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Original Article

Upregulation of Angiogenic Factors via Protein Kinase C and Hypoxia-induced Factor-1α Pathways under High-glucose Conditions in the Placenta

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Abnormal glucose metabolism during pregnancy is an established risk factor for preeclampsia (PE). Disruption of the balance between placental angiogenic factors is linked to PE pathophysiology. We examined whether hypoxia-induced factor-1α (HIF-1α) and protein kinase Cβ (PKCβ) are involved in the regulation of placental angiogenic factors under high-glucose conditions *in vitro*. The human choriocarcinoma cell lines BeWo and JEG-3, and the human trophoblast cell line HTR-8/SVneo were cultured with 10 and 25 mmol/L glucose [control glucose group (CG) and high-glucose group (HG), respectively]. We examined the changes in HIF-1α, soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), and vascular endothelial growth factor (VEGF) expression in the CG and HG by real-time PCR and ELISA. PKC activation was also measured by ELISA. The expressions of HIF-1α, sFlt-1, PlGF, and VEGF were significantly higher in the HG than in the CG. PKC activity was significantly increased in the HG. High glucose affected the expression of angiogenic factors in choriocarcinoma cells via the PKCβ and HIF-1α pathways, suggesting their involvement in PE pathogenesis.

Key words: high-glucose condition, preeclampsia, protein kinase C

Preeclampsia (PE) is defined as hypertension accompanied by albuminuria occurring between 20 weeks of gestation and 12 weeks post-partum in pregnant women [1-8]. PE causes severe complications such as death and cerebral hemorrhage in the mother, and death and growth retardation in the fetus. PE has a major influence on the prognosis of pregnancy. The pathogenesis of PE is explained by the two-stage disorder theory proposed by Roberts, according to which an increase in the expression of hypoxia-induced factor-1α (HIF-1α) and the collapse of the balance between angiogenic and antiangiogenic factors are involved in the development of PE [9]. The pathogenesis of PE has been reported to differ depending on the severity [10,11] and onset time [12,13], and the influence on the mother's body and fetus differs depending on the type of disease [14-16]. Therefore, some hitherto unknown mechanism might also play a role in the pathogenesis of PE.

Several risk factors have been associated with PE, and abnormal glucose metabolism during pregnancy, such as that in pregnancy with diabetes and gestational diabetes, complicates PE [17]. In abnormal glucose metabolism, a large amount of glucose incorporated into cells might activate protein kinase C (PKC)

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through a *de novo* diacylglycerol synthesis pathway [18]. PKC is a protein family comprising 10 or more isozymes [19]. It phosphorylates the hydroxyl group of the serine-threonine residue and plays a central role in intracellular signaling pathways such as those involving mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF-κB), and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [20-22]. The activation of PKC is intimately involved in the development of diabetic microangiogenic complications such as diabetic retinopathy and diabetic nephropathy, and it has been reported that administration of the PKCβ-specific inhibitor ruboxistaurin hydrochloride (LY333531) improves or suppresses the development of diabetic microangiogenic complications [23-26].

In this study, we examined whether HIF-1α and PKC are involved in the regulation of angiogenic factors of the placenta under high-glucose conditions *in vitro*. In addition, we examined whether the production of HIF-1α, an angiogenic factor, is inhibited by LY333531.

Materials and Methods

Cell culture. The choriocarcinoma cell line BeWo (Japanese Collection of Research Bioresources Cell Bank, Saitoasagi, Ibaraki, Osaka) was cultured in Ham's F-12K medium containing 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (all from Gibco, Grand Island, NY, USA). The choriocarcinoma cell line JEG-3 (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin/amphotericin B (all from Gibco). The trophoblast cell line HTR-8/SVneo (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI containing 5% FBS and 1% penicillin/streptomycin/amphotericin B (all from Gibco). BeWo and JEG-3 were plated in medium supplemented with 10 mmol/L glucose [control glucose concentration; control group (CG)] or 25 mmol/L glucose [high-glucose concentration; high-glucose group (HG)] and cultured for 6 and 24 h. HTR-8/SVneo was plated in medium supplemented with 10 mmol/L glucose [control glucose concentration; control group (CG)] or 25 mmol/L glucose [high-glucose concentration; high-glucose group (HG)] and cultured for 24 h. 1.0×10^6 cells were plated per well. Where indicated, cultures were treated with 200 nM ruboxistaurin hydrochloride [24], a PKCβ-specific inhibitor (LY333531; Tocris Bioscience, Avonmouth, Bristol, UK), and 10 μM methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate, a HIF-1α inhibitor (Santa Cruz Biotechnology, Dallas, TX, USA). The cells were cultured under an atmosphere of humidified 5% CO_2/air at 37°C. After exposure to various culture conditions, the cells were harvested. Total RNA was extracted from the cells according to the protocol included in the RNeasy Mini Kit (Qiagen, Hilden, Land Nordrhein-Westfalen, Germany) and stored at −80°C until analysis. Supernatants were collected and stored at −30°C until analysis.

