Full Length Research Paper

Overexpression of hypoxia-inducible factor prolyl-hydoxylase attenuated by HCG-induced vascular endothelial growth factor expression in luteal cells

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Vascular endothelial growth factor (VEGF)-dependent angiogenesis plays a crucial role in the corpus leteum formation and their functional maintenances in mammalian ovaries. We recently reported that the 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 (HCG). This study was designed to test the hypothesis that, HIF prolyl-dydoxylases (PHDs) express in LCs and overexpression of PHD attenuates the expression of VEGF induced by HCG in LCs. By real-time PCR and western blot analysis, we examined the expression of PHDs, confirmed the plasmid transfection and their expression and also investigated the changes of HIF-1α and VEGF expression after treatment with HCG and PHD2 transgenes. PHD2 expression was significantly higher than the others, indicating its main roles. Moreover, a significant increase of VEGF mRNA was found after HCG treatment, while this increased VEGF mRNA was also blocked by PHD2 overexpression in LCs. Further analysis also found that, this HCG-induced increase of VEGF mRNA was consistent with the level of HIF-1α protein, which is regulated by HIF prolyl-dydoxylase -mediated degradation. Taken together, our results indicated that, PHD2 mainly expressed in LCs and HCG-induced VEGF expression can be blocked by PHD2 overexpression through HIF-1α -mediated mechanism in LCs. This PHD2-mediated transcriptional activation may be one of the important mechanisms regulating VEGF expression in LCs during mammalian corpus leteum development.

Key words: Hypoxia-inducible factor-1α, HIF prolyl-dydoxylase, vascular endothelial growth factor, human chorionic gonadotropin, luteal cells.

INTRODUCTION

Vascular endothelial growth factor (VEGF) plays a fundamental role in the physiological angiogenesis and the vascularization of the follicular luteinizing granulosa layer during corpus luteum (CL) formation (Christenson

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Abbreviations: VEGF, Vascular endothelial growth factor; **HIF,** hypoxia-inducible factor; **HCG,** human chorionic gonadotropin; **PHDs,** prolyl-dydoxylases; **LC,** luteal cells.

and Stouffer, 1997; Kaczmarek et al., 2005; Shimizu et al., 2007a; Shimizu and Miyamoto, 2007b). Because inhibition of VEGF *in vivo* during the luteal phase will prevent luteal angiogenesis and subsequent proges-terone secretion (Duncan et al., 2008; Fraser et al., 2005; Fraser et al., 2006; Wulff et al., 2001), the excess VEGF generation during the vascularization of multiple follicles is also thought to cause ovarian hyperstimulation syndrome (OHSS) (Nastri et al., 2010; Neulen et al., 1995; Zhang et al., 2010a). Therefore, the molecular regulation of luteal VEGF expression becomes more and more important.

We recently reported that, HIF-1α contributes to the

transcriptional regulation of VEGF in LCs (Zhang et al., 2010b). 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 many oxygen-sensitive genes, such as erythropoietin, heme oxygenases, transferrin and several glycolytic enzymes (Wang et al., 1995; Wang and Semenza, 1993a, b, 1995; Wenger et al., 1996). It has been indicated that, HIF-1α is an inducible protein by a decrease in tissue or cellular O_2. HIF-1β is not inducible, but it can be bound to HIF-1α to form a dimer to activate the transcription of many genes containing cis hypoxia-response element (HRE) in their promoter or enhancer regions. Previous chromatin immuno-precipitation (ChIP) results have already indicated that, estrogen can simultaneously induce the recruitment of both HIF-1 (α and β) to the upstream HRE and ERα to the proximal GC-rich region of the VEGF promoter, which mediates transcriptional activation of the mouse VEGF gene (Kazi et al., 2005; Kazi and Koos, 2007; Molitoris et al., 2009).

