Roles of low oxygen condition and hypoxia-inducible factor 1α during luteinization of bovine granulosa cells

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PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Environmental and Life Science (Doctor’s course), Okayama University, Japan, from October, 2014 to September, 2017, under the supervision of Professor Emeritus Kiyoshi OKUDA (October, 2014-December, 2015) and Professor Koji KIMURA (January, 2016-September, 2017).

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

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Finally, I would like to express my gratitude to my parents, my husband, my younger brother and big family. None of this would have been possible without their constant love, support and strength.
Ovarian follicular vascularization is restricted to the theca cell layer with the basement membrane as the borderline during folliculogenesis. As a result, the granulosa cell layer and oocyte seem to grow in a non-vascular environment which is under low oxygen condition or hypoxia. After ovulation, the ruptured follicle is also thought to be under hypoxic conditions due to bleeding and immature vascularization. Simultaneously, follicular cells start differentiating into luteal cells and producing a large amount of progesterone (P4), a hormone which is essential for establishing pregnancy. Based on those findings, the luteinization of granulosa cells (GCs) may occur under low oxygen condition. However, it is unclear whether low oxygen condition contributes to P4 synthesis during luteinization.

Luteinization causes important changes in follicular function, as the main product of the luteinized cells is changed from estrogen to P4. These changes include modifications of the rate-limiting elements of steroid synthesis. The key protein and enzymes in P4 biosynthesis include steroidogenic acute regulatory protein (StAR; \textit{STAR}), which transports cholesterol from outer mitochondrial membrane to inner mitochondrial membrane, cytochrome P450 side-chain cleavage (P450scc; \textit{CYP11A1}), which converts cholesterol into pregnenolone and 3β-hydroxysteroid dehydrogenase (3β-HSD; \textit{HSD3B}), which converts pregnenolone into P4.

Hypoxia is defined as a reduction in available oxygen whether in a whole organism, tissue or cell. This condition is known to cause accumulation of hypoxia-inducible factor 1α (HIF-1α) protein in cells and to enhance transcription of hypoxia-inducible genes. There is increasing evidence that HIFs participate in ovulation and follicular differentiation. Human chorionic gonadotropin (hCG) was found in synergy with hypoxic conditions to up-regulate HIF-1α activity within luteinizing GCs both \textit{in vivo} and \textit{in vitro}; these findings suggest the fundamental roles for HIFs in follicle differentiation. However, whether HIF-1α plays a role in hypoxia-driven enhancement of P4 synthesis during luteinization is not clear.

In the present study, the effects of hypoxia in P4 production and protein expression of key steroidogenic factors in P4 biosynthesis; StAR, P450scc, and 3β-HSD, in
cultured bovine luteinizing GCs was investigated. A model of bovine luteinizing and non-luteinizing GCs in a culture system was used. GCs obtained from bovine small antral follicles were treated for 24 h with insulin (2 µg/ml), forskolin (10 µM) or insulin in combination with forskolin at 20% O₂ to mimic the effect of luteinizing hormone surge. After 24 h, progesterone (P4) production was higher in the treated cells, which we defined as luteinizing GCs, than in non-treated cells, which we defined as non-luteinizing GCs. P4 production by non-luteinizing GCs was not affected by low oxygen condition (24 h at 10% and 5% O₂), while P4 production by luteinizing GCs was significantly increased under low oxygen condition (24 h at 10% and 5% O₂). Because low oxygen condition affected P4 production by the luteinizing GCs but not by the non-luteinizing GCs, low oxygen condition seems to promote P4 production during, rather than before, luteinization. In the luteinizing GCs, mRNA and protein expression of StAR and protein expression of 3β-HSD increased under 10% O₂, while mRNA and protein expressions of key protein and enzymes in P4 biosynthesis did not increase under 5% O₂. The overall results suggest that low oxygen condition plays a role in progressing and completing the luteinization by enhancing P4 production through StAR as well as 3β-HSD expressions in the early time of establishing the corpus luteum.

Secondly, by using the same model of luteinizing and non-luteinizing GCs, the effect of changes in protein expression of the α-subunit of HIF-1 (HIF-1α) on P4 production and on the expression levels of StAR, P450scc, and 3β-HSD was investigated to clarify whether HIF-1α mediates hypoxia-induced increase in P4 synthesis during luteinization in bovine GCs. Cobalt chloride (CoCl₂), a hypoxia-mimicking chemical, was used because treatment with this compound has been shown to successfully mimic hypoxia and induce the accumulation of HIF-1α in other studies. In the present study, 100 µM CoCl₂ increased HIF-1α protein expression in luteinizing GCs. After the upregulation of HIF-1α, there was an increase in P4 production and in the gene and protein expression levels of StAR in CoCl₂-treated luteinizing GCs. In contrast, CoCl₂ did not affect the expression of either P450scc or 3β-HSD. Echinomycin, a small-molecule inhibitor of HIF-1’s DNA-binding activity, attenuated the effects of CoCl₂ and of low oxygen tension (10% O₂) on P4 production and StAR expression in luteinizing GCs. Overall, these findings suggest that HIF-1α is one of the factors that upregulate P4 in GCs during luteinization.
In conclusion, the overall findings suggest that low oxygen condition induced by lower oxygen tension (10% O₂) and a hypoxia-mimicking chemical (CoCl₂) promotes the P4 synthesis during luteinization by enhancing the expression of StAR. Moreover, the hypoxia-induced increase in P4 production and in StAR expression in bovine cultured luteinizing GCs is mediated by HIF-1α. In other words, by enhancing P4 synthesis, HIF-1α may play as an important factor in the progression of luteinization by enhancing P4 synthesis.
CHAPTER 1

Low oxygen condition promotes progesterone synthesis during luteinization in bovine granulosa cells

INTRODUCTION

In the ovary, follicular vascularization is restricted to the theca cell layer, while the granulosa cell layer and oocyte develop in an avascular environment. As the follicle develops, the blood vessels in the theca cell layer increase in number and size but do not penetrate the granulosa cell layer [1-3]. Ovarian blood flow decreases toward ovulation, and gradually increases with luteal development [4]. In addition, the O₂ concentration in the follicular fluid in large follicles is less than in small follicles [5]. These conditions seem to represent a physiological hypoxia or low oxygen condition during follicular growth. Furthermore, immediately after ovulation, the ruptured follicle is also thought to be under a hypoxic condition due to bleeding and immature vascularization [6].

Hypoxia is defined as a reduction in available oxygen whether in a whole organism or in a tissue or cell. Hypoxia response elements of target genes are recognized and regulated by hypoxia-inducible factor 1 (HIF-1), comprising the subunit factors HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT; HIF-1β) [7-9]. Hypoxia and HIF-1α have been studied on luteal function related to the steroidogenesis at various stages in cows [10-12]. Expression of HIF-1α in the corpus luteum (CL) was highest at the early luteal stage in cattle [12], humans [13] and monkeys [14]. In granulosa cells, HIF-1α expression peaks around the time of ovulation [1, 14, 15] and is upregulated by low oxygen conditions (2% O₂) in synergy with human chorionic gonadotrophin (hCG), a mimic of luteinizing hormone (LH) [15]. The above findings indicate that the follicle, specifically the granulosa cell layer, is in a hypoxic condition around the time of ovulation.

Granulosa cells and theca cells start to be luteinized after an LH surge, and after ovulation, they differentiate into luteal cells and then produce a large amount of progesterone (P₄), which is essential for establishing pregnancy [16]. Luteinization causes important changes in follicular function, as the main product of the luteinized
cells is changed from estrogen (E2) to P4. These changes include modifications of the rate-limiting elements of steroid synthesis. The key protein and enzymes in P4 biosynthesis include steroidogenic acute regulatory protein (StAR; STAR), which transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, cytochrome P450 side-chain cleavage (P450scc; CYP11A1), which converts cholesterol into pregnenolone and 3β-hydroxysteroid dehydrogenase (3β-HSD; HSD3B), which converts pregnenolone into P4 [17-21]. A common process in luteinization involves rupture and collapse of the follicle at ovulation and also the invasion of some elements, including theca cells and blood vessels [22]. Based on the above findings, luteinization and hypoxia may take place simultaneously. However, it is unclear whether hypoxia contributes to P4 synthesis during luteinization.