Real-time reverse transcriptase polymerase chain reaction. Total RNA (10 μg) was reverse-transcribed in 20 μL of reaction solution according to the protocol of a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Amplification of soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), vascular endothelial growth factor (VEGF), HIF-1α, and β-actin genes was performed. β-actin was used as an internal control. Amplification was performed on a STEP ONE PCR system (Applied Biosystems) with initial denaturation at 95°C for 15 sec, followed by 50 cycles of annealing at 60°C with a final extension at 60°C for 1 min. The results of real time PCR were expressed by the comparative CT. The value of CG in each experiment was set to 1, and the value of HG in each experiment was calculated as the ratio to the value of CG.

Enzyme-linked immunosorbent assay. Culture supernatants were assayed for sFlt-1 (Aviscera Bioscience, Santa Clara, CA, USA), PlGF (Aviscera Bioscience), and VEGF (Aviscera Bioscience), using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. Assays for sFlt-1, PlGF, and VEGF were performed in BeWo and JEG-3 cells. The concentration of CG in each experiment was set to 1, and the concentration of HG in each experiment was calculated as the ratio to the value of CG.

Protein kinase C kinase activity. The kinase activity of PKC was assayed in BeWo and JEG-3 cells according to the manufacturer's protocol of the PKC kinase activity assay kit (Abcam, Cambridge, UK). Activation of PKC was calculated as absorbance value of the ELISA assay detecting phosphorylated form of spe-

cific synthetic peptide as a substrate divided by quantity of crude protein used per assay. The activation of PKC of CG in each experiment was set to 1, and the activation of PKC of HG in each experiment was calculated as the ratio to the value of CG. Samples were collected with lysis buffer including protease inhibitor and stored at −80°C until analysis.

Statistical analysis. All data are presented as the mean percentage of the control ± SE. Statistical analyses were performed by the Student's *t*-test and ANOVA for comparison with the control. The analyses were performed using the Software Package for Social Science (Armonk, NY, USA). Differences were considered significant at $p < 0.05$.

Results

mRNA expressions of sFlt-1, PlGF, and VEGF in BeWo and JEG-3 cells under control and high-glucose conditions. BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose conditions, and the mRNA expressions of sFlt-1, PlGF, and VEGF were examined by real-time PCR. Expression levels in the CG and HG cultured for 6 h did not differ significantly. However, the mRNA expressions of sFlt-1, PlGF, and VEGF were significantly higher in the HG than in the CG cultured for 24 h (Fig. 1A, B, C).

Protein expressions of sFlt-1, PlGF, and VEGF under control and high-glucose conditions in BeWo and JEG-3 cells. BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose condi-

