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Oxygen Concentration Is an Important Factor for Modulating Progesterone Synthesis in Bovine Corpus Luteum

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Oxygen deficiency caused by a decrease in the blood supply is known to induce various responses of cells. Because luteal blood flow has been shown to decrease during luteolysis, a low-oxygen condition seems to be an integral part of the environment during luteolysis. To determine whether a low-oxygen condition is associated with functional luteolysis, we examined the influence of reduced oxygen tension on the luteal progesterone (P4) generating system in cultured bovine midluteal cells. Luteal cells obtained from midcycle corpus luteum (d 8–12) were incubated under different O_2 concentrations (20, 10, 5, 3% O_2) with or without LH for 24 h. P4 production decreased with decreasing O_2 concentration but was significantly stimulated by LH regardless of O_2 concentration. After 8 h of culture, both basal and LH-stimulated P4 production was significantly lower under 3% O_2 than under 20% O_2. Low-oxygen condition also inhibited pregnenolone production. Cytochrome P450 side-chain cleavage enzyme (*P450scc*) mRNA expression, measured by quantitative PCR, decreased under low-oxygen condition in both non-LH-treated and LH-treated cells. Low-oxygen condition did not affect the expressions of steroidogenic acute regulatory protein mRNA or protein, whereas steroidogenic acute regulatory protein mRNA expression was stimulated by LH during 4 h of culture. Low-oxygen condition also did not affect 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase mRNA expression or the activity of the enzyme in the cells, regardless of the incubation period. The overall results indicate that a low-oxygen condition decreases P4 synthesis by attenuating P450scc production and P450scc activity in bovine luteal cells and suggest that oxygen deficiency is an essential condition for the progression of luteolysis in cattle. (*Endocrinology* 147: 4273–4280, 2006)

IN MAMMALS, LUTEOLYSIS consists of two phases, functional luteolysis and structural luteolysis (1). A rapid functional regression of corpus luteum (CL) is characterized by a decrease of progesterone (P4) production, followed by a phase of structural regression (1). In the cow, luteolysis is initiated by prostaglandin (PG)F2α released from the uterus at the late luteal stage or by an exogenous injection of PGF2α given at the midluteal stage. A decrease in luteal blood flow begins in parallel with systemic P4 concentrations during both spontaneous and PGF2α-induced luteolysis (2–6). Furthermore, the oxygen content in the blood collected from the ovarian vein began to decrease at the late luteal stage (6). Recently, blood flow within the CL during PGF2α-induced luteolysis has been shown to decrease significantly 8 h after PGF2α injection (7). The above findings suggest that a low oxygen condition is an integral part of the environment during luteolysis and that oxygen concentration is closely associated with P4 production.

The CL is the major site of P4 production during the luteal phase of the estrous cycle in cattle. Cholesterol mobilization is considered to be the rate-limiting step in the acute response of steroidogenic cells to tropic hormone stimulation (8). Steroidogenic acute regulatory protein (StAR) is known to transport cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (9–11). Cytochrome P450 side-chain cleavage enzyme (P450scc) converts cholesterol into pregnenolone, and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) converts pregnenolone into P4. Thus, P450scc and 3β-HSD are recognized as the key enzymes in P4 biosynthesis (12, 13). Previous studies showed that StAR expression at both the mRNA and protein levels in the CL were lowest in the regressed stage (14) and that enzymatic activity and mRNA expression for both P450scc (14–16) and 3β-HSD (17) were lower in the regressed stage than the developing stage in bovine CL. Based on these findings, we hypothesized that oxygen deficiency prevents P4 production during luteolysis by modifying StAR, P450scc, and 3β-HSD expression and/or their activities.

In the present study, to determine the influence of oxygen deficiency on luteal function, we investigated the effects of reduced oxygen tension on P4 production and mRNA expressions of key steroidogenic factors (*StAR*, *P450scc*, and *3β-HSD*). In addition, we examined the effects of reduced oxygen tension on StAR protein expression and the enzymatic activity of P450scc and 3β-HSD in cultured bovine luteal cells. Furthermore, to test whether a low oxygen condition used in the present study is hypoxic, we determined

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Abbreviations: CL, Corpus luteum; EIA, enzyme immunoassay; Hif-1α, hypoxia-inducible factor-1α; HRP, horseradish peroxidase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P4, progesterone; PG, prostaglandin; PLSD, protected least significant difference procedure; P450scc, P450 side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TBS, Tris-buffered saline; TBS-T, Tween 20 in TBS.

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the expression of hypoxia-inducible factor-1α (Hif-1α) protein because Hif-1α is known to accumulate in cells and function specifically under hypoxic conditions (18, 19).

Materials and Methods

Collection of CL

Ovaries with a midstage CL from Holstein cows were collected at a local abattoir within 10–20 min after exsanguination. Midstage CLs were identified by macroscopic observation of the ovary and uterus as described previously (20). For cell culture experiments, the ovaries with CL were submerged in ice-cold physiological saline and transported to the laboratory.