In the present study, we hypothesized that hypoxia plays some roles in luteinization by stimulating the P4 generating system. To test this hypothesis, we used a model of bovine luteinizing and non-luteinizing granulosa cells in a culture system. We induced hypoxic conditions (10% and 5% O2) in the culture system and examined P4 production as well as mRNA and protein expression of StAR, P450scc and 3β-HSD. Furthermore, it has been confirmed that the conditions used in the present study are hypoxic by determining the protein expression of HIF-1α, which is known to accumulate in cells and function specifically under hypoxic conditions [23, 24].
MATERIALS AND METHODS

Granulosa cell isolation and culture

Bovine ovaries were obtained from a local slaughterhouse and were transported to the laboratory in ice-cold sterile physiological saline. The ovaries with healthy follicles were washed several times in a sterile saline containing 100 IU/ml of penicillin (Meiji Seika Pharma, Tokyo, Japan; 611400D3051) and 100 µg/ml streptomycin (Meiji Seika Pharma; 6161400D1034). Granulosa cells in follicular fluid were aspirated aseptically from healthy small follicles (≤ 6 mm in diameter) using 2.5-ml disposable syringe and 24-gauge needle, pooled and transferred to a plastic Petri disc filled with Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F-12 medium (1:1 [v/v]; Invitrogen, Carlsbad, CA, USA; 12400-024) containing 10% calf serum (Invitrogen; 16170078), 20 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA; G1397) and 2 µg/ml amphotericin B (Sigma-Aldrich; A9528) along with 50 IU heparin sodium salt (Nacalai Tesque, Kyoto, Japan; 17513-41). After removing cumulus-oocyte complexes (COCs) with a fine glass pipet under a dissecting microscope, granulosa cells in follicular fluid were centrifuged (800 × g; 5 min at 4°C) and then resuspended in Tris NH₄Cl to break the blood cells after discarding the supernatant. Cell suspensions were centrifuged again and resuspended in DMEM (Sigma-Aldrich; D1152) with 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.1% bovine serum albumin (BSA; Roche Diagnostics, Manheim, Germany; 10735086001) after the supernatant was discarded. This washing step was done two times. Cell suspensions were then centrifuged, filtered through metal meshes (100 µm × 2, 80 µm × 2) to avoid cell aggregation and resuspended in a suitable volume of culture medium (DMEM and Ham’s F-12 containing 10% calf serum and 20 µg/ml gentamicin). The cell viability of granulosa cells was assessed by trypan blue dye exclusion.

The dispersed granulosa cells were seeded at 0.5 × 10⁵ viable cells per 1 ml in culture medium in 75 cm² culture flasks (20 ml/ flask; Greiner Bio-One, Frickenhausen, Germany; 658175) and cultured in a humidified atmosphere of 5% CO₂ in air at 37.5 °C in a N₂-O₂-CO₂-regulated incubator (ESPEC Corp., Osaka, Japan; no. BNP-110) for 3-4 days. When the cultured cells reached 80-90% confluence, cell passage was done using
0.1% bovine trypsin (Sigma-Aldrich; T92012) and sterile phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd. Tokyo, Japan; 05913). The granulosa cells were seeded at a concentration of $2.0 \times 10^5$ viable cells per 1 ml in 48-well cluster dishes (0.5 ml/well; Greiner Bio-One; 662160) for determination of P4 production, in 24-well cluster dishes (1.0 ml/well; Greiner Bio-One; 677180) for determination of gene expression and in 75 cm$^2$ culture flasks (20 ml/flask; Greiner Bio-One; 658175) for determination of protein expression.

**Model of luteinizing and non-luteinizing granulosa cells and hypoxic culture conditions**

To prepare luteinizing and non-luteinizing granulosa cells, the culture medium was replaced with fresh medium containing 0.1% BSA, 5 ng/ml sodium selenite (Sigma-Aldrich; S5261), 5 µg/ml transferrin (Sigma-Aldrich; T4132) and 0.5 mM ascorbic acid (Wako-Pure Chemical Industries, Osaka, Japan; 031-12061), and the cells were then incubated under a normal culture atmosphere (20% O$_2$, 5% CO$_2$, 75% N$_2$) without or with insulin (2 µg/ml; Sigma-Aldrich; I4011), forskolin (10 µM; Research Biochemicals International, Natick, MA, USA; 70-0501-05) or both for 24 h. The concentration of insulin and forskolin was selected based on a previous report [25]. The culture media from these cultured cells were collected to determine the effect of insulin and forskolin treatment on P4 production for 24 h.

To determine the effect of hypoxia on P4 production, mRNA and protein expressions of STAR, CYP11A1 and HSD3B, the luteinizing and non-luteinizing granulosa cells were incubated under various O$_2$ concentrations, 20% O$_2$ (normoxia), 10% O$_2$ (hypoxia) or 5% O$_2$ (hypoxia), for 24 h in small individual culture chambers. The chambers were refilled with a nonstandard gas mixture, as described previously [10], containing the indicated O$_2$ level (20%, 10% or 5% O$_2$) and 5% CO$_2$ in an N$_2$ base.

**P4 production determination**

To determine P4 production, enzyme immunoassay (EIA) and DNA assay were performed. The conditioned media were collected and stored at -30°C until assayed for
determining the P4 concentration after the granulosa cells were incubated under normoxic or hypoxic conditions without or with insulin (2 µg/ml), forskolin (10 µM) or insulin (2 µg/ml) in combination with forskolin (10 µM) for 24 h. The concentration of P4 was determined by EIA as described previously [26]. The standard curve ranged from 0.391 to 100 ng/ml. To fit the range of the standard concentration, the culture media were diluted. The cultured cells were also stored at -30°C until the DNA content was measured by the spectrophotometric method of Labarca and Paigen [27] and were used to standardize the P4 concentration. Four experiments were performed, and each treatment was tested in triplicate wells in each experiment. Insulin treatment increased the cell number, while the hypoxic conditions did not alter the cell number (data not shown).

**RNA isolation, cDNA synthesis and real-time PCR**

Total RNA of cultured luteinizing and non-luteinizing granulosa cells under normoxic and hypoxic conditions for 24 h was extracted to determine mRNA expression of *STAR, CYP11A1* and *HSD3B*. Total RNA was prepared from granulosa cells using TRIsure (Bioline, London, UK; BIO-38033) according to the manufacturer’s directions. Extracted RNA from each sample was quantified using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was reverse transcribed using a ThermoScript RT-PCR system (Invitrogen; 11146-016).

*STAR, CYP11A1* and *HSD3B* gene expressions were measured by real-time PCR using a MyiQ (Bio-Rad, Tokyo, Japan) and iQ SYBR Green supermix (Bio-Rad; No. 170-8880) starting with 1 ng reverse-transcribed total RNA as described previously [28]. 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. In a preliminary experiment, *18SrRNA* was confirmed not to be influenced by luteinization and hypoxia (data not shown). Twenty-base pair primers with 50-60% GC-contents were synthesized (Table 1).

The PCR conditions were: 95°C for 30 sec, followed by 45 cycles of 94°C for 6 sec, 60°C for 30 sec and 65°C for 6 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. The melting curve analysis was checked to verify that only the target amplicon was amplified.

**HIF-1α, StAR, P450scc and 3β-HSD protein expressions**

The luteinizing and non-luteinizing granulosa cells cultured under normoxic and hypoxic conditions for 24 h were washed with ice-cold PBS and scraped from the culture flask in 1 ml ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete [protease inhibitor cocktail; Roche Diagnostics; 11697498001], pH 7.4). The cell suspension was centrifuged at 19,000 × g for 30 min, the supernatant was discarded, and the suspension was then lysed in 100 µl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol [Sigma; G7757], Complete, pH 7.4). The protein samples were then stored at -80°C until HIF-1α, StAR, P450scc and 3β-HSD protein analyses were performed by Western blotting.

The protein concentration was determined by the method of Osnes et al. [29] using BSA as a standard. The protein samples were solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [Nacalai Tesque; 31607-94], 10% glycerol, 1% β-mercaptoethanol [Wako Pure Chemical Industries; 137-06862], pH 6.8) and heated at 95°C for 10 min. Samples (50 µg protein) were subjected to electrophoresis on a 7.5% SDS-PAGE gel that included a pre-stained molecular weight marker (Bio-Rad; 161-0374) for 1 h at 200 V.