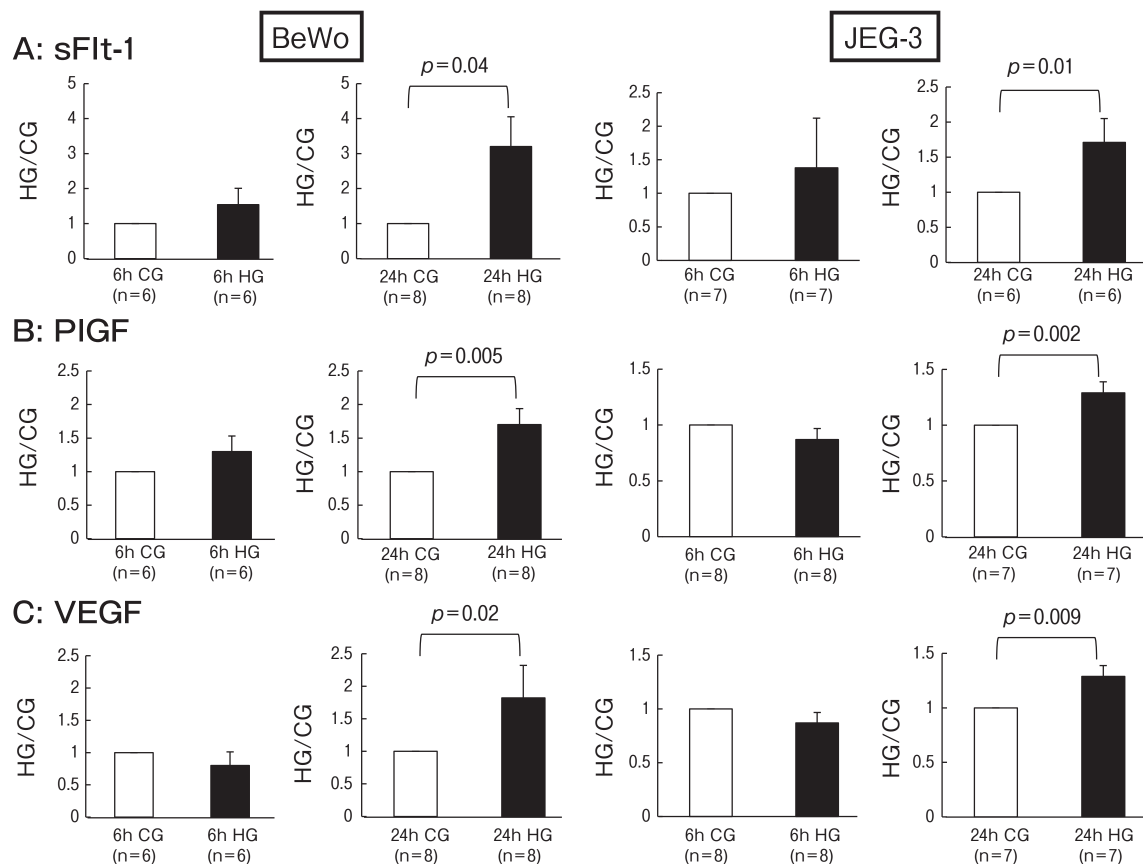

Fig. 1 BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose conditions (CG and HG, respectively), and the mRNA expressions of sFlt-1, PlGF, and VEGF were examined by real-time PCR. **A**, mRNA expression of sFlt-1; **B**, mRNA expression of PlGF; **C**, mRNA expression of VEGF. White bars = CG, black bars = HG. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results were analyzed by Student's *t*-test, 24 h CG vs. 24 h HG.

tions, and the expression levels of the sFlt-1, PlGF, and VEGF proteins were examined by ELISA. The levels of the sFlt-1, PLGF, and VEGF proteins did not differ between the CG and HG cultured for 6 h; however, the protein levels were higher in the HG than in the CG after 24 h of culture (Fig. 2A, B, C).

Activation of protein kinase C under control and high-glucose conditions in BeWo and JEG-3 cells. BeWo and JEG-3 cells were cultured for 24 h under control and high-glucose conditions, and activation of PKC was examined by ELISA. PKC activation was significantly higher in the HG than in the CG after 24 h of culture (Fig. 3A). Moreover, the increase in the mRNA expressions of sFlt-1, PLGF, and VEGF in the HG was suppressed by treatment with 200 nM LY333531 (Fig. 3B, C, D).

mRNA expression of HIF-1α under control and high-glucose conditions in BeWo and JEG-3 cells. BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose conditions, and the mRNA expression of HIF-1α was assessed by real-time PCR. HIF-1α mRNA expression was significantly higher in the HG than in the CG after 24h of culture (Fig. 4A). Moreover, treatment with 200 nM LY333531 suppressed the increase in HIF-1α mRNA expression in the HG (Fig. 4B). The increase in the mRNA expressions of HIF-1α, sFlt-1, PlGF, and VEGF in the HG was suppressed by treatment with 10 μM methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate, a HIF-1α inhibitor (Fig. 5A, B, C, D).

mRNA expressions of sFlt-1, PlGF, VEGF and HIF-1α in HTR-8/SVneo cells under control and

