G-protein-coupled receptor 30 mediates the effects of estrogen on endothelial cell tube formation *in vitro*

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Abstract. The placenta is the exchange organ between the mother and the fetus. The inadequate function of this organ is associated with a number of pregnancy disorders. Hypoxia and oxidative stress during placental development may induce endothelial dysfunction, resulting in the reduction in the perfusion of the placenta. During pregnancy, the levels of estrogen are increased. Decreased estrogen levels have been reported in women with preeclampsia. However, whether estrogen is involved in placental angiogenesis remains unclear. In this study, we aimed to investigate the effects of estrogen on endothelial cell tube formation and to elucidate the underlying mechanisms. For this purpose, human umbilical vein endothelial cells (HUVECs) were cultured with 17-β-estradiol under conditions of hypoxia/reoxygenation (H/R). The total pipe length of the tube-like structure on endothelial cells was measured. The expression levels of G-protein-coupled receptor 30 (GPR30) and endothelial nitric oxide synthase (eNOS) and Akt were also measured in the endothelial cells following treatment with 17-β-estradiol under H/R conditions by western blot analysis and immunostaining. We found that the total pipe length of the tube-like structure on endothelial cells was significantly reduced. This reduction was reversed by treatment with 17-β-estradiol. The expression of GPR30 in endothelial cells was significantly increased following treatment with 17-β-estradiol under H/R conditions. Furthermore, the levels of eNOS and Akt in endothelial cells were also significantly increased following treatment with 17-β-estradiol under H/R conditions. The activation of eNOS was inhibited by wortmannin, an inhibitor of PI3K/Akt. Our data thus demonstrate that estrogen prevents the failure of endothelial cell tube formation induced by H/R. GPR30 plays an important role in these protective effects through the activation of eNOS and Akt in endothelial cells. Our data suggest that increased levels of estrogen are important for placental angiogenesis.

Introduction

Successful pregnancy requires normal placental function from adequate placentation and placental vascularization. It may increase proportionally to the organ volume to maintain the number of placental vessels throughout the gestation. Therefore, the study of placental vascularization can enhance our understanding of the physiopathological process of increased resistance in umbilical arteries associated with placental insufficiency. Abnormal placentation causes inadequate utero-placental blood flow (1), resulting in a number of pregnancy complications, including preeclampsia, a leading cause of maternal and perinatal mortality and morbidity (2), and intrauterine growth restriction (IUGR) (3,4).

During normal pregnancy, women experience changes in hormone levels. The levels of both estrogen and progesterone are increased during pregnancy. The blocking of estrogen production results in pregnancy loss in animal models, suggesting that estrogen plays a role in the maintenance of healthy pregnancy. It has been suggested that in addition to its anti-inflammatory effect (5), estrogen also stimulates endothelial cell function and angiogenesis, in particular in vessel formation [reviewed in (6)]. There is growing evidence to suggest that lower levels of estrogen and an increase in progesterone are associated with the pathogenesis of preeclampsia (7-9). However, whether estrogen plays an important role in the regulation of vascular growth in the placenta during pregnancy has not yet been fully investigated.

The biological effects of estrogen are usually mediated by estrogen receptors (ERs)α and β (10,11). However, previous studies have suggested that mediating the function of estrogen can be achieved not only through ERα and ERβ. G-protein-coupled receptor 30 (GPR30), identified as a novel estrogen receptor in 2005 has been suggested to mediate the

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action of estrogen (12,13). Recently, we also reported that GPR30 is involved in the development of preeclampsia (14,15). GPR30 is a specific receptor for 17β-estradiol, a form of estrogens (16), and is expressed in endothelial cells. It is a regulator of the inflammatory response in endothelial cells (5).

Abnormal placentation causes placental ischemia and hypoxia, and it is well known that hypoxia/reoxygenation (H/R) may be a potential mechanism which contributes to the development of preeclampsia (17). Therefore, we undertook this *in vitro* study to investigate the effects of estrogen on endothelial cell tube formation, as well as the potential mechanisms responsible for these effects.