It has been demonstrated that, HIF prolyl-hydroxylases are the major enzymes to promote the degradation of hypoxia inducible factor (HIF)-1α (Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001). HIF prolyl-hydroxylases catalyze site-specific proline hydroxylation of HIF-1α and prolyl-hydroxylated HIF-1α is recognized and targeted for degradation by the ubiquitin-proteasome pathway. Three isoforms of HIF prolyl-hydroxylase, including prolyl hydroxylase domain-containing proteins 1, 2 and 3 (PHD1, 2 and 3), have been identified (Bruick et al., 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). This study indicated that, PHD2 is most abundantly expressed in LCs. Our previous studies have also indicated that, PHD2 participates in the functional regulation through regulating HIF-1α level (Wang et al., 2010) and the importance of PHD2 in angiogenesis (Chan and Giaccia, 2010).

Given the important role of PHD2 in the regulation of HIF-1α levels, we hypothesize that, PHD2 signaling pathway exists in ovarian angiogenesis and over-expression of PHD2 attenuates the expression of VEGF induced by HCG in LCs. We examined the effect of HCG on the expression of HIF-1α and determined the role of PHD2 and the primary isoform of PHDs, in HCG-induced activation of HIF-1α by transfection of PHD2 transgenes into LCs. We also revealed the changes of VEGF mRNA level in each group. Our result demonstrated that, PHD2 was the mediator of cellular HIF-1α and its target gene VEGF in LCs, which may be an important mechanisms regulating VEGF-dependent angiogenesis during mammalian corpus leteum development.

MATERIALS AND METHODS

Animals

25-day-old female Sprague-Dawley (SD) rats (Fuzhou Animal Center, Fuzhou, China) were used in this study. All animals were maintained under a 12 h light/12 h dark schedule with food and water available *ad libitum*. The study was conducted in accordance with guidelines of the Institutional Animal Care and Use Committee and was approved by the Ethics Committee on Animal Experimentation of the 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 as methods described previously (Conti et al., 1977; Pepperell et al., 2003; Thomas et al., 1978). Briefly, the rats received a subcutaneous injection of equine chorionic gonadotropin (eCG; Sigma-Aldrich, St. Louis, MO, US; 50 international units (IU)) and an ovulatory dose (25 IU) of human chorionic gonadotropin (HCG; Sigma) 64 h later. Ovaries was obtained at 5 days after HCG injection and minced with a razor blade. Tissue was digested in medium 199 (GIBCO, Grand Island, NY, US; which contains Earle's salts but no L-glutamine and no sodium bicarbonate.) containing 1% fetal calf serum (FCS; GICBO) and 2,000 IU collagenase (GIBCO) plus 3,000 IU of DNase (Sigma) per gram of tissue for 1 h at 37°C under 95% air with 5% CO_2. The contents of the flask were filtered through nylon mesh (BD, Franklin Lakes, NJ, US) and centrifuged (100 g, 5 min); the supernatant fraction was discarded and the pellet was washed three times with fresh medium. The final cell concentration was 10^6 cells per ml and cell incubation was carried out in 6-well culture plates. Cell numbers were determined with a hemocytometer and cell viability was >90% as assessed by exclusion of Trypan blue dye.

### Transfection of plasmids expressing rat PHD2 into the cells

Plasmids encoding rat full-length PHD2 cDNA are generous gifts from Dr. Ningjun Li (Virginia Commonwealth University). The expression and function of rat PHD2 protein by the plasmids has been validated by previous studies (Huang et al., 2002; Li et al., 2007; Percy et al., 2006; Wang et al., 2010). Plasmids transfections were performed using lipids (DOTAP/DOPE, Avanti Polar Lipids, Inc.) according to the manufacturer's instructions. In brief, 5 μg of DNA was mixed with lipids solution at a ratio of 1:10 (DNA:lipid, w/w) in serum free culture medium (5 ml for a 10 cm dish). Cells were incubated with this transfection medium for 5 h and switched to normal medium for another 16 h. The cells were then ready for experiment.