The separated proteins were electrophoretically transblotted to a PVDF membrane (GE Healthcare, Limited, Buckinghamshire, UK; RPN1416LFP) for 1 h at 25 V in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]) for 10 min and was incubated in PVDF blocking buffer (Toyobo, Co., Ltd., Osaka, Japan; NYBR01) for 1 h at room temperature. The membranes were then incubated separately with a primary antibody in immunoreaction enhancer solution (Toyobo, Co., Ltd., Osaka, Japan; NKB-101) specific to each protein, HIF-1α antibody (Sigma-Aldrich; SAB2104366; 1:500), StAR antibody (Abcam; ab96637; 1:3,000), P450scc antibody (Abcam; ab75497; 1:1,000), 3β-HSD antibody (Abcam; ab75710; 1:3,000) and β-actin antibody (ACTB; Sigma-Aldrich; A2228; 1:8,000), for overnight at
4°C. The membranes were washed three times for 5 min in TBS-T at room temperature, incubated with a secondary antibody in immunoreaction enhancer solution (for HIF-1α, StAR and P450scc [1:5,000], anti-rabbit Ig, HRP-linked whole antibody produced in donkey; Amersham Biosciences Corp., Piscataway, NJ, USA; NA934; for 3β-HSD and ACTB [1:40,000], anti-mouse Ig, HRP-linked whole antibody produced in sheep; Amersham Biosciences Corp.; NA931) for 1 h and washed three times in TBS-T for 5 min at room temperature. The signal was detected with an ECL Western blotting detection system (Amersham Biosciences Corp.; RPN2109). The intensity of the immunological reaction (HIF-1α, StAR, P450scc, 3β-HSD, ACTB) in the 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).

**Statistical analysis**

All experimental data are shown as the mean ± SEM. The statistical analysis was performed using the GraphPad Prism 4 computer program. The statistical significance of differences in P4 production was assessed by ANOVA followed by a Fisher’s protected least-significant difference procedure (PLSD) as a multiple comparison test, while the statistical significance of differences in the amounts of STAR, CYP11A1 and HSD3B mRNA and the StAR, P450scc and 3β-HSD protein levels were assessed by two-way ANOVA with replications followed by Bonferroni post-tests to compare replicate means. \( P<0.05 \) was considered statistically significant.
RESULTS

P4 production by luteinizing and non-luteinizing granulosa cells

Insulin and forskolin increased P4 production by granulosa cells cultured for 24 h under normoxia (20% O2) (Fig. 1; \(P<0.05\)), with the highest P4 production was shown in granulosa cells cultured with insulin in combination with forskolin. Non-treated granulosa cells produced only a low level of P4. Based on these results, the treated and non-treated granulosa cells were used as models of luteinizing and non-luteinizing granulosa cells for further experiments to determine the effect of hypoxia.

Effects of hypoxia on HIF-1α protein expression

The expressions of HIF-1α protein were increased under 10% O2 (Fig. 2A) and 5% O2 (Fig. 2B) after 24 h.

Effects of hypoxia on P4 production by non-luteinizing and luteinizing granulosa cells

Hypoxia, both 10% and 5% O2, increased P4 production by luteinizing granulosa cells, while the same conditions did not affect P4 production by non-luteinizing granulosa cells (Fig. 3). The culture conditions under 10% O2 significantly increased P4 production both in granulosa cells treated with insulin and those treated with insulin in combination with forskolin compared with normoxia (20% O2) (Fig. 3A; \(P<0.05\)). However, under 5% O2, P4 production was significantly increased only in granulosa cells treated with insulin in combination with forskolin (Fig. 3B; \(P<0.05\)).

Effects of hypoxia on \textit{STAR}, \textit{CYP11A1} and \textit{HSD3B} mRNA expressions in non-luteinizing and luteinizing granulosa cells

A real-time PCR analysis showed that the culture conditions under 10% O2 significantly increased \textit{STAR} mRNA expression in granulosa cells treated with insulin
in combination with forskolin compared with normoxia (20% O₂) (Fig. 4A; P<0.05). However, the conditions under 5% O₂ did not affect *STAR, CYP11A1 and HSD3B* mRNA expressions (Fig. 4B).

**Effects of hypoxia on StAR, P450scc and 3β-HSD protein expressions in non-luteinizing and luteinizing granulosa cells**

The culture conditions under 10% O₂ significantly increased StAR and 3β-HSD protein expressions in granulosa cells treated with insulin in combination with forskolin compared with normoxia (Fig.5A and 5B; P<0.05), while the conditions under 5% O₂ did not affect StAR, P450scc and 3β-HSD protein expressions (Fig. 5C and 5D).
Fig. 1. Progesterone (P4) production by granulosa cells for 24 h in the presence or absence of insulin (2 µg/ml), forskolin (10 µM) or both under 20% O₂ (Cont, control; Ins, insulin; Fors, forskolin; Ins + Fors, insulin in combination with forskolin). All values represent mean ± SEM of four separate experiments. Different letters indicate significant differences (P<0.05), as determined by a Fisher’s PLSD as a multiple comparison test.
Fig. 2. Effects of hypoxia on HIF-1α protein. Representatives samples of Western blotting for HIF-1α protein expressions under 20% O₂ or 10% O₂ (A) and 20% O₂ or 5% O₂ (B) for 24 h in non-treated granulosa cells.
Fig. 3. Effects of hypoxia on progesterone (P4) production by non-luteinizing and luteinizing granulosa cells. The cells were cultured under 20% O$_2$ or 10% O$_2$ (A) and 20% O$_2$ or 5% O$_2$ (B) for 24 h in the presence or absence of insulin (2 µg/ml), forskolin (10 µM) or both (mean ± SEM). All values are expressed as a percentage of normoxia (20% O$_2$) of four separate experiments. Asterisks indicate significant differences (P<0.05) as determined by ANOVA followed by Fisher’s PLSD as a multiple comparison test.
Fig. 4. Effects of hypoxia on *STAR*, *CYP11A1* and *HSD3B* mRNA by non-luteinizing and luteinizing granulosa cells. The cells were cultured under 20% O$_2$ or 10% O$_2$ (A) and 20% O$_2$ or 5% O$_2$ (B) for 24 h in the presence or absence of insulin (2 µg/ml), forskolin (10 µM) or both. The amounts of *STAR*, *CYP11A1* and *HSD3B* mRNA are expressed relative to the amounts of 18S rRNA. The asterisk indicates a significant difference (*P*<0.05) within the same treatment group, as determined by two-way
ANOVA with replications (n = 3) followed by Bonferroni post-tests to compare replicate means.
Fig. 5. Effects of hypoxia on StAR, P450scc and 3β-HSD protein by non-luteinizing and luteinizing granulosa cells. The cells were cultured under 20% O\textsubscript{2} or 10% O\textsubscript{2} (A and B) and 20% O\textsubscript{2} or 5% O\textsubscript{2} (C and D) for 24 h in the presence or absence of insulin (2...
µg/ml), forskolin (10 µM) or both. Representative samples of Western blotting for StAR, P450scc, 3β-HSD and β-actin are shown in Fig. 5A for 10% O₂ and in Fig. 5C for 5% O₂. The blot was incubated with primary antibodies against StAR, P450scc, 3β-HSD or β-actin and then incubated with secondary antibody conjugated to HRP. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. All protein levels are expressed relative to the amounts of β-actin. Asterisks indicate significant differences (P<0.05) within the same treatment groups, as determined by two-way ANOVA with replications (n = 3) followed by Bonferroni post-tests to compare replicate means.
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</table>
Luteinization occurs after an LH surge, and the follicle differentiates into corpus luteum (CL) after ovulation. Meanwhile, the follicle is under its most hypoxic conditions around the time of ovulation [1, 14, 15]. Since hypoxia and luteinization occur at the same time, it raises the question whether hypoxia plays some roles during luteinization. In the present study, we used a model of luteinizing granulosa cells induced by insulin (2 µg/ml) and forskolin (10 µM). Bovine granulosa cells obtained from small antral follicles are known to differentiate into large luteal-like cells during culture in vitro [25]. Our results showed that the cultured granulosa cells treated with insulin in combination with forskolin showed increased P4 production after 24 h, and the production was the highest among the groups (Fig. 1). Insulin or insulin-like growth factor-I (IGF-I) is known to stimulate proliferation and P4 production in granulosa cells [30-34]. In addition, forskolin induces an increased intracellular cyclic AMP concentration via activation of adenylate cyclase [35]. Furthermore, insulin in combination with forskolin mimics the effect of LH and activates adenylate cyclase through upregulation of P4 production [36]. Therefore, these granulosa cells were used for further experiments in the present study.

