Hypoxia is Important for Establishing Vascularization During Corpus Luteum Formation in Cattle

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Abstract. Hypoxia-inducible factor 1 (HIF1) has been demonstrated to have critical roles in angiogenesis via transcriptional regulation of angiogenic factors, such as vascular endothelial growth factor (VEGF). In the ovary, angiogenesis is known to occur after ovulation in the developing corpus luteum (CL) in mammals. To determine whether HIF1 participates in angiogenesis in bovine CL, the present study investigated the mRNA and protein expressions of the HIF1 alpha subunit (HIF1A) and VEGF in bovine CL during the estrous cycle. The effects of hypoxia on the expressions of HIF1A protein, VEGF mRNA and VEGF protein in bovine luteal cells were also examined by using a cell culture system. HIF1A mRNA expression was lower at the regressed stage than at the other stages, whereas protein expression of HIF1A was highest at the early luteal stage and decreased thereafter. VEGF mRNA expression was highest at the developing luteal stage and decreased thereafter. VEGF protein expression was highest at the early luteal stage and decreased significantly at the regressed luteal stage. Hypoxia increased the amounts of HIF1A protein, VEGF mRNA and VEGF protein in cultured bovine luteal cells. Furthermore, we found that hypoxia inhibited progesterone production in the mid luteal phase, but not in the early luteal phase. The overall findings indicate that HIF1 is one of the factors promoting VEGF-induced angiogenesis during luteal development, and suggest that the hypoxic conditions formed after follicle rupture contribute to establishing luteal vascularization in cattle.

Key words: Cattle, Corpus luteum, Hypoxia, Hypoxia-inducible factor 1, Vascular endothelial growth factor

T he corpus luteum (CL) is an organ that is temporarily formed and regressed during the female reproductive cycle. It is formed from a ruptured follicle after ovulation with rapid angiogenesis [1-3]. Angiogenesis is known to be stimulated by a variety of growth factors [1, 2, 4], one of the strongest which is vascular endothelial growth factor (VEGF) [5]. VEGF has a role in the angiogenesis of newly formed CL in cattle [6, 7]. Another factor, hypoxia-inducible factor 1 (HIF1), is a strong inducer of the transcription of erythropoietin [8] and VEGF [9]. A ruptured follicle just after ovulation is thought to be under hypoxic conditions because of bleeding and because it has an immature vasculature [10]. Therefore, we hypothesized that hypoxic conditions are important for establishing the vascular system during luteal development.

HIF1 is an obligatory heterodimeric protein composed of two members of the basic-helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family, HIF1A and the aryl hydrocarbon receptor nuclear translocator (ARNT) [11]. ARNT expression is not affected by oxygen concentration, whereas HIF1A is rapidly ubiquitinated under hypoxia, which targets the protein for degradation by the proteasome [12-14]. In bovine CL, HIF1 expression has been reported only in cultured bovine luteal endothelial cells [15] and steroidogenic cells [16]. However, since the expression of HIF1 in bovine CL during the estrous cycle has not been determined, it is unclear whether HIF1 participates in regulating luteal angiogenesis during the estrous cycle in cattle.

In the present study, in order to investigate possible roles of HIF1 and VEGF in the regulation of bovine CL function, especially in development of the CL, we examined mRNA and protein expressions of HIF1A and VEGF in the CL during the estrous cycle. The effects of hypoxia on the expression of HIF1A protein, VEGF mRNA and protein in cultured bovine luteal cells were examined. Furthermore, to examine whether hypoxia has stage-dependent effects on bovine luteal function, we checked the difference in the effects of hypoxia on progesterone (P4) production between early and mid luteal cells.

Materials and Methods

Collection of CLs
Ovaries with CLs from Holstein cows were collected at a local abattoir within 10-20 min after exsanguination. Luteal stages were classified as the early, developing, mid, late or regressed stages by macroscopic observation of the ovary and uterus as described previously [17]. After determination of the stages, the CLs (n=4/stage) were immediately separated from the ovaries, frozen rapidly in liquid nitrogen, and stored at −80°C until being processed for RNA isolation. For cell culture experiments, the ovaries with CLs were submerged in ice-cold physiological saline and transported to the laboratory.

Cell isolation
Early, developing and mid luteal tissues were enzymatically dis-
sociated, and the luteal cells were cultured as described previously [18]. The luteal cells were suspended in a culture medium, DMEM and Ham’s F-12 medium (1:1 [v/v]; Sigma-Aldrich, St. Louis, MO, USA, No. D8900) containing 5% calf serum (Life Technologies, Grand Island, NY, USA, No. 16170-078) and 20 μg/ml gentamicin (Life Technologies, No. 15750-060). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small luteal cells, 20% large luteal cells, 10% endothelial cells or fibrocytes, and no erythrocytes. Developing luteal cells were used for an experiment to compare the effect of hypoxia on HIF1A protein, VEGF mRNA and protein expression. Early and mid luteal cells were used for an experiment to compare the effect of hypoxia on P4 production between the early and mid luteal stages.