Cell treatment and experimental groups

After plasmid transfection, the cells were switched to serum-free medium containing 100 μM of HCG. After HCG treatment for 18 h, the cells were harvested for protein and RNA isolation as described further.

RNA extraction and quantitative RT-PCR analysis of the mRNA levels of PHD1, 2 and 3, HIF- 1α and VEGF

Total RNA was extracted using TRIzol solution (Life Technologies, Inc. Rockville MD, US) and then reverse-transcribed (RT) (cDNA synthesis kit, Bio-Rad, Hercules, CA, US). The RT products were amplified using SYBR green for PHD1 (forward primer 5'-GCT GCT GCG TTG GTT AC-3' and reverse primer 5'-GCC TCC TGG TTC TCT TG-3'; GenBank accession no.: NM001004083), PHD2 (forward primer 5'-CTG GGA CGC CAA GGT GA-3' and reverse primer 5'- CAA TGT CAG CAA ACT GG-3'; GenBank accession no.: NM178334), PHD3 (forward primer 5'-GTT CAG CCC TCC
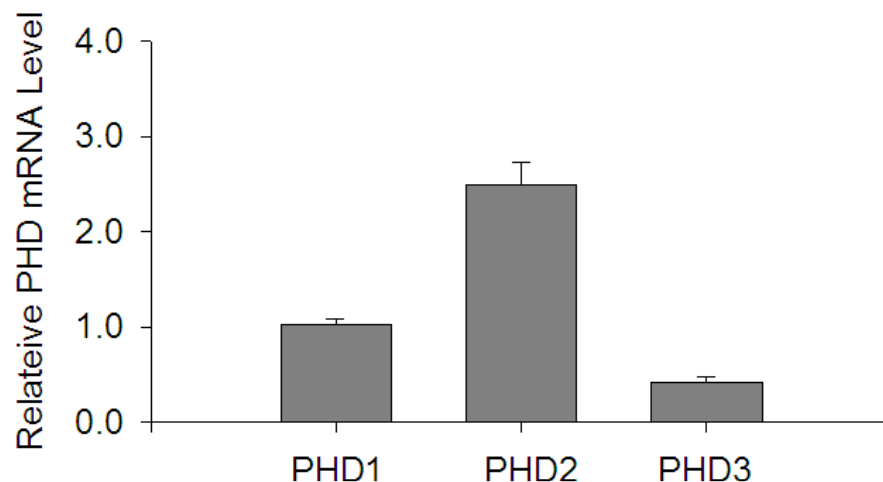
Figure 1. PHD2 mRNA expression in LCs. The relative mRNA levels of PHDs by Real-time PCR analysis. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other groups (P < 0.05) by Duncan's multiple range test. n = 6 batches of cells.

TAT GC-3' and reverse primer 5'-ACC ACC GTC AGT CTT TA-3'; GenBank accession no.: NM019371), HIF-1α(forward primer 5'-CTG GCA CGG GGA TGA TAC AGC-3' and reverse primer 5'-TCT CAT CCA TTG ACT GCC CCA G-3'; GenBank accession no.: AF057308) and VEGF (forward primer 5'-ACG AAG CGC AAG AAA TCC C-3' and reverse primer 5'-TTA ACT CAA GCT GCC TCG CC-3'; GenBank accession no.: M32167) with an iCycler iQ real time PCR detection system (Bio-Rad). The level of 18 rRNA (forward primer 5'-CGC CGC TAG AGG TGA AAT TC-3' and reverse primer 5'-TCT TGG CAA ATG CTT TCG C-3'; GenBank accession no.: M11188) was used as an endogenous control. The relative gene expressions were calculated in accordance with the $\Delta\Delta Ct$ method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Preparation of nuclear extracts and cytosolic protein and western blot analyses for protein levels of HIF-1α and PHD2

Nuclear protein was prepared as we described previously (Li et al., 2007; Wang et al., 2010; Zhang et al., 2010b). Cytosolic protein and nuclear protein were separately collected. The cytosolic protein was used for western blot analyses of PHD2. The nuclear fraction was used for western blot analyses of HIF-1α. Primary antibodies used in this study included HIF-1α (monoclonal, Novus Biologicals, 1:300 dilution) and PHD2 (rabbit polyclonal, Novus Biologicals, 1:300).

