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FULL PAPER

Theriogenology

BNIP3 expression in bovine follicle and corpus luteum

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ABSTRACT. BNIP3 (BCL2/adenovirus E1B nineteen kilodalton interacting protein-3), a member of the BCL2 family, is activated under hypoxic conditions and induces apoptosis or mitochondrial autophagy for adapting cells to hypoxia. The physiological roles of BNIP3 in the mammalian ovary are still unclear. In order to understand the role of BNIP3 in the bovine ovary, we examined its mRNA and protein expressions of BNIP3 in follicular granulosa cells and corpus luteum (CL). BNIP3 mRNA and protein expressions in granulosa cells from large follicles (>10 mm) at the follicular stage were much higher than those in small follicles (2–8 mm). BNIP3 mRNA and protein expressions in the CL peaked at the early luteal stage. In bovine granulosa cells cultured for 6 hr under hypoxia (3% O_2) and normoxia (20% O_2), BNIP3 mRNA expression was higher under hypoxia. These results of the present study suggest that BNIP3 has some roles in luteal formation in the bovine ovary, and that the highly expressed BNIP3 in the granulosa cells from large follicles at the follicular stage is related to the roles of BNIP3 in the luteal formation.

KEY WORDS: bovine, granulosa cell, hypoxia, luteal cell, ovary

In mammals, the ovarian cycle has several phases: follicular growth, ovulation, luteal formation and regression. Blood flow to the ovary changes with the phase of the ovarian cycle, and has been indicated to regulate ovarian cycles [1, 23, 28, 41]. In cows, ovarian blood flow has been reported to decrease during luteal regression, and after ovulation, gradually increase during luteal formation [41]. Since follicles develop during luteal regression, follicular growth before ovulation occurs in parallel with the decrease of blood flow to the ovary. Furthermore, the oxygen content in the ovarian venous blood begins to decrease at the late luteal stage [41]. These findings indicate that the low oxygen condition (hypoxia) caused by the decreased blood supply is a characteristic part of the ovarian environment during follicular growth, ovulation and luteal formation. We previously found that hypoxia has one of the important roles in luteal formation [26] and regression in the cow [25, 27]. Hypoxia promoted apoptosis via increasing the expression of a BCL2 family protein, BCL2/adenovirus E1B nineteen kilodalton interacting protein-3 (BNIP3) during luteal regression [25]. BNIP proteins, including BNIP3 and BNIP3L, are a relatively new subgroup of the BCL2 family [44]. These proteins are classified into this family based on limited sequence homology with the BCL2 homology domain 3 and carboxyl terminal transmembrane domain [44]. BNIP proteins were discovered through their interaction with the adenovirus E1B nineteen kilodalton protein and then known to promote apoptosis by binding to BCL2, an antiapoptotic protein of BCL2 family [4, 44]. BNIP3 is also known to be transcriptionally activated by hypoxia-inducible factor-1 (HIF1) [3]. Recently, BNIP3 has been suggested to promote mitochondrial autophagy (mitophagy) to reduce intracellular reactive oxygen species, for adapting cells to hypoxic conditions [34, 43]. During ovulation and luteal formation, both the blood flow in the ovarian artery and the oxygen concentration in the ovarian venous blood are low [41], indicating that oxygen levels in the intraovarian environment are

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Table 1. Primers used in real-time PCR

Gene	Primer	Sequence	Accession No.	Product (bp)
BNIP3	Forward	5′-GAAGGAATGCCGACACTAGG-3′	XM_867122	176
	Reverse	5′-CAAAGCCAGCAGACACTCAG-3′		
18SrRNA	Forward	5′-TCGCGGAAGGATTTAAAGTG-3′	AY779625	141
	Reverse	5′-AAACGGCTACCACATCCAAG-3′		

relatively low oxygen. Thus, in the granulosa cells before ovulation, and in the early luteal cells just after ovulation, BNIP3 has been suggested to help cells adapt to hypoxic conditions to support luteal formation. However, whether BNIP3 takes part in luteal formation is unknown.