Materials and methods

Reagents. 17-β-estradiol (E2; ab120657), a general ER agonist, and monoclonal anti-GPR30 (ab154069) antibody were purchased from Abcam (Cambridge, MA, USA). The selective GPR30 agonist, G1, the selective GPR30 inhibitor, G15, and the specific PI3K inhibitor, wortmannin, were purchased from Sigma-Aldrich, St. Louis, MO, USA (G6798, G6748 and W1628, respectively). Anti-endothelial nitric oxide synthase (eNOS; Cat. no. 5880), monoclonal anti-phosphorylated (p-)PI3K (p85; Cat. no. 4228), monoclonal anti-p-eNOS Ser[1177] (Cat. no. 9570) and monoclonal anti-p-Akt (p-Akt Ser[473]; Cat. no. 4060) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

Cell culture under H/R conditions. Human umbilical vein endothelial cells (HUVECs) were purchased from Biomics Biotechnologies Co., Ltd. (Nantong, China) and cultured in Medium-1640 (Gibco Life Technologies, Beijing, China) with 10% (v/v) fetal bovine serum (FBS) (Gibco Life Technologies) at 37°C in 5% CO_2 in air. The HUVECs were seeded and grown to 80% confluence under H/R conditions, as previously described (18-21). In brief, to decrease the influence of stimulation by serum mitogens, the HUVECs were incubated for 12 h in low-serum medium which was supplemented with 1% FBS prior to exposure to H/R. The HUVECs were then incubated in a hypoxic environment (5% CO_2, 94% N_2 and 1% O_2) in a tri-gas cell culture incubator (Thermo Fisher Scientific Oxoid, Ltd., Basingstoke, UK) for 4 h, and subsequently moved to a normoxic incubator with 5% CO_2 in air with normal culture medium (10% FBS) for a further 18 h. The oxygen concentration inside the tri-gas incubator was monitored by an oxygen analyser (Vascular Technology Inc., Nashua, NH, USA).

In some experiments, the HUVECs were individually pre-treated with 17-β-estradiol (E2) (100 nM) or G1 (1 mM) or G15 (2 mM) for 1 h (5,22), and then exposed to 4 h of hypoxia followed by 18 h of reoxygenation.

Immunofluorescence. The immunofluorescence staining of GPR30 and p-eNOS in the HUVECs was performed as previously described (23). Following treatment, the HUVECs were fixed in 4% formaldehyde (Aladdin, Shanghai, China) and blocked with 10% normal goat serum (Sigma-Aldrich), then incubated with anti-GPR30 antibody (1:80 dilution) or anti-p-eNOS Ser[1177] antibody (1:25 dilution). A fluorescein

isothiocyanate-conjugated goat anti-rabbit antibody (1:50 dilution; sc-2012; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was then used. The nuclei were stained with propidium iodide (3 mg/ml; Santa Cruz Biotechnology, Inc.). Images were acquired using a confocal microscope (FV10i; Olympus Corp., Tokyo, Japan).

Western blot analysis. The relative activation levels of eNOS and Akt in the HUVECs following treatment were measured by western blot analysis as described (18,24). Briefly, the HUVECs were homogenised in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonident P40 substitute, protease inhibitor, 1 mM phenylmethanesulfonylfluoride). All the samples (10 μl) were loaded on 6-10% SDS-PAGE gels and electrophoresed then transferred onto nitrocellulose membranes. Non-specific binding was blocked by incubating membranes in 5% non-fat milk for 1 h and the membranes were then incubated with monoclonal anti-GPR30 (1:1,000 dilution) or monoclonal anti-p-PI3K (p85) (1:500 dilution) or polyclonal anti-PI3K (p85) (1:500 dilution; sc-292114; Santa Cruz Biotechnology, Inc.) or monoclonal anti-p-Akt (p-Akt Ser[473]) (1:1,000 dilution) or polyclonal anti-Akt (1:500 dilution; sc-5298; Santa Cruz Biotechnology, Inc.) or monoclonal anti-p-eNOS (Ser[1177]) (1:1,000 dilution) or monoclonal anti-eNOS (1:1,000 dilution) antibodies. After washing with PBS-T, the membranes were incubated with goat anti-mouse (1:2,000 dilution; ZB-2305; ZSGB-BIO, Beijing, China) or goat anti-rabbit (1:2,000 dilution; ZB-2301; ZSGB-BIO) secondary antibodies for 1 h at room temperature. After washing with PBS-T, the membranes were then incubated with streptavidin-conjugated horseradish peroxidase (1:3,000) for 1 h at room temperature. After washing with (PBS-T), the membranes were incubated with Amersham™ ECL™ Prime Western blotting detection reagent. β-actin (1:500 dilution; A1978; Sigma-Aldrich) was used as a loading control.

