GPR30 expressed in endothelial cells [17, 18], moreover GPR30 may also possess anti-inflammatory[18] and proliferative [19] functions in human umbilical vein endothelial cells (HUVECs) and may mediate, in part, endothelium-dependent relaxation [10]. However, the role of GPR30 in pathophysiology of PE is poorly understood. In this study, our aim was to determine the role of GPR30 in mediating the pathophysiological changes of PE, as well as

any underlying molecular mechanisms. We also attempted to gain an insight into the therapeutic potential of GPR30 for the treatment of PE.

Materials and Methods

Ethics

This study was approved by the Ethics Committee of Human Experimentation of the First Affiliated Hospital of Chongqing Medical University, China. Informed consent was received from all subjects.

Study population

A total of 41 singleton pregnant women participated in this study. Fresh placental, decidua and umbilical cord tissues were collected from PE patients (n=21) and from normotensive control pregnant women, matched for gestational age at delivery (n=20). All participants were scheduled to undergo elective Caesarean section in the Department of Gynecology and Obstetrics in The First Affiliated Hospital of Chongqing Medical University between January 2013 and May 2014. The definition and diagnostic criteria of PE that of the American College of Obstetrics and Gynecology [20]. In brief, PE was identified as a pregnancy specific syndrome which occurred after 20 weeks of pregnancy and was characterized by a systolic blood pressure ≥140 mm Hg or diastolic pressure ≥90 mm Hg, with proteinuria (0.3g or more protein in 24 hrs or a random dip-stick urine was measured >1+protein). All subjects with chronic medical disorders such as diabetes mellitus, cardiovascular disease, collagen disorder, chronic renal disease, chronic hypertension, and metabolic diseases were excluded. Subject characteristics are shown in Table 1.

Tissue preparation

Sample collection was carried out as described previously [21]. Placental (maternal side), decidua and umbilical cord tissues were harvested within 1 hr of delivery. After collection, tissues taken from placenta, decidua and umbilical cord were snap frozen and stored at -80° or fixed in 4%

formaldehyde (Aladdin) before being embedded in paraffin for immunohistochemical analysis (IHC).

Reagents

17-β-estradiol (E2), a general oestrogen receptors agonist, was purchased from Abcam (ab120657); the selective GPR30 agonist G1, the selective GPR30 inhibitor G15 and the specific PI3K inhibitor wortmannin were purchased from Sigma-Aldrich (G6798; G6748; W1628;)

Cell culture and H/R application

HUVECs (Biomics Biotech; JiangSu; China) were cultured in Medium-1640 (Gibco; Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) at 37°C in 5% CO_2 in air. Experiments were performed when cells reached 80% confluence. The hypoxia/reoxygenation (H/R) treatment was performed as previously described [21-24]. In brief, to decrease the influence of stimulation by serum mitogens, HUVECs were pre-incubated for 12 hrs in low-serum medium which was supplemented with 1% FBS before H/R treatment. HUVECs were then incubated in a hypoxic environment (5% CO_2 and 95% N_2) in a tri-gas cell culture incubator (Thermo Fisher Scientific; Basingstoke; UK) for 4 hrs, and subsequently moved to a normoxic incubator with 5% CO_2 in air with normal culture media (10% FBS) for another 18 hrs. The oxygen concentration inside the tri-gas incubator was monitored by an oxygen analyzer (Vascular Technology; N.H.; USA).

Cell treatments

HUVECs were pretreated with E2 (100 nM) or G1 (1 μM) or G15 (2 μM) then exposed to 4 hrs of hypoxia followed by 18 hrs of reoxygenation. Detailed cell treatments and classification of groups are shown in Table 2.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as reported previously [25]. Briefly, 5μm paraffin embedded tissue sections were deparaffinized in xylene, then rehydrated in a serial gradient of ethanol and washed in PBS. Tissue sections were further quenched sequentially by the use of 3%

hydrogen peroxide for 15 min and incubated in 10% normal goat serum (Sigma) for 45 min at room temperature. The slides were then incubated at 4°C overnight with polyclonal rabbit anti-GPR30 antibody (1:150 dilution; ab39742; Abcam, Inc.) or polyclonal rabbit anti-CD31 antibody (1:100 dilution; #GTX110602; GeneTex). The slides were rinsed with PBS, and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min at 37°. 3,3′-diaminobenzidine (DAB; chromogenic reagent, ZSGB-BIO; China) was used as the chromogen, and hematoxylin (Sigma) was used for nuclear counterstain. For negative controls, the primary antibodies were omitted. The Image-Pro Plus software (version 6.0; NIH image) was used to calculate the integrated optical density values; experiments were repeated three times.