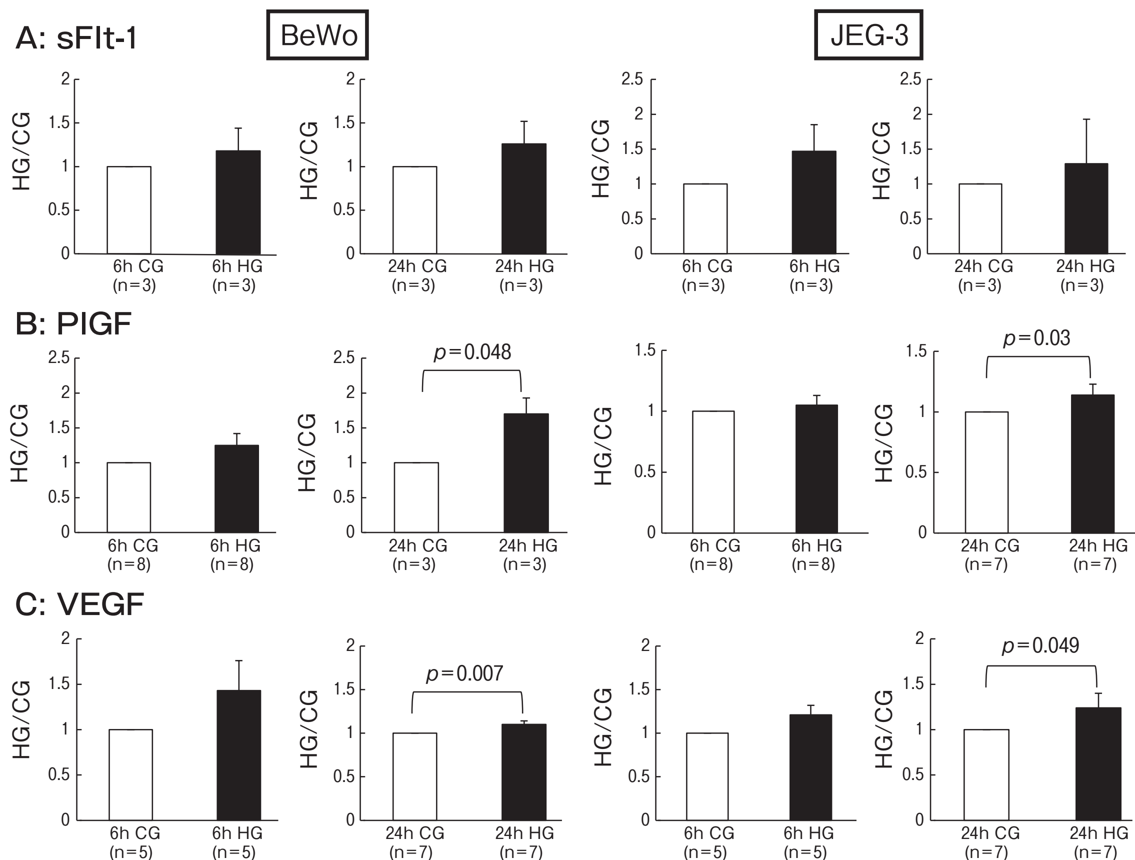

Fig. 2 BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose conditions (CG and HG, respectively), and sFlt-1, PlGF, and VEGF protein expressions were examined by ELISA. **A**, Expression of sFlt-1; **B**, Expression of PlGF; **C**, Expression of VEGF. White bars = CG, black bars = HG. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results were analyzed by Student's *t*-test, 24 h CG vs. 24 h HG.

