The expression and activation of sex steroid receptors in the preeclamptic placenta

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Abstract. Estrogen and progesterone are the main pregnancy hormones produced by the placenta. It is well understood that estrogen stimulates angiogenesis in the uterus during the reproductive cycle. Although the estrogen and progesterone signaling pathways are assumed to be associated with placental vascularization and preeclampsia, expression of estrogen receptors (ESRs) and progesterone receptor (PGR) in the placenta have not been well studied. The present study examined the expression patterns of steroid hormone receptors in placentas. Human placenta samples were collected and divided into normal and preeclampsia groups. Results revealed that expression levels of ESR1 were reduced, whereas ESR2 and PGR were elevated in preeclamptic placentas. To generate an *in vitro* preeclampsia environment, human placenta-derived BeWo cells were incubated under hypoxic conditions, or treated with catechol-O-methyl transferase inhibitor (COMT-in) or L-NG-nitroarginine methyl ester (L-NAME). Expression levels of ESR1, ESR2 and PGR in hypoxic cells demonstrated similar regulation as those in placentas from women with preeclampsia. Although COMT-in and L-NAME did not significantly regulate the expression levels of the receptors, COMT-in translocated ESR2 and PGR from the nucleus to the cytoplasm, indicating that these receptors were inactivated. These results suggested that ESRs and PGR are associated with symptoms of preeclampsia in the placenta. The expression of ESR1 was reduced in preeclamptic placenta and hypoxic BeWo cells. In addition, the activation of ESR2 and PGR was blocked in placenta cells subjected to COMT-in treatment. The reduced ESR1 expression and inactivation of ESR2 and PGR proteins may affect the physiological complications of preeclampsia in the placenta.

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Introduction

Preeclampsia is a hypertensive pregnancy disorder that affects 2-7% of pregnancies, resulting in notable maternal and fetal morbidities and mortalities, as well as greater susceptibility and earlier onset of future cardiovascular diseases in mothers and infants (1-4). This condition is characterized by elevated blood pressure and proteinuria, developing after 20 weeks of gestation (5).

There are multiple theories resulting in little agreement concerning the ultimate cause of preeclampsia. However, all forms of preeclampsia are characterized by disruption of vascular remodeling and a systemic anti-angiogenic response in the placenta (6). Trophoblast cells of the placenta invade the uterus, together with blood vessels (7), in order to establish efficient fetomaternal exchange of molecules, including nutrients and oxygen. In the placenta of a patient with preeclampsia, interstitial invasion by trophoblast cells is shallow and limited, resulting in the generation of an insufficient blood flow and sustained hypoxic conditions (8). Due to this, hypoxia is used to mimic preeclampsia conditions *in vitro* and *in vivo* (9).

Despite numerous clinical trials (10,11), the only standard and effective treatment for preeclampsia is preterm delivery

of the infant, which is the major cause of perinatal morbidity and mortality associated with preeclampsia (12). Beyond the delivery, the only successful medication used in the treatment of preeclampsia is magnesium sulfate for prevention of convulsions associated with eclampsia (13).

Estradiol and progesterone are critical female sex steroid hormones that regulate growth, differentiation and function in a broad range of target tissues, including the breasts, uterus and placenta (14). Concentrations of estrogen and progesterone, which are major pregnancy hormones produced by the placenta, increase throughout pregnancy, peaking at the end of gestation (15). These hormones potentially exist in placental tissue at considerably higher concentrations than in maternal blood (15). Notably, estrogen concentrations are significantly lower in placental tissue from pregnancies complicated by preeclampsia (16), and this evidence supports the hypothesis that estrogen serves an important role in the development of preeclampsia (17). It is well known that estrogen stimulates expression of vascular endothelial growth factor (VEGF) and angiogenesis in the uterus during the normal reproductive cycle (18). Furthermore, estrogen has been demonstrated to enhance uteroplacental blood flow (18-20) and microvascular volume in sheep (21), presumably as a result of enhanced angiogenesis and vasodilation. In addition, a previous study has indicated that estrogen serves an important role in regulating these processes in the primate placenta (18). This stimulatory action of estrogen requires estrogen receptors (ESRs), as chronic estrogen treatment has been demonstrated to induce angiogenesis in uterus tissue of normal but not ESR-null mice (18). The specific receptors of estrogen, ERα (ESR1) and ERβ (ESR2), act as ligand-activated transcription factors. The classical mechanism of ESR activation involves estrogen binding to receptors in the nucleus, after which the receptors dimerize and bind to specific response elements, known as estrogen response elements, located in the promoters of target genes (14).