Hypoxic conditions are known to cause accumulation of HIF-1α protein in cells and to enhance transcription of hypoxia-inducible genes [23, 24]. In the present study, we induced hypoxic conditions in our culture system by using low oxygen tension. We selected 10% and 5% O2 as the hypoxic conditions based on the following previous studies. The O2 levels in antral follicles of humans and pigs are around 7-11% [37, 38]. Basini et al. [5] demonstrated that severe hypoxic conditions (lowering the level to 1% O2) decreased both E2 and P4 production by swine granulosa cells, while partial hypoxia (5% O2) did not affect them. Hillier [39] also reported that partial hypoxic conditions are possibly more comparable to the conditions of follicular development, which relies on E2 and P4 production. The finding that the protein expression of HIF-1α was increased by 10% and 5% O2 conditions in the present study (Fig. 2A and 2B) shows that the cells were hypoxic. Furthermore, newly formed CLs after ovulation increase P4 production [16] and express high levels of HIF-1α [12]. In the present study, both P4 production and HIF-1α expression of luteinizing granulosa cells were increased...
under 10% O₂. Thus, the O₂ conditions in the cells cultured under 10% O₂ may be similar to the O₂ conditions in the cells under luteinization.

P4 production by the CL is essential for establishing and maintaining pregnancy. During luteinization, granulosa cells and theca cells differentiate into luteal cells, and P4 starts to be produced in large amounts [16]. The present results showed that under 10% O₂ and 5% O₂ for 24 h, P4 production by cultured granulosa cells treated with insulin in combination with forskolin increased (Fig. 3A and 3B). Furthermore, under 10% O₂, P4 production by the granulosa cells treated with only insulin also increased. Interestingly, hypoxic conditions did not affect the P4 production by non-luteinizing granulosa cells. These results suggest that hypoxic conditions promote P4 production during, rather than before, luteinization.

In our previous study [10], hypoxia inhibited basal and LH-stimulated P4 production by cultured bovine mid luteal cells, suggesting that hypoxia facilitates luteolysis. On the other hand, in the present study, hypoxia increased P4 production and seemed to promote P4 production during luteinization of granulosa cells. We have no clear explanation for these contradictory effects of hypoxia on P4 production between luteinizing granulosa cells and luteal cells. Hypoxia may differently affect cells depending on the differentiation status of granulosa and luteal cells. Mid luteal cells are matured luteal cells that produce the highest level of P4, while in the present study, we used luteinizing granulosa cells, which are immature luteal cells that have just begun to produce P4. The difference in cell status may be the reason for the contradictory action of hypoxia on luteolysis and luteinization.

Luteinization includes modification of steroidogenic enzymes and the steroidogenic acute regulatory protein expressions to bring about large-scale synthesis of P4. StAR, P450scc and 3β-HSD are known as the key protein and enzymes involved in P4 biosynthesis [20, 21, 40]. The expression of StAR and 3β-HSD are upregulated in theca and granulosa cells during the luteinization process [41-45]. The development of P450scc also characterizes the differentiation of follicular granulosa cells because the enzyme is not present or present only in low abundance in granulosa cells of the preovulatory follicle [46]. Our findings that 10% O₂ increased the mRNA and protein expressions of StAR (Fig. 4A) suggest that hypoxia enhances P4 production by increasing StAR expression. StAR is essential for steroidogenesis because it imports
cholesterol, a precursor of all steroids, into mitochondria [47]. Expression of StAR has also been shown to undergo luteinization-dependent upregulation in both pigs [40, 48] and cows [43]. Thus, its expression is an important marker for the luteinization process. Under 10% O₂, the protein expression of 3β-HSD was also increased but not the mRNA expression. The protein expression of 3β-HSD may be more highly stabilized under 10% O₂ than the mRNA expression. Under 5% O₂, there was no significant increase in StAR, P450scc and 3β-HSD mRNA and protein expressions in luteinizing granulosa cells; however, we could see that the mRNA and protein expressions of this protein and the enzymes were slightly increased under 5% O₂ in granulosa cells treated with insulin in combination with forskolin (Fig. 4B). These results suggest that 10% O₂ may reflect the O₂ concentration in vivo in the follicle around the time of ovulation. We previously showed that 3% O₂ decreases P4 production [10]. If we applied this lower O₂ concentration to our present model, P4 production and steroidogenesis may decrease too. Further studies are needed to confirm the relationship between HIF-1α and steroidogenic factors.

In conclusion, the overall findings suggest that hypoxia (10% O₂) promotes the P4 synthesis during luteinization by enhancing the expression of StAR and partly the expression of 3β-HSD, and this condition is important for establishing the CL.
SUMMARY

To determine whether hypoxia has an effect on luteinization, we examined the influence of hypoxia on a model of bovine luteinizing and non-luteinizing granulosa cell culture. The granulosa cells were obtained from small antral follicles (≤ 6 mm in diameter). To induce luteinization, the cells were treated for 24 h with insulin (2 µg/ml), forskolin (10 µM) or both at 20% O₂. After 24 h, progesterone (P4) production was higher in the treated cells, which we defined as luteinizing granulosa cells, than in non-treated cells, which we defined as non-luteinizing granulosa cells. P4 production by non-luteinizing granulosa cells was not affected by hypoxia (24 h at 10% and 5% O₂), while P4 production by granulosa cells treated with insulin in combination with forskolin was significantly increased under hypoxia (24 h at 10% and 5% O₂). Because hypoxia affected P4 production by the luteinizing granulosa cells but not by the non-luteinizing granulosa cells, hypoxia seems to promote P4 production during, rather than before, luteinization. In the cells treated with insulin in combination with forskolin, mRNA and protein expression of steroidogenic acute regulatory protein (StAR) and protein expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) increased under 10% O₂, while mRNA and protein expressions of key protein and enzymes in P4 biosynthesis did not increase under 5% O₂. The overall results suggest that hypoxia plays a role in progressing and completing the luteinization by enhancing P4 production through StAR as well as 3β-HSD expressions in the early time of establishing the corpus luteum.
CHAPTER 2
Hypoxia-inducible factor 1α mediates hypoxia-enhanced synthesis of progesterone during luteinization of granulosa cells

INTRODUCTION

During follicular growth, blood vessels that develop during follicular maturation in the theca cell layer do not penetrate the basement membrane [49]. The granulosa cell (GC) layer remains avascular until the breakdown of the basement membrane; thus, GCs are believed to develop under low oxygen (O₂) tension or hypoxic conditions, as compared to atmospheric O₂ tension [1-3]. Immediately after ovulation, the ruptured follicle is also thought to be under low oxygen tension due to bleeding and immature vascularization [6].

Cellular responses to hypoxic conditions are mediated by hypoxia-inducible factor 1 (HIF-1), an oxygen-regulated transcriptional activator [8]. HIF-1 is composed of two subunits: the oxygen-sensitive HIF-1α subunit and the constitutively expressed HIF-1β subunit [7, 8]. Under hypoxic conditions, the HIF-1α protein is stabilized and translocated from the cytoplasm to the nucleus, where it dimerizes with HIF-1β. This heterodimer then binds to a hypoxia response element (HRE) in target gene promoters and activates transcription of HIF-controlled genes involved in many physiological functions [7, 8]. There is increasing evidence that HIFs participate in ovulation and follicular differentiation. HIF-1α, induced by various stimuli, is suggested to serve as a key mediator of endothelin 2 expression, which performs a crucial function in ovulation in mammals [50]. In mice, HIFs control follicular rupture by regulating the expression of a specific subset of progesterone receptor (PGR)’s target genes, whereas blocking of HIF activity impairs ovulation [1]. Human chorionic gonadotropin (hCG) in synergy with hypoxic conditions has been demonstrated to up-regulate HIF-1α activity within luteinizing GCs both in vivo and in vitro; these findings suggest the fundamental roles for HIFs in follicle differentiation [15].
The protein level of HIF-1α increases in response to several stimuli, including hypoxia, proteasomal inhibitors, transition metals (Co\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\)), iron chelators [hydrophilic desferrioxamine (DFO) and lipophilic 2,2’-dipiridyl (DP)] and other stressors [51-53]. Iron chelators and transition metals suppress the interaction between iron-mediated hydroxylation of HIF-1α and pVHL binding and inhibit hydroxylation of a key proline residue within the ODD domain of HIF-1α, thus resulting in accumulation of the HIF-1α protein [52]. Treatment with cobalt chloride (CoCl\(_2\)) was found to mimic HIF-1 activation through inhibition of HIF-1α degradation. In latter process, HIF-1 activation strongly induces vascular endothelial growth factor (VEGF), which represents the most important mechanism for hypoxia-induced angiogenesis in GCs of several species [13, 14, 53-56]. On the other hand, HIF-1’s DNA-binding activity in the promoter region of target genes can be inhibited by echinomycin, a cyclic peptide that was originally discovered as a sequence-specific DNA-binding agent [57].