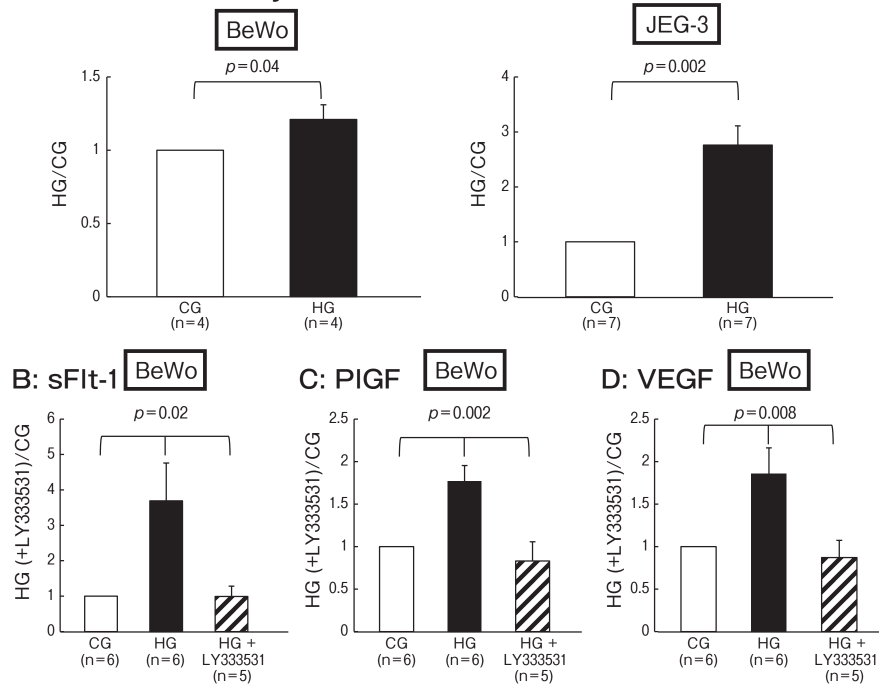

Fig. 3 BeWo and JEG-3 cells were cultured for 24 h under control and high-glucose conditions (CG and HG, respectively), and the activation of PKC was examined by ELISA. **A**, Activation of PKC kinase. White bars = CG, black bars = HG. The PKC*β*-specific inhibitor ruboxistaurin hydrochloride (LY333531; 200 nM) was administered in the cultures, and the mRNA expressions of sFlt-1, PlGF, and VEGF in BeWo cells were examined by real-time PCR; **B**, mRNA expression of sFlt-1; **C**, mRNA expression of PlGF; **D**, mRNA expression of VEGF. White bars = CG, black bars = HG, black shaded bars = HG + LY333531. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results of **A** were analyzed by Student's *t*-test, CG vs. HG. The results of **B**, **C**, **D** were analyzed by ANOVA, CG vs. HG vs. HG + LY333531.

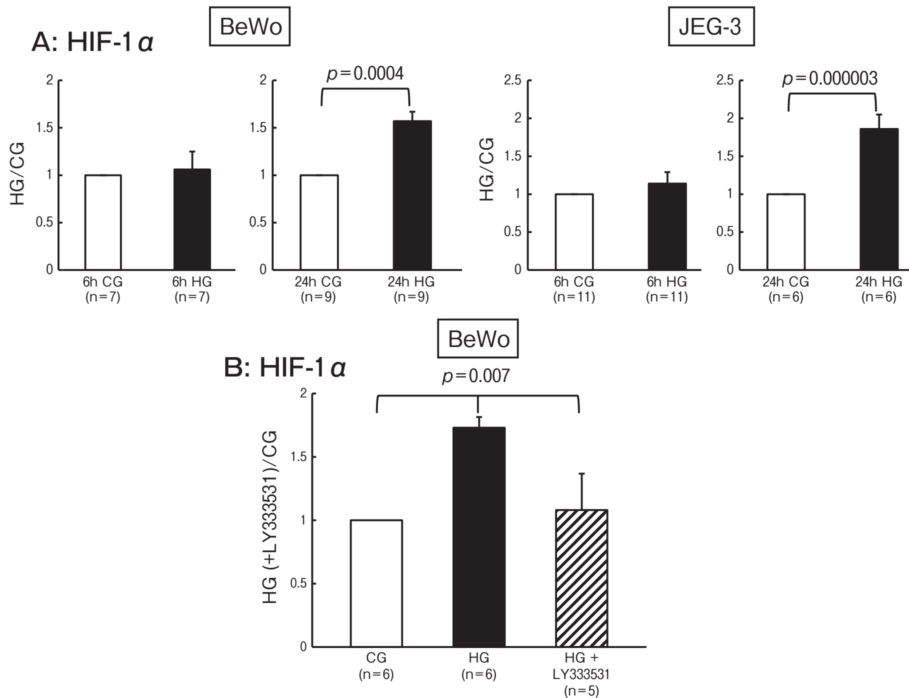

Fig. 4 BeWo and JEG-3 cells were cultured for 6 h and 24 h under control and high-glucose conditions (CG and HG, respectively), and the mRNA expression of HIF-1*α* was examined by real-time PCR. **A**, mRNA expression of HIF-1*α*. The PKC*β*-specific inhibitor ruboxistaurin hydrochloride (LY333531; 200 nM) was administered in the cultures, and the mRNA expression of HIF-1*α* in BeWo cells was examined by real-time PCR; **B**, mRNA expression of HIF-1*α*. White bars = CG, black bars = HG, black shaded bars = HG + LY333531. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results of **A** were analyzed by Student's *t*-test, 24 h CG vs. 24 h HG. The results of **B** were analyzed by ANOVA, CG vs. HG vs. HG + LY333531.