Cell isolation

Luteal tissue was enzymatically dissociated and luteal cells were cultured as described previously (21). The luteal cells were suspended in a culture medium, DMEM, and Ham's F-12 medium [1:1 (vol/vol); Sigma-Aldrich, Inc., St. Louis, MO; no. D8900] containing 5% calf serum (Life Technologies, Inc., Grand Island, NY; 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.

Cell culture

The dispersed luteal cells were seeded at 2.0×10^5 viable cells in 1 ml in 24-well cluster dishes (Costar, Cambridge, MA; no. 3524) for experiments 1, 3, and 5, or in a 80 cm^2 culture flask (Greiner Bio-One, Frickenhausen, Germany; no. 658175) for experiments 2 and 4 and cultured in a humidified atmosphere of 5% CO_2 in air at 37.5 C in a N_2-O_2-CO_2-regulated incubator (ESPEC Corp., Osaka, Japan; no. BNP-110). After 12 h of culture, the medium was replaced with fresh medium (5% calf serum), and the following experiments were carried out. To culture cells under conditions with different levels of O_2 (3, 5, 10, or 20%), an individual small culture chamber was used for each oxygen concentration and 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 nonstandard gas mixture containing each level of O_2 (3, 5, or 10%) and 5% CO_2 in a N_2 base. After repeating this evacuation and inflow five times, the incubations were started.

Experiment 1: effects of reduced oxygen tensions on P4 production

Luteal cells were incubated under various O_2 concentrations (20, 10, 5, 3% O_2) with or without LH (10 ng/ml, USDA-bLH-B6) for 24 h. Conditioned media were collected and stored at −30 C until assayed for P4.

Experiment 2: effects of reduced oxygen tension on Hif-1α protein expression

Hif-1α is known to accumulate in cells and function specifically under hypoxic conditions (18, 19). To test whether a condition with 3% O_2 is hypoxic, we determined the expression of Hif-1α protein. Luteal cells were incubated under commonly used (normal) culture atmosphere (20% O_2, 5% CO_2, 75% N_2) or low-oxygen concentration (3% O_2, 5% CO_2, 92% N_2) for 2, 4, and 24 h. 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, Mannheim, Germany; no. 1697498) (pH 7.4)] and then frozen in liquid nitrogen and stored at −80 C until Hif-1α protein analysis by Western blotting.

Experiment 3: time-dependent effects of reduced oxygen tension

Luteal cells were incubated under a normal culture atmosphere (20% O_2) or low-oxygen concentration (3% O_2) with or without LH (10 ng/ml) for 1, 2, 4, 8, 12, and 24 h. Conditioned media from each culture period were collected and stored at −30 C until assayed for P4 and pregnenolone. After each incubation, total RNA was extracted for determination of *StAR*, *P450scc*, and *3β-HSD* mRNA expressions.

Experiment 4: effect of reduced oxygen tension on StAR protein expression

Luteal cells were incubated under a normal culture atmosphere (20% O_2) or low-oxygen concentration (3% O_2) with or without LH (10 ng/ml) for 8 and 24 h. The cultured cells were scraped and placed in ice-cold homogenization buffer and then frozen in liquid nitrogen and stored at −80 C until StAR protein analysis by Western blotting.

Experiment 5: time-dependent effect of reduced oxygen tension on 3β-HSD activity

Luteal cells were incubated under a normal culture atmosphere (20% O_2) or low-oxygen concentration (3% O_2) with or without LH (10 ng/ml) in the presence or absence of pregnenolone (a substrate of 3β-HSD, 1 μg/ml, no. P9129; Sigma) for 4, 8, 12, and 24 h. The conditioned media from each culture period were collected and stored at −30 C until assayed for P4.

P4 determination

Concentrations of P4 were determined directly from the cell culture media with an enzyme immunoassay (EIA) as described previously (22). The standard curve ranged from 0.391 to 100 ng/ml, and the effective dose of the assay for 50% inhibition (ED_{50}) was 4.5 ng/ml. The intra- and interassay coefficients of variation were 7.0 and 10.8%, respectively.

RNA isolation and cDNA synthesis

Total RNA was prepared from cultured luteal cells using TRIZOL reagent according to the manufacturer's directions (Invitrogen, Carlsbad, CA; no. 15596–026). Total RNA (1 μg) was reverse transcribed using a ThermoScript RT-PCR system (Invitrogen; no. 11146-016).