During the differentiation of GCs and theca cells into luteal cells, called the luteinization process, the main steroid product of ovaries (estrogen synthesized by follicles) is replaced by progesterone (P4) produced by the corpus luteum [16]. These changes are mediated by differentiation-dependent modification of the steroidogenic pathway. The key proteins and enzymes in P4 synthesis include steroidogenic acute regulatory protein (StAR; \(\text{STAR}\)), which transports cholesterol from the outer to inner mitochondrial membrane; cytochrome P450 side chain cleavage enzyme (P450scc; \(\text{CYP11A1}\)), which converts cholesterol into pregnenolone; and 3β-hydroxysteroid dehydrogenase (3β-HSD; \(\text{HSD3B}\)), which converts pregnenolone into P4 [17-21]. The rupture and collapse of a follicle at ovulation and the invasion by some elements, including theca cells and blood vessels, also commonly take place during luteinization [22].

Luteinization is thought to occur in a hypoxic environment. We previously suggested that hypoxia promotes P4 synthesis in our model of bovine luteinizing GCs [58]. Nevertheless, whether HIF-1α plays a role in hypoxia-driven enhancement of P4 synthesis during luteinization is not clear.

In the present study, we hypothesized that the hypoxia-induced increase in P4 synthesis during luteinization in bovine GCs is mediated by HIF-1α. To test this
hypothesis, we evaluated the effect of changes in the protein level of the α-subunit of HIF-1 by means of a hypoxia-mimetic compound (CoCl$_2$) and by means of low-oxygen-tension culture with or without echinomycin in bovine cultured luteinizing and non-luteinizing GCs. We then quantified the P4 production as well as mRNA and protein expression of StAR, P450scc, and 3β-HSD in these cells.
MATERIALS AND METHODS

GC isolation and culture

Bovine ovaries were obtained from a local slaughterhouse and were transported to the laboratory in ice-cold sterile physiological saline. The ovaries with healthy follicles were washed several times in sterile saline containing 100 IU/ml penicillin (Meiji Seika Pharma, Tokyo, Japan; 611400D3051) and 100 µg/ml streptomycin (Meiji Seika Pharma; 6161400D1034) as described previously [26]. GCs in follicular fluid were aspirated aseptically from healthy small follicles (≤ 6 mm in diameter) using a 2.5-ml disposable syringe and a 24-gauge needle, were pooled, then transferred to a plastic Petri dish filled with Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F-12 medium (1:1 [v/v]; Invitrogen, Carlsbad, CA, USA; 12400-024) containing 10% of calf serum (Invitrogen; 16170078), 20 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA; G1397), 2 µg/ml amphotericin B (Sigma-Aldrich; A9528), and 50 IU heparin sodium salt (Nacalai Tesque, Kyoto, Japan; 17513-41). After removal of cumulus-oocyte complexes with a fine glass pipet under a dissecting microscope, GCs in follicular fluid were centrifuged (800 × g, 5 min at 4°C), then resuspended in Tris-HCl buffer (25 mM, pH 7.4) to rupture the blood cells after discarding the supernatant. The cell suspensions were centrifuged again and resuspended in DMEM (Sigma-Aldrich; D1152) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1% of bovine serum albumin (BSA; Roche Diagnostics, Manheim, Germany; 10735086001) after the supernatant was discarded. This washing step was performed twice. The cell suspensions were then centrifuged, filtered through metal meshes (100 µm × 2, 80 µm × 2) to avoid cell aggregation, and were resuspended in a suitable volume of the culture medium (DMEM and Ham’s F-12 containing 10% of calf serum and 20 µg/ml gentamicin). The viability of GCs was assessed by a trypan blue dye exclusion assay.

The dispersed GCs were seeded at 0.5 × 10^5 viable cells per 1 ml in the culture medium in 75-cm² culture flasks (20 ml/flask; Greiner Bio-One, Frickenhausen, Germany; 658175) and cultured in a humidified atmosphere containing 5% CO₂ at 37.5°C in a N₂-O₂-CO₂-regulated incubator (ESPEC Corp., Osaka, Japan; BNP-110) for
3–4 days. When the cultured cells reached 80-90% confluence, cell passaging was conducted using 0.1% bovine trypsin (Sigma-Aldrich; T92012) and sterile phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; 05913). The GCs were seeded at the concentration of $2.0 \times 10^5$ viable cells per 1 ml in 48-well cluster dishes (0.5 ml/well; Greiner Bio-One; 662160) for quantification of P4 production, in 96-well cluster dishes (0.1 ml/well; Iwaki, Chiba, Japan; 3860-096) for the cell viability assay, in 24-well cluster dishes (1.0 ml/well; Greiner Bio-One; 677180) for determination of gene expression, and in 75 cm$^2$ culture flasks (20 ml/flask; Greiner Bio-One; 658175) for analysis of protein expression.

**Preparation of luteinizing and non-luteinizing GCs**

To prepare luteinizing and non-luteinizing GCs, the culture medium was replaced with a fresh medium containing 0.1% of BSA, 5 ng/ml sodium selenite (Sigma-Aldrich; S5261), 5 µg/ml transferrin (Sigma-Aldrich; T4132), and 0.5 mM ascorbic acid (Wako-Pure Chemical Industries Osaka, Japan; 031-12061), and the cells were then incubated in a normal culture atmosphere (20% O$_2$, 5% CO$_2$, and 75% N$_2$) with or without insulin (2 µg/ml; Sigma-Aldrich; I4011) in the medium in combination with forskolin (10 µM; Research Biochemicals International, Natick, MA, USA; 70-0501-05) for 24 h. Insulin and insulin-like growth factor I (IGF-I) are known to stimulate proliferation of (and P4 production in) GCs [30, 32-34, 59]. In addition, forskolin increases intracellular cyclic AMP concentration via activation of adenylate cyclase [35]. Insulin in combination with forskolin mimics the effects of luteinizing hormone (LH) and activates adenylate cyclase via upregulation of P4 [36]. The concentration of insulin and forskolin was selected according to other reports [25, 58].

**Experiment 1: Effects of CoCl$_2$ on P4 production and cell viability**

To determine the effects of hypoxia, a hypoxia-mimicking agent (CoCl$_2$; Sigma-Aldrich; C8661) was used. The luteinizing and non-luteinizing GCs were cultured in the presence or absence of CoCl$_2$ (100 or 250 µM) for 2, 6, or 24 h. The conditioned media and the cultured cells were then collected to quantify P4 production by an enzyme
immunoassay (EIA) and a spectrophotometric method. The cell viability after 24 h culture was also determined by Dojindo Cell Counting Kit including WST-1 (Dojindo, Kumamoto, Japan; 345-06463).

**Experiment 2: Effects of CoCl₂ on mRNA and protein expression levels of StAR, P450scC, and 3β-HSD**

To measure the effect of CoCl₂ on P₄ synthesis, mRNA and protein expression levels of StAR, P450scC, and 3β-HSD were also evaluated. The luteinizing and non-luteinizing GCs were cultured in the presence or absence of CoCl₂ (100 or 250 µM) for 2 or 6 h. The cells were then collected for real-time PCR and western blotting.

**Experiment 3: The effect of CoCl₂ on HIF-1α protein expression**

Because HIF-1α expression is known to be strongly regulated by hypoxic conditions, we determined the effect of CoCl₂ on HIF-1α protein expression. The luteinizing and non-luteinizing GCs were cultured in the presence or absence of CoCl₂ (100 or 250 µM) for 2, 6, or 24 h. The cultured cells were then washed with PBS and harvested for western blotting.