Western blotting

After treatment, protein samples of HUVECs were analysed using Western blotting techniques, as previously established in our laboratory [21, 25]. The primary antibodies used were as follows: monoclonal anti-GPR30 (1:1000 dilution; ab154069; Abcam), monoclonal anti-phosphor-PI3K (p85) (1:500 dilution; #4228; Cell Signaling Technology), polyclonal anti-PI3K(p85) (1:500 dilution; sc-292114; Santa Cruz Biotechnology), monoclonal anti-phosphor-Akt (p-Akt Ser^{473}) (1:1000 dilution; #4060; Cell Signaling Technology), polyclonal anti-Akt (1:500 dilution; sc-5298; Santa Cruz Biotechnology), monoclonal anti-phosphor-eNOS (p-eNOS Ser^{1177}) (1:1000 dilution; #5880; Cell Signaling Technology), monoclonal anti-eNOS (1:1000 dilution; #9570; Cell Signaling Technology). β-actin (Sigma) was used as the loading control.

HUVEC migration assay

The HUVEC migration assay was performed as reported previously [21, 26]. In brief, 24-well plates were used as the outer chambers and polycarbonate filters (8-μm pores; Transwell chamber; Millipore) were used as the inner chambers. The inner chambers were coated with 70 ml of 10 μg/ml Matrigel (BD Biosciences) for 2 hrs in 37°. 1.0×10^5 HUVECs were inoculated into each of the inner chambers and 650 μl medium supplemented with 10% FBS was added to the outer chambers of each well. The cells were then exposed to H/R (4 hrs of hypoxia followed by 2hrs of

reoxygenation) or normoxia at 37° for 6hrs. Next, the cells on the upper surface of the filter were completely removed by gently scraping with a cotton swab; these cells were then fixed in 90% ethanol (10 min) and stained with crystal violet. A light microscope (IX51; Olympus; Japan) was used to count the number of migrated cells, and the experiment was performed three times. The relative migration rate was calculated by comparing with non-treated cells, which were thought to have an average of 100% of the comparison.

In vitro tube formation assay

The *in vitro* tube formation assay was as previously described [21, 26]. Briefly, Matrigel (BD Biosciences) was thawed at 4°C overnight and then diluted with serum-free medium at a ratio of 1:2; the mixture was distributed into a 96-well plate (65 μl/well) and incubated at 37°C for 2 hrs. HUVECs (1.0×10^4), with a variety of pretreatments, were added to wells in triplicate; this was followed by H/R (hypoxia for 4 hrs and reoxygenation for 18 hrs) or normal culture for 22 hrs. At the end of the incubation period, digital images were captured from each well (Olympus). The total pipe length of the tube-like structure was calculated by using the image-Pro Plus software (version 6.0; NIH image). Tracks of HUVECs organized into networks of cellular cords were counted and averaged in 5 randomly selected view fields (100X magnifications). The tube formation indexes were expressed as tube length (mm)/mm^2 area.

Immunofluorescence

The immunofluorescence staining of GPR30 and p-eNOS in HUVECs was performed as previously described [27]. After treatment, cells were fixed in 4% formaldehyde (Aladdin) and blocked with 10% normal goat serum (Sigma), then incubated with anti-GPR30 antibody (1:80 dilution, #ab154069, Abcam) or anti-p-eNOS Ser^{1177} antibody (1:25 dilution; #5880; Cell Signaling Technology). A fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:50 dilution; Santa Cruz Biotechnology) was then applied. Propidium iodide (3 mg/mL; Santa-cruz) was used to visualise the nucleus. Images were acquired by confocal microscopy (FV10i; Olympus; Japan).

Statistical analysis

All data was expressed as mean ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism software (PRISM software version 5.0; GraphPad). Differences were assessed by independent t-tests or a one-way ANOVA; $P<0.05$ was considered statistically significant.

Results

Clinical characteristics

Clinical data from all subjects is summarized in Table 1. There were no significant differences in maternal age, body mass index (BMI), parity, and length of gestation; all subjects denied having a history of smoking. The women with PE had significantly higher mean arterial pressure (MAP), 24-hour proteinuria, and significantly lower infant birth weights and placental weights ($P<0.05$).

The expression of GPR30 was significantly lower in placenta, decidua and umbilical cord from pregnancies complicated with PE

GPR30 immunoreactivity was expressed in both vascular endothelial cells and trophoblast cells in third trimester placentas, as well as in vascular endothelial cells of the third trimester decidua and umbilical cords (Fig.1). GPR30 expression in all tissue (placenta, decidua, and umbilical cord), was significantly lower in cases of PE compared with controls （Fig.1M *$P<0.01$; N *$P<0.05$; O *$P<0.01$).

The expression of GPR30 and activation of eNOS was reduced by H/R treatment in HUVECs; eNOS phosporylation at Ser^{1177} is GPR30 dependent.