Real-time PCR

Gene expression was measured by real-time PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green PCR system (QIAGEN GmbH, Hilden, Germany) starting with 1 ng reverse-transcribed total RNA. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1000). 18S ribosomal RNA expression was used as an internal control. Twenty-base pair primers with 50–60% GC contents were synthesized (Table 1). PCR conditions

TABLE 1. Primers used in real-time PCR

Gene	Primer	Sequence (5′–3′)	Accession no.	Product (bp)
StAR	Forward	CCCATGGAGAGGCTTTATGA	Y17259	115
	Reverse	TGATGACCGTGTCTTTTCCA		
P450scc	Forward	CTGGCATCTCCACAAAGACC	J05245	131
	Reverse	GTTCTCGATGTGGCGAAAGT		
3β-HSD	Forward	CCAAGCAGAAAACCAAGGAG	X17614	109
	Reverse	ATGTCCACGTTCCCATCATT		
18SrRNA	Forward	TCGCGGAAGGATTTAAAGTG	AY779625	141
	Reverse	AAACGGCTACCACATCCAAG		

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 $r > 0.99$).

P450scc activity

P450scc activity was assessed by total pregnenolone production (nanograms per hour/2×10^5 cells) at various time points (0–4, 4–8, 8–12, and 12–24 h). Because pregnenolone is metabolized to P4, total pregnenolone production in an interval i (P_i) (nanograms per hour/2×10^5 cells) was calculated as follows:

$$P_i = [A_{preg,i} + A_{P4,i} * (M_{preg}/M_{P4})]/t_i$$

where $A_{preg,i}$ and $A_{P4,i}$ are the accumulated amounts of pregnenolone and P4 (nanograms per milliliter) in the interval, M_{preg} and M_{P4}, are their respective molecular weights (316.48 and 314.47, respectively), and t_i is the length of the interval (4 or 12 h).

Pregnenolone concentration in conditioned media was measured by an EIA that was identical with the EIA for P4. Standards or samples (20 μl) were incubated with 100 μl polyclonal antibody (raised in a rabbit against pregnenolone-3-succ-BSA; Cosmo Bio Co., Tokyo, Japan; no. FKA316E) solution (1:10,000) and 100 μl pregnenolone-3-succ-horseradish peroxidase (HRP; 1:100,000; Cosmo Bio; no. FKA315) for 3 h at room temperature. The standard curve ranged from 0.391 to 100 ng/ml, and ED_{50} was 4.5 ng/ml. The intra- and interassay coefficients of variation were 6.7 and 9.6%, respectively. The cross-reactivities of the antibody were 100% for pregnenolone, 6% for P4, 1% for 17α-hydroxy-pregnenolone, 0.3% for 17α-hydroxy-progesterone, 0.01% for cortisol, 0.016% for cortisone, 0.02% for 4-androstenedione, and 0.03% for testosterone.

3β-HSD activity

3β-HSD activity was determined by measuring the conversion of pregnenolone to P4 in a bovine luteal cell culture. 3β-HSD activity was assessed by subtracting the mean P4 concentration in the pregnenolone-untreated wells from the mean P4 concentration in the pregnenolone-treated wells. The activity was expressed as a percentage of the value in non-LH-treated cells incubated under 20% O_2 for 4 h.

Hif-1α and StAR protein analysis

Hif-1α and StAR (9) protein levels in cultured bovine luteal cells were assessed by Western blotting analysis. The cultured cells (experiments 2 and 4) were ultrasonicated on ice in the homogenization buffer. For Hif-1α protein analysis, nuclei were isolated from the cell homogenates by centrifugation at $600 \times g$ for 30 min. For StAR protein analysis, the resultant supernatant was recentrifuged at $9000 \times g$ for 30 min to isolate mitochondria. Protein concentration was determined by the method of Osnes *et al.* (23), using BSA as a standard. The proteins were then solubilized in NuPAGE LDS sample buffer (Invitrogen; no. NP0007) and heated at 95 C for 10 min. Samples (Hif-1α: 50 μg protein; StAR: 25 μg protein) were subjected to electrophoresis on a NuPAGE 4–12% Bis-Tris gel (Invitrogen; no. NP0322BOX) for 45 min at 200 V.

The separated proteins were electrophoretically transblotted to a 0.2-μm nitrocellulose membrane (Invitrogen; no. LC2000) at 100 V for 2 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 Tris-buffered saline (TBS) (25 mM Tris-HCl [pH 7.5], 137 mM NaCl)] and cut into two pieces: one piece was used for a target protein [Hif-1α (120 kDa) or StAR (30 kDa)], and the other piece was used for β-actin (42 kDa). The piece for Hif-1α was incubated in blocking buffer (4% nonfat dry milk in TBS-T) for 1 h at room temperature, whereas the pieces for StAR and β-actin 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: Hif-1αantibody (Novus Biologicals, Inc., Littleton, CO; no. NB 100–105; 1:500 in blocking buffer, overnight at 4 C), StAR antibody (kindly donated by Dr. Stocco, Texas Tech Health Sciences Center; 1:1000 in blocking buffer, 1 h at room temperature), β-actin 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 [StAR (1:20,000 in TBS-T): antirabbit Ig, HRP-linked whole antibody produced in donkey; Amersham Biosciences Corp., Piscataway, NJ; no. NA934; Hif-1α (1:2,000 in blocking buffer) and β-actin (1:20,000 in TBS-T): antimouse Ig, HRP-linked whole antibody produced in sheep, Amersham Biosciences; no. NA931] for 1.5 h and washed three times in TBS for 10 min at room temperature. The signal was detected by ECL Western blotting detection system (Amersham Biosciences; no. RPN2109).