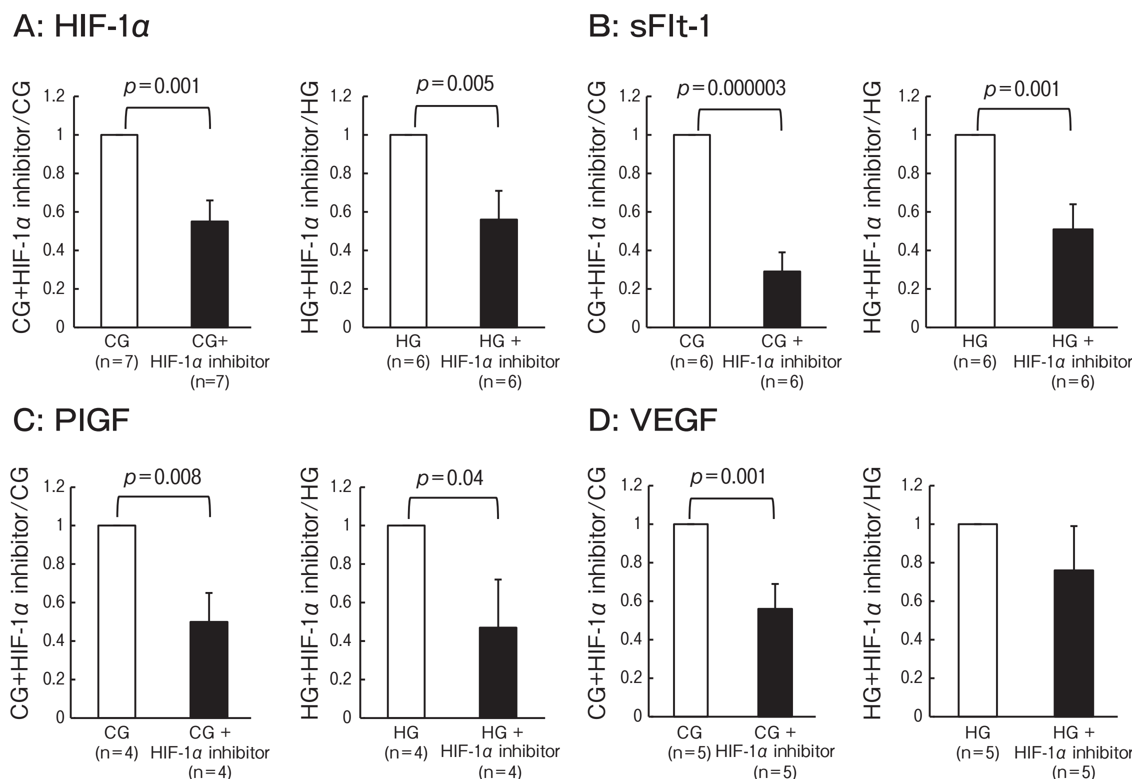

Fig. 5 BeWo cells were cultured for 24 h under control and high-glucose conditions (CG and HG, respectively), and the HIF-1α inhibitor methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate was administered in the cultures. We examined the mRNA expressions of HIF-1α, sFlt-1, PlGF, and VEGF by real-time PCR. **A**, mRNA expression of HIF-1α; **B**, mRNA expression of sFlt-1; **C**, mRNA expression of PlGF; **D**, mRNA expression of VEGF. White bars = CG and HG, black bars = CG + HIF-1α inhibitor and HG + HIF-1α inhibitor. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results were analyzed by Student's *t*-test, CG vs. CG + HIF-1α inhibitor and HG vs. HG + HIF-1α inhibitor.

high-glucose conditions. HTR-8/SVneo cells were cultured for 24 h under control and high-glucose conditions, and the mRNA expressions of sFlt-1, PlGF, VEGF and HIF-1α were examined by real-time PCR. The mRNA expressions of sFlt-1, PlGF, VEGF and HIF-1α were significantly higher in the HG than in the CG cultured for 24 h (Fig. 6A, B, C, D).

