Overexpression of hypoxia-inducible factor prolyl hydroxylase-2 attenuates hypoxia-induced vascular endothelial growth factor expression in luteal cells

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Abstract. Vascular endothelial growth factor (VEGF)-dependent angiogenesis has a crucial role in the corpus luteum formation and their functional maintenances in mammalian ovaries. A previous study by our group reported that activation of hypoxia-inducible factor (HIF)-1α signaling contributes to the regulation of VEGF expression in the luteal cells (LCs) in response to hypoxia and human chorionic gonadotropin. The present study was designed to test the hypothesis that HIF prolyl-hydroxylases (PHDs) are expressed in LCs and overexpression of PHD2 attenuates the expression of VEGF induced by hypoxia in LCs. PHD2-overexpressing plasmid was transfected into LC2 cells, and successful plasmid transfection and expression was confirmed by reverse transcription quantitative polymerase chain reaction and western blot analysis. In addition, the present study investigated changes of HIF-1α and VEGF expression after incubation under hypoxic conditions and PHD2 transfection. PHD2 expression was significantly higher expressed than the other two PHD isoforms, indicating its major role in LCs. Moreover, a significant increase of VEGF mRNA expression was identified after incubation under hypoxic conditions, which was, however, attenuated by PHD2 overexpression in LCs. Further analysis also indicated that this hypoxia-induced increase in the mRNA expression of VEGF was consistent with increases in the protein levels of HIF-1α, which is regulated by PHD-mediated degradation. In conclusion, the results of the present study indicated that PHD2 is the main PHD expressed in LCs and hypoxia-induced VEGF expression can be attenuated by PHD2 overexpression through HIF-1α-mediated mechanisms in LCs. This PHD2-mediated transcriptional activation may be one of the mechanisms regulating VEGF expression in LCs during mammalian corpus luteum development.

Introduction

Vascular endothelial growth factor (VEGF) has a fundamental role in the physiological angiogenesis and the vascularization of the follicular luteinizing granulosa layer during corpus luteum (CL) formation (1-4). Inhibition of VEGF in vivo during the luteal phase prevents luteal angiogenesis and subsequent progesterone secretion (5-8), while an excess VEGF generation during the vascularization of multiple follicles is also thought to cause ovarian hyperstimulation syndrome (OHSS) (9-11). Therefore, the molecular regulation of luteal VEGF expression becomes more and more important.

A previous study by our group reported that hypoxia-inducible factor (HIF)-1α contributes to the transcriptional regulation of VEGF in luteal cells (LCs) (12). HIF-1, a helix-loop-helix transcriptional factor, which consists of HIF-1α and HIF-1β, has been cloned and characterized as a transcriptional activator of numerous oxygen-sensitive genes, including erythropoietin (13,14), heme oxygenases (15,16), transferrin (16), and several glycolytic enzymes (13-17). It has been indicated that HIF-1α is inducible by a decrease in tissue or cellular O2 (15,16). HIF-1β is not inducible, but it can bind to HIF-1α to form a dimer to activate the transcription of numerous genes containing cis hypoxia-response element (HRE) in their promoter or enhancer regions (15,16). Previously, a chromatin immunoprecipitation (ChIP) assay indicated that estrogen can simultaneously induce the recruitment of HIF-1α as well as -β to the upstream HRE and ERα to the proximal GC-rich region of the VEGF promoter, which mediates transcriptional activation of the murine VEGF gene (18-20).

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It has been demonstrated that PHDs are the major enzymes to promote the degradation of HIF-1α (21-23). PHD catalyzes site-specific proline hydroxylation of HIF-1α, and PHD is recognized and targeted for degradation by the ubiquitin-proteasome pathway. Three isoforms of PHD, including PHD1, -2, and -3, have been identified (21-24). Previous studies by our group have indicated that PHD2 participates in functional regulation, including sensing high-salt intake and maintaining normal blood pressure, through regulating HIF-α levels (25) and also has a role in angiogenesis (26).

Given the role of PHD2 in the regulation of HIF-1α levels, it was hypothesized in the present study that the PHD2 signaling pathway has a role in ovarian angiogenesis and that overexpression of PHD2 attenuates the expression of VEGF induced by hypoxia in LCs. The present study examined the effect of hypoxia on the expression of HIF-1α and determined the role of PHD2, the primary isoform of PHDs, in hypoxia-induced activation of HIF-1α by transfection of PHD2-expressing vectors into LCs. The effect on VEGF mRNA expression was also examined. The results indicated that PHD2 is a mediator of cellular HIF-1α and its target gene VEGF in LCs, which may be an important regulatory mechanism of VEGF-dependent angiogenesis during mammalian CL development.