The intensity of the immunological reaction (Hif-1α, StAR, β-actin) in the cells was estimated by measuring the OD in the defined area by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD).

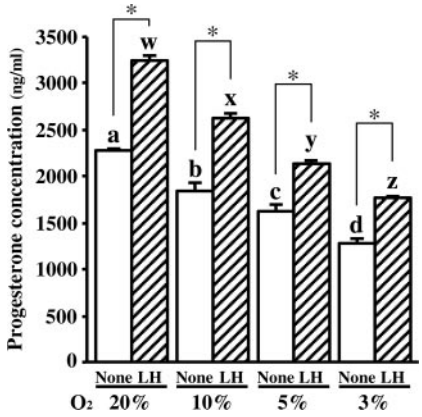

FIG. 1. Effects of reduced oxygen tensions on P4 production by cultured bovine midluteal cells. The cells were cultured under various oxygen tensions (20, 10, 5, 3% O_2) with or without LH (10 ng/ml) for 24 h (n = 3). All values represent mean ± SEM of three separate experiments. *Asterisks* indicate that LH showed a significant effect on P4 production within each oxygen tension ($P < 0.01$) and *different letters* indicate significant differences ($P < 0.01$) among oxygen tensions within non-LH-treated cells (a–d) or within LH-treated cells (w–z), as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

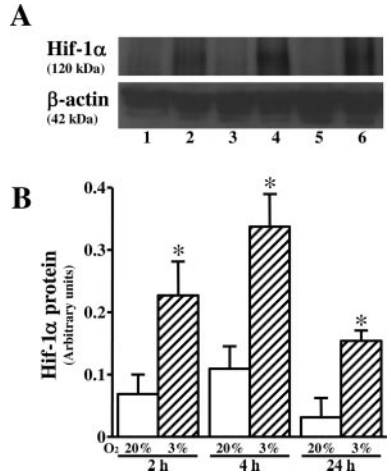

FIG. 2. Time-dependent effects of reduced oxygen tension on Hif-1α protein expression in cultured bovine midluteal cells. The cells were cultured under 20% O_2 or 3% O_2 for 2, 4, and 24 h. A, Representative samples of Western blot for Hif-1α and β-actin are shown in *upper panels* [lane 1, 20% O_2 (2 h); lane 2, 3% O_2 (2 h); lane 3, 20% O_2 (4 h); lane 4, 3% O_2 (4 h); lane 5, 20% O_2 (24 h); lane 6, 3% O_2 (24 h)]. The blot was incubated with primary antibodies against Hif-1α or β-actin and then incubated with second antibody conjugated to HRP. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. The Hif-1α protein levels are expressed relative to the amounts of β-actin protein (B; n = 3).

Statistical analysis

All experimental data are shown as the mean ± SEM. The statistical significance of differences in the concentration of P4, the amounts of *StAR*, *P450scc*, and *3β-HSD* mRNA, the activity of P450scc and 3β-HSD, and the StAR protein levels was assessed by ANOVA followed by a Fisher protected least significant difference procedure (PLSD) as a multiple comparison test.

Results

Experiment 1: effects of reduced oxygen tensions on P4 production

LH stimulated P4 production by luteal cells cultured for 24 h, regardless of the O_2 concentration (Fig. 1; $P < 0.05$). P4 production was inhibited by decreasing O_2 in a concentration-dependent manner in both non-LH-treated and LH-treated cells (Fig. 1; $P < 0.001$). Based on this result, further experiments were performed under 20% O_2 and 3% O_2 to more clearly determine the effects of low O_2 on P4 synthesis in bovine luteal cells.

Experiment 2: time-dependent effects of reduced oxygen tension on Hif-1α protein expression

Hif-1α protein expression significantly increased under a condition with 3% O_2 at 2, 4, and 24 h of culture (Fig. 2; $P < 0.05$).

Experiment 3–1: time-dependent effect of reduced oxygen tension on P4 production

LH stimulated P4 production by cultured luteal cells regardless of the incubation period or O_2 concentration (Fig. 3A; $P < 0.05$). As shown in Fig. 3A, a low-oxygen condition decreased basal and LH-stimulated P4 production by bovine luteal cells after 8, 12, and 24 h of culture ($P < 0.05$). Analysis of P4 production for each time interval revealed that the rate of P4 production was reduced by low-oxygen tension during 2–24 h in LH-treated cells and during 4–24 h in non-LH-treated cells (Fig. 3B; $P < 0.05$).