Discussion

In this study, we demonstrated that the expressions of sFlt-1, PlGF, and VEGF increased significantly under high levels of glucose. The activation of PKC was increased in the HG, and 200 nM LY333531 suppressed the increase in the mRNA expressions of sFlt-1, PlGF, and VEGF in the HG. In addition, HIF-1α mRNA expression was also significantly elevated in the HG, and LY333531 administration suppressed this increase. The expressions of HIF-1α, sFlt-1, PlGF, and VEGF in the HG were also suppressed by administration of 10 μM methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate.

The expression of HIF-1α and antiangiogenic factors such as sFlt-1 and soluble endoglin (sEng) might be closely linked with the pathogenesis of PE [27-29]. The levels of antiangiogenic factors such as sFlt-1 are known to be elevated [30-32] — and those of angiogenic factors such as PlGF to be decreased [33] — in the pathogenesis of PE. There have been conflicting reports in regard to the vasculogenic and angiogenic factor VEGF (*i.e.*, whether the levels of VEGF are increased or decreased in PE) [34, 35]. However, it is clear that the collapse of the balance between these angiogenic and antiangiogenic factors is important in the pathogenesis of PE,

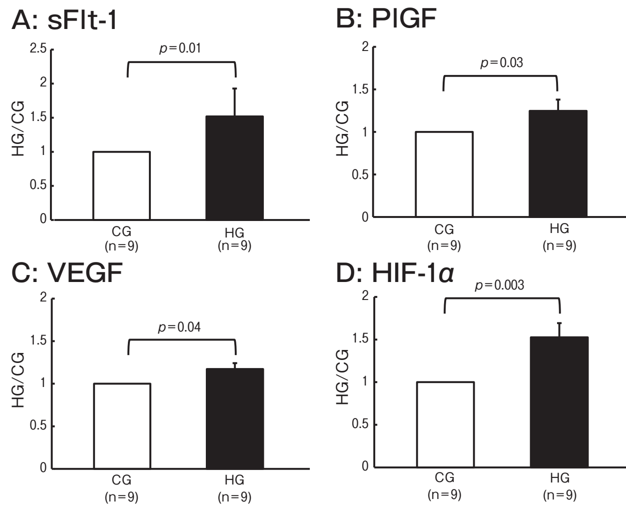

Fig. 6 HTR-8/SVneo cells were cultured for 24 h under control and high-glucose conditions (CG and HG, respectively), and the mRNA expressions of sFlt-1, PlGF, VEGF and HIF-1*α* were examined by real-time PCR. **A**, mRNA expression of sFlt-1; **B**, mRNA expression of PlGF; **C**, mRNA expression of VEGF; **D**, mRNA expression of HIF-1*α*. White bars = CG, black bars = HG. Results in all figures are shown as the mean ± SEM of data from at least 3 separate experiments, each performed with triplicate samples. Differences were considered significant at $p < 0.05$. The results were analyzed by Student's *t*-test, 24 h CG vs. 24 h HG.

and might be responsible for various clinical findings in the disorder.

In this study, HIF-1α, sFlt-1, PlGF, and VEGF levels were elevated in BeWo, JEG-3 and HTR-8/SVneo cells in the HG. In the placenta of pregnant women with abnormal glucose metabolism, when maternal blood glucose control is poor, the number of immature villi of the narrow intervillous space increases and ischemic changes occur in peripheral villi in the placenta [36]. Angiogenesis and chorangiosis occur as a result of persistent hypoxia and ischemia in the villi [37], whereas maternal blood pressure might be elevated and fetal growth restriction might occur in such cases. These pathological changes in the placenta resemble those occurring in the placenta of pregnant women with PE [36]. In the placenta exposed to high glucose levels, increases in the levels of HIF-1α and the angiogenic factors sFlt-1, PlGF, and VEGF are expected.

It has been reported that PlGF levels are low in cases of PE caused by remodeling failure of the spiral artery in early pregnancy. However, in an earlier study, we reported that sFlt-1 levels were lower and PlGF levels were higher in an obese group of pregnant women who developed PE than in a non-obese group of pregnant women who developed PE. Moreover, the pathogenesis of PE in pregnant women with high insulin resistance (*e.g.*, obesity) differs from the pathogenesis of PE caused by remodeling failure of the spiral artery in early pregnancy [38]. In the present study, the levels of PlGF and VEGF were also increased, similarly to those of HIF-1α and sFlt-1, in the HG. Therefore, the placenta under high levels of glucose exhibits increased production of PlGF and VEGF as well as HIF-1α and sFlt-1, suggesting that the pathogenesis of PE in pregnant women with high insulin resistance (including those with conditions such as obesity) and abnormal glucose metabolism might differ from the pathogenesis of PE caused by remodeling failure of the spiral artery in early pregnancy. Thus, an imbalance between these angiogenic and antiangiogenic factors is implicated in the development of PE.