**Experiment 4: The effect of echinomycin on CoCl₂-enhanced P₄ synthesis**

To demonstrate the involvement of HIF-1α in CoCl₂-enhanced P₄ synthesis, echinomycin (Sigma-Aldrich; SML0477), a small-molecule inhibitor of HIF-1 activity, was used. The luteinizing and non-luteinizing GCs were exposed to CoCl₂ (100 µM) in the presence or absence of echinomycin for 2 or 6 h. P₄ production and mRNA and protein expression levels of *STAR, P450scC*, and *3β-HSD* were then quantified.

**Experiment 5: The effect of echinomycin on 10% O₂-enhanced P₄ synthesis**

In our previous study, we found that 10% O₂ increases P₄ synthesis [26]. To determine the role of HIF-1 in 10% O₂-enhanced P₄ synthesis, echinomycin—a small
molecule inhibitor of HIF-1 activity [57]—was added to the culture medium of luteinizing and non-luteinizing GCs incubated at 10% O₂ for 24 h. P4 production and the mRNA and protein expression levels of STAR, P450scc, and 3β-HSD were then measured.

**Quantification of P4 production**

To measure P4 production, EIA and DNA assay were performed. The conditioned media were collected and stored at -30°C until analysis of P4 concentration. This concentration was determined by EIA as described previously [26]. The standard curve had a range from 0.391 to 100 ng/ml. To fit the range of concentrations of the standards, the culture media were diluted 1:10. The cultured cells were also stored at -30°C until the DNA content was measured by spectrophotometry as described previously [27] and was used to normalize the P4 concentrations. Four experiments were conducted, and each treatment was tested in triplicate wells in each experiment. Neither CoCl₂ nor 10% O₂ altered the cell number (data not shown).

**RNA isolation, cDNA synthesis, and real-time PCR**

Total RNA was extracted to determine mRNA expression of *STAR, CYP11A1*, and *HSD3B*. For this purpose, we used TRIsure (Bioline, London, UK; BIO-38033). The extracted RNA from each sample was quantified using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was reverse-transcribed on a ThermoScript RT-PCR system (Invitrogen; 11146-016).

*STAR, CYP11A1*, and *HSD3B* mRNA expression levels were measured by real-time PCR using the MyiQ (Bio-Rad, Tokyo, Japan) and the iQ SYBR Green supermix (Bio-Rad; 170-8880) starting with 1 ng of reverse-transcribed total RNA as described previously [28]. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1,000). Expression of the 18S ribosomal RNA gene (*18SrRNA*) served as an internal control. In a preliminary experiment, *18SrRNA* was confirmed to not be
influenced by luteinization and hypoxia (data not shown). Twenty-base pair primers with 50–60% GC content were synthesized for PCR (Table 2).

The PCR conditions were as follows: 95°C for 30 sec, followed by 45 cycles of 94°C for 6 sec, 60°C for 30 sec, and 65°C for 6 sec. The use of the QuantiTect SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the PCR products, with high linearity. The melting curve analysis was used to confirm that only the target amplicon was amplified.

Western blotting

The cells were washed with ice-cold PBS, scraped from the culture flask in 1 ml of ice-cold homogenization buffer (25 mM Tris-HCl pH 7.4, 300 mM sucrose, 2 mM EDTA, and Complete [protease inhibitor cocktail; Roche Diagnostics; 11697498001]). The cell suspension was centrifuged at 19,000 × g for 30 min, the supernatant was discarded, and the pellet was lysed in 100 µl of lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% of Triton X-100, 10% of glycerol [Sigma; G7757], and Complete). The protein samples were then stored at -80°C until protein expression of HIF-1α, StAR, P450scc, and 3β-HSD was analyzed by western blotting.

The protein concentration was determined by the method described elsewhere [29], using BSA as a standard. The protein samples were solubilized in SDS gel-loading buffer (50 mM Tris-HCl pH 6.8, 2% of SDS [Nacalai Tesque; 31607-94], 10% of glycerol, and 1% of β-mercaptoethanol [Wako Pure Chemical Industries; 137-06862]) and heated at 95°C for 10 min. Next, the samples (50 µg protein) were subjected to SDS-PAGE in a 7.5% gel with pre-stained molecular weight markers (Bio-Rad; 161-0374) for 1 h at 200 V.

The separated proteins were electrophoretically transblotted to a PVDF membrane (GE Healthcare, Limited, Buckinghamshire, UK; RPN1416LFP) for 1 h at 25 V in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% of methanol). The membrane was washed in TBS-T (0.1% of Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]) for 10 min and was incubated in PVDF blocking buffer (Toyobo, Co., Ltd., Osaka, Japan; NYBR01) for 1 h at room temperature. The membranes were
then incubated separately with a primary antibody in an immunoreaction enhancer solution (Toyobo, Co., Ltd., Osaka, Japan; NKB-101) specific to each protein: an anti-HIF-1α antibody (Sigma-Aldrich; SAB2104366; 1:500), anti-StAR antibody (Abcam; ab96637; 1:3,000), anti-P450scs antibody (Abcam; ab75497; 1:1,000), anti-3β-HSD antibody (Abcam; ab75710; 1:3,000), and an anti-β-actin antibody (ACTB; Sigma-Aldrich; A2228; 1:8,000) overnight at 4°C. The membranes were washed three times for 5 min in TBS-T at room temperature, incubated with a secondary antibody in the immunoreaction enhancer solution (for HIF-1α, StAR, and P450scs [1:5,000 dilution]: an anti-rabbit IgG horseradish peroxidase [HRP]-conjugated whole antibody produced in donkey; Amersham Biosciences Corp., Piscataway, NJ; NA934; 3β-HSD and ACTB [1:40,000]: an anti-mouse IgG HRP-conjugated whole antibody produced in sheep; Amersham Biosciences Corp. NA931) for 1 h and were washed three times in TBS-T 5 min each at room temperature. The signals were detected by means of the ECL Western Blotting Detection System (Amersham Biosciences Corp.; RPN2109). The intensity of the immunological reaction (HIF-1α, StAR, P450scs, 3β-HSD, and ACTB) in the cells was estimated by measuring optical density of a defined area by computerized densitometry in the NIH Image software (National Institutes of Health, Bethesda, MD, USA).

**WST-1 Assay**

WST-1, a version of MTT (3-[4,5-dimethyl-2 thiazolyl]-2,5-diphenyl-2 H-tetrazolium bromide), is a yellow tetrazolium salt that is reduced to formazan by viable cells containing active mitochondria. The culture medium was replaced with 100 μl of the D/F medium with BSA without phenol red, and a 10-μl aliquot of the assay reagent (0.3% WST-1, 0.2 mM 1-methoxy phenazine methosulfate in PBS, pH 7.4) was added to each well. The cells were then incubated for 4 h at 38°C. The absorbance was read at 450 nm using a microplate reader (Model 450; Bio-Rad Laboratories). The measured absorbance directly correlates with the number of viable cells [60]. In this assay, data were expressed as a percentage of the appropriate control values.
**Statistical analyses**

All data are shown as mean ± SEM. The statistical analyses were performed in the GraphPad Prism 4 software. Statistical significance of differences in all experiments was assessed by one-way analysis of variance (ANOVA) followed by Fisher’s protected least-significant difference procedure as a multiple-comparison test for each group: the group of non-luteinizing GCs and the group of luteinizing GCs. Differences with $P < 0.05$ were considered statistically significant.
RESULTS

P4 production by luteinizing and non-luteinizing GCs

Insulin increased P4 production by GCs cultured for 24 h under 20% O₂ (Fig. 6A; \( P < 0.05 \)). GCs cultured with insulin in combination with forskolin produced more P4. According to these results, the treated and untreated GCs were used as a model of luteinizing and non-luteinizing GCs in further experiments.

Effects of CoCl₂ on P4 production and cell viability

To characterize the effects of hypoxia and HIF-1α, we cultured luteinizing and non-luteinizing GCs with 100 or 250 µM CoCl₂ for 2, 6, or 24 h. After 2 and 6 h of culture, 100 and 250 µM CoCl₂ increased P4 production by luteinizing GCs, but these treatments did not have any effect on P4 production by non-luteinizing GCs (Fig. 6B and 6C). On the other hand, after 24 h of culture, 100 and 250 µM CoCl₂ tended to decrease P4 production by luteinizing GCs (Fig. 6D). CoCl₂ did not affect cell viability within 24 h after CoCl₂ addition, meaning that CoCl₂ under these conditions did not cause cell toxicity in this study (Fig. 6E).