Experiment 3–2: time-dependent effects of reduced oxygen tension on StAR, P450scc, and 3β-HSD mRNA expressions

A real-time PCR analysis showed that LH increased the expression of *StAR* mRNA 2.7-fold under 20% O_2 and 2.0-fold under 3% O_2 at 4 h of culture (Fig. 4A; $P < 0.01$); no differences were observed between different oxygen tensions in any incubation period. After 24 h of culture of non-LH-treated cells, the expression of *P450scc* mRNA under 3% O_2 (Fig. 4B, *hatched bar on right*) was only 31% of the expression under 20% O_2 (*white bar on right*). Similarly, after 12 and 24 h of culture of LH-treated cells, the expressions of *P450scc* mRNA under 3% O_2 (*striped bars on right*) were 47 and 27% of the expression under 20% O_2 (*black bars on right*), respectively ($P < 0.05$). The expression of *3β-HSD* mRNA was not

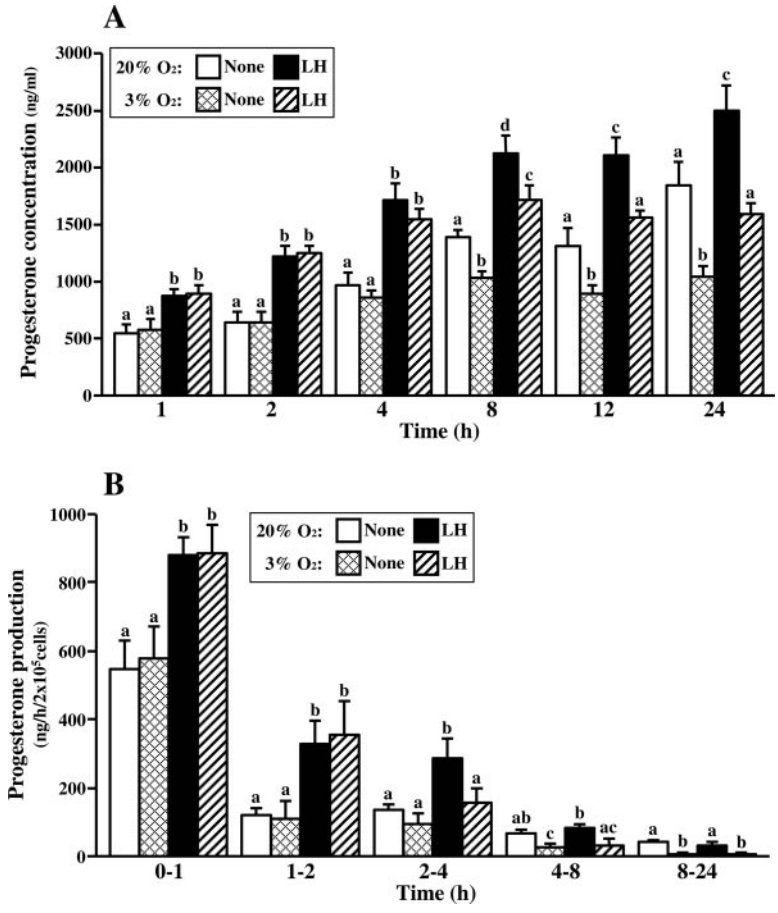

FIG. 3. Time-dependent effects of reduced oxygen tension on P4 production [accumulated value (A); production per hour (B)] by cultured bovine midluteal cells. The cells were cultured under 20% O_2 or 3% O_2 with or without LH (10 ng/ml) for different time periods (1, 2, 4, 8, 12, and 24 h; n = 5). All values represent mean ± SEM of five separate experiments. *Different letters* indicate significant difference ($P < 0.05$) within each culture period, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