In vitro tube formation assay. The *in vitro* tube formation assay in the HUVECs was performed as previously described (18,25). Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) 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 h. The HUVECs ($1.0x10^4$), subjected to a variety of pre-treatments, were added to wells in triplicate under H/R conditions (hypoxia for 4 h and reoxygenation for 18 h) or culture in normal oxygen for 22 h. Following treatment, the cultures were captured from each well using a microscope (Olympus Corp.) (5 for each, x100 magnification). The total pipe length of the tube-like structure was calculated by using image-Pro Plus software (version 6.0; NIH image). Tracks of HUVECs organised into networks of cellular cords were counted and averaged in 5 randomly selected view fields (x100 magnification). The tube formation indexes were expressed as tube length (mm)/mm^2 area.

Statistical analysis. All the experiments in this study were performed at least 3 times and the data are expressed as the means ± standard deviation (SD). Statistical analyses were performed using Graph Pad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences between 2 groups were anal-
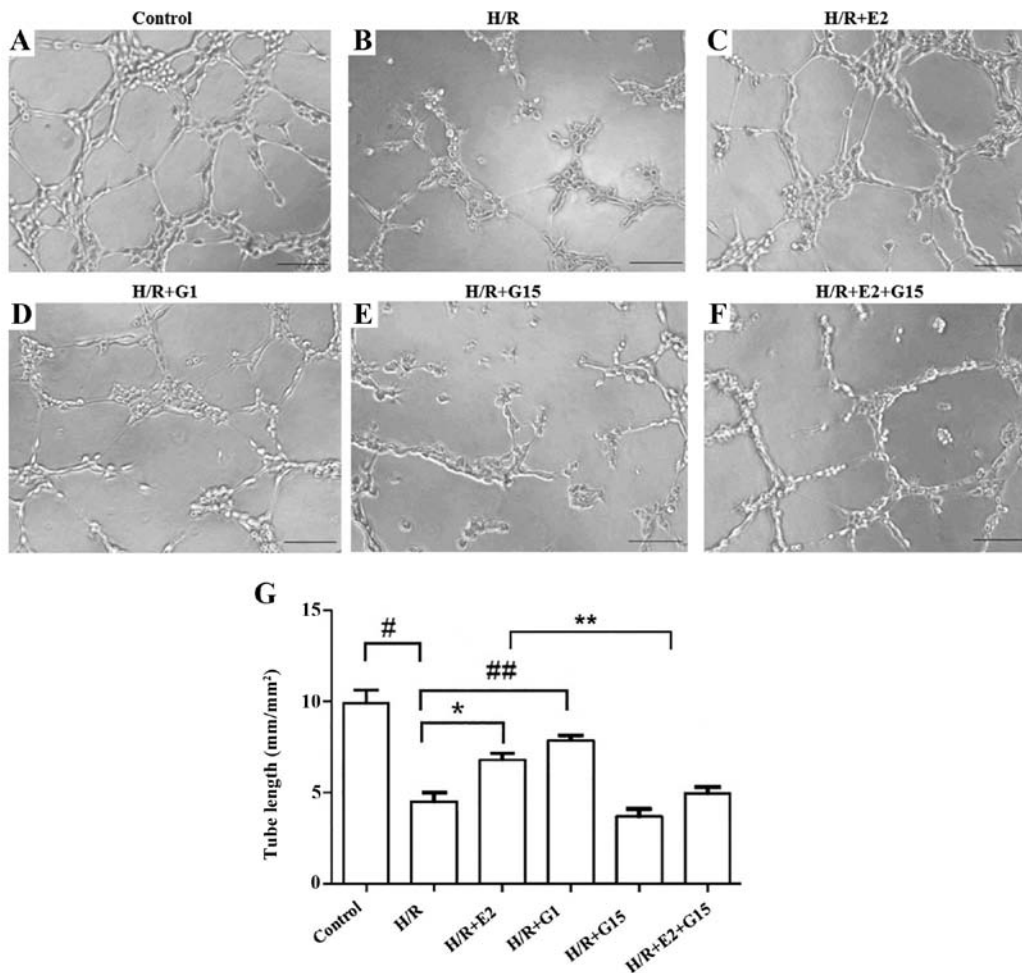
Figure 1. (A-F) Representative images showing endothelial cell tube formation. (A) Endothelial cell tube formation under normoxic conditions; (B) endothelial cell tube formation under hypoxia-reoxygenation (H/R) conditions; (C) endothelial cell tube formation under H/R conditions in the presence of 17-β-estradiol (E2); (D) endothelial cell tube formation under H/R in the presence of G1; (E) endothelial cell tube formation under H/R conditions in the presence of G15; (F) endothelial cell tube formation under H/R in the presence of E2 with G15; and (G) showing the quantification of endothelial cell tube formation. ([#]P=0.003, [*]P=0.017, [##]P=0.003, and [**]P=0.021).