**Cell culture**

The dispersed luteal cells were seeded at 2.0 × 10^5 viable cells per ml in 24-well cluster dishes (Costar, Cambridge, MA, USA, No. 3524) for determination of mRNA expression and P4 production, or in a 80 cm² culture flask (Greiner Bio-One, Frickenhausen, Germany, No. 658175) for determination of protein expression, and cultured in a humidified atmosphere of 5% CO₂ in air at 37.5°C in a N₂-O₂-CO₂-regulated incubator (ESP ECO, Osaka, Japan; No. BNP-110). After 12 h of culture, the medium was replaced with fresh medium containing 0.1% BSA, 5 ng/ml sodium selenite, 5 μg/ml transferrin and 2 μg/ml insulin, and the following experiments were carried out. Cell culture under conditions with different levels of O₂ (3 or 20%) was described previously [16]. Briefly, an individual small culture chamber was used for each oxygen concentration and for each time point. After setting the culture plates in the chamber, the atmospheric air was evacuated by a vacuum pump, and the chamber was refilled with a non-standard gas mixture containing 3% O₂ and 5% CO₂ in an N₂ base. After repeating this evacuation and inflow 5 times, the cells were cultured for 6 or 24 h. After each incubation, total RNA was extracted for determination of VEGF mRNA. For HIF1A and VEGF protein determination, the cultured cells were scraped and placed in ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete [protease inhibitor cocktail; Roche, No. 1697498], pH 7.4), frozen in liquid nitrogen, and then stored at −80°C until Western blotting analysis.

**RNA isolation and cDNA synthesis**

Total RNA was prepared from CL tissue and cultured luteal cells using TRIZOL Reagent according to the manufacturer’s directions (Invitrogen, Carlsbad, CA, USA; No. 15596-026). Total RNA (1 μg) was reverse transcribed using a ThermoScript RT-PCR System (Invitrogen, No. 11146-016).

**Real-time Polymerase Chain Reaction (PCR)**

Gene expression was measured by real-time PCR using a MyiQ thermal cycler (BIO-RAD, Tokyo, Japan) and the iQ SYBR Green supermix (BIO-RAD, No. 170-8880) starting with 1 ng of reverse-transcribed total RNA. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1000). The expression of 18S ribosomal RNA (18SrRNA) was used as an internal control. Twenty-bp primers with 50–60% GC-contents were synthesized (Table 1). The PCR conditions were 95°C for 15 min, followed by 55 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Use of the QuantiTect SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the PCR products with high linearity (Pearson correlation coefficient r>0.99).

### Table 1. Primers used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Accession No.</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1A</td>
<td>Forward</td>
<td>5'-CAGCCACCAGTGATGAATTG-3'</td>
<td>AB018398</td>
<td>111 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGGCACAAGGAGGTCTCTTAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward</td>
<td>5'-ATTTCGAAGGCTCCTGGTGT-3'</td>
<td>M32976</td>
<td>138 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TATGGGCTGCGTCTTTGTTAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18SrRNA</td>
<td>Forward</td>
<td>5'-TCGCGGAAGGATTTAAGTG-3'</td>
<td>AY779625</td>
<td>141 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AAACGGCTACCACATCCAAG-3'</td>
<td></td>
<td></td>
</tr>
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**HIF1A and VEGF protein analysis**