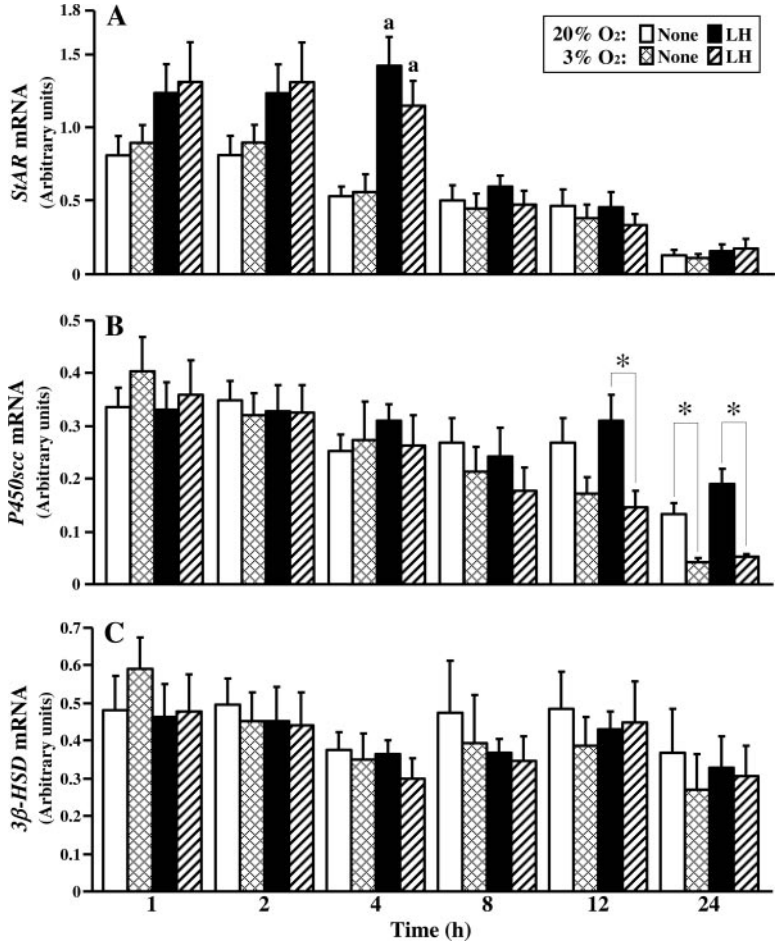

FIG. 4. Time-dependent effects of reduced oxygen tension on the amounts of *StAR* (A), *P450scc* (B), and *3β-HSD* (C) mRNA in cultured bovine midluteal cells. The cells were cultured under 20% O_2 or 3% O_2 with or without LH (10 ng/ml) for different time periods (1, 2, 4, 8, 12, and 24 h). The amounts of *StAR*, *P450scc*, and *3β-HSD* mRNA are expressed relative to the amounts of *18S rRNA* (n = 3). In each time period, *letters* (a) indicate significant differences between non-LH-treated and LH-treated cells within each oxygen tension ($P < 0.05$). *Asterisks* indicate significant differences between oxygen tensions within non-LH-treated cells or within LH-treated cells ($P < 0.05$). Significant differences were determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

affected by O_2 concentration or LH in any incubation period (Fig. 4C).

Experiment 3–3: time-dependent effects of reduced oxygen tension on P450scc activity

P450scc activity was significantly stimulated by LH during 0–4 h of culture (Fig. 5; $P < 0.05$). On the other hand, reduced oxygen tension decreased P450scc activity during 4–24 h of culture in both non-LH-treated and LH-treated cells (Fig. 5; $P < 0.05$).

Experiment 4: effect of reduced oxygen tension on StAR protein expression

Under all four conditions (20% O_2 and 3% O_2, with and without LH treatment), two StAR-specific bands were expressed after both 8 and 24 h incubation (Fig. 6A), in agreement with previous results (14). Intensities of the bands, after normalization to β-actin-specific bands, showed no effect of reduced oxygen tension or LH on StAR protein expression after either incubation period (Fig. 6B).

Experiment 5: time-dependent effect of reduced oxygen tension on 3β-HSD activity

Neither reduced oxygen tension nor LH had any significant effect on 3β-HSD activity during any incubation period (Fig. 7).

Discussion

The present study demonstrated for the first time that reduced oxygen tension inhibits basal and LH-stimulated P4 production by bovine luteal cells. This inhibitory effect is closely associated with suppression of enzymatic activity

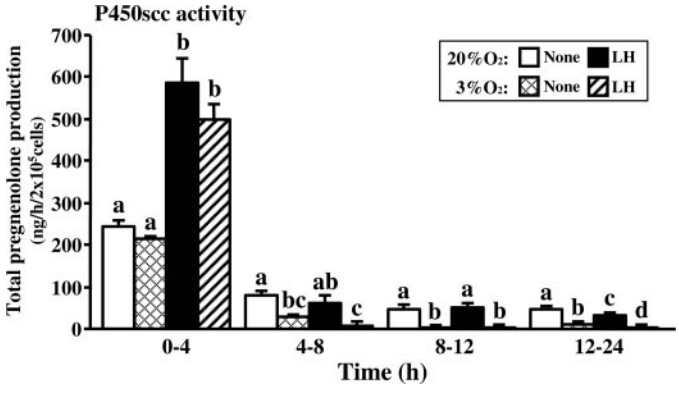

FIG. 5. Time-dependent effects of reduced oxygen tension on P450scc activity in cultured bovine midluteal cells. P450scc activity was assessed by total pregnenolone production (defined in *Materials and Methods*). The cells were cultured under 20% O_2 or 3% O_2 with or without LH (10 ng/ml) for different time periods (4, 8, 12, and 24 h). All values represent mean ± SEM of four separate experiments. *Different letters* indicate significant difference ($P < 0.05$) within each culture period, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