Materials and methods

Animals. 25-day-old Female Sprague-Dawley (SD) rats (Fuzhou Animal Center, Fuzhou, China) were used in the present study. All animals were maintained under a 12-h light/dark cycle with food and water available ad libitum. The study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee and was approved by the Ethics Committee on Animal Experimentation of Fujian Normal University. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

Isolation and culture of rat LCs. Rat LCs were isolated and cultured according to previously described procedures (27-29). Briefly, the rats received a subcutaneous injection of equine chorionic gonadotropin (eCG; Sigma-Aldrich, St. Louis, MO, USA; 50 international units (IU)) and an ovariolytic dose (25 IU) of human chorionic gonadotropin (hCG; Sigma-Aldrich) 64 h later. Ovaries were obtained at day 5 after hCG injection and minced with a razor blade. Tissue was digested in medium 199 (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) containing 1% fetal calf serum (FCS; Gibco-BRL) plus 3,000 IU of DNase (Sigma-Aldrich) 64 h later. The culture medium was rapidly replaced by TRIzol solution (Invitrogen Life Technologies), and total RNA was extracted for determination of HIF-1α and VEGF mRNA. For HIF-1α protein determination, the cultured cells were scraped immediately and placed in ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 μM EDTA, protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), pH 7.4), frozen in liquid nitrogen and then stored at -80°C for western blot analysis.

Cell hypoxia. Cell culture under hypoxia with 3% O2 was performed as described previously (33,34). Briefly, LCs were plated in six-well plates 24 h prior to the experiments to reach sub-confluence. To decrease O2 pressure in the culture medium, the plates and dishes were transferred to a sealed, humidified modular chamber, the atmospheric air was evacuated by a vacuum pump, and the chamber was re-filled with a non-standard gas mixture containing 3% O2 and 5% CO2 in an N2 base. After repeating this evacuation and inflow five times, the cells were cultured for 6 h. After hypoxia, the culture medium was rapidly replaced by TRIzol solution (Invitrogen Life Technologies), and total RNA was extracted for determination of HIF-1α and VEGF mRNA. For HIF-1α protein determination, the cultured cells were scraped immediately and placed in ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 μM EDTA, protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), pH 7.4), frozen in liquid nitrogen and then stored at -80°C for western blot analysis.

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of PHD1, 2 and 3, HIF-1α and VEGF. Total RNA was extracted using TRIzol solution and then reverse-transcribed using a cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The RT products were amplified using SYBR green (Bio-Rad Laboratories, Inc.) and the following primers: PHD1 forward, 5'-GCTGTGCTGTTGTTGTTAC-3' and reverse, 5'-GCCCTCTGGTCTTCTTG-3' (GenBank accession no.: NM001004083); PHD2 forward, 5'-CTGGGACCGCAA GTGTA-3' and reverse, 5'-CAATGTCAAGAAACTGG-3' (GenBank accession no.: NM178334); PHD3 forward, 5'-GTCGCCCCCTCTATGC-3' and reverse, 5'-ACCACGTCAGTCTTA-3' (GenBank accession no.: NM019371); HIF-1α forward, 5'-CTGGCCACGGGATGATACGC-3' and reverse, 5'-TCTCATCACTTGGACTCCAG-3' (GenBank accession no.: AF057308) and VEGF forward, 5'-ACGAAACGC AAGAATCCC-3' and reverse, 5'-TAAACTCAAGCTGCC TCGCC-3' (GenBank accession no.: M32167) with an iCypher iQ Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). The levels of 18S rRNA (forward, 5'-GGCGGCTAGAG TGAATTC-3' and reverse, 5'-TCTTGGGAAATGCTT TGCC-3' (GenBank accession no.: M11888)) were used as an endogenous control. The 25 μl PCR reaction mix included 12.5 μl SYBR Green PCR Master Mix, 2.5 μl 10X primers, 1 μl cDNA template and 9 μl RNase-free water. The PCR conditions were as follows: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Relative gene expression was calculated in
according with the ΔΔCt method, with relative mRNA levels calculated as 2^-ΔΔCt.