Effects of CoCl₂ on mRNA and protein expression levels of StAR, P450sc, and 3β-HSD

To determine the effects of hypoxia and HIF-1 on P4 synthesis, we also analyzed the expression of key steroidogenic factors involved in P4 synthesis: StAR, P450sc, and 3β-HSD. The real-time PCR analysis showed that 100 µM CoCl₂ after 6 h significantly increased STAR mRNA expression in luteinizing GCs (Fig. 7A; \( P < 0.05 \)) but did not affect CYP11A1 and HSD3B mRNA expression levels (data not shown).

Western blotting analyses revealed that 100 µM CoCl₂ after 2 and 6 h significantly increased StAR protein expression in luteinizing GCs (Fig. 7B; \( P < 0.05 \)) but did not affect CYP11A1 and HSD3B protein expression (Fig. 7C and 7D).
The effect of CoCl$_2$ on HIF-1α protein expression

After 2 and 6 h of culture in the presence of 100 µM CoCl$_2$, we observed the highest expression of the HIF-1α protein concomitant with an increase in StAR protein expression (Fig. 8). Although the expression of HIF-1α was detectable, there was no significant difference between the presence and the absence of 100 µM CoCl$_2$ in culture. Based on these results, we chose 100 µM CoCl$_2$ for 2 and 6 h as the conditions mimicking hypoxia in all subsequent experiments.

The effect of echinomycin on CoCl$_2$-enhanced P4 synthesis

0.5 nM echinomycin inhibited P4 production (Fig. 9A; $P < 0.05$) and the expression of $STAR$ mRNA (Fig. 9B; $P < 0.05$) and protein (Fig. 9C and 9D; $P < 0.05$) under hypoxic conditions induced by CoCl$_2$ (incubation for 2 or 6 h) in luteinizing GCs.

The effect of echinomycin on 10% O$_2$-enhanced P4 synthesis

We also evaluated the involvement of HIF-1 in 10% O$_2$-enhanced P4 synthesis by means of echinomycin. In agreement with our previous findings [58], culturing of luteinizing GCs under 10% O$_2$ significantly increased P4 production after 24 h (Fig. 10A; $P < 0.05$). Culturing of luteinizing GCs under 10% O$_2$ also increased the expression of $STAR$ mRNA (Fig. 10C; $P < 0.05$) and protein (Fig. 10D and 10E; $P < 0.05$) in comparison with cultivation under 20% O$_2$. Echinomycin attenuated the 10% O$_2$ effects that increased P4 production (Fig. 10B; $P < 0.05$) and the increased expression of $STAR$ mRNA (Fig. 10C; $P < 0.05$) and protein (Fig. 10D and 10E; $P < 0.05$) after 24 h in luteinizing GCs.
Fig. 6. Effects of CoCl₂ on P4 production by luteinizing and non-luteinizing GCs and on cell viability. To prepare luteinizing and non-luteinizing GCs, we cultured the cells at 20% O₂ in the presence of insulin (2 µg/ml) and/or forskolin (10 µM) for 24 h. P4 production was then quantified (A). To measure P4 production, the culture media were collected for an enzyme immunoassay (EIA) of P4, while the cultured cells were collected for measurement of DNA content by spectrophotometry to normalize the P4 concentration. The cells cultured without insulin and forskolin were defined as non-
luteinizing GCs, while the cells cultured in the presence of insulin in combination with forskolin were defined as luteinizing GCs (control: non-luteinizing GCs, insulin + forskolin: luteinizing GCs). After 24 h, the luteinizing and non-luteinizing GCs were cultured with or without CoCl$_2$ (100 or 250 µM) for 2 (B), 6 (C), or 24 h (D) to determine the effect of CoCl$_2$ on P4 production. P4 production value was shown as a percentage of control (cultured non-luteinizing GCs without CoCl$_2$). (E) The effect of CoCl$_2$ on cell viability was also determined. The cultured cells were incubated with the WST-1 reagent for 4 h at 38°C, then the absorbance was read using a microplate reader. The data are shown as a percentage of control (cultured cells without CoCl$_2$) for each group: the group of luteinizing GCs and the group of non-luteinizing GCs. Different letters (A) and asterisks (B, C) indicate significant differences ($P < 0.05$) between groups as determined by one-way ANOVA. All data represent mean ± SEM of four independent experiments.
All the protein expression levels are expressed relative to the level of β-actin protein.
expression. The blot was incubated with primary antibodies against StAR, P450scc, 3β-HSD, or β-actin and then incubated with a secondary antibody conjugated with HRP. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. Asterisks indicate significant differences ($P < 0.05$) between groups—the group of luteinizing GCs and the group of non-luteinizing GCs—as determined by one-way ANOVA. All data represent mean ± SEM of four independent experiments.
Fig. 8. Effects of CoCl$_2$ on HIF-1α and StAR protein expression levels. The luteinizing and non-luteinizing GCs were incubated with or without CoCl$_2$ (100 or 250 µM) for 2 h or 6 h. The cells were then collected to determine HIF-1α and StAR protein expression by western blotting. The blot was incubated with primary antibodies against HIF-1α, StAR, or β-actin and then with a secondary antibody conjugated with HRP. The resultant signals were detected by chemiluminescence and quantitated by computer-assisted densitometry.
Fig. 9. The effect of echinomycin (Ech) on CoCl$_2$-enhanced P4 synthesis. The luteinizing and non-luteinizing GCs were cultured with or without 0.5 nM echinomycin in the presence of 100 µM CoCl$_2$ for 2 or 6 h. To determine P4 production, the culture media were collected for EIA to measure P4 concentration, while the cultured cells were
collected for analysis of DNA content by a spectrophotometric method to normalize the P4 concentrations. P4 amounts are shown as a percentage of the control (cultured non-luteinizing GCs without CoCl₂) (A). *STAR* mRNA expression was determined by real-time PCR (B). The amount of *STAR* mRNA is expressed relative to the amount of 18SrRNA mRNA. The protein expression of StAR was determined by western blotting (C). The protein level is expressed relative to the protein expression of β-actin. Asterisks indicate significant differences (*P* < 0.05) between groups—the group of luteinizing GCs and the group of non-luteinizing GCs—as determined by one-way ANOVA. All data represent mean ± SEM of four independent experiments. Representative examples of western blotting for StAR and β-actin are shown in (D).
Fig. 10. The effect of echinomycin (Ech) on 10% O₂-enhanced P4 synthesis. The luteinizing and non-luteinizing GCs were cultured at 20% O₂ as a control or at 10% O₂ as low-oxygen tension for 2, 6, or 24 h to quantify P4 production (A). To this end, the culture media were collected for EIA of P4, while the cultured cells were collected for measurement of DNA content by a spectrophotometric method to normalize the P4 concentrations. Other batches of luteinizing and non-luteinizing GCs were cultured at
20% or 10% O₂ with or without 0.5 nM echinomycin for 24 h, then P4 production was measured (B). P4 amounts are shown as a percentage of the control (cultured non-luteinizing GCs cells at 20% O₂ without echinomycin). *STAR* mRNA expression was quantified by real-time PCR (C), and StAR protein expression was determined by western blotting (D). Asterisks indicate significant differences (\( P < 0.05 \)) between groups—the group of luteinizing GCs and the group of non-luteinizing GCs—as determined by one-way ANOVA. All data represent mean ± SEM of four independent experiments. Representative examples of western blotting for StAR and β-actin are shown in (E).
<table>
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</table>
DISCUSSION

LH pulses increase GCs and the oocyte to develop in an avascular environment that is considered to be hypoxic [1-3]. The microenvironment of the ruptured follicle is also thought to be hypoxic immediately after ovulation [6]. Thus, luteinization and hypoxia seem to occur simultaneously gradually and result in an LH surge. The increasing LH pulses stimulate dominant follicle growth, ovulation, and luteinization [61, 62]. In the follicle compartment before ovulation, the time of ovulation. Luteinization is defined as the process in which GCs and theca cells differentiate into luteal cells and then produce a large amount of P4, which is important for establishing pregnancy [51, 63-65]. We previously suggested that hypoxic conditions promote the progression and completion of luteinization by enhancing P4 synthesis in bovine GCs [58]. Using the same model of bovine cultured luteinizing GCs, we found in the present study that enhancement of P4 synthesis either by hypoxia (10% O2) or by CoCl2 is attenuated by the addition of echinomycin, the inhibitor of HIF-1 DNA binding [57], suggesting that HIF-1α-mediated P4 upregulation takes place during luteinization. The present results strongly support the idea that hypoxic conditions are important for luteinization and for steroidogenesis during this period.