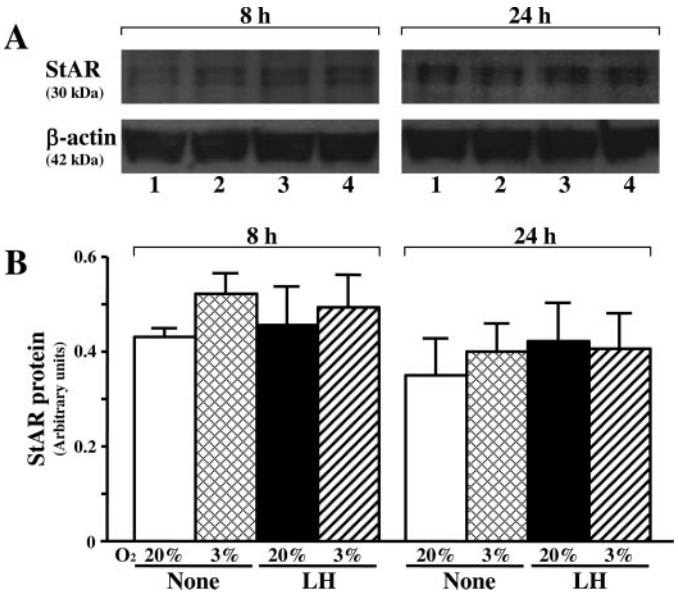

FIG. 6. Effects of reduced oxygen tension on StAR protein levels in cultured bovine midluteal cells. The cells were cultured under 20% O_2 or 3% O_2 with or without LH (10 ng/ml) for 8 and 24 h. A, Representative samples of Western blot for StAR and β-actin are shown in *upper panels* [lane 1, non-LH-treated (20% O_2); lane 2, non-LH-treated (3% O_2); lane 3, LH-treated (20% O_2); lane 4, LH-treated (3% O_2)]. The blot was incubated with primary antibodies against StAR or β-actin and then incubated with second antibody conjugated to HRP. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. The StAR protein levels are expressed relative to the amounts of β-actin protein (B; n = 3).

and mRNA expression of *P450scc*. More than 20 yr ago, several researchers reported a decreased ovarian blood flow during spontaneous luteolysis in cows using electromagnetic flow probes implanted around the ovarian artery (5, 6). They also demonstrated a close correlation between the decrease of blood supply to the ovary and the concentration of P4 in the circulation (5, 6). Close temporal correlations between ovarian blood flow and plasma P4 concentration during luteolysis were also observed in sheep (2, 3) and guinea pigs

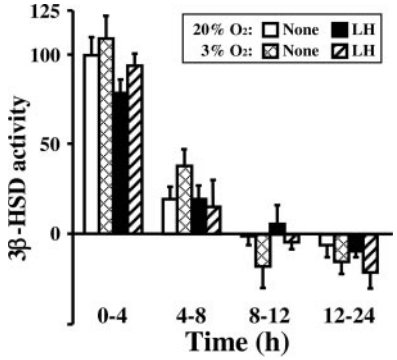

FIG. 7. Time-dependent effects of reduced oxygen tension on 3β-HSD activity in cultured bovine midluteal cells. The cells were cultured under 20% O_2 or 3% O_2 with or without LH (10 ng/ml) in the presence or absence of pregnenolone (1 μg/ml) for different time periods (4, 8, 12, and 24 h; n = 5). All values are expressed as a percentage of the activity of non-LH-treated cells incubated under 20% O_2 for 4 h (defined in *Materials and Methods*).

(4). A recent study using color Doppler ultrasonography showed that the blood flow within the mid-CL significantly decreased 8 h after injection of a luteolytic dose of PGF2α and that circulating P4 concentration continuously decreased until 16 h after a significant reduction of blood flow during PGF2α-induced luteolysis in cattle (7). The present results provide strong evidence that a low-oxygen condition is involved in the inhibition of P4 synthesis by decreasing P450scc activity, which consequently reduced pregnenolone production in bovine luteal cells. The above findings suggest that a low-oxygen condition, caused by a decreased blood supply, is essential for the progression of the luteolytic cascade in cattle.

The oxygen concentration in bovine luteal tissue has not been reported yet. However, because mean oxygen concentration in the ovarian venous blood ipsilateral to the CL during the midluteal stage has been shown to be 15.1% (6), the oxygen concentration in the CL is presumably higher than 15.1%. In the present study, the amounts of P4 produced after 24 h of culture under the four oxygen concentrations were significantly different, indicating that luteal P4 synthesis is sensitive to oxygen concentration and suggesting that an oxygen deficiency caused by the decreased blood supply induces functional luteolysis. However, in the time-course experiments, low-oxygen tension (3% O_2) did not affect P4 synthesis during 0–2 h of culture, although cellular responses to hypoxic condition have been suggested to be acute (24). We do not have a clear explanation for the lack of effects of low O_2 during the first 2 h of culture. It might be due to the time lag for dissolved oxygen in the culture medium to be brought to the same level as the culture atmosphere. Under hypoxic conditions, Hif-1α protein is known to accumulate in the cells and enhance transcription of hypoxia-inducible genes (18, 19). Because the protein expression of Hif-1α was increased by low O_2 (3% O_2) starting at 2 h in the present study, the culture condition with 3% O_2 is a hypoxic condition.