Determination of HIF-4-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 (Richard-Fiardo et al., 2008; Wang et al., 2010; Yi et al., 2009).

Statistics analysis

Data are presented as mean ± SE. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA followed by a Duncan's multiple range tests.

Student's t-test was used to evaluate statistical significance of differences between two groups. P < 0.05 was considered statistically significance.

RESULTS

PHDs mRNA expression in LCs

This study showed that, PHD2 mRNA level was almost three times higher than PHD1 mRNA level, while PHD3 was just half of PHD2 mRNA level, indicating that all of the three PHDs were expressed and PHD2 is most abundantly expressed in LCs (Figure 1).

### Effects of HCG and PHD2 transgene on PHD2 mRNA level in LCs

HCG treatment increased PHD2 mRNA level significantly than the control group in LCs (Figure 2), while PHD2 mRNA level also increased dramatically in PHD2 transgene group after HCG treatment than those in the control group and HCG-treated group (Figure 2), indicating high transfection efficiency and high expression level of PHD2 plasmid.

### Effects of HCG and PHD2 transgene on PHD2 protein level and PHD2 activity in LCs

To further confirm the role of PHD2 in PHD2-transfected LCs, we also performed the experiments to detect PHD2 protein level and PHD2 biological activity in each group (Figures 3 and 4). Our results showed that, HCG increased PHD2 protein level in some degree (Figure 3),
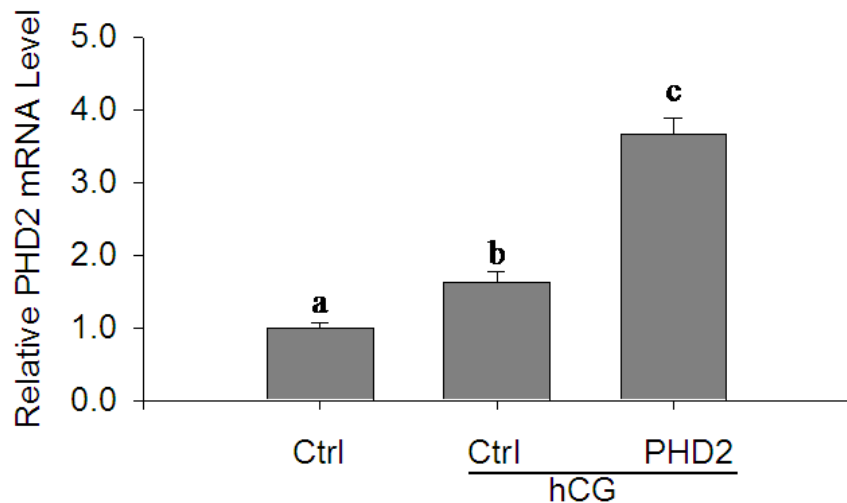
Figure 2. Effects of HCG and PHD2 plasmid on PHD2 mRNA level in LCs. The relative mRNA levels of PHD2 by real-time RT-PCR analysis. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other groups (P < 0.05) by Duncan's multiple range test. n = 6 batches of cells.
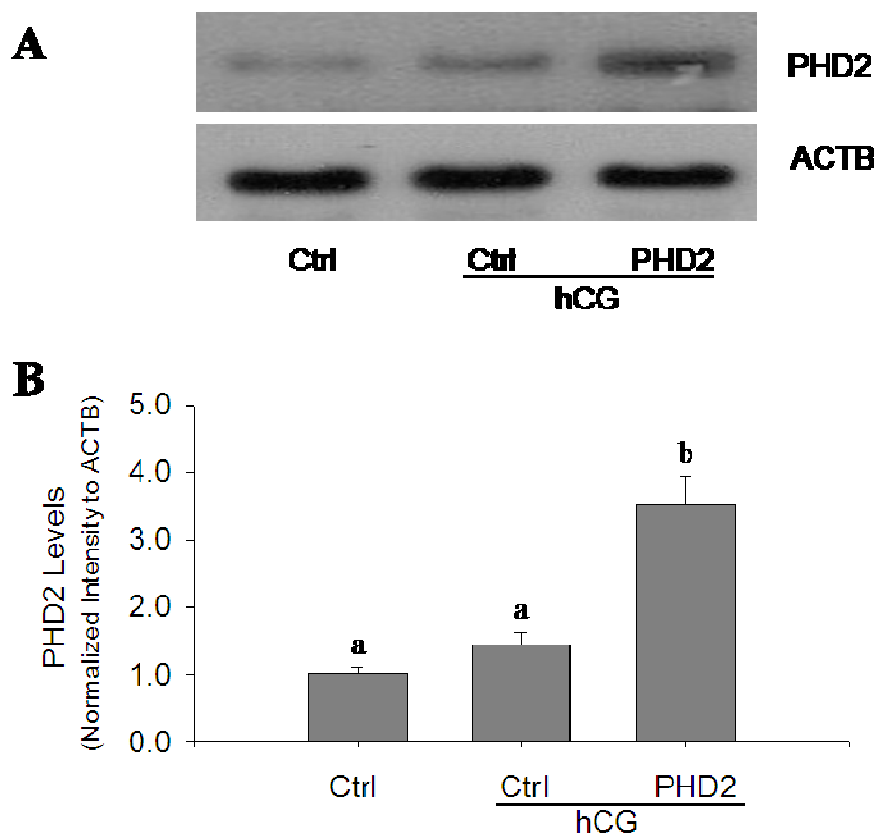
Figure 3. Effects of HCG and PHD2 plasmid on PHD2 protein level in LCs. Panel A: Representative ECL gel documents of western blot analyses depicting the protein level of PHD2; panel B: summarized intensities of PHD2 blot normalized to control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other groups (P < 0.05) by Duncan's multiple range test. n = 6 batches of cells.
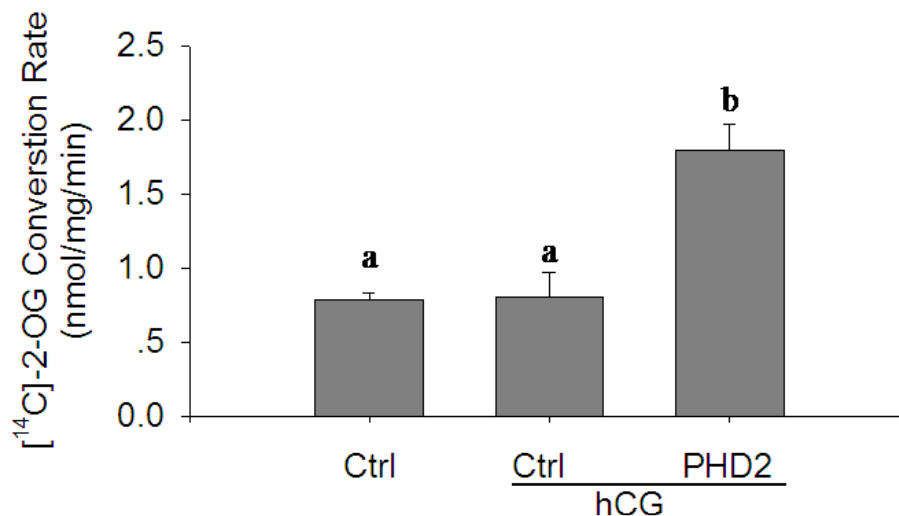
Figure 4. Effects of HCG and PHD2 plasmid on PHD2 activity in LCs. HIF-4-prolyl hydroxylase activity in each group by (^{14}C)-2-OG conversation rate. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other groups ($P < 0.05$) by Duncan's multiple range test. n = 6 batches of cells.