ysed by independent t-test assuming, while differences between multi-groups were analysed by one-way ANOVA. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

A previous study suggested that estrogen has an effect on endothelial cell angiogenesis (6). In this study, to further investigate the effects of estrogen on endothelial cell tube formation, the total pipe length of the tube-like structure in HUVECs that had been treated with 17-β-estradiol (E2) or the selective GPR30 agonist, G1, was measured after 22 h of culture under H/R conditions (Fig. 1A-F). The total pipe length of the tube-like structure in the HUVECs that had been cultured under H/R conditions was significantly reduced compared to the HUVECs that were cultured under normoxic control conditions (Fig. 1G; P=0.003). This reduction was reversed by pre-treatment with 17-β-estradiol (E2) (Fig. 1G; P=0.017) or G1 (Fig. 1G; P=0.003). However, the protective effects of 17-β-estradiol (E2) on tube formation were inhibited by G15 (Fig. 1G; P=0.021).

In order to investigate whether GPR30, one of the estrogen receptors, is involved in the protective effects of 17-β-estradiol (E2) on endothelial cell tube formation, GPR30 expression in HUVECs was measured in the presence or absence of 17-β-estradiol (E2) or G1 in culture under H/R conditions. The expression of GPR30 was significantly decreased under H/R conditions compared with that under normoxic conditions (Fig. 2). However, this reduction of GPR30 expression in the HUVECs induced by H/R conditions was significantly reversed when the cells were pre-treated with 17-β-estradiol (E2) or G1 (Fig. 2). Howeveer, the effects of 17-β-estradiol (E2) and G1 were inhibited by treatment with the specific GPR30 inhibitor, G15 (Fig. 2). The decrease in GPR30 expression in the HUVECs was also confirmed by western blot analysis (Fig. 3; P<0.001).