During pregnancy, progesterone stimulates growth and differentiation of the endometrium for implantation, induces immunological tolerance to the fetus, inhibits uterine contractions, and maintains pregnancy (22). Research has suggested that progesterone at least partially induces vascular adaptations during normal pregnancy by reducing responsiveness of blood vessels to vasoconstrictors and by inducing vasodilatation (23-26). Progesterone has been proposed to prevent preeclampsia in previous studies, although evidence that supports this hypothesis is relatively weak (27-29). Although the estrogen and progesterone signaling pathways are assumed to be associated with placental vascularization and preeclampsia, expression and regulation of ESRs and progesterone receptor (PGR) in the placenta have not been well studied. Therefore, the present study examined the expression patterns of ESRs and PGR in placentas from normal patients and patients with preeclampsia, and in a preeclampsia-like *in vitro* system. In addition, the localization of the receptors in a preeclampsia-like condition was examined.

Materials and methods

Tissue collection and processing. The biospecimens and data used for the present study were provided by the Biobank of Pusan National University Hospital (Busan, Korea), a member of the Korea Biobank Network. The present study was approved by the Institutional Review Board of the Pusan National University Hospital Clinical Trials Center (H-1302-005-015), and all participants from Pusan National University Hospital provided written informed consent. Placental tissues (n=41) were obtained from patients, between January and December of 2015, with hypertension (systolic blood pressure of ≥140 mmHg and diastolic blood pressure of ≥90 mmHg at least 6 h apart) and proteinuria (≥300 mg/24 h or >1+ by dipstick). The placental samples were divided into normal (n=21) and preeclampsia (n=20) placentas, which were obtained from 29-40-week gestation. The clinical characteristics of the patients are demonstrated in Table I.

Table I. Clinical characteristics of preeclamptic pregnancies.

	Pregnancy type	
Characteristics	Normal (n=21)	Preeclampsia (n=20)
Gestational age, weeks	35.1±1.8	35.6±2.6
Birth weight, g	2,485.7±439.3	2,130.0±686.2
Systolic BP, mmHg	107.1±7.8	147.9±12.7[a]
Diastolic BP, mmHg	67.1±7.2	96.8±10.0[a]
Parity	0.8±0.7	0.6±0.7
Gravidity	2.6±1.4	2.3±1.3
Maternal body mass index, kg/m^2	25.8±3.0	28.6±5.8

[a]P<0.05 vs. the normal group. Data are presented as the mean ± standard deviation. BP, blood pressure.

Cell culture and in vitro model of preeclampsia. The BeWo human choriocarcinoma-derived cell line was purchased from the Korean Cell Line Bank (Seoul, Korea), cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 100 IU/ml penicillin and 100 μg/ml streptomycin, and grown in 5% CO_2 at 37˚C. The BeWo human choriocarcinoma cell line was used as a model for trophoblast. The BeWo cell line is frequently used as a trophoblast model as it maintains several characteristics of cytotrophoblasts, including human chorionic gonadotropin secretion, preservation of cytotrophoblast morphology and cytokeratin-7 expression (30). To establish an *in vitro* model of preeclampsia, hypoxic conditions were generated in a Modular Incubator Chamber (Billups-Rothenberg, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Briefly, cells were placed in a hypoxia chamber with inflow and outflow connectors, and hypoxic gas consisted of 2% O_2, 5% CO_2 and 93% N_2. Experiments were conducted in a constant 37˚C environment by placing the chambers in an incubator. In order to create oxidative stress, cells were either treated with catechol-O-methyl transferase inhibitor (COMT-in; 10 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or L-NG-nitroarginine methyl ester (L-NAME;

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction analyses.

Gene name	Primer	Sequence (5'-3')	Fragment, bp
β-actin	Forward	GGACTTCGAGCAAGAGATGG	234
	Reverse	AGCACTGTGTTGGCGTACAG	
Cytochrome c1	Forward	CCAGCTACCATGTCCCAGAT	185
	Reverse	TATGCCAGCTTCCGACTCTT	
Vascular endothelial growth factor	Forward	GGCCAGCACATAGGAGAGAT	216
	Reverse	ACGCTCCAGGACTTATACCG	
Soluble fms-like tyrosine kinase-1	Forward	GTCGTGTAAGGAGTGGACCA	211
	Reverse	GCAGATTTCTCAGTCGCAGG	
ESR1	Forward	AGCACCCTGAAGTCTCTGGA	153
	Reverse	GATGTGGGAGAGGATGAGGA	
ESR2	Forward	AAGAAGATTCCCGGCTTTGT	173
	Reverse	TCTACGCATTTCCCCTCATC	
Progesterone receptor	Forward	AAATCATTGCCAGGTTTTCG	209
	Reverse	TGCCACATGGTAAGGCATAA	

ESR, estrogen receptor.