Preparation of nuclear extracts and cytosolic protein, and western blot analysis of protein levels of HIF-1α and PHD.
Nuclear protein was prepared as previously described (12,25,32). Cytosolic protein and nuclear protein were collected separately (Beyotime Institute of Biotechnology, Haimen, China). The cytosolic protein was used for western blot analysis of PHD2, while the nuclear fraction was used for western blot analyses of HIF-1α. Briefly, protein concentration was determined using a Bio-Rad assay (Bio-Rad Laboratories, Inc.) with bovine serum albumin standards. The protein samples (30 µg) were separated by 8% SDS-PAGE, and were electrophoretically transferred to a polyvinylidene fluoride membrane (Pall Corporation, Pensacola, FL, USA). The membrane was washed with phosphate-buffered saline with 0.2% Tween-20 (PBST; Sigma-Aldrich), blocked with 5% nonfat dried milk in PBST and probed with the following primary antibodies (Novus Biologicals, Littleton, CO, USA): Mouse monoclonal anti-HIF-1α (1:300; cat. no. NB100-105), rabbit polyclonal anti-PHD2 (1:300; cat. no. NB100-137), and rabbit polyclonal anti-β-actin (cat. no. NB600-503H) overnight at 4°C. After washing, the membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse (1:5,000; cat. no. NB7570) or goat anti-rabbit (1:5,000, cat. no. NBP2-30348H) immunoglobulin G secondary antibodies (Novus Biologicals) for 1 h at room temperature. β-actin was used as a loading control (1:5,000, cat. no. NB600-501H). To detect the immunoblotting signal 2 ml enhanced chemiluminescence detection solution (Thermo Scientific, Rockford, IL, USA) was used, and the membrane was exposed to Kodak OMAT film (Eastman Kodak, Rochester, NY, USA). The blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA)

Determination of HIF prolyl hydroxylase activity. HIF-1α peptide-specific conversion of 2-oxoglutarate into succinate provides a hydroxyl group for HIF-1α to be prolyl hydroxylated. This reaction has been widely used for the determination of PHD activity by measuring HIF-1α-dependent conversion rate of 2-oxoglutarate into succinate (25,35,36).

Statistics. Values are expressed as the mean ± standard error. The significance of differences in mean values within and between multiple groups was evaluated using analysis of variance followed by a Duncan's multiple range test. Statistical analysis was conducted using Sigmastat 3.02 (Sigma-Aldrich). P<0.05 was considered to indicate a statistically significant difference.

Results
PHD2 is the most abundantly expressed PHD in LCs, and is not affected by hypoxia. RT-qPCR indicated that all of three PHDs were present, among which PHD2 was most abundantly expressed in LCs (Fig. 1), while no significant changes of these mRNA levels were found after hypoxia treatment (Fig. 1).

Efficient PHD2 transfection in LCs is not affected by hypoxia. PHD2 mRNA levels increased significantly after PHD2 trans-
Infection, even following hypoxia treatment (Fig. 2). However hypoxia was found to have no marked effect on PHD2 mRNA expression (Fig. 2), indicating the high transfection efficiency and the high expression levels of PHD2 plasmid.

Furthermore, PHD2 protein levels were detected by western blot analysis (Fig. 3). The results indicated that, similar to mRNA levels, PHD2 protein levels were enhanced following transfection, but that hypoxia had no effects on PHD2 protein levels (Fig. 3).

**Effects of hypoxia and PHD2 transfection on PHD2 protein levels and PHD2 activity in LCs.** To further confirm stable transfection of PHD2 into LCs, PHD2 biological activity was tested using the 2-oxoglutarate conversion assay (Fig. 4). In the PHD2-transfected groups, 2-oxoglutarate conversion was enhanced, however, not to a statistically significant extent. Of note, a significant decrease of PHD activity was found in LCs under hypoxic conditions, even following transfection with PHD2 plasmids (Fig. 4).