In the present study, in order to determine whether BNIP3 has some roles in luteal formation, we investigated the expressions of BNIP3 mRNA and protein in follicular granulosa cells and in corpus luteum (CL) in the bovine ovaries at different stages of the estrous cycle.

MATERIALS AND METHODS

Collection of granulosa cells and CL

Ovaries with CL from Holstein cows were collected at a local abattoir within 10–20 min after exsanguinations. The stages of the estrous cycle were identified by macroscopic observation of the ovary as described previously [21]. After 60–90 min of exsanguinations, granulosa cells were collected from small follicles (diameter: 2–8 mm) and the largest follicle (diameter: >10 mm) at the follicular stage (regressed luteal stage): Days 19–21 (Day 0=day of ovulation). After aspiration of follicular fluid by syringe, granulosa cells were collected by removing cumulus-oocyte complexes, and used for cell culture, RNA and protein isolation. CL tissues were collected from cows at five different stages of the estrous cycle; early: Days 2–3, developing: Days 5–6, mid: Days 8–12, late: Days 15–17, regressed luteal stage: Days 19–21. The CL tissues were immediately separated from the ovaries, frozen rapidly in liquid nitrogen, and stored at −80°C until processed for RNA and protein isolation.

Cell isolation

Bovine granulosa cells were isolated with the method based on our previous report [7]. Briefly, The collected granulosa cells from small follicles (diameter: 2–8 mm) were suspended in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Grand Island, NY, U.S.A., No. 11885) containing 5% fetal bovine serum (Life Technologies, No. 26140-087) and antibiotic-antimycotic solution (Life Technologies, No. 15240-096; 400 U/m*l* penicillin, 400 μg/m*l* streptomycin, 1 μg/m*l* amphotericin B). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells consisted of 100% granulosa cells, confirmed by microscopic observation and stimulating effects of FSH on estrogen synthesis in culture.

Cell culture

The dispersed granulosa cells were seeded at 1.0×10^5 viable cells in 1 m*l*, in 35 mm culture dishes (Becton Dickinson Labware, NJ, U.S.A., No. 351008) for detection of *BNIP3* mRNA expression, and cultured in a humidified atmosphere of 5% CO_2 in air at 38°C in a N_2-O_2-CO_2-regulated incubator (Bio-Labo, Tokyo, Japan, No. BL-40M). After 12 hr of culture, the medium was replaced with fresh medium containing 0.1% BSA, 5 *n*g/m*l* sodium selenite and 5 μg/m*l* transferrin, and the following experiments were carried out. The cell culture under conditions with different levels of O_2 (3 or 20%) was described previously [27]. By using this system, we previously confirmed the presence of hypoxic conditions in bovine luteal cells by observing an increase in hypoxia-inducible factor 1α (HIF1A) protein under 3% O_2 [27].

Quantitative reverse transcription polymerase chain reaction (quantitative RT-PCR)

Total RNA was prepared from granulosa cells and luteal tissues using TRIZOL Reagent according to the manufacturer's directions (Invitrogen, Carlsbad, CA, U.S.A.; No. 15596-026). Total RNA (1 μg) was reverse transcribed using a ThermoScript™ RT-PCR System (Invitrogen, No. 11146-016).

Gene expression was measured by real-time PCR using a MyiQ thermal cycler (Bio-Rad, Tokyo, Japan) and the iQ SYBR Green Supermix (Bio-Rad, No. 170-8880) starting with 1 *n*g of reverse-transcribed total RNA. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1,000). The expression of 18S ribosomal RNA (*18SrRNA*) was used as an internal control. Twenty-bp primers with 50–60% GC-contents were synthesized (Table 1). The PCR conditions were 95°C for 15 min, followed by 55 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Use of the QuantiTect SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the PCR products with high linearity (Pearson correlation coefficient r>0.99).