100 μM; Sigma-Aldrich; Merck KGaA), respectively, for 24 h in a 37˚C incubator. All *in vitro* experiments were performed at least three times in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from BeWo cells and placenta tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The concentration of total RNA was measured using a spectrophotometer. First-strand cDNA was prepared from total RNA (3 μg) by reverse transcription at 37˚C using a M-MLV reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) and random primers (9-mers; Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. qPCR was performed with cDNA template (2 μl) and 2X Power SYBRGreen (6 μl; Toyobo Life Sciences, Osaka, Japan) containing specific primers. Primer sequences for cytochrome c1 *(CYC1), β-actin, VEGF,* soluble fms-like tyrosine kinase-1 *(sFlt1), ESR1, ESR2* and *PGR* are demonstrated in Table II. qPCR was conducted for 40 cycles using the following parameters: Denaturation at 95˚C for 15 sec, followed by annealing and extension at 70˚C for 60 sec. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold during the exponential phase of PCR amplification was considered to be the threshold cycle. Expression of the target gene was quantified relative to that of β-actin and CYC1, which are housekeeping genes, according to the $2^{-\Delta\Delta Cq}$ method (31).

Western blot analysis. Protein samples were extracted with Pro-prep solution (Intron Biotechnology, Inc., Seongnam, Korea), following the manufacturer's protocol. The concentration of the protein was determined using a bicinchoninic acid assay. A total of 20 μg protein sample was loaded and separated by 8-10% SDS-PAGE and then transferred to nitrocellulose membranes (Daeil Lab Services Co., Ltd., Seoul, Korea). Membranes were subsequently blocked for 2 h with 5% skim milk in PBS with 0.05% Tween-20 (PBS-T) at room temperature. Following blocking, membranes were incubated with hypoxia-inducible factor 1-α (HIF1A; 1:500; cat. no. sc-53546; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-ESR1 (1:500; cat. no. sc-542; Santa Cruz Biotechnology, Inc.), anti-ESR2 (1:500; cat. no. sc-8974; Santa Cruz Biotechnology, Inc.) and anti-PGR (1:500; cat. no. sc-538; Santa Cruz Biotechnology, Inc.) antibodies overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. sc-2313; Santa Cruz Biotechnology, Inc.) in 5% skim milk with PBS-T for 1 h at room temperature. Luminol reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to visualize antibody binding. Each blot was then stripped by incubation with 2% SDS and 100 mM mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50-60˚C. Membranes were subsequently probed with antibody against β-actin (1:3,000; cat. no. 4967; Cell Signaling Technology, Inc., Danvers, MA, USA) as an internal control for the same duration and temperature as with the other primary antibodies. Blots were scanned using Gel Doc 1000 version 1.5 (Bio-Rad Laboratories, Inc.), and band intensities were normalized to β-actin levels.

Immunocytochemical analysis. BeWo cells were seeded at $5x10^4$ cells with 200 μl DMEM in 8-well chamber slides (Thermo Fisher Scientific, Inc.). When the cells attained 70-80% confluence, they were either exposed to hypoxic conditions or treated with COMT-in or L-NAME for 24 h in a 37˚C incubator. Following three washes with 1X PBS, the cells were fixed with 4% formaldehyde solution (200 μl) for 10 min at room temperature, permeated with 1% Triton X-100 (Biosesang, Inc., Seonam, Korea) for 10 min at room temperature, and subsequently washed with 1X PBS three times.