Confocal microscopy was performed to capture immunostaining of GPR30 and p-eNOS (Ser^{1177}). HUVECs were exposed to 4 hrs of hypoxia followed by 18 hrs of reoxygenation, or maintained as normoxic controls. GPR30 expression was reduced following H/R compared with normoxic controls (Fig.2). Moreover, the H/R induced reduction in GPR30 expression was inhibited by

pretreatment with a general oestrogen receptor agonist E2 or the selective GPR30 agonist G1 (Fig.2). This effect was further abolished by co-incubation with the specific GPR30 inhibitor G15 (Fig.2).

It was observed that p-eNOS (Ser^{1177}) protein was mainly localized in the cytoplasm of HUVECs (Fig.3). Both H/R treatment and G15 treatment reduced p-eNOS (Ser^{1177}) levels in HUVECs compared with the normal control group (Fig.3).

Western blot quantification of protein levels of GPR30 and p-eNOS in HUVECs identified that 22 hrs of H/R treatment significantly reduced GPR30 protein levels compared with the normoxic control group (Fig.4A; $p<0.01$). Activated eNOS, which is phosphorylated at Ser^{1177}, was also reduced by H/R treatment in HUVECs (Fig.4B; $p<0.05$). H/R-induced changes in GPR30 and activated eNOS expression were inhibited by pretreatment with the general oestrogen receptor agonist E2 (Fig.4A and 4B; $p<0.01$) or the selective GPR30 agonist G1 (Fig.4A; $p<0.01$ and Fig.4B; $p<0.05$ respectively). Further, the inhibitory effects of the ER agonists were abolished by co-incubation with G15 (Fig. 4A and 4B; $p<0.01$).

PI3K/Akt signaling pathway mediated GPR30 dependent eNOS activation in HUVECs during H/R.

Western blot quantification of protein levels of p-Akt (Ser^{473}) and phosphorylation of PI3K (p85) in HUVECs identified that after 22 hrs H/R treatment, expression of p-PI3K (p85) and p-Akt (Ser^{473}) was significantly increased by pretreatment with either G1 (Fig.5A and 5B; $p<0.01$) or E2 (Fig. 5A and 5B; $p<0.01$). This effect of E2 was inhibited by co-incubation with G15 (Fig. 5A and 5B; $p<0.01$). In order to verify the role of the PI3K/Akt signaling pathway in mediating GPR30 dependent eNOS activation in HUVECs during H/R, eNOS phosphorylation at Ser^{1177} was assessed (Fig. 5C). After 22 hrs of H/R treatment, eNOS phosphorylation at Ser^{1177} was significantly decreased compared with the normoxia conditions (Fig.5C, $P<0.05$); H/R-induced changes in GPR30 and activated eNOS expression were inhibited by pretreatment with the general oestrogen

receptor agonist E2 (Fig.5C; $p<0.01$) or the selective GPR30 agonist G1 (Fig.5C; $p<0.05$). The effects of ER agonists (E2 or G1) on eNOS phosphorylation were blocked by the presence of PI3K inhibitor wortmannin (Wort) (Fig.5C; $P<0.01$).

GPR30 is critical for in vitro tube formation of HUVECs during H/R

In order to determine the role of GPR30 in angiogenesis, the effect of E2 and the selective GPR30 agonist G1 on the formation of *in vitro* tube-like structures in HUVECs were examined (representative images are shown in Fig. 6A-F). Following 22 hrs of incubation under H/R, *in vitro* tube formation of HUVECs was significantly reduced compared to normoxic control conditions (Fig. 6G; $p<0.01$). The H/R induced effect was inhibited by pretreatment with E2 (Fig. 6G; $p<0.05$) or G1 (Fig. 6G; $p<0.01$); further, those effects of E2 were further abolished by co-incubation with G15 (Fig.6G; $p<0.05$).

GPR30 is critical for HUVECs migration during H/R

Representative images of HUVEC migration are shown in Fig. 7A-F. When exposed to 4hrs of hypoxia, followed by 2 hrs of reoxygenation, the migratory capability of HUVECs was significantly impaired compared with the normoxic control conditions (Fig.7G; $p<0.01$). However, the H/R-induced reduction of migration in HUVECs was restored by pretreatment with E2 (Fig. 7G; $p<0.01$) or G1 (Fig.7G; $p<0.05$). This protective effect was inhibited by co-incubation with the specific GPR30 inhibitor G15 (Fig.7G; $p<0.05$).

