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—Original Article—

Hypoxia-inducible factor 1 mediates hypoxia-enhanced synthesis of progesterone during luteinization of granulosa cells

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Abstract. 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 1 (HIF-1). 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, we determined 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. $CoCl_2$ (100 μM), a hypoxia-mimicking chemical, increased HIF-1α protein expression in luteinizing GCs. After the upregulation of HIF-1α, we observed an increase in P4 production and in the gene and protein expression levels of StAR in $CoCl_2$-treated luteinizing GCs. In contrast, $CoCl_2$ 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 $CoCl_2$ and of low oxygen tension (10% O_2) on P4 production and StAR expression in luteinizing GCs. Overall, these findings suggest that HIF-1 is one of the factors that upregulate P4 in GCs during luteinization.

Key words: Corpus luteum, Granulosa cell, Ovary, Progesterone

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During follicular growth, blood vessels that develop during follicular maturation in the theca cell layer do not penetrate the basement membrane [1]. The granulosa cell (GC) layer remains avascular until the breakdown of the basement membrane; thus, GCs are believed to develop under low oxygen (O_2) tension or hypoxic conditions, as compared to atmospheric O_2 tension [2–4]. Immediately after ovulation, the ruptured follicle is also thought to be under low oxygen tension due to bleeding and immature vascularization [5].

Cellular responses to hypoxic conditions are mediated by hypoxia-inducible factor 1 (HIF-1), an oxygen-regulated transcriptional activator [6]. HIF-1 is composed of two subunits: the oxygen-sensitive HIF-1α subunit and the constitutively expressed HIF-1β subunit [6, 7]. 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 [6, 7]. 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 [8]. 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 [2]. 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 [9].

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 [10–12]. 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 [11]. Treatment with cobalt chloride ($CoCl_2$) was found to mimic HIF-1 activation through inhibition of HIF-1α degradation. In latter process, HIF-1activation strongly induces vascular endothelial growth factor (VEGF), which represents the most important mechanism for hypoxia-induced angiogenesis in GCs of several species [12–17]. 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 [18].

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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 [19]. 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; *STAR*), which transports cholesterol from the outer to inner mitochondrial membrane; cytochrome P450 side chain cleavage enzyme (P450scc; *CYP11A1*), which converts cholesterol into pregnenolone; and 3β-hydroxysteroid dehydrogenase (3β-HSD; *HSD3B*), which converts pregnenolone into P4 [20–24]. 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 [25].

Luteinization is thought to occur in a hypoxic environment. We previously suggested that hypoxia promotes P4 synthesis in our model of bovine luteinizing GCs [26]. Nevertheless, whether HIF-1 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-1. 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-cm^2 culture flasks (20 ml/flask; Greiner Bio-One, Frickenhausen, Germany; 658175) and cultured in a humidified atmosphere containing 5% CO_2 at 37.5°C in a N_2-O_2-CO_2-regulated incubator (ESPEC, 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, Tokyo, Japan; 05913). The GCs were seeded at the concentration of 2.0×10^5 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 and hypoxic culture conditions

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 [27–31]. In addition, forskolin increases intracellular cyclic AMP concentration via activation of adenylate cyclase [32]. Insulin in combination with forskolin mimics the effects of luteinizing hormone (LH) and activates adenylate cyclase via upregulation of P4 [33]. The concentration of insulin and forskolin was selected according to other reports [26, 34].

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 $CoCl_2$ on mRNA and protein expression levels of StAR, P450scc, and 3β-HSD

To measure the effect of $CoCl_2$ 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 $CoCl_2$ (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 $CoCl_2$ on HIF-1α protein expression

Because HIF-1α expression is known to be strongly regulated by hypoxic conditions, we determined the effect of $CoCl_2$ on HIF-1α protein expression. 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 cultured cells were then washed with PBS and harvested for western blotting.

Experiment 4: The effect of echinomycin on $CoCl_2$-enhanced P4 synthesis

To demonstrate the involvement of HIF-1α in $CoCl_2$-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 $CoCl_2$ (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% O_2-enhanced P4 synthesis

In our previous study, we found that 10% O_2 increases P4 synthesis [26]. To determine the role of HIF-1 in 10% O_2-enhanced P4 synthesis, echinomycin — a small molecule inhibitor of HIF-1 activity [18] — was added to the culture medium of luteinizing and non-luteinizing GCs incubated at 10% O_2 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 [35]. 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 [36] and was used to normalize the P4 concentrations. Four experiments were conducted, and each treatment was tested in triplicate wells in each experiment. Neither $CoCl_2$ nor 10% O_2 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 [37]. 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 1).

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.

Table 1. Primers used in real-time PCR

Gene	Primer	Sequence (5'–3')	Accession no.	Product (bp)
STAR	Forward	CCCATGGAGAGGCTTTATGA	Y17259	115
	Reverse	TGATGACCGTGTCTTTTCCA		
CYP11A1	Forward	CTGGCATCTCCACAAAGACC	J05245	131
	Reverse	GTTCTCGATGTGGCGAAAGT		
HSD3B	Forward	CCAAGCAGAAAACCAAGGAG	X17614	109
	Reverse	ATGTCCACGTTCCCATCATT		
18SrRNA	Forward	TCGCGGAAGGATTTAAAGTG	AY779625	141
	Reverse	AAACGGCTACCACATCCAAG		

The protein concentration was determined by the method described elsewhere [38], 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, 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; 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-P450scc 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 P450scc [1:5,000 dilution]: an anti-rabbit IgG horseradish peroxidase [HRP]-conjugated whole antibody produced in donkey; Amersham Biosciences, Piscataway, NJ; NA934; 3β-HSD and ACTB [1:40,000]: an anti-mouse IgG HRP-conjugated whole antibody produced in sheep; Amersham Biosciences; 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; RPN2109). The intensity of the immunological reaction (HIF-1α, StAR, P450scc, 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).