It has previously been suggested that GPR30 activates eNOS. The activation of eNOS is involved in angiogenesis (26-28). In this study, we further investigated whether eNOS is involved in tube formation of endothelial cells *in vitro* under H/R conditions. Immunofluorescence revealed that the levels of p-eNOS (Ser[1177]) in the HUVECs were decreased under H/R conditions and following treatment with G15 compared
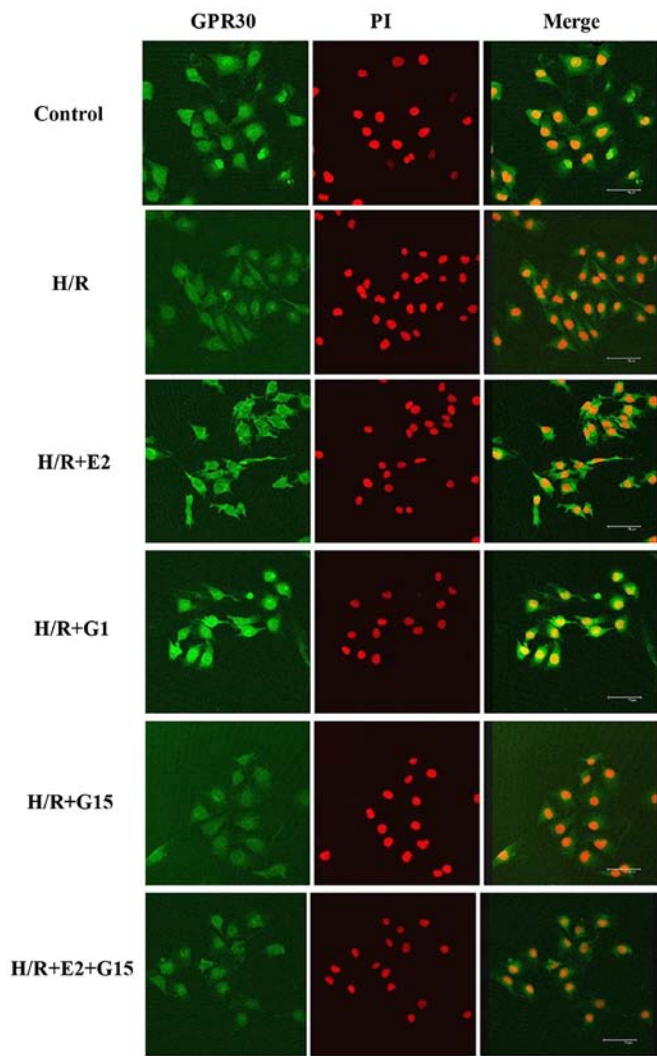
Figure 2. Immunofluorescence images demonstrating the staining of G-protein-coupled receptor 30 (GPR30) in human umbilical vein endothelial cells (scale bar, 75 μm). E2, 17-β-estradiol; H/R, hypoxia-reoxygenation.
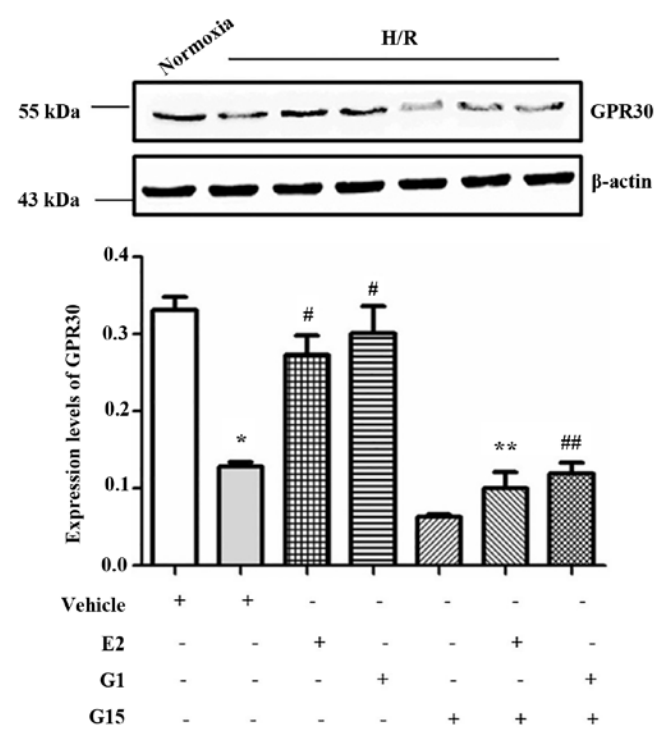

Figure 3. Western blot analysis demonstrating that the levels of G-protein-coupled receptor 30 (GPR30) were decreased under hypoxia-reoxygenation (H/R) conditions in human umbilical vein endothelial cells (HUVECs) compared to normoxic conditions. The levels of GPR30 were increased in the presence of 17-β-estradiol (E2) in HUVECs under H/R conditions. Semi-quantitative analysis revealed that when normalised to the levels of β-actin, the levels of GPR30 were significantly decreased in HUVECs under H/R conditions ($^*P<0.001$); however, these levels were significantly increased in the presence of E2 or G1 ($^\#P=0.002$, or $^\#P=0.0004$). These effects were inhibited by the addition of G15 ($^{**}P=0.0004$ or $^{\#\#}P=0.0002$).

with the control group (Fig. 4A). The decrease in the expression of p-eNOS at Ser[1177] in the HUVECs under H/R conditions was also confirmed by western blot analysis (Fig. 4B; P=0.0132). However, the decrease in p-eNOS expression was reversed by pre-treatment with 17-β-estradiol (E2) (Fig. 4B; P=0.0052) or the selective GPR30 agonist, G1 (Fig. 4B; P=0.0123). However, the effects of 17-β-estradiol (E2) were inhibited by treatment with G15 (Fig. 4B; P=0.0005).

We further investigated the estrogen-mediated phosphorylation of PI3K (p85) and Akt (Ser[473]) in the HUVECs under H/R conditions. The expression of p-PI3K (p85) and p-Akt (Ser[473]) in the HUVECs was significantly increased by pre-treatment with either G1 (Fig. 5A and B; P=0.0001 and P=0.0027, respectively) or 17-β-estradiol (E2) (Fig. 5A and B; P=0.0001). This effect of 17-β-estradiol (E2) was inhibited by treatment with G15 (Fig. 5A and B; P<0.0001 and P=0.0098).