The HIF1A and VEGF protein levels in CL tissues and in cultured luteal cells were assessed by Western blotting analysis. CL tissues were homogenized on ice in the homogenization buffer by a tissue homogenizer (Physcotron; NITI-ON, Chiba, Japan; NS-50), and then filtered with a metal wire mesh (150 μm). For HIF1A protein analysis, nuclei were isolated from the tissue homogenates by centrifugation at 600 × g for 30 min. The resultant supernatant was used for VEGF protein analysis. The cultured cells were lysed in 200 μl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol [Sigma, No. G7757], Complete, pH 7.4). The cell lysate was used for both HIF1A and VEGF protein analysis. The protein concentration was determined by the method of Osnes et al. [19] using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [Nacalai Tesque, Kyoto, Japan, No. 31607-94], 10% glycerol, 1% β-mercaptoethanol [Wako Pure Chemical Industries, Osaka, Japan, No. 137-06862], pH 6.8) and heated at 95°C for 10 min. Samples (30 μg protein) were subjected to SDS-PAGE electrophoresis (HIF1A: 10% SDS-PAGE, VEGF: 15% SDS-PAGE) for 1 h at 200 V. The separated proteins were electrophoretically transblotted to a 0.2 μm nitrocellulose membrane (Invitrogen, No. LC2000) at 100 V for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was then washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, pH 7.5, 137 mM NaCl]) and cut into two pieces; one piece was used for a target pro-
tein (HIF1A [120 kDa] or VEGF [27 kDa]), and the other piece was used for β-actin (ACTB; 42 kDa). The piece for HIF1A was incubated in blocking buffer (4% nonfat dry milk in TBS-T) for 1 h at room temperature, while the pieces for VEGF and ACTB were incubated in blocking buffer overnight at 4°C. After the blocking incubation, the pieces of membrane were separately incubated with a primary antibody specific to each protein, HIF1A antibody (Novus Biologicals, LLC, Littleton, CO, USA, No. NB 100-105; 1:500 in blocking buffer, overnight at 4°C), VEGF antibody (kindly donated by Dr D Schams of Technical University Munich, Freising-Weihenstephan, Germany; 1:1000 in TBS-T, 1 h at room temperature), or ACTB antibody (Sigma, No. A2228; 1:1000 in TBS-T, 1 h at room temperature). After incubation, the membrane pieces were washed three times for 10 min in TBS-T at room temperature, and then incubated with secondary antibody (for VEGF [1:20000 in TBS-T], anti-rabbit Ig, HRP-linked whole antibody produced in donkey, Amersham Biosciences Corp., Piscataway, NJ, USA, No. NA934; for HIF1A [1:2000 in blocking buffer] and ACTB [1:20000 in TBS-T], anti-mouse Ig, HRP-linked whole antibody produced in sheep, Amersham Biosciences Corp., No. NA931) for 1.5 h, and washed three times in TBS for 10 min at room temperature. The signal was detected using an ECL Western Blotting Detection System (Amersham Biosciences, No. RPN2109).

The intensity of the immunological reaction (HIF1A, VEGF, ACTB) in the tissues or cells was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

P4 determination

Concentrations of P4 were determined directly from the cell culture media with an enzyme immunoassay (EIA) as described previously [20]. The standard curve ranged from 0.391 to 100 ng/ml, and the effective dose of the assay for 50% inhibition (ED50) was 4.5 ng/ml. The intra- and interassay coefficients of variation were 5.6 and 6.9%, respectively.

Statistical analysis

All experimental data are shown as the mean ± SEM. The statistical significance of differences in the amounts of HIF1A (Fig. 1A) and VEGF mRNA (Fig. 2A) and in the protein levels of HIF1A (Fig. 1C) and VEGF (Fig. 2C) during the estrous cycle was assessed by analysis of variance (ANOVA) followed by a multiple comparison with Bonferroni correction. Statistical significance of differences between different oxygen conditions in the protein levels of HIF1A (Fig. 3) and VEGF (Fig. 4B), in the amounts of VEGF mRNA (Fig. 4A) and in P4 production (Fig. 5) was assessed by Student’s t-test.

Results

Expression of HIF1A and VEGF mRNA and protein

There was less expression of HIF1A mRNA at the regressed stage than at the other stages (Fig. 1A; P<0.05), whereas the protein expression of HIF1A was highest at the early luteal stage and decreased at the latter stages (Fig. 1B, C; P<0.05). VEGF mRNA expression was highest at the developing luteal stage and decreased thereafter (Fig. 2A; P<0.05). At all stages, two VEGF164-specific bands (glycosylated and unglycosylated) were expressed (Fig. 2B), in agreement with previous results [6]. Based on the band intensities after normalization to ACTB-specific bands, the VEGF protein expression was highest at the early luteal stage and
Effects of hypoxia on HIF1A protein, VEGF mRNA and protein expression in developing luteal cells

The expressions of HIF1A protein (Fig. 3; P<0.05) and VEGF mRNA significantly increased (P<0.05) after 6 and 24 h of hypoxia (Figs. 3 and 4A, respectively). VEGF protein expression increased after 24 h of hypoxia (Fig. 4B; P<0.05).

Effects of hypoxia on P4 production in early and mid luteal cells

Hypoxia inhibited P4 production by mid luteal cells (P<0.05), whereas it did not affect P4 production by early luteal cells (Fig. 5).

Discussion

The present study demonstrates that HIF1A mRNA and protein were expressed in the bovine CL throughout the estrous cycle. The HIF1A protein content in the CL was greatest at the early luteal stage. In the cultured bovine developing luteal cells, HIF1A protein, VEGF mRNA and protein expressions increased under hypoxic conditions (3% O2) compared with normal culture conditions (20% O2). These findings suggest that the early CL exists under hypoxic conditions, and that HIF1- and VEGF-stimulated angiogenesis induced by the hypoxic conditions is required for establishing CL.