The 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 [39]. 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% O_2 (Fig. 1A; $P < 0.05$). 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 $CoCl_2$ 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 $CoCl_2$ for 2, 6, or 24 h. After 2 and 6 h of culture, 100 and 250 μM $CoCl_2$ increased P4 production by luteinizing GCs, but these treatments did not have any effect on P4 production by non-luteinizing GCs (Fig. 1B and 1C). On the other hand, after 24 h of culture, 100 and 250 μM $CoCl_2$ tended to decrease P4 production by luteinizing GCs (Fig. 1D). $CoCl_2$ did not affect cell viability within 24 h after $CoCl_2$ addition, meaning that $CoCl_2$ under these conditions did not cause cell toxicity in this study (Fig. 1E).

Effects of $CoCl_2$ on mRNA and protein expression levels of StAR, P450scc, 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, P450scc, and 3β-HSD. The real-time PCR analysis showed that 100 μM $CoCl_2$ after 6 h significantly increased *STAR* mRNA expression in luteinizing GCs (Fig. 2A; $P < 0.05$) but did not affect *CYP11A1* and *HSD3B* mRNA expression levels (data not shown).

Western blotting analyses revealed that 100 μM $CoCl_2$ after 2 and 6 h significantly increased StAR protein expression in luteinizing GCs (Fig. 2B; $P < 0.05$) but did not affect CYP11A1 and HSD3B protein expression (Fig. 2C and 2D).

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. 3). 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. 4A; $P < 0.05$) and the expression of STAR mRNA (Fig. 4B; $P < 0.05$) and protein (Fig. 4C and 4D; $P < 0.05$) 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