but a significant increase of PHD2 protein level was found in LCs transfected with PHD2 plasmids (Figure 3). The results of PHD activity had also demonstrated that these proteins have biological activity in PHD2-transfected LCs (Figure 4).

Effects of HCG and PHD2 transgene on VEGF and HIF-1α mRNA level in LCs

In this study, HCG significantly increased VEGF and HIF-1α mRNA expression in LCs. To better understanding of the important role of PHD2 in the LCs, we also examined the level of VEGF and HIF-1α mRNA in LCs transfected with PHD2 plasmid (Figure 5). Interestingly, a dramatic decrease of VEGF was found in PHD2-transfected LCs (Figure 5b), while HIF-1α mRNA had no obvious changes compare with HCG-treated LCs (Figure 5a).

Effects of HCG and PHD2 transgene on HIF-1α protein level in LCs

It has been demonstrated that, HIF prolyl-dydroxylases are major enzymes to promote the degradation of HIF-1α (Bruick et al., 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001); therefore, we also examined the HIF-1α protein levels in each group (Figure 6). A significant decrease of HIF-1α protein was found in PHD2-transfected LCs, which was consistent with our previous reports (Li et al., 2007; Wang et al., 2010), indicating that, PHD2 may regulate VEGF expression via HIF-1α pathway in LCs during corpus leteum development.

DISCUSSION

The results of our present study clearly demonstrated that HCG induced VEGF and HIF-1α expression in LCs, which was blocked by overexpression of PHD2, suggesting PHD2-mediated VEGF expression via HIF-1α pathway could be an important mechanism of VEGF-dependent angiogenesis during mammalian corpus luteum development.