*Hypoxia increases and PHD2 transfection decreases VEGF mRNA levels in LCs, while HIF-1α mRNA is unaffected.* In the present study, hypoxia significantly increased VEGF mRNA expression in LCs (Fig. 5). To better understand the role of PHD2 in LCs, the mRNA levels of VEGF and HIF-1α in LCs transfected with PHD2 plasmid were also examined (Figs. 5 and 6). Of note, a marked decrease in VEGF levels was observed in PHD2-transfected LCs, which was consistent with the results of previous studies by our group (25,32), indicating that PHD2 may regulate VEGF expression via the HIF-1α pathway in LCs during CL development.
Discussion

The results of the present study clearly demonstrated that hypoxia induced VEGF through inhibiting HIF-1α signaling in LCs, which was attenuated by overexpression of PHD2, suggesting that PHD2-mediated VEGF expression via the HIF-1α pathway may be an important mechanism of VEGF-dependent angiogenesis during mammalian CL development.

The CL is a temporary endocrine structure in mammals, which has an important role in the female reproductive cycle and is formed temporarily from a ruptured and ovulated follicle with rapid angiogenesis (5,6,11,37,38). VEGF is thought to have a paramount role in the regulation of normal and abnormal angiogenesis in the ovary (2-4,6,9,11,39,40), particularly in the newly formed CL. Numerous studies have shown that reproductive hormones such as hCG also take part in the primary regulation of VEGF expression in the ovary. For example, VEGF mRNA expression in human luteinized granulosa cells has been shown to be dose- and time-dependently enhanced by hCG in vitro (9,10). Chronic or acute exposure to hCG directly stimulates VEGF production and secretion by monkey (1) and human luteinized granulosa cells (5,9,10,40).

Furthermore, the administration of a gonadotropin-releasing hormone antagonist decreased VEGF mRNA expression in the CL of monkeys (41). In addition, luteal vascularization and the development of ovarian hyperstimulation syndrome (OHSS) are absolutely dependent on LH/hCG stimulation (9,10). Furthermore, in a fully formed, highly vascular CL hCG also up-regulates VEGF expression (5). However, a previous study by our group has already provided direct evidence that VEGF is transcriptionally activated by a HIF-1-mediated mechanism in LCs under hypoxia (12), which is caused by ovulation of the ruptured follicle with bleeding and an immature vasculature (2,38). Therefore, the present study examined the induced effect of hypoxia and PHD2 overexpression on VEGF mRNA expression in LCs. Of note, VEGF expression was induced by hypoxia in LCs and hypoxia-stimulated HIF-1α protein expression was highly correlated with VEGF expression, indicating hypoxia stimulated VEGF expression via the HIF-1α signaling pathway.

Numerous studies have indicated that HIF-1α regulates the expression of numerous genes whose protein products have critical roles in developmental and physiological processes, including angiogenesis, erythropoiesis, glycolysis, iron transport and cell proliferation/survival (5,38,42-45). Because HIF-1α activates the transcription of VEGF, which is required for angiogenesis, it is possible that hypoxia may mediate angiogenesis via the HIF-1α/VEGF pathway. In addition to a detailed exploration of the downstream mechanism of HIF-1α (12,46-48), recent studies have clarified the upstream process modulated by PHDs (49), which regulates HIF-1α degradation by the ubiquitin-proteasome pathway. In particular, PHD2 has been drawing considerable attention because PHD2 is considered to be the key oxygen sensor of all identified PHD enzymes (25,26), as knockdown of PHD2 resulted in elevated HIF protein levels (49), and several recent studies have also highlighted the importance of PHD2 in tumourigenesis (26).

The present study reported that hypoxia induced the activation of HIF-1α and overexpression of PHD2 blocked this activation of HIF-1α and its target gene VEGF following hypoxia treatment. These results indicated that PHD2 is involved in HIF-1α-mediated gene activation in LCs under hypoxia, which may present a novel mechanism of VEGF-dependent angiogenesis during mammalian CL development.

In conclusion, the present study clearly demonstrated that hypoxia induces HIF-1α and VEGF expression in LCs. To the best of our knowledge, the present study was also the first to provide direct evidence indicating that PHDs are expressed in rat LCs and that hypoxia-induced VEGF expression can be blocked by overexpression of PHD2 in LCs. This PHD2-mediated VEGF expression may be one of the important mechanisms of VEGF-dependent angiogenesis during CL formation in the mammalian ovary. Furthermore, this PHD2 antagonism presents an opportunity for the development of novel treatments for fertility control and for certain types of ovarian dysfunction (26,44,45), particularly conditions characterized by pathological angiogenesis and excessive vascular permeability, including polycystic ovarian syndrome (PCOS), OHSS and ovarian neoplasia.

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