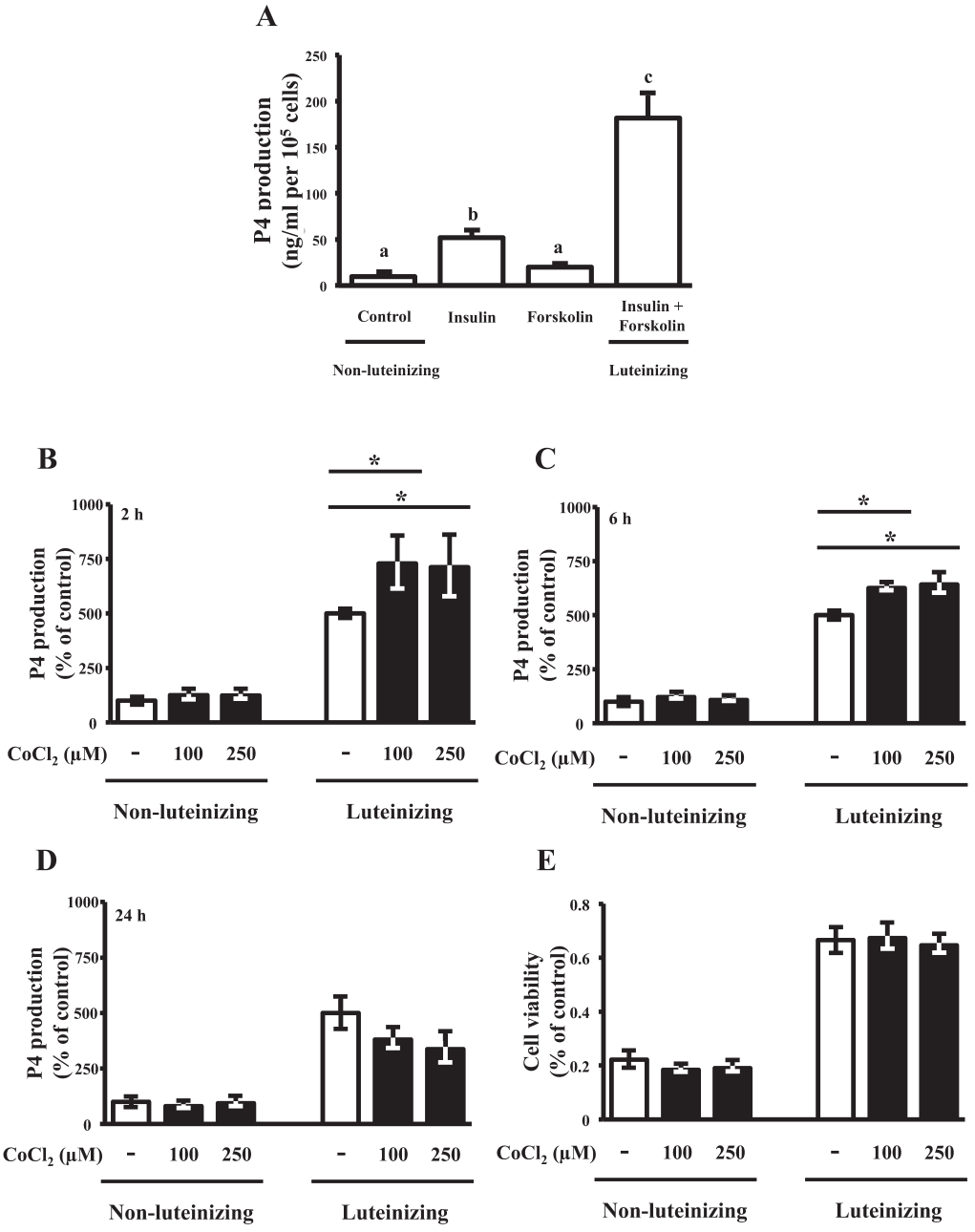

Fig. 1. Effects of $CoCl_2$ on P4 production by luteinizing and non-luteinizing GCs and on cell viability. To prepare luteinizing and non-luteinizing GCs, we cultured the cells at 20% O_2 in the presence of insulin (2 μg/ml) and/or forskolin (10 μM) for 24 h. P4 production was then quantified (A). To 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.

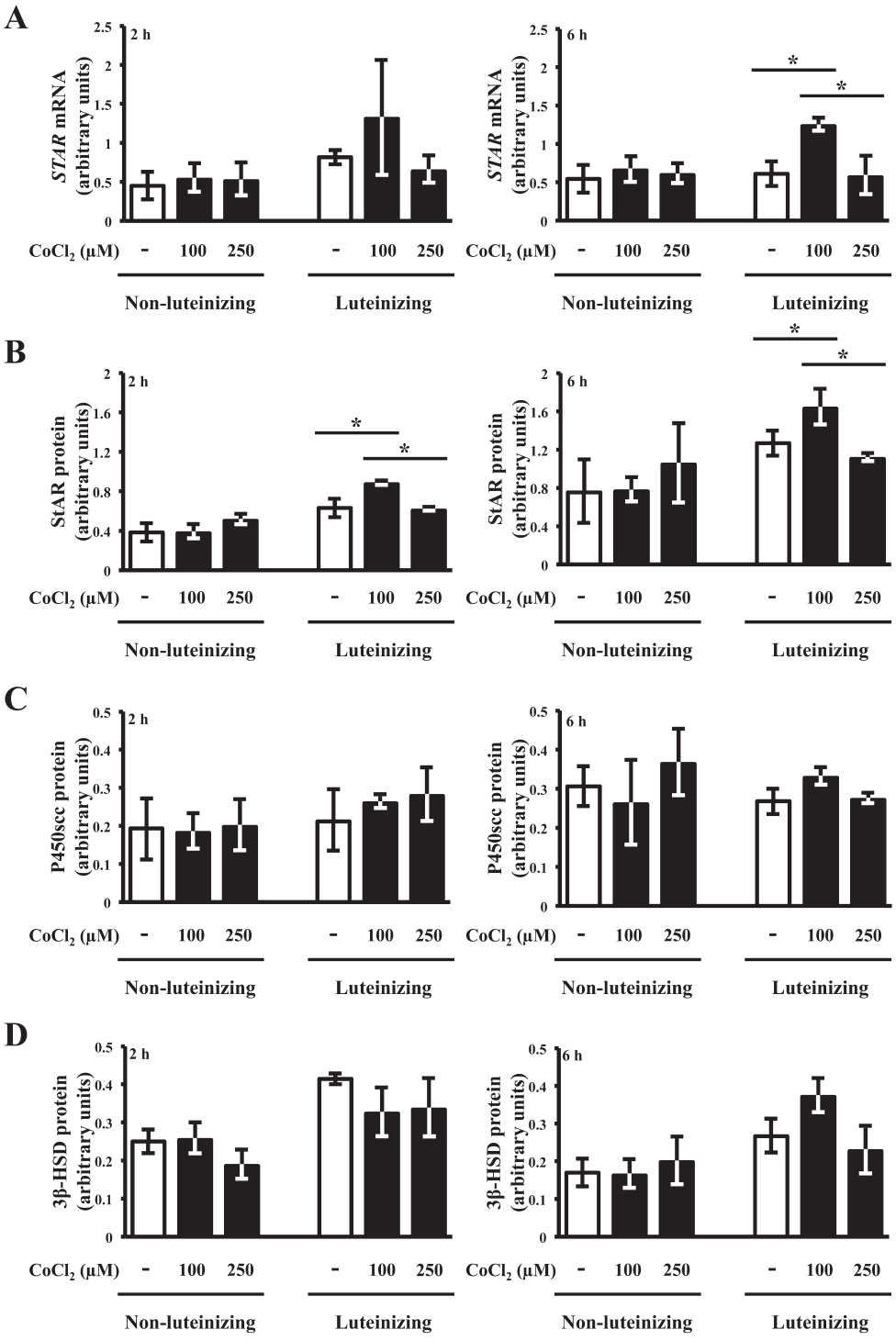

Fig. 2. Effects of $CoCl_2$ on the expression levels of StAR, P450scc, and 3β-HSD in non-luteinizing and luteinizing GCs. These cells were incubated with or without $CoCl_2$ (100 or 250 μM) for 2 or 6 h. Total RNA was then extracted from harvested cells to determine mRNA expression of *STAR* (A) by real-time PCR. The amount of *STAR* mRNA is expressed relative to the amount of *18SrRNA* mRNA. The protein expression levels of StAR (B), P450scc (C), and 3β-HSD (D) were determined by western blotting. 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.