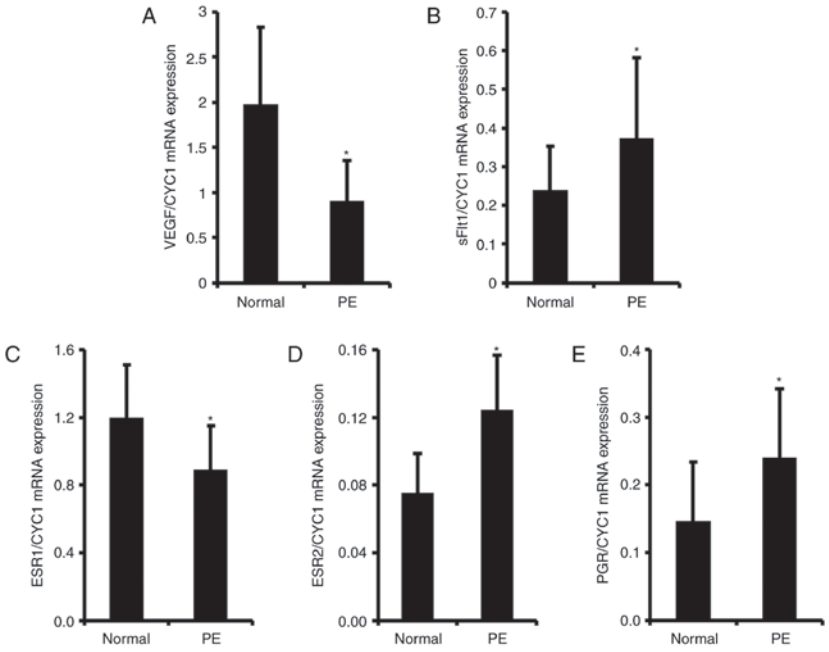

Figure 1. mRNA expression levels of VEGF, sFlt1, ESR1, ESR2 and PGR in human normal and preeclamptic placenta. Total mRNA was harvested from human normal and preeclamptic placentas following the onset of labor. Transcriptional levels of (A) *VEGF*, (B) *sFlt1*, (C) *ESR1*, (D) *ESR2* and (E) *PGR* were analyzed by reverse transcription-quantitative polymerase chain reaction. Total mRNA expression levels were normalized to that of *CYC1*. Data are expressed as the mean ± standard deviation. *P<0.05 vs. normal placenta. PE, preeclampsia; VEGF, vascular endothelial growth factor; sFlt1, soluble fms-like tyrosine kinase-1; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; PGR, progesterone receptor; CYC1, cytochrome c1.

The cells were then blocked in 2% bovine serum albumin (GenDEPOT, Inc., Barker, TX, USA), in PBS for 1 h at room temperature, and subsequently specific primary antibodies [anti-ERα (cat. no. sc-542), anti-ERβ (cat. no. sc-8974) and anti-PGR (cat. no. sc-538); Santa Cruz Biotechnology, Inc.] were added at a dilution of 1:50 overnight at 4˚C. Following washing, cells were incubated with secondary antibody (goat anti-rabbit immunoglobulin G-Texas red; 1:100; cat. no. sc-2313; Santa Cruz Biotechnology, Inc.) for 1 h in the dark at room temperature. Following one wash with 1X PBS, the fluorochrome DAPI (2 μg/ml; Santa Cruz Biotechnology, Inc.) was then applied to each well, after which samples were incubated for 10 min in the dark at room temperature. Finally, the cells were washed three times with 1X PBS and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). All cells were observed and analyzed using a fluorescent microscope (Eclipse TX100; Nikon Corporation, Tokyo, Japan) at a magnification of x400.

Statistical analysis. Results were presented as the mean ± standard deviation. Data were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test using SPSS version 10.10 for Windows (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Confirmation of preeclampsia conditions by biomarker genes. To confirm preeclampsia conditions, the expression levels of *VEGF* and *sFlt1*, which are well known biomarkers of preeclampsia in the human placenta, were examined (32). As expected, transcription levels of *VEGF* and *sFlt1* were altered in the preeclamptic placenta. Expression levels of *VEGF* were significantly reduced up to 2.2-fold in preeclampsia samples compared with the levels in normal placentas (Fig. 1A). The expression of *sFlt1* significantly increased up to 1.5-fold in the preeclamptic placenta samples compared with the level in normal samples (Fig. 1B). These results suggest that the normal and preeclamptic placentas were properly separated in the present study.

Expression of ESR1 is reduced in preeclamptic placentas, and ESR2 and PGR expression is increased. For the next experiment, the expression levels of *ESR1*, *ESR2* and *PGR* in normal and preeclamptic placenta tissues were evaluated by RT-qPCR. As demonstrated in Fig. 1C, the mRNA expression level of *ESR1* was significantly reduced by 1.4-fold, whereas that of *ESR2* was significantly elevated by 1.5-fold in preeclamptic placenta samples, compared with the expression levels in the normal group (Fig. 1D). Expression of *PGR*, another steroid hormone receptor, was significantly elevated up to 1.6-fold in the preeclamptic placenta samples compared with the level in the normal group (Fig. 1E). To examine protein expression levels of ESRs and PGR, western blotting was performed, and their photographic figures and schematic graphs are represented in Fig. 2. It was demonstrated that protein expression levels of all steroid receptors were similar to their mRNA expression level patterns in the preeclampsia group; however, the only significant result obtained was for ESR1.