The corpus luteum (CL) is a temporary endocrine structure in mammals, which plays an important role in the female reproductive cycle and is formed temporarily from a ruptured and ovulated follicle with rapid angio-genesis (Fraser et al., 2005; Nishimura and Okuda, 2010; Wulff et al., 2001; Young et al., 2000; Zhang et al., 2010a). Vascular endothelial growth factor (VEGF) is thought to play a paramount role in the regulation of normal and abnormal angiogenesis in the ovary (Fraser et al., 2005; Kaczmarek et al., 2005; Lee et al., 1997; Neulen et al., 1995; Shimizu et al., 2007a, b; van den Driesche et al., 2008; Zhang et al., 2010a), especially in the newly formed CL. Our previous experiments have already provided the direct evidences that VEGF is transcriptionally activated by HIF-1-mediated mechanism in LCs under hypoxia (Zhang et al., 2010b), which is caused by ovulation of the ruptured follicle with bleeding and an immature vasculature (Kaczmarek et al., 2005; Nishimura and Okuda, 2010). However, many reports have also shown that, reproductive hormones like 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
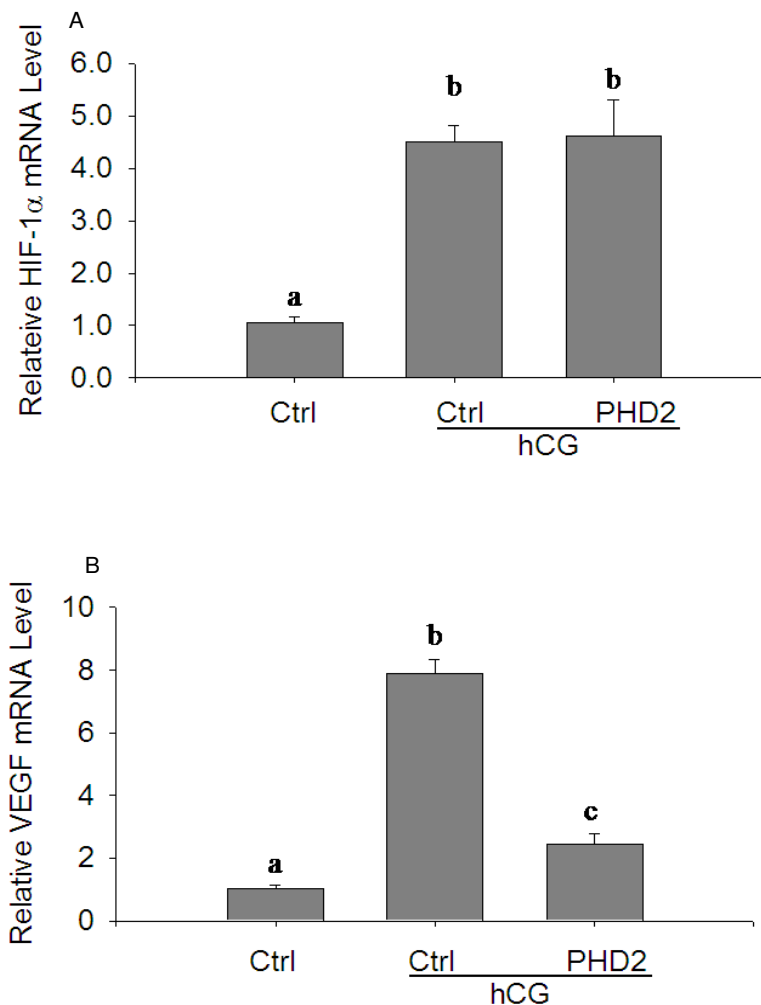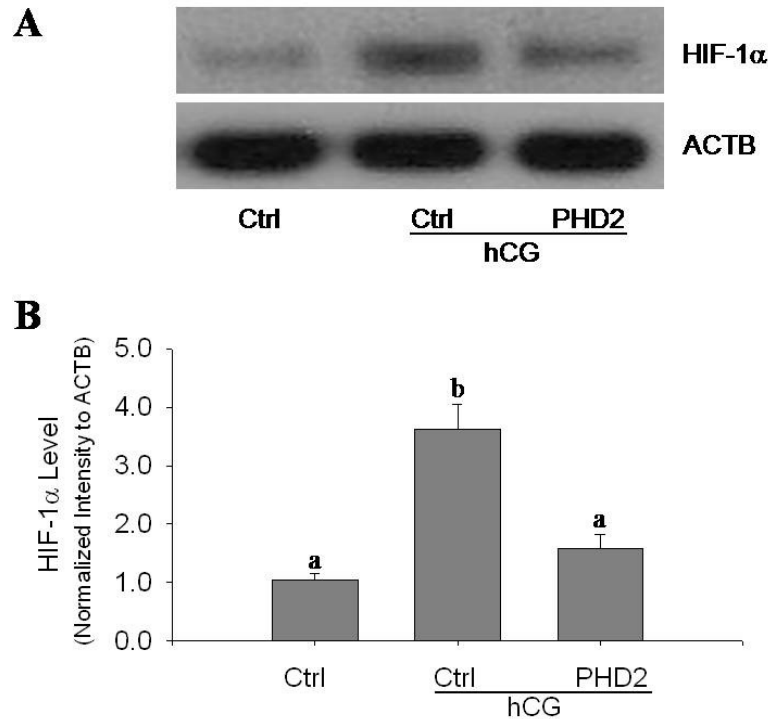
Figure 5. Effects of HCG and PHD2 plasmid on HIF-1α and VEGF mRNA level in LCs. Panel A: the relative mRNA levels of HIF-1α by real-time PCR analysis. Panel B: the relative mRNA levels of VEGF by real-time PCR analysis. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other group ($P < 0.05$) by Duncan's multiple range test. n = 6 batches of cells.