In the present study, we used CoCl2 to mimic hypoxia because treatment with this compound has been shown to successfully mimic hypoxia in other studies [17, 50, 66]. CoCl2 (100 and 250 µM) increased P4 production after 2 and 6 h in luteinizing GCs. Concentrations of CoCl2 up to 500 µM have been used in some studies on HIF-1α [67, 68]. Simultaneously with the increase in P4 production, 100 µM CoCl2 can also be sufficient to increase the HIF-1α protein level after 2 and 6 h. In addition, the present results indicated that CoCl2 has the same effects as 10% O2 does on HIF-1α protein expression and on P4 production in luteinizing GCs, as shown in our previous study [58]. This low-oxygen condition may represent the O2 condition in the cells during luteinization. This finding suggests that low oxygen conditions promote P4 production during rather than before luteinization.

Our results showed that treatment of luteinizing GCs with CoCl2 for 24 h tended to decrease their P4 production. Similarly, 500 µM CoCl2 and 1% O2 for 24 h inhibited P4 synthesis in bovine luteinized GCs obtained from the largest follicle [69]. Hypoxia
seems to have a biphasic effect on steroidogenesis depending on oxygen tension and on duration of exposure to hypoxia. The induction of apoptosis by hypoxia was found to be most pronounced after exposure of normal cells or tissues to severe hypoxia [70, 71]. Hypoxia-mimicking agents, such as CoCl$_2$, and low oxygen tension are also known to induce apoptosis in a number of cell types [70]. Nevertheless, the degrees of apoptosis in the GCs exposed to CoCl$_2$ or to low oxygen tension was not assessed in the present study, and further studies would be required to address this issue.

StAR is one of the key proteins in P4 synthesis. In GCs, StAR expression signals early functional maturation of an ovarian antral follicle [72]. During luteinization, StAR expression is upregulated in order to transfer cholesterol from the outer to inner mitochondrial membrane [41-45, 52]. We confirmed that luteinizing GCs in our present model show higher expression of StAR in comparison with non-luteinizing GCs as shown in our previous study [58]. In luteinizing GCs, expression of the StAR protein is increased by 100 µM CoCl$_2$ after 2 or 6 h of incubation and by 10% O$_2$ after 24 h. The observed exposure duration necessary for CoCl$_2$ or 10% O$_2$ to increase StAR expression in luteinizing GCs is in agreement with their exposure time necessary to increase P4 production. One of the reasons why CoCl$_2$ increased P4 production (Fig. 6B and 6C) together with mRNA (Fig. 7A) and protein expression of StAR (Fig. 7B) during shorter culture periods (2 and 6 h) than 10% O$_2$ did (24 h; Fig. 10A–E) is that the direct inhibition of HIF-1α protein degradation by CoCl$_2$ [20] is more effective than the influence of 10% O$_2$ on HIF-1α protein accumulation. On the other hand, neither CoCl$_2$ nor 10% O$_2$ affected P450scc and 3β-HSD expression levels. These results suggest that hypoxic conditions promote P4 production via the upregulation of StAR during luteinization.

Echinomycin is a DNA-binding agent that binds to the HRE site within the promoters of HIF-1’s target genes and selectively inhibits the binding activity of HIF-1α [57]. In rats undergoing gonadotropin-induced ovulation, blockage of HIF-1 activity by echinomycin profoundly impairs the rupture of preovulatory follicles and reduces VEGF expression [1], suggesting that HIF-1 performs important functions in ovulation, especially in steroidogenesis during luteinization. Our finding in the present study that echinomycin attenuated hypoxia-enhanced P4 production and StAR expression in luteinizing GCs suggests that the increase in P4 synthesis during luteinization is
stimulated by the transcription-regulatory activity of HIF-1. All these results may explain additional roles of hypoxia and HIF-1 around approximately at the time of ovulation, especially steroidogenesis, which is required not only for ovulation but also for luteinization.

In our previous studies [11, 12], we reported that hypoxia also affects luteolysis. Hypoxia has been shown to induce corpus luteum regression and to promote apoptosis of luteal cells, in which an oxygen deficiency or low-oxygen conditions suppress P4 production. It seems that hypoxia has a biphasic effect on P4 production during luteinization and in luteolysis depending on the differentiation status of the cells. The cells used in the present study were early-growing luteal cells, which start producing a large amount of P4, whereas the cells analyzed in our previous study were luteal cells obtained from a mid-stage corpus luteum, which produce the largest amount of P4.

In agreement with our present results, it has been reported that 10% O2 stimulates \textit{STAR} gene transcription in immortalized (KK1) murine GCs, and that HIF-1α seems to be actively involved in direct regulation of basal and dibutyryl cyclic AMP-stimulated StAR protein expression by binding to the proximal murine \textit{STAR} promoter [73]. Further studies are needed to explain why hypoxic conditions and HIF-1α affected P4 synthesis in luteinizing GCs but not in non-luteinizing GCs in the present study. In conclusion, overall, our findings suggest that the hypoxia-induced increase in P4 production and in StAR expression in bovine cultured luteinizing GCs is mediated by HIF-1α. In other words, by enhancing P4 synthesis, HIF-1α may play as an important factor in the progression of luteinization by enhancing P4 synthesis.
SUMMARY

Hypoxia has been suggested to enhance progesterone (P4) synthesis in luteinizing granulosa cells (GCs), but the mechanism is unclear. The present study was designed to test the hypothesis that the hypoxia-induced increase in P4 synthesis during luteinization in bovine GCs is mediated by hypoxia-inducible factor 1α (HIF-1α). GCs obtained from small antral follicles were cultured with 2 µg/ml insulin in combination with 10 µM forskolin for 24 h as a model of luteinizing GCs. To examine the influence of HIF-1 on P4 synthesis, the effect of changes in protein expression of the α-subunit of HIF-1 (HIF1A) on P4 production and on the expression levels of StAR, P450scc, and 3β-HSD was determined. Cobalt chloride (CoCl₂, 100 µM), a hypoxia-mimicking chemical, increased HIF-1α protein expression in luteinizing GCs. After the upregulation of HIF-1α, increases in P4 production and in the gene and protein expression levels of StAR in CoCl₂-treated luteinizing GCs were observed. In contrast, CoCl₂ did not affect the expression of either P450scc or 3β-HSD. Echinomycin, a small-molecule inhibitor of HIF-1’s DNA-binding activity, attenuated the effects of CoCl₂ and of low oxygen tension (10% O₂) on P4 production and StAR expression in luteinizing GCs. Overall, these findings suggest that HIF-1α is one of the factors that upregulate P4 in GCs during luteinization.
CONCLUSION

Progesterone (P4) is a hormone which is essential for establishing the pregnancy. During luteinization, the follicular cells start to produce a large amount of progesterone. At the same time, this process is occurred under low oxygen condition. The present study investigated the possible roles of low oxygen tension and hypoxia-inducible factor 1α (HIF-1α) transcription factor on P4 synthesis during luteinization in bovine granulosa cells. A model of luteinizing GCs was conducted by the induction by insulin and forskolin for 24 h. Low oxygen condition was induced by a lower oxygen tension (10% O₂) and a hypoxia-mimicking chemical (cobalt chloride, CoCl₂). P4 production as well as mRNA and protein expression of StAR, P450scc, and 3β-HSD, a key protein and enzymes involved in P4 synthesis, were then measured in luteinizing GCs. Either 10% O₂ or 100 µM CoCl₂ increased HIF-1α protein expression in luteinizing GCs. After the upregulation of HIF-1α, there was an increase in P4 production and in the gene and protein expression levels of StAR in luteinizing GCs cultured under 10% O₂ and luteinizing GCs treated with CoCl₂. Further, to confirm whether HIF-1α mediates low oxygen condition-induced P4 synthesis, the luteinizing GCs were treated with echinomycin, a small-molecule inhibitor of HIF-1’s DNA-binding activity. Echinomycin attenuated the effects of CoCl₂ and of low oxygen tension (10% O₂) on P4 production and StAR expression in luteinizing GCs. Overall, these data provide an insight on the effect of low oxygen condition and HIF-1α in promoting P4 synthesis on granulosa cells during luteinization which is important for formation of corpus luteum.
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