Induction of preeclamptic conditions in vitro. To generate an *in vitro* preeclamptic environment, human placenta-derived BeWo cells were incubated in a hypoxia chamber, or under oxidative stress, induced by treatment with COMT-in and

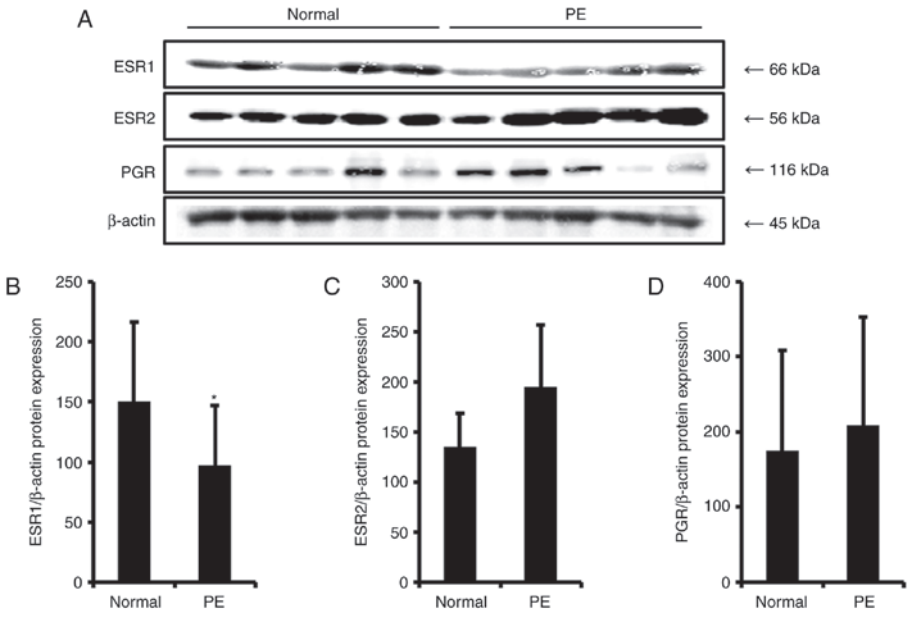

Figure 2. Protein expression levels of ESR1, ESR2 and PGR in human normal and preeclamptic placentas. Total proteins were harvested from human normal and preeclamptic placentas following the onset of labor. Proteins were processed for (A) western blot analysis, and the values are represented as schematic graphs for (B) ESR1, (C) ESR2 and (D) PGR. Total protein expression levels were normalized to that of β-actin. Data are expressed as the mean ± standard deviation. *P<0.05 vs. normal placenta. PE, preeclampsia; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; PGR, progesterone receptor.

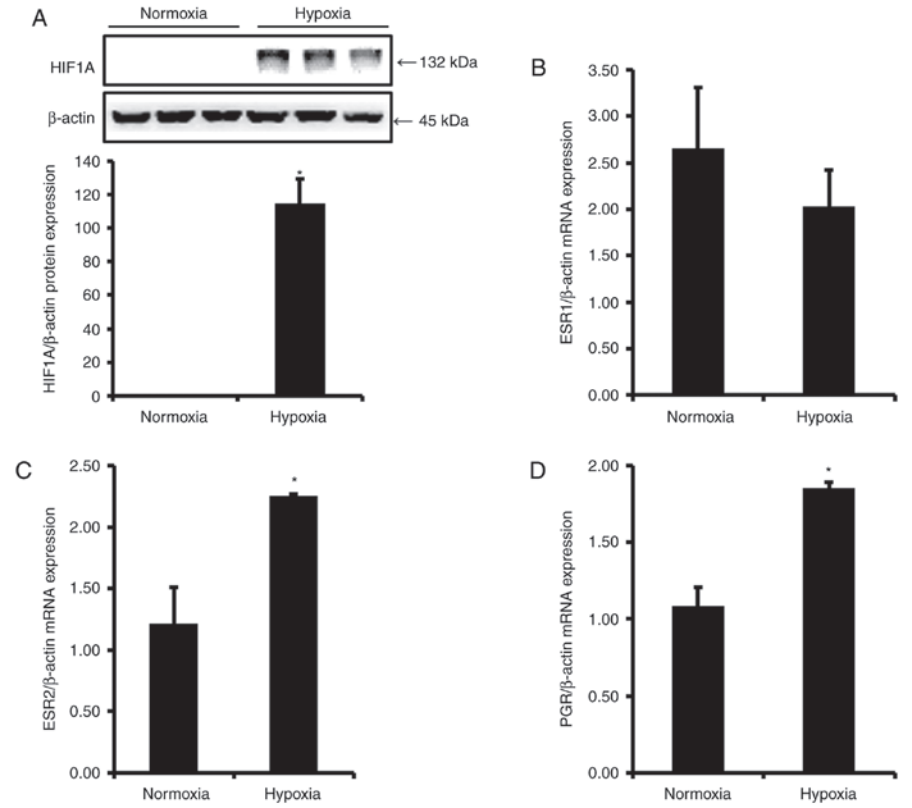

Figure 3. Protein expression levels of HIF1A, and mRNA expression levels of ESR1, ESR2 and PGR in a preeclampsia-like *in vitro* model. Total protein and mRNA were harvested from BeWo cells under hypoxic conditions. Translational levels of (A) HIF1A were determined by western blotting. Transcriptional levels of (B) *ESR1*, (C) *ESR2* and (D) *PGR* were analyzed by reverse transcription-quantitative polymerase chain reaction in the normoxia and hypoxia groups. Total protein and mRNA expression levels were normalized to that of β-actin. Data are expressed as the mean ± standard deviation. *P<0.05 vs. normoxia group. HIF1A, hypoxia-inducible factor 1-α; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; PGR, progesterone receptor.