The effects of reduced oxygen tension on steroidogenesis were previously studied in human placental villi (25), rat (26), and porcine (27) granulosa cells and bovine (28–30), rabbit (31), and rat (32) adrenocortical cells. A low-oxygen condition (1% O_2) did not significantly reduce cortisol production by cultured bovine adrenocortical cells after either short (2 h) or long (24 h) culture (30). Studies on rat (26) and porcine (27) granulosa cells revealed that a low-oxygen condition (1–2% O_2) significantly reduced P4 production. In the present study, a low-oxygen condition (3% O_2) significantly inhibited basal and LH-stimulated P4 production in cultured bovine luteal cells. Based on these findings, the biosynthesis of P4 seems to be more sensitive to low-oxygen concentrations than the biosynthesis of cortisol. Furthermore, the present results are coincident with those of Gafvels *et al.* (33), who used an isolated rat whole CL culture, demonstrating that LH-stimulated P4 production decreased under a low-oxygen condition. However, under a low-oxygen condition, P4 production of LH-treated cells was still significantly higher than that of non-LH-treated cells in the present study. Therefore, we assume that functional luteolysis is a result of both reduced oxygen and reduced LH delivery.

Our findings that a low-oxygen condition decreased

P450scc mRNA expression in both non-LH-treated and LH-treated cells, but not mRNA expressions of *StAR* and *3β-HSD*, suggest that a low-oxygen condition inhibits P4 production, mainly by suppressing *P450scc* expression. In addition, the inhibition of P4 and pregnenolone production by a low-oxygen condition that we observed could be at least partly due to reduced P450scc activity. This is because P450scc is a key enzyme in the synthesis of pregnenolone so that pregnenolone production can act as an indicator of P450scc activity (32). Thus, the inhibition of P4 production by a low-oxygen condition observed in the present study could be due to the reduced P450scc activity at least. A low-oxygen condition has been suggested to inhibit the process of side-chain cleavage of cholesterol because molecular oxygen is required for this process (34), and the importance of oxygen in the process has been shown in different cell types (32, 35). Moreover, mRNA expression and enzymatic activity of P450scc in CL were less in the regressed stage than the developing stage in cattle (14–16). These findings suggest that a low-oxygen condition causes P4 suppression in functional luteolysis by inhibiting P450scc activity. In addition to P450scc activity, mRNA expression of this enzyme was decreased under a low-oxygen condition. Further studies are needed to clarify whether the amount of P450scc protein is inhibited by reduced oxygen tension.

In the present study, the activity of P450scc was decreased by a low-oxygen condition starting at 4–8 h of culture, whereas a low-oxygen condition did not start to decrease mRNA expression of *P450scc* until 12 h of culture. The reason for the decrease in enzyme activity occurred earlier than the decrease in mRNA expression is unclear. Protein synthesis was demonstrated to be strongly inhibited by a low-oxygen condition in rat hepatocytes *in vitro* (36, 37). Thus, it is possible that a low-oxygen condition also inhibits the synthesis of proteins, including P450scc, in bovine luteal cells, resulting in a decrease in P4 production. On the other hand, a low-oxygen condition (3% O_2) did not affect the expression of StAR protein or 3β-HSD activity in the present study, suggesting that StAR and 3β-HSD do not have significant roles in P4 inhibition by low O_2. Based on these findings, a low-oxygen condition seems to decrease P4 production mostly by regulating P450scc activity in bovine luteal cells.

Luteal regression is characterized by a phase of inhibition of P4 production (functional luteolysis), followed by a phase of decrease in luteal size (structural luteolysis), when cells of the CL undergo apoptosis (1). We previously demonstrated that CL volume significantly decreased 8 h after PGF2α injection when the blood flow within the CL was significantly decreased (7). In addition, we recently showed that a specific P4 receptor antagonist induced apoptosis of bovine luteal cells and suggested that P4 suppresses luteal cell apoptosis in bovine CL (38). Because a low-oxygen condition decreased P4 production in bovine luteal cells in the present study, oxygen deficiency may promote luteal cell apoptosis by inhibiting P4 production. Further studies are in progress to clarify whether a low-oxygen condition induces apoptosis in bovine luteal cells.

In conclusion, our results indicate that a low-oxygen condition inhibits P4 production by suppressing P450scc activity in bovine luteal cells and suggest that an oxygen deficiency caused by a decreased blood supply is an important condition for progression of the luteolytic cascade in cattle.

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