HCG *in vitro* (Nastri et al., 2010; Neulen et al., 1995). Chronic or acute expo-sure to HCG directly stimulates VEGF production and secretion by monkey (Christenson and Stouffer, 1997) and human luteinized granulosa cells (Lee et al., 1997; Nastri et al., 2010; Neulen et al., 1995; Wulff et al., 2001). And the administration of a GnRH antagonist decreased VEGF mRNA expression in the monkey corpus luteum (Ravindranath et al., 1992). In addition, luteal vascularization and the development of OHSS are absolutely dependent on LH/HCG stimulation (Nastri et al., 2010; Neulen et al., 1995). Furthermore, in a fully formed highly vascular, corpus luteum HCG also up-regulates VEGF expression (Wulff et al., 2001). Therefore, this study examined the induced effect of HCG on VEGF mRNA expression in LCs. Interestingly; we found that VEGF expression is induced by HCG in LCs

and HCG-stimulated HIF-1α protein expression is highly correlated with VEGF expression, indicating HCG stimulated VEGF expression via HIF-1α signaling pathway.

Many studies have already indicated that, HIF-1α regulates the expression of many genes whose protein products play critical roles in the developmental and physiological processes including angiogenesis, erythro-poiesis, glycolysis, iron transport and cell proliferation/ survival (Miyazawa et al., 2009, 2010; Nishimura et al., 2010; Wulff et al., 2001; Yaba et al., 2008; Zhong et al., 2000). Because HIF-1α activates the transcription of VEGF, which is required for angiogenesis, it is possible that, HCG may mediate angiogenesis via HIF-1α/VEGF pathway. In addition to a detailed exploration of the downstream mechanism of HIF-1α, studies have clarified the upstream process modulated by PHDs, which regulates

A

HIF-1α

ACTB

Ctrl   Ctrl   PHD2
              hCG

B

Figure 6. Effects of HCG and PHD2 plasmid on HIF-1α protein level in LCs. Panel A: representative ECL gel documents of western blot analyses depicting the protein level of HIF-1α; panel B: summarized intensities of HIF-1α blot normalized to control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values between the other groups (P < 0.05) by Duncan's multiple range test. n = 6 batches of cells.

HIF-1α degradation by the ubiquitin-protea-some pathway. In particular, PHD2 has been drawing considerable attention because PHD2 is considered to be the key oxygen sensor of all identified PHD enzymes (Chan et al., 2010; Wang et al., 2010), as knockdown of PHD2 results in elevated HIF protein and several recent studies have highlighted the importance of PHD2 in tumourigenesis (Chan et al., 2010). This study found that, HCG induced the activation of HIF-1α and over-expression of PHD2 transgenes blocked this activation of HIF-1α and its target gene VEGF, after HCG treatment. These results indicate that, PHD2 is involved in HIF-1α-mediated gene activations in LCs treated by HCG, which may reveal a novel mechanism about VEGF-dependent angiogenesis during mammalian corpus luteum development.

In summary, this study clearly demonstrates that HCG induces HIF-1α and VEGF expression in LCs and is also the first time to provide direct evidences indicating PHDs exists in rat LCs and HCG-induced VEGF expression can be blocked by overexpression of PHD2 transgenes. This PHD2-mediated VEGF expression may be one of the important mechanisms about VEGF-dependent angiogenesis during the corpus leteum formation in the mammalian ovary. Furthermore, PHD2 antagonism affords an opportunity for the development of novel treatments for fertility control and for some types of ovarian dysfunction (Chan and Giaccia, 2010; Miyazawa et al., 2009, 2010), particularly those conditions characterized by pathological angiogenesis and excessive vascular permeability, such as polycystic ovarian syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS) and ovarian neoplasia.

ACKNOWLEDGEMENTS

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