L-NAME for 24 h. As HIF1A is a well-known biomarker of hypoxia, HIF1A protein expression was examined to test the present experimental conditions. As expected, HIF1A was not expressed in the normoxia group, and the expression level increased significantly in the hypoxia group compared with the level in the normoxia group (Fig. 3A).

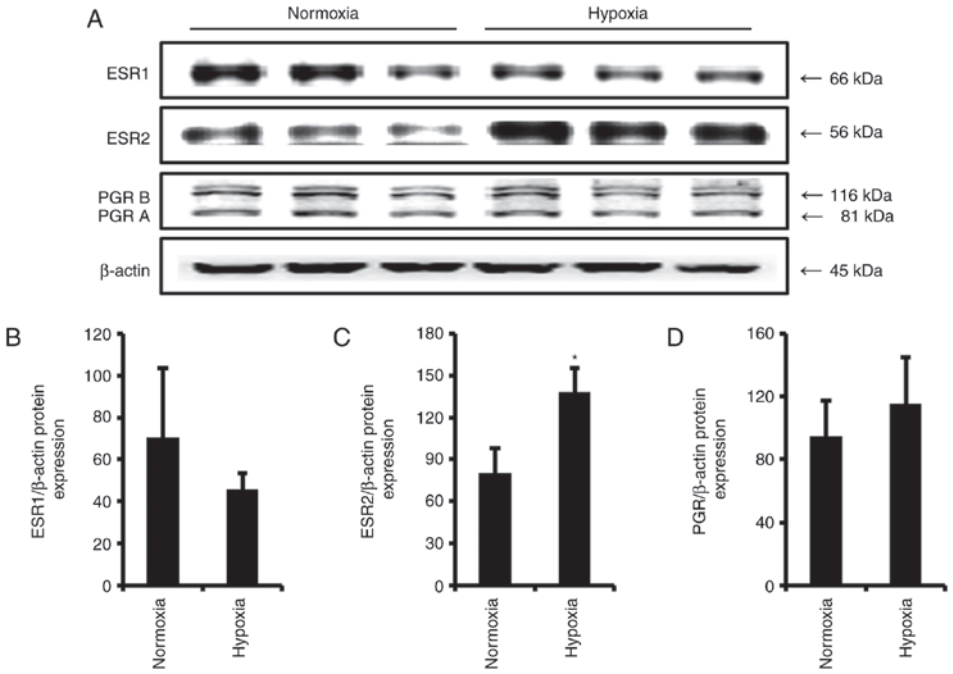

Figure 4. Protein expression levels of ESR1, ESR2 and PGR in a preeclampsia-like in vitro model. Total proteins were harvested from BeWo cells under hypoxic conditions. Proteins were processed for (A) western blot analysis, and the values are represented as schematic graphs for (B) ESR1, (C) ESR2 and (D) PGR. Total protein expression levels were normalized to that of β-actin. Data are expressed as the mean ± standard deviation. *P<0.05 vs. normoxia group. ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; PGR, progesterone receptor.

Expression of ESR1 is reduced in an in vitro model of preeclampsia, and ESR2 and PGR expression is increased. To evaluate expression levels of *ESRs* and *PGR* in hypoxic conditions, BeWo cells were grown in the absence and presence of hypoxia stress, and mRNA levels were examined by RT-qPCR. Notably, expression levels of *ESR1*, *ESR2* and *PGR* after hypoxic stress in BeWo cells demonstrated similar regulation to those in placentas from patients with preeclampsia. As indicated in Fig. 3, mRNA expression levels of *ESR1* were reduced by 1.3-fold following hypoxia (Fig. 3B), whereas *ESR2* and *PGR* levels were significantly elevated by 1.9- and 1.7-fold (Fig. 3C and D), respectively, compared with the levels in the normoxia group. Protein expression levels of ESRs and PGR were also analyzed (Fig. 4A). ESR1 protein expression following hypoxia was reduced (Fig. 4B), while ESR2 and PGR expression levels were evaluated compared with the levels in the normoxia group (Fig. 4C and D). These results were consistent with the transcriptional results. In other *in vitro* preeclampsia conditions, the expression levels of ESRs and PGR were not significantly altered by COMT-in or L-NAME (data not shown).