VEGF has been suggested to be an important factor for luteal formation in cattle [6, 7], as it has been shown to be in the rat [21] and human [22]. Although HIF1 as well as hypoxia has been shown to be a major inducer of VEGF [9], the expression of HIF1 in the bovine CL has not been reported. Our finding that the HIF1A protein expression was highest at the early luteal stage suggests that the early bovine CL exists under hypoxic conditions, and that HIF1 actively functions in the early stage CL. Furthermore, hypoxia markedly increased HIF1A protein expression, VEGF mRNA expression and VEGF protein expression in developing luteal cells. Based on these results, we hypothesize that hypoxia,
formed in the early CL after follicle rupture, induces HIF1 activation and VEGF production, resulting in angiogenesis for luteal formation in cattle. Expression of HIF1A protein has been found to be increased at the early luteal stage in the human [23] and monkey [24]. Therefore, the HYPOXIA-HIF1-VEGF system seems to function in formation of the CL across species.

Little is known about HIF1A protein expression in the CL. HIF1A protein was recently immunohistochemically detected in the nuclei of the marmoset CL [24]. Staining was strong in the early CL and weak in the fully formed CL [24]. Similarly, in the present immunoblotting study, abundant expression of HIF1A protein was detected in the early and developing CL, and the expression decreased in the later stages. Furthermore, in the pig ovary, expression of HIF1A mRNA tends to decrease as the CL matures [25]. Based on these findings, we suggest that HIF1 functions in the angiogenesis required for luteal formation across several species. In addition to hypoxic conditions, insulin-like growth factor (IGF)-1, IGF-2 and gonadotropins have been reported to stimulate VEGF production in monkey granulosa cells [26] and luteal cells [27]. HIF1 protein has also been demonstrated to mediate VEGF transcription by IGF-1 and insulin [28]. Furthermore, because the full transcriptional induction of VEGF gene expression by hypoxia requires activator protein (AP)-1 binding [29], some factors such as LH and FSH, which up-regulate an AP-1 transcription factor [30], may contribute to VEGF gene expression in response to hypoxia. Thus, it is also possible that a variety of factors, such as IGFs and gonadotropins, cooperate with hypoxic conditions to stimulate VEGF production followed by angiogenesis during luteal formation in bovine CL.

During the luteal phase, the changes in the expression of HIF1A mRNA and protein were not consistent. HIF1A mRNA expression was significantly higher at the early, developing, mid and late luteal stages than at the regressed luteal stage, whereas HIF protein expression was much higher at the early and developing stages and significantly decreased thereafter. HIF1A expression is tightly regulated at the protein level. HIF1A protein is degraded by an ubiquitin-proteasome pathway under normoxic conditions [12–14]. Thus, the protein content of HIF1A is more closely related to oxygen conditions than the HIF1A mRNA expression, and an abundant expression of HIF1A protein indicates a hypoxic condition in the tissue. Therefore, the finding that the protein expression of HIF1A was abundant at the early and developing luteal stages suggests that early and developing CLs develop under hypoxic conditions and that HIF1 functions in luteal formation.

We have previously reported that hypoxia is related to luteal
regression, such as inhibition of P4 synthesis [16] and promotion of luteal cell apoptosis [31]. In the present study, however, we found that the expression of HIF1A protein was highest at the early luteal stage. This result suggests that HIF1 is more active in luteal formation than in luteal regression. We recently hypothesized [31] that hypoxia induces luteal cell apoptosis via a proapoptotic member of BCL2 family proteins, BNIP3, whose expression is induced by hypoxia. Interestingly, we previously found that the expression of BNIP3 in the bovine CL is highest at the early luteal stage during the estrous cycle (unpublished data), suggesting that BNIP3-induced apoptosis is important in luteal formation as well as in luteal regression. Further studies are needed to clarify the role of BNIP3 in luteal formation.

In the present study, hypoxia did not affect P4 production by early luteal cells, whereas it decreased P4 production by mid luteal cells, which is consistent with our previous study [16]. These findings indicate that the response of the cells to hypoxia changes from the early to mid luteal stage, and suggest that early luteal cells are more resistant to the hypoxia-induced P4 decrease than mid luteal cells. It has also been suggested that early luteal cells maintain the ability for P4 production by luteotropic factors highly produced by them, such as prostaglandins (PGs) [32–36] and IGF-1 [37]. In general, injection of cows with PGF2α at the early luteal stage (within 5 days after ovulation) does not induce luteolysis, whereas injection 6 days after ovulation induces luteolysis [38, 39]. In the present study, the luteolytic effect of hypoxia observed in the mid luteal cells was not clearly observed in the early luteal cells. This change in the response to hypoxia is possibly related to the resistance to PGF2α injection at the early luteal stage in the cow.

In conclusion, our present study indicates that the early CL exists under hypoxic conditions, and suggests that HIF1, activated by the hypoxic conditions, contributes to establishment of the CL via VEGF-induced angiogenesis in cattle.

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