Discussion

PE is a life threatening pregnancy complication with multi-systemic complications. The aetiology and pathogenesis of PE have not yet been fully elucidated, although pathological changes in the placental vasculature are thought to play an important role in meditating the various clinical manifestations [33]. Consequently, disruption of the oxygen supply to the placenta could be a

major causative factors [2]. Specifically, intermittent placental perfusion induces H/R injury in placental vascular endothelial cells in PE [1, 22, 31]. In the present study, we established an *in vitro* cellular H/R model by using the immortalized human umbilical vein endothelial cell (HUVEC) line. Pharmacological manipulation of GPR30 activity provided an in-depth insight into the impact of this novel oestrogen receptor on H/R-induced vascular endothelial cells dysfunction.

Any damage to vascular endothelial cells leads to endothelial dysfunction, which includes the unregulated release of vasoconstrictor and vasodilator substances. The production and/or release of vasodilatory substances, such as nitric oxide (NO) and prostacyclin (PGI2) decreases, while the production and/or release of vasoconstrictors such as thromboxane A2 (TXA2) increases. This results in increased blood pressure, and leads to a series of pathological changes in PE [2, 34]. Accumulative evidence shows that the circulating levels of NO are decreased in PE patients, and diminished nitric oxide synthesis is thought to contribute to the pathophysiological changes in PE [35, 36].

Previous studies have suggested that oestrogens and oestrogen metabolites are associated with alterations in vascular function in PE [4, 5]. As a novel oestrogen receptor, GPR30 has been shown to regulate many of the rapid vascular effects attributable to oestrogens [16], moreover, multiple studies have demonstrated that GPR30 is associated with the pathogenesis of hypertension [9,11-15]. Together, these findings suggest a potential role of GPR30 in PE development. GPR30 is able to mediate vasodilation and decrease blood pressure in both rats and normal humans [38], and the deletion of GPR30 a knockout mouse model results in hypertension [39]. However, the role of GPR30 in mediating the pathophysiological changes of PE is still poorly understood. In this study, we discovered that GPR30 is expressed in vascular endothelial cells and trophoblasts in third trimester placentas, and that GPR30 is expressed in vascular endothelial cells in third trimester decidua and umbilical cord. The widespread expression of GPR30 suggests that it could play a role in mediation of angiogenesis at the maternal-fetal interface. Intriguingly, in vascular endothelial cells from these tissues, expression of GPR30 was significantly lower in PE patients than in normal

pregnant women, indicating that that compromised GPR30 expression may be associated with the pathogenesis of PE.

It has been proposed that oestrogen regulates NO synthesis through GPR30 [29] and a recent study confirmed that the NO-dependent beneficial effects of endogenous oestrogens is partly mediated by GPR30 in the cardiovascular system [30]. The PI3K/Akt pathway plays an important role in regulating vascular endothelial cells function, including NO production [32, 40], cell migration [41] and cell survival [42]. It is known that PI3K/Akt is an upstream signaling modulator of the activation of eNOS by phosphorylating Ser^{1177} [30, 32, 37]. In the present study, p-eNOS at Ser^{1177} and p-PI3K (p85) protein levels were impaired in HUVECs following an H/R insult. It was observed that the phosphorylation of eNOS at Ser^{1177} induced by the specific GPR30 agonist G1 and general oestrogen receptor agonist E2 in HUVECs was blocked by the specific PI3K inhibitor wortmannin, strongly suggesting that the PI3K/Akt signaling pathway mediates GPR30 dependent eNOS activation.

In addition to previous observations that GPR30 displays anti-inflammatory [18], proliferative [19] and vasorelaxant functions [13] in HUVECs, we were able to demonstrate migratory and angiogenic properties. Following an H/R insult, *in vitro* tube formation and migration of HUVECs was compromised, however, pharmacological activation of GPR30 was able to preserve *in vitro* tube formation and the migratory properties of HUVECs. This protective effect was diminished by the GPR30 specific inhibitor G15. Taken together, these findings demonstrate that GPR30 plays an important role in angiogenesis in HUVECs during H/R. Notably, G15 treatment almost fully blocked the beneficial effects of the general oestrogen receptor agonist E2, indicating that GPR30 is the predominant receptor that mediates the oestrogen-eNOS signaling axis, rather than the classic oestrogen receptors.

In conclusion, the present study has shown that GPR30 is expressed in human placenta, decidua, and umbilical cord, and that the expression of GPR30 is significantly lower in tissues from patients with pregnancies complicated by PE compared to normal pregnant women. Further, GPR30

regulates eNOS phosphorylation through the PI3K/Akt pathway, which is critical for protecting vascular endothelial cells against H/R induced dysfunction. Therefore, GPR30 could be a potential therapeutic target of PE and other related pregnancy complications characterised by placental endothelial dysfunction.

Acknowledgements

This study was supported by National Natural Science Foundation of China (No. 81100444, 81370732,81170585), Chongqing Science and Technology Commission of China (No. 2011BB5121) and Chongqing Municipal Health Bureau of China (No.2011-2-046).

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