The activation of PKC caused by hyperglycemia has been found to be related to blood vessel abnormalities in the retina, kidneys, and cardiovascular system [20]. Activation of PKC has also been reported to activate MAPK, NF-κB, and NADPH oxidase [20-22]. MAPK, the phosphoinositide 3-kinase-Akt (PI3K-Akt) pathway, and the mammalian target of rapamycin pathway (mTOR) pathway are key signaling pathways in angiogenesis. Ras in the MAPK pathway is activated by PKC [39]. Activated Ras in turn activates the Pl3K-Akt pathway, and the Pl3K-Akt pathway increases the production of HIF-1α via the mTOR pathway [40,41]. That is, activation of PKC increases the production of HIF-1α via the MAPK, PI3K-Akt, and mTOR pathways. NF-κB also increases the production of HIF-1α [42]. In this study, the activation of PKC was significantly higher in the HG than in the CG after 24 h of culture. Further, the increase in the mRNA expression levels of HIF-1α, sFlt-1, PlGF, and VEGF in the HG with LY333531 treatment was suppressed, and the increase in the mRNA expression levels of sFlt-1, PlGF, and VEGF in the HG with methyl 3-[[2-[4-(2-adamantyl) phenoxy]acetyl]amino]-4-hydroxybenzoate treatment was also suppressed. Therefore, the activation of PKC in villous cells under high-glucose conditions might increase the production of HIF-1α, angiogenic factors and antiangiogenic factors, and cause the collapse between angiogenic factors and antiangiogenic factors via the MAPK, NF-κB, PI3K-Akt, and mTOR path-

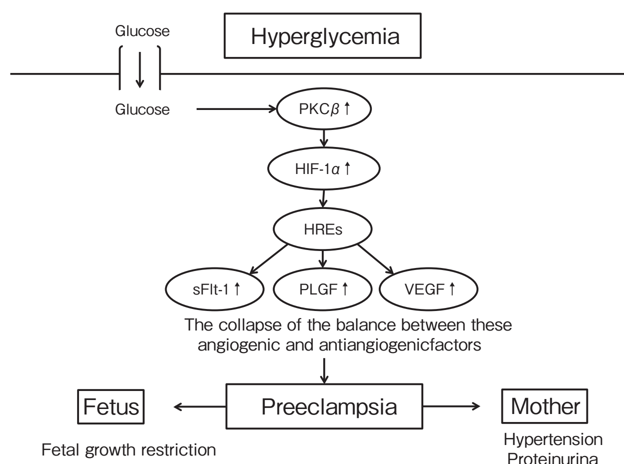

Fig. 7 Activation of PKC in hyperglycemia increases the expression of HIF-1*α* via the MAPK, NF-*κ*B, PI3K-Akt, and mTOR pathways. HIF-1*α* binds to hypoxia responsive elements (HREs). The production of HIF-1*α* increases angiogenic and antiangiogenic factors in human choriocarcinoma cells and human trophoblast cells, and causes the collapse of the balance between these angiogenic and antiangiogenic factors.

ways (Fig. 7).

Important limitations of this study should be acknowledged. First, the study was performed only *in vitro*, and thus the extensibility of the findings to an *in vivo* setting is uncertain. In addition, we used human choriocarcinoma cell lines and a human trophoblast cell line, rather than normal placental cells. In the future, these experiments should be replicated using cells derived from the normal placenta.

In conclusion, we demonstrated that in human choriocarcinoma cells and human trophoblast cells under high-glucose conditions, the production of HIF-1α and angiogenic factors increased and PKC was activated. In addition, the inhibition of PKC and HIF-1α suppressed the production of angiogenic factors, suggesting the possibility of controlling the vascular lesions of the placenta in pregnancies complicated with abnormal glucose metabolism via the PKCβ and HIF-1α pathways. Although further *in vivo* study will be needed, our results suggest that in the clinical setting, agents such as the PKCβ-specific inhibitor LY333531 could control or prevent the development of PE in pregnant women with abnormal glucose metabolism.

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