Immunofluorescent localization of ESR1, ESR2 and PGR proteins. To analyze the spatial localization of ESR1, ESR2 and PGR in BeWo cells according to *in vitro* preeclamptic conditions, immunocytochemistry was performed (Fig. 5). First, the localization of ESR1 was examined and it was revealed that the protein was predominantly localized in the nucleus in the control group, and the localization was not changed by hypoxia, COMT-in or L-NAME (Fig. 5A). In the case of ESR2, the localization was limited to the nucleus in the control, hypoxia and L-NAME groups. However, when the cells were treated with COMT-in, the ESR2 protein was observed to be located in the cytoplasm (Fig. 5B). The expression pattern of PGR was similar to that of ESR2, indicating that PGR was present in the nucleus in the control, hypoxia and L-NAME groups, and in the cytoplasm in the COMT-in-treated group (Fig. 5C).

Discussion

Preeclampsia is a common pregnancy-specific syndrome characterized by hypertension and proteinuria (2). Despite the fact that preeclampsia is regarded as a major disorder in pregnant women, its actual cause and physiological characteristics are not clearly defined. Although it is assumed that sex steroid hormones, including estrogen and progesterone, may be associated with preeclampsia, expression and regulation of steroid receptors in the preeclamptic placenta have not been well studied. The present study examined the expression levels of sex steroid receptors, including *ESR1, ESR2* and *PGR*, in the preeclamptic placenta. Initially, to confirm that the placenta samples were derived from normal and preeclamptic placentas, mRNA expression levels of *VEGF* and *sFlt1*, biomarker genes of preeclampsia, were examined. Angiogenesis requires complex interplay between VEGF and its cognate receptor, VEGF receptor 1 (VEGFR1), which is alternatively named fms-like tyrosine kinase (Flt1) (33). Placental cells also secrete a soluble isoform of Flt1 (sFlt1), which is generated through alternative splicing of *Flt1* mRNA, and acts as an anti-angiogenic factor by interacting with, and thereby neutralizing, VEGF (34). There is strong evidence to suggest increased placental expression of *sFlt1* and reduced expression of free bioactive *VEGF* in patients with preeclampsia (35). In the present results, *VEGF* expression was decreased while *sFlt1* expression was increased in the preeclampsia group compared with the levels in the normal group, as expected,