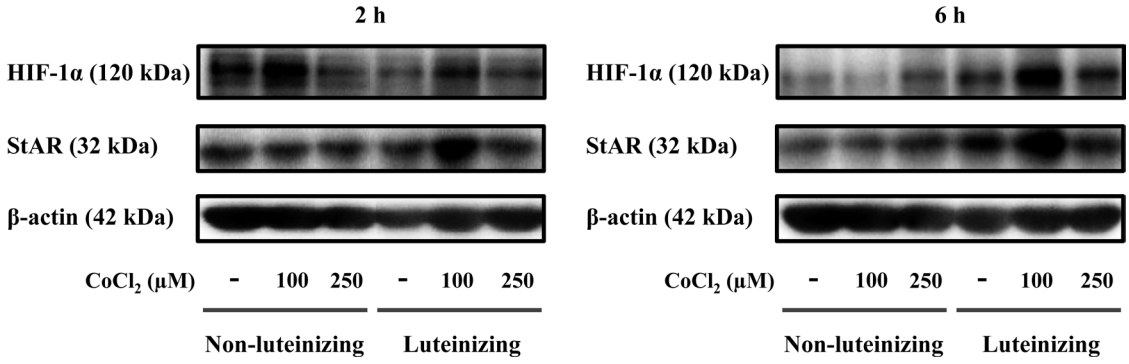

Fig. 3. Effects of $CoCl_2$ on HIF-1α and StAR protein expression levels. The luteinizing and non-luteinizing GCs were incubated with or without $CoCl_2$ (100 or 250 μM) for 2 h or 6 h. The cells were then collected to determine HIF-1α and StAR protein expression by western blotting. The blot was incubated 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.

previous findings [26], culturing of luteinizing GCs under 10% O_2 significantly increased P4 production after 24 h (Fig. 5A; $P < 0.05$). Culturing of luteinizing GCs under 10% O_2 also increased the expression of STAR mRNA (Fig. 5C; $P < 0.05$) and protein (Fig. 5D and 5E; $P < 0.05$) in comparison with cultivation under 20% O_2. Echinomycin attenuated the 10% O_2 effects that increased P4 production (Fig. 5B; $P < 0.05$) and the increased expression of STAR mRNA (Fig. 5C; $P < 0.05$) and protein (Fig. 5D and 5E; $P < 0.05$) after 24 h in luteinizing GCs.

Discussion

LH pulses increase gradually and result in an LH surge. The increasing LH pulses stimulate dominant follicle growth, ovulation, and luteinization [40, 41]. In the follicle compartment before ovulation, GCs and the oocyte develop in an avascular environment that is considered to be hypoxic [2–4]. The microenvironment of the ruptured follicle is also thought to be hypoxic immediately after ovulation [5]. Thus, luteinization and hypoxia seem to occur simultaneously 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 [10, 42–44]. We previously suggested that hypoxic conditions promote the progression and completion of luteinization by enhancing P4 synthesis in bovine GCs [26]. Using the same model of bovine cultured luteinizing GCs, we found in the present study that enhancement of P4 synthesis either by hypoxia (10% O_2) or by $CoCl_2$ is attenuated by the addition of echinomycin, the inhibitor of HIF-1 DNA binding [18], suggesting that HIF-1-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 $CoCl_2$ to mimic hypoxia because treatment with this compound has been shown to successfully mimic hypoxia in other studies [8, 20, 45]. $CoCl_2$ (100 and 250 μM) increased P4 production after 2 and 6 h in luteinizing GCs. Concentrations of $CoCl_2$ up to 500 μM have been used in some studies on HIF-1α [46, 47]. Simultaneously with the increase in P4 production, 100 μM $CoCl_2$ can also sufficient to increase the HIF-1α protein level after 2 and 6 h. In addition, the present results indicated that $CoCl_2$ has the same effects as 10% O_2 does on HIF-1α protein expression and on P4 production in luteinizing GCs, as shown in our previous study [26]. This low-oxygen condition may represent the O_2 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 $CoCl_2$ for 24 h tended to decrease their P4 production. Similarly, 500 μM $CoCl_2$ and 1% O_2 for 24 h inhibited P4 synthesis in bovine luteinized GCs obtained from the largest follicle [48]. 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 [49, 50]. Hypoxia-mimicking agents, such as $CoCl_2$, and low oxygen tension are also known to induce apoptosis in a number of cell types [49]. 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 [51]. During luteinization, StAR expression is upregulated in order to transfer cholesterol from the outer to inner mitochondrial membrane [11, 52–56]. 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 [26]. 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. 1B and 1C) together with mRNA (Fig. 2A) and protein expression of StAR (Fig. 2B) during shorter culture periods (2 and 6 h) than 10% O_2 did (24 h; Fig. 5A–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