We then investigated whether the PI3K/Akt signalling pathway mediates GPR30-dependent eNOS activation in HUVECs under H/R conditions. The effects of 17-β-estradiol (E2) and G1 on eNOS phosphorylation in

the HUVECs were blocked by the PI3K inhibitor, wortmannin (Wort, 100 nM) (Fig. 5C; P<0.0001).

Discussion

During human pregnancy, the placenta is supplied with maternal blood via the uterine spiral arteries. The fetus requires an increasing supply of oxygen and nutrients, suggesting that uterine spiral artery remodelling is necessary for a successful pregnancy. However, the failure of spiral artery remodelling impacts the oxygen concentration analogous to hypoxia-reperfusion within the placental environment. A decreased feto-placental perfusion and restricted oxygen delivery causes placental insufficiency, resulting in a number of complications of pregnancy, such as preeclampsia and IUGR. H/R is a secondary to intermittent perfusion of the intervillous space and plays an important role in placental development (17). Studies have suggested that H/R causes apoptotic changes in syncytiotrophoblasts, which is another characteristic feature of the preeclamptic placenta (29,30). During normal pregnancy, women experience changes in hormone levels and the levels of both estrogen and progesterone are increased. Lower levels of estrogen have been reported in women with preeclampsia (7-9). However, whether estrogen has an effect on placental vasculature is unknown.
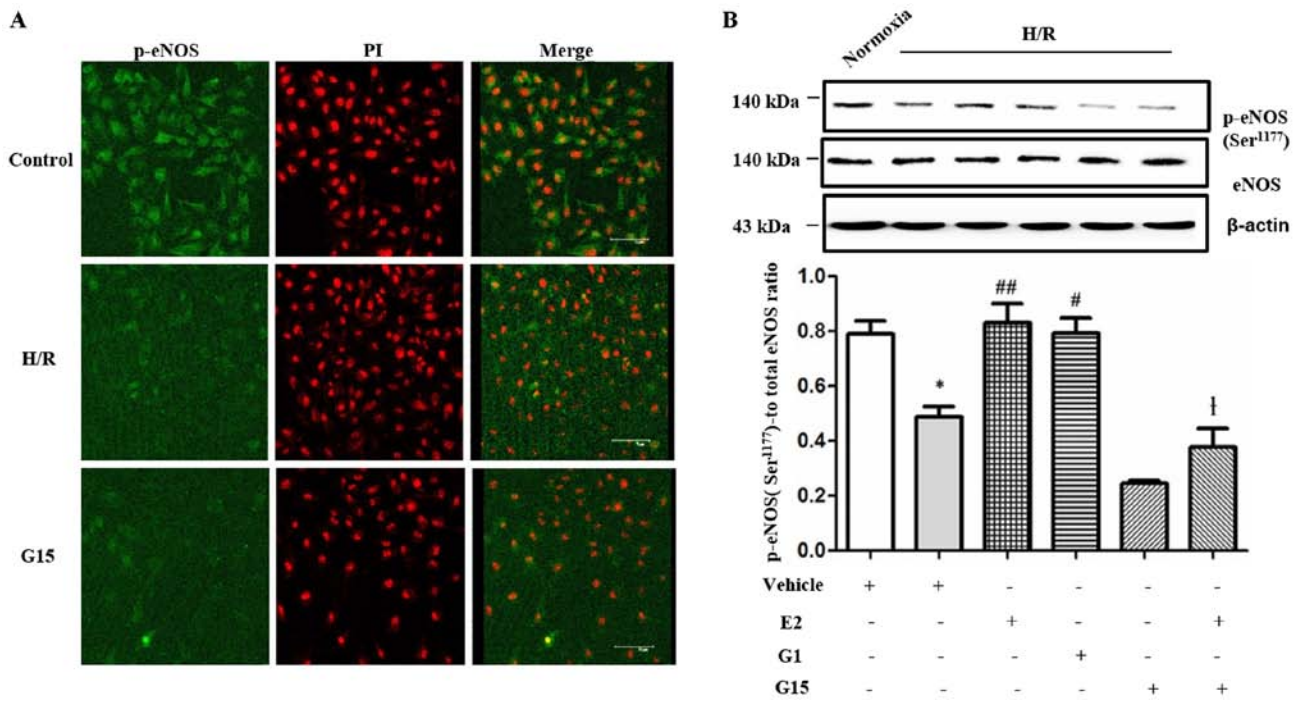
Figure 4. (A) Immunofluorescence images demonstrating the staining of p-eNOS (Ser^{1177}) in human umbilical vein endothelial cells (HUVECs) (scale bar, 75 µm). (B) Western blot analysis demonstrating that the levels of p-eNOS (Ser^{1177}) were decreased under hypoxia-reoxygenation (H/R) conditions in HUVECs compared to normoxic conditions. The levels of p-eNOS (Ser^{1177}) were increased in the presence of E2 in HUVECs under H/R conditions. Semi-quantitative analysis revealed that when normalised to the levels of β-actin, the levels of p-eNOS (Ser^{1177}) were significantly decreased in HUVECs under H/R conditions (*P=0.132), but were significantly increased in the presence of E2 or G1 ($^{##}$P=0.0052, or $^#$P=0.0123). These effects were inhibited by the addition of G15 (**P=0.0005). eNOS, endothelial nitric oxide synthase; E2, 17-β-estradiol.
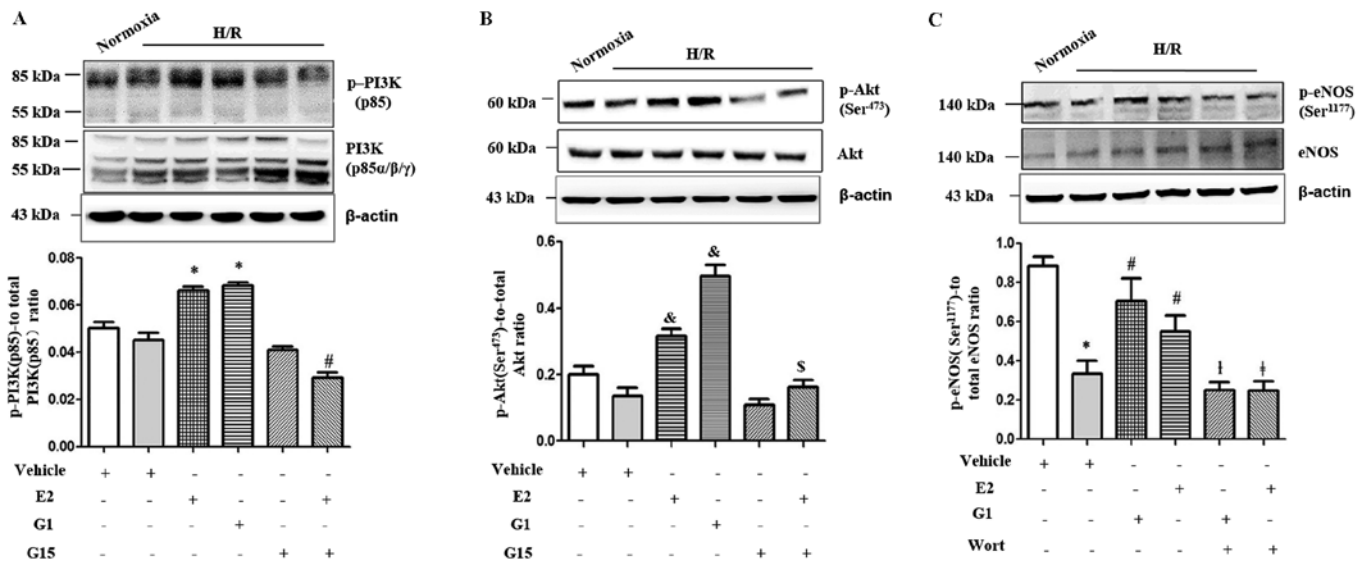