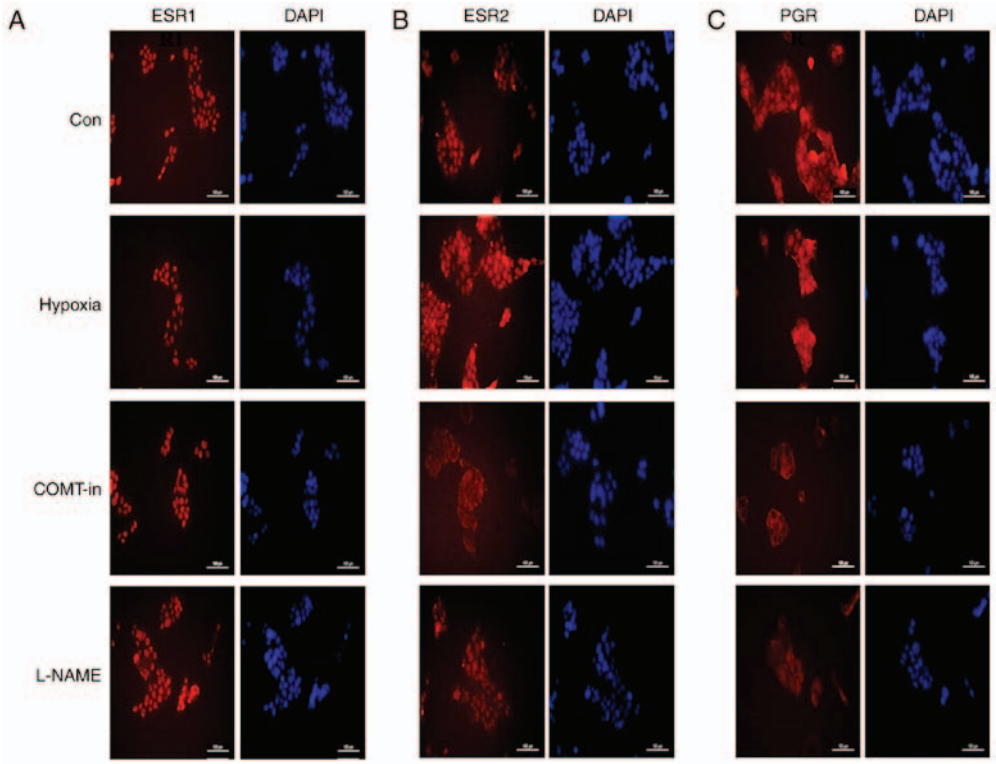

Figure 5. Immunocytochemistry analyses for ESR1, ESR2 and PGR proteins in BeWo cells under hypoxic conditions and following treatment with COMT-in and L-NAME. Cells were cultivated under hypoxic conditions, or treated with COMT-in (10^{-5} M) or L-NAME (10^{-4} M) for 24 h. Cells were stained with primary antibodies against total (A) ESR1, (B) ESR2 and (C) PGR. DAPI served as a counter stain. Scale bar, 100 μm. ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; PGR, progesterone receptor; COMT-in, catechol-O-methyl transferase inhibitor; L-NAME, L-NG-nitroarginine methyl ester; Con, control.

suggesting that the present placental samples were classified correctly.

To the best of our knowledge, expression of PGR has not been previously evaluated in the placentas of women with preeclampsia, while there have been a few studies that have examined the expression of *ESRs* in the preeclamptic placenta (36). A study by Yin *et al* (36) reported a significant increase of *ESR1* mRNA in preeclamptic placentas compared with that in normal pregnancies. However, Schiessl *et al* (37) reported that ESR protein levels were not significantly altered in preeclamptic placentas, although only immunostaining was performed in their study. In contrast to the report by Yin *et al* (36), the present study demonstrated reduced *ESR1* expression in the preeclampsia group compared with the normal group, whereas *ESR2* and *PGR* expression were increased at the mRNA and protein levels. It is well established that *ESR1* and *ESR2* are differentially expressed in various human tissues. Significantly elevated *ESR1* expression was observed in macroadenomas compared with microadenomas, and in non-invasive compared to invasive tumors (38). *ESR1* expression significantly increased while *ESR2* decreased in human non-functional pituitary adenomas, suggesting that the balance between ESR1 and ESR2 may affect the invasiveness of these tumors (38).

To study the association between steroid receptors and preeclampsia, the present study generated *in vitro* preeclamptic conditions. Although the accurate cause of preeclampsia remains unclear, lowered placental vascularization and hypoxia are the most common model for preeclampsia (39,40). Therefore, placental cells were incubated in a hypoxic chamber and the expression levels of steroid receptors were examined. Although the effects of hypoxia were not robust, the expression pattern of steroid receptors was similar to those in the placenta of women with preeclampsia, with decreased *ESR1* and increased *ESR2* and *PGR* expression levels. In addition to hypoxia, preeclampsia models may be constructed by stimulating the cells with L-NAME and COMT-in (39). L-NAME is a potent inhibitor of nitric oxide synthase and induces oxidative stress by reducing nitric oxide production (41). COMT is a key enzyme in the metabolism of estrogen, and generates 2-methoxyestradiol (2ME) that is increased during pregnancy and is related to cytotrophoblast invasion and preeclampsia (42). A study by Kanasaki *et al* (43) demonstrated that the plasma concentration of 2ME and placental COMT activity were reduced in women with preeclampsia (43). In addition, other previous studies have suggested that treatments with COMT and L-NAME may be used as models of preeclampsia (43,44). However, the mechanism of 2ME has not been sufficiently studied. In the present study, L-NAME and COMT-in did not significantly alter the expression of the steroid receptors.

Steroid receptors, including ESR and PGR, are classified as nuclear receptors, which directly bind to DNA and regulate the expression of target genes (45). Therefore, the localization of the steroid receptors is critical for their activation and function. In normal conditions, the ESR1, ESR2 and PGR proteins were present in the nucleus when the cells were immunostained in the present study. The hypoxia and L-NAME treatment did not alter the localization of the receptors; however, COMT-in

translocated ESR2 and PGR from the nucleus to the cytoplasm, suggesting that ESR2 and PGR may be inactivated when the action of 2ME is blocked in placental cells. It is also possible that *ESR2* and *PGR* expression was enhanced in the preeclamptic placenta to compensate for their inactivation caused by reduction of 2ME.

Taken together, the present study suggested that ESRs and PGR were dynamically associated with preeclampsia. In preeclamptic placenta samples and hypoxic conditions, the mRNA and protein expression levels of ESR1 were reduced. Furthermore, *in vitro* study indicated that the activation of ESR2 and PGR was blocked by treatment with COMT-in. The reduced ESR1 signals and inactivation of ESR2 and PGR may be associated with the physiological symptoms of preeclampsia.

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Competing interests

The authors declare that they have no competing interests.

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