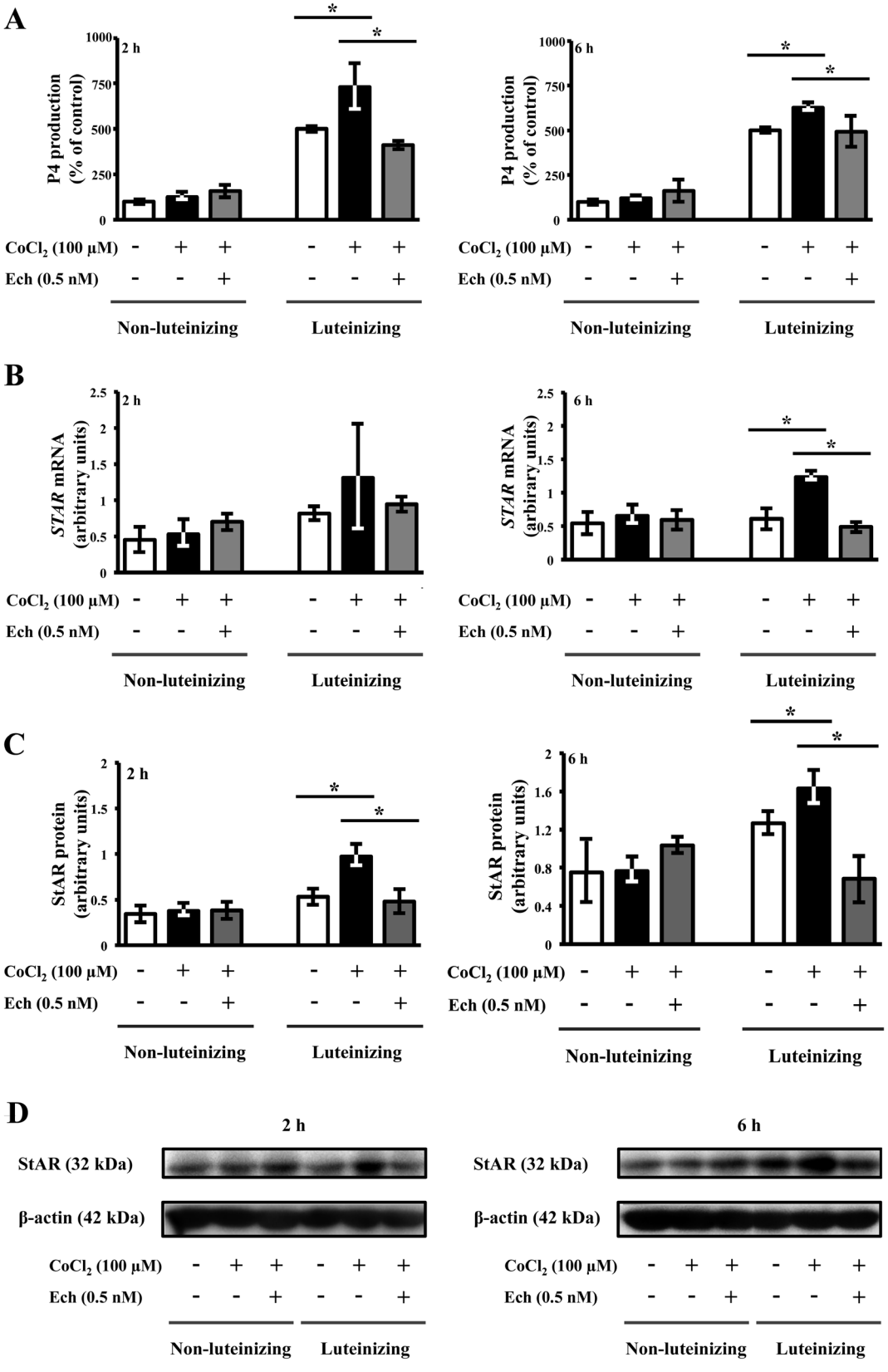
A
2 h
6 h
P4 production (% of control)
1000
750
500
250
0
*
CoCl2 (100 µM) - + + - + +
Ech (0.5 nM) - - + - - +
Non-luteinizing
Luteinizing
B
STAR mRNA (arbitrary units)
2.5
2
1.5
1
0.5
0
C
StAR protein (arbitrary units)
1.6
1.2
0.8
0.4
D
StAR (32 kDa)
β-actin (42 kDa)

Fig. 4.

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 [18]. In rats undergoing gonadotropin-induced ovulation, blockage of HIF-1 activity by echinomycin profoundly impairs the rupture of preovulatory follicles and reduces VEGF expression [2], 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 [57, 58], 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% O_2 stimulates *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 *STAR* promoter [59]. Further studies are needed to explain why hypoxic conditions and HIF-1 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-1. In other words, by enhancing P4 synthesis, HIF-1 may play as an important factor in the progression of luteinization by enhancing P4 synthesis.