Figure 5. Western blot analysis demonstraing that the levels of (A) p-PI3K (p85) and (B) p-Akt (Ser^{473}) were significantly increased in the presence of E2 (A and B, *P=0.0001 and $^&$P=0.0001, respectively) or G1 (A and B, *P=0.0001 and $^&$P=0.0027, respectively) in human umbilical vein endothelial cells (HUVECs) under hypoxia-reoxygenation (H/R) conditions. The effect was blocked by treatment with G15 (A and B, $^#$P<0.0001 and $^$$P=0.0098, respectively). Western blot analysis also demonstrated that the levels of (C) p-eNOS (Ser^{1177}) were significantly decreased under H/R conditions in HUVECs compared to normoxic conditions (*P=0.0111). This decrease was significantly increased in the presence of E2 or G1 in HUVECs under H/R conditions ($^#$P<0.0043 and P=0.0104). This effect was significantly blocked by treatment with wortmannin (Wort, 100 nM) (*P<0.0001, $^{##}$P<0.0001). eNOS, endothelial nitric oxide synthase; E2, 17-β-estradiol.

Placental vasculature expands in both maternal and fetal placental tissue during pregnancy (31). Endothelial cell tube formation assay is a common tool to study angiogenesis. In this *in vitro* study, we found that the supplementation of

17-β-estradiol (E2), a form of estrogen reversed the failure of endothelial cell tube formation induced by H/R. Another study demonstrated that estrogen, such as 17-β-estradiol (E2) plays a role in the modulation of endothelial cell function and in

the promotion of angiogenesis (6). That study also suggested that the effect of 17-β-estradiol (E2) on angiogenesis may also apply to *in vivo* (6). A recent study further suggested that 17-β-estradiol (E2) was able to protect cardiomyocytes against H/R injury (32). To understand the specific mechanisms of estrogen by which receptors improve endothelial cell tube formation, in this *in vitro* study, we found that the expression of GPR30, a novel estrogen receptor was significantly reduced in endothelial cells under H/R conditions. However, this decreased expression of GPR30 was reversed by the supplementation of 17-β-estradiol (E2) or a selective GPR30 agonist (G1) in endothelial cells under H/R conditions. GPR30 is associated with the protective effects of estrogen in breast cancer (12,13). A recent study also suggested that the GPR30 agonist, G1, improves cerebral microvascular function following H/R injury in animal model (33). Taken together, our data suggest that 17-β-estradiol (E2) may have a similar function as the GPR30 agonist, G1. 17-β-estradiol (E2) is involved in the regulation of endothelial cell tube formation in H/R injury and its protective effects are associated with estrogen receptor, GPR30, in endothelial cells.

Reactive oxygen species (ROS) play an important role in endothelial cell dysfunction (34) and multiple ROS systems are activated during H/R, including NOS (35). A number of studies have suggested that the activation of eNOS is involved in angiogenesis (26,36). Endogenous estrogens mediate protective effects at least partially due to the activation of eNOS and the bioactivity of NO is regulated by GPR30 in the cardiovascular system. In addition, the protective effects of the GPR30 agonist, G1, in endothelial cells following H/R injury, at least partially also depends on eNOS (33). In this *in vitro* study, consistent with other studies, we found that the decreased expression of eNOS induced by H/R was reversed by the addition of 17-β-estradiol (E2) or the GPR30 agonist, G1, in endothelial cell culture.

A previous study suggested that in addition to the association with the expression of eNOS, GPR30 also mediated the activation of Akt in endothelial cells (37) and is involved in cell proliferation (38). In this *in vitro* study, we also found that the expression of Akt was decreased by H/R in endothelial cells; however, this decrease in Akt expression was reversed by supplementation with either 17-β-estradiol (E2) or the GPR30 agonist, G1. We further found that the activation of eNOS induced by 17-β-estradiol (E2) or the GPR30 agonist, G1, was inhibited by an inhibitor of PI3K/Akt (wortmannin), suggesting the activation of eNOS induced by 17-β-estradiol (E2) is Akt-dependent in endothelial cells under H/R conditions. Taken together our data suggest that both eNOS and PI3K/Akt activation are involved in the prevention of endothelial cell tube formation by 17-β-estradiol (E2), and the PI3K/Akt signalling pathway regulates the activation of eNOS in the prevention of endothelial cell tube formation by 17-β-estradiol (E2).

In conclusion, to the best of our knowledge, in the present *in vitro* study, we report for the first time that the supplementation of estrogen 17-β-estradiol (E2) may prevent the failure of endothelial cell tube formation induced by H/R. The estrogen receptor, GPR30, is at least partially involved in this protective effect through the activation of eNOS and the Akt signalling pathway in endothelial cells. Lower levels of estrogen are reported in complications of pregnancy, such as preeclampsia. Therefore, our data suggest that increased levels of estrogen during normal pregnancy promote placental vasculature development, thus exerting positive effects.

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