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References

1. **Moor RM, Seamark RF.** Cell signaling, permeability, and microvasculatory changes during antral follicle development in mammals. *J Dairy Sci* 1986; **69**: 927–943. [Medline] [CrossRef]
2. **Kim J, Bagchi IC, Bagchi MK.** Signaling by hypoxia-inducible factors is critical for ovulation in mice. *Endocrinology* 2009; **150**: 3392–3400. [Medline] [CrossRef]
3. **Suzuki T, Sasano H, Takaya R, Fukaya T, Yajima A, Nagura H.** Cyclic changes of vasculature and vascular phenotypes in normal human ovaries. *Hum Reprod* 1998; **13**: 953–959. [Medline] [CrossRef]
4. **Redmer DA, Reynolds LP.** Angiogenesis in the ovary. *Rev Reprod* 1996; **1**: 182–192. [Medline] [CrossRef]
5. **Amselgruber WM, Schäfer M, Sinowatz F.** Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. *Anat Histol Embryol* 1999; **28**: 157–166. [Medline] [CrossRef]
6. **Wang GL, Jiang BH, Rue EA, Semenza GL.** Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 1995; **92**: 5510–5514. [Medline] [CrossRef]
7. **Salceda S, Caro J.** Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997; **272**: 22642–22647. [Medline] [CrossRef]
8. **Yalu R, Oyesiji AE, Eisenberg I, Imbar T, Meidan R.** HIF1A-dependent increase in endothelin 2 levels in granulosa cells: role of hypoxia, LH/cAMP, and reactive oxygen species. *Reproduction* 2015; **149**: 11–20. [Medline] [CrossRef]
9. **Tam KK, Russell DL, Peet DJ, Bracken CP, Rodgers RJ, Thompson JG, Kind KL.** Hormonally regulated follicle differentiation and luteinization in the mouse is associated with hypoxia inducible factor activity. *Mol Cell Endocrinol* 2010; **327**: 47–55. [Medline] [CrossRef]
10. **Gradin K, McGuire J, Wenger RH, Kvietikova I, fhitelaw ML, Toftgård R, Tora L, Gassmann M, Poellinger L.** Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol* 1996; **16**: 5221–5231. [Medline] [CrossRef]
11. **Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH.** Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 1998; **92**: 2260–2268. [Medline]
12. **Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH.** The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. *FASEB J* 2003; **17**: 761–763. [Medline]
13. **Yuan Y, Hilliard G, Ferguson T, Millhorn DE.** Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J Biol Chem* 2003; **278**: 15911–15916. [Medline] [CrossRef]
14. **Boonyaprakob U, Gadsby JE, Hedgpeth V, Routh PA, Almond GW.** Expression and localization of hypoxia inducible factor-1α mRNA in the porcine ovary. *Can J Vet Res* 2005; **69**: 215–222. [Medline]
15. **van den Driesche S, Myers M, Gay E, Thong KJ, Duncan WC.** HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *Mol Hum Reprod* 2008; **14**: 455–464. [Medline] [CrossRef]
16. **Duncan WC, van den Driesche S, Fraser HM.** Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and

Fig. 4. 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 $CoCl_2$) (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).

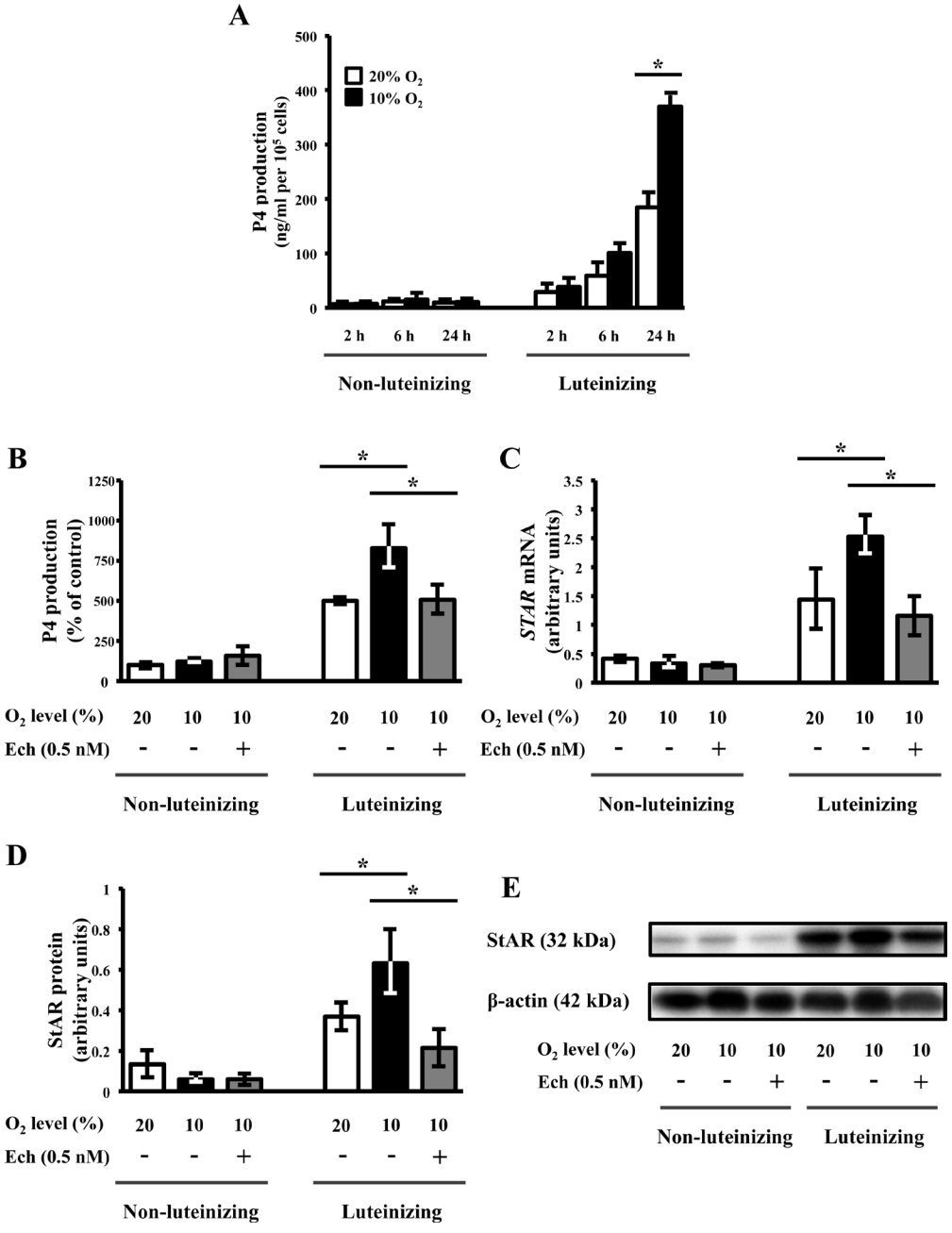

Fig. 5. The effect of echinomycin (Ech) on 10% O_2-enhanced P4 synthesis. The luteinizing and non-luteinizing GCs were cultured at 20% O_2 as a control or at 10% O_2 as low-oxygen tension for 2, 6, or 24 h to quantify P4 production (A). To this end, the culture media were collected for EIA of P4, while 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% O_2 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% O_2 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).

corpus luteum. *Endocrinology* 2008; **149**: 3313–3320. [Medline] [CrossRef]

17. **Meidan R, Klipper E, Zalman Y, Yalu R.** The role of hypoxia-induced genes in ovarian angiogenesis. *Reprod Fertil Dev* 2013; **25**: 343–350. [Medline] [CrossRef]
18. **Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, Fisher RJ, Shoemaker RH, Melillo G.** Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Res* 2005; **65**: 9047–9055. [Medline] [CrossRef]
19. **Concannon P, Hansel W, Mcentee K.** Changes in LH, progesterone and sexual behavior associated with preovulatory luteinization in the bitch. *Biol Reprod* 1977; **17**: 604–613. [Medline] [CrossRef]
20. **Clark BJ, Wells J, King SR, Stocco DM.** The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* 1994; **269**: 28314–28322. [Medline]
21. **Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL, Stocco DM.** Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Mol Endocrinol* 1995; **9**: 1346–1355. [Medline]
22. **Stocco DM.** A StAR search: implications in controlling steroidogenesis. *Biol Reprod* 1997; **56**: 328–336. [Medline] [CrossRef]
23. **Juengel JL, Niswender GD.** Molecular regulation of luteal progesterone synthesis in domestic ruminants. *J Reprod Fertil Suppl* 1999; **54**: 193–205. [Medline]
24. **Niswender GD.** Molecular control of luteal secretion of progesterone. *Reproduction* 2002; **123**: 333–339. [Medline] [CrossRef]
25. **Murphy BD, Gévry N, Ruiz-Cortés T, Coté F, Downey BR, Sirois J.** Formation and early development of the corpus luteum in pigs. *Reprod Suppl* 2001; **58**(Suppl): 47–63. [Medline]
26. **Fadhillah YS, Yoshioka S, Nishimura R, Okuda K.** Hypoxia promotes progesterone synthesis during luteinization in bovine granulosa cells. *J Reprod Dev* 2014; **60**: 194–201. [Medline] [CrossRef]
27. **Savion N, Lui GM, Laherty R, Gospodarowicz D.** Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* 1981; **109**: 409–420. [Medline] [CrossRef]
28. **Veldhuis JD, Kolp LA.** Mechanisms subserving insulins differentiating actions on progestin biosynthesis by ovarian cells: studies with cultured swine granulosa cells. *Endocrinology* 1985; **116**: 651–659. [Medline] [CrossRef]
29. **McArdle CA, Holtorf AP.** Oxytocin and progesterone release from bovine corpus luteal cells in culture: effects of insulin-like growth factor I, insulin, and prostaglandins. *Endocrinology* 1989; **124**: 1278–1286. [Medline] [CrossRef]
30. **Saumande J.** Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. *J Steroid Biochem Mol Biol* 1991; **38**: 189–196. [Medline] [CrossRef]
31. **Gong JG, McBride D, Bramley TA, Webb R.** Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on the proliferation of bovine granulosa cells in vitro. *J Endocrinol* 1993; **139**: 67–75. [Medline] [CrossRef]
32. **Lioutas C, Einspanier A, Kascheike B, Walther N, Ivell R.** An autocrine progesterone positive feedback loop mediates oxytocin upregulation in bovine granulosa cells during luteinization. *Endocrinology* 1997; **138**: 5059–5062. [Medline] [CrossRef]
33. **Meidan R, Altstein M, Girsh E.** Biosynthesis and release of oxytocin by granulosa cells derived from preovulatory bovine follicles: effects of forskolin and insulin-like growth factor-I. *Biol Reprod* 1992; **46**: 715–720. [Medline] [CrossRef]
34. **Fujita Y, Okuda K, Horikawa K, Naito C.** In vitro differentiation of bovine granulosa cells obtained from small antral follicles. *J Reprod Dev* 1996; **42**: 133–138. [CrossRef]
35. **Okuda K, Uenoyama Y, Lee KW, Sakumoto R, Skarzynski DJ.** Progesterone stimulation by prostaglandin F2α involves the protein kinase C pathway in cultured bovine luteal cells. *J Reprod Dev* 1998; **44**: 79–84. [CrossRef]
36. **Labarca C, Paigen K.** A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 1980; **102**: 344–352. [Medline] [CrossRef]
37. **Sakumoto R, Komatsu T, Kasuya E, Saito T, Okuda K.** Expression of mRNAs for interleukin-4, interleukin-6 and their receptors in porcine corpus luteum during the estrous cycle. *Domest Anim Endocrinol* 2006; **31**: 246–257. [Medline] [CrossRef]
38. **Osnes T, Sandstad O, Skar V, Osnes M, Kierulf P.** Total protein in common duct bile measured by acetonitrile precipitation and a micro bicinchoninic acid (BCA) method. *Scand J Clin Lab Invest* 1993; **53**: 757–763. [Medline] [CrossRef]
39. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63. [Medline] [CrossRef]
40. **Walters DL, Schams D, Schallenberger E.** Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the luteal phase of the oestrous cycle in the cow. *J Reprod Fertil* 1984; **71**: 479–491. [Medline] [CrossRef]
41. **Schallenberger E, Schams D, Bullermann B, Walters DL.** Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during prostaglandin-induced regression of the corpus luteum in the cow. *J Reprod Fertil* 1984; **71**: 493–501. [Medline] [CrossRef]
42. **Lipner H.** Mechanism of ovulation. *In*: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 447−488.
43. **Niswender GD, Nett TM.** The corpus luteum and its control *In*: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 489−525.
44. **Cavender JL, Murdoch WJ.** Morphological studies of the microcirculatory system of periovulatory ovine follicles. *Biol Reprod* 1988; **39**: 989–997. [Medline] [CrossRef]
45. **Zhang J, Zhang Z, Wu Y, Chen L, Luo Q, Chen J, Huang X, Cheng Y, Wang Z.** Regulatory effect of hypoxia-inducible factor-1α on hCG-stimulated endothelin-2 expression in granulosa cells from the PMSG-treated rat ovary. *J Reprod Dev* 2012; **58**: 678–684. [Medline] [CrossRef]
46. **Cho J, Kim D, Lee S, Lee Y.** Cobalt chloride-induced estrogen receptor alpha down-regulation involves hypoxia-inducible factor-1alpha in MCF-7 human breast cancer cells. *Mol Endocrinol* 2005; **19**: 1191–1199. [Medline] [CrossRef]
47. **Ardyanto TD, Osaki M, Tokuyasu N, Nagahama Y, Ito H.** $CoCl_2$-induced HIF-1alpha expression correlates with proliferation and apoptosis in MKN-1 cells: a possible role for the PI3K/Akt pathway. *Int J Oncol* 2006; **29**: 549–555. [Medline]
48. **Jiang YF, Tsui KH, Wang PH, Lin CW, Wang JY, Hsu MC, Chen YC, Chiu CH.** Hypoxia regulates cell proliferation and steroidogenesis through protein kinase A signaling in bovine corpus luteum. *Anim Reprod Sci* 2011; **129**: 152–161. [Medline] [CrossRef]
49. **Greijer AE, van der Wall E.** The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* 2004; **57**: 1009–1014. [Medline] [CrossRef]
50. **Hang X, Li P, Li Z, Qu W, Yu Y, Li H, Shen Z, Zheng H, Gao Y, Wu Y, Deng M, Sun Z, Zhang C.** Transcription and splicing regulation in human umbilical vein endothelial cells under hypoxic stress conditions by exon array. *BMC Genomics* 2009; **10**: 126. [Medline] [CrossRef]
51. **Thompson WE, Powell J, Thomas KH, Whittaker JA.** Immunolocalization and expression of the steroidogenic acute regulatory protein during the transitional stages of rat follicular differentiation. *J Histochem Cytochem* 1999; **47**: 769–776. [Medline] [CrossRef]
52. **LaVoie HA, Garmey JC, Veldhuis JD.** Mechanisms of insulin-like growth factor I augmentation of follicle-stimulating hormone-induced porcine steroidogenic acute regulatory protein gene promoter activity in granulosa cells. *Endocrinology* 1999; **140**: 146–153. [Medline]
53. **Bao B, Calder MD, Xie S, Smith MF, Salfen BE, Youngquist RS, Garverick HA.** Expression of steroidogenic acute regulatory protein messenger ribonucleic acid is limited to theca of healthy bovine follicles collected during recruitment, selection, and dominance of follicles of the first follicular wave. *Biol Reprod* 1998; **59**: 953–959. [Medline] [CrossRef]
54. **Pescador N, Soumano K, Stocco DM, Price CA, Murphy BD.** Steroidogenic acute regulatory protein in bovine corpora lutea. *Biol Reprod* 1996; **55**: 485–491. [Medline] [CrossRef]
55. **Kerban A, Boerboom D, Sirois J.** Human chorionic gonadotropin induces an inverse regulation of steroidogenic acute regulatory protein messenger ribonucleic acid in theca interna and granulosa cells of equine preovulatory follicles. *Endocrinology* 1999; **140**: 667–674. [Medline]
56. **Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA, Reynolds LP.** Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 α-hydroxylase during follicular and luteal development in pigs, sheep, and cows. *Biol Reprod* 1995; **52**: 1081–1094. [Medline] [CrossRef]
57. **Nishimura R, Sakumoto R, Tatsukawa Y, Acosta TJ, Okuda K.** Oxygen concentration is an important factor for modulating progesterone synthesis in bovine corpus luteum. *Endocrinology* 2006; **147**: 4273–4280. [Medline] [CrossRef]
58. **Nishimura R, Komiyama J, Tasaki Y, Acosta TJ, Okuda K.** Hypoxia promotes luteal cell death in bovine corpus luteum. *Biol Reprod* 2008; **78**: 529–536. [Medline] [CrossRef]
59. **Kowalewski MP, Gram A, Boos A.** The role of hypoxia and HIF1α in the regulation of STAR-mediated steroidogenesis in granulosa cells. *Mol Cell Endocrinol* 2015; **401**: 35–44. [Medline] [CrossRef]