BNIP3 protein analysis

The BNIP3 protein levels in granulosa cells and in CL tissues were assessed by Western blotting analysis. CL tissues were homogenized on ice in the homogenization buffer by a tissue homogenizer (Physcotron; NITI-ON Inc., Chiba, Japan; NS-50), and

then filtered with a metal wire mesh (150 μm). For BNIP3 protein analysis, nuclei were removed from the tissue homogenates by centrifugation at 600 × g for 30 min. Mitochondria were isolated from the resultant supernatant by centrifugation at 8,000 × g for 30 min for BNIP3 protein analysis. The granulosa cells aspirated from antral follicles were lysed in 200 μl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol [Sigma-Aldrich, St. Louis, MO, U.S.A., No. G7757], Complete, pH 7.4). The cell lysate was used for BNIP3 protein analysis. The protein concentration was determined by the method of Osnes *et al*. [29] using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [Nacalai Tesque, Inc., Kyoto, Japan, No. 31607-94], 10% glycerol, 1% β-mercaptoethanol [Wako Pure Chemical Industries, Ltd., Osaka, Japan, No. 137-06862], pH 6.8), and heated at 95°C for 10 min. Samples (50 μg protein) were subjected to electrophoresis on a 10% SDS-PAGE for 1 hr at 200 V.

The separated proteins were electrophoretically transblotted to a 0.2 μm nitrocellulose membrane (Invitrogen, No. LC2000) at 250 mA for 3 hr 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]), and incubated in blocking buffer (4% nonfat dry milk in TBS-T) overnight at 4°C. After blocking incubation, the membrane was cut into two pieces: one piece was used for detecting BNIP3 protein (21.5 kDa), and the other piece was used for β-actin (ACTB; internal standard; 42 kDa). The membranes were then incubated separately with a primary antibody specific to each protein: BNIP3 antibody (Sigma, No. B7931; 1:1,000 in TBS-T), ACTB antibody (Sigma, No. A2228; 1:4,000 in TBS-T) for 1 hr at room temperature, washed three times for 10 min in TBS-T at room temperature, incubated with secondary antibody (anti-mouse Ig, HRP-linked whole antibody produced in sheep, Amersham Biosciences Corp., Piscataway, NJ, U.S.A., No. NA931; 1:20,000 in TBS-T) for 1.5 hr, and washed three times in TBS for 10 min at room temperature. The signal was detected by ECL Western Blotting Detection System (Amersham Biosciences Corp., No. RPN2109).

The intensity of the immunological reaction 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, U.S.A.).

Expression of BNIP3 mRNA and protein in the bovine granulosa cells from small and large follicles at follicular stage (experiment 1)

Granulosa cells were collected from small (2–8 mm) and large (>10 mm) follicles at follicular stage as described above, and total RNA and cell lysate were used for determination of *BNIP3* mRNA expression by quantitative RT-PCR and for determination of BNIP3 protein expression by Western blotting.

Expression of BNIP3 mRNA and protein in bovine CL at different stages of the estrous cycle (experiment 2)

After homogenization of CL tissues at different stages of the estrous cycle, total RNA and mitochondria were extracted for determination of *BNIP3* mRNA expression by quantitative RT-PCR and BNIP3 protein expression by Western blotting, respectively.

Effect of hypoxia on BNIP3 mRNA expression in cultured bovine granulosa cells (experiment 3)

Granulosa cells were incubated under normal culture atmosphere (20% O_2) or hypoxia (3% O_2) for 6 hr. After the culture, cells were collected, and total RNA was extracted for determination of *BNIP3* mRNA expression by quantitative RT-PCR.

Statistical analysis

All experimental data are shown as the mean ± SEM. The statistical significance of differences in *BNIP3* mRNA expression (Figs. 1 and 3) was assessed by Student's *t*-test. Statistical significance of differences in *BNIP3* mRNA expression (Fig. 2A) and BNIP3 protein expression (Fig. 2B) was assessed by analysis of variance (ANOVA) followed by a multiple comparison with Bonferroni correction.

RESULTS

Expression of BNIP3 mRNA and protein in granulosa cells

At the follicular stage, *BNIP3* mRNA expression was much higher in the large follicles (diameter: >10 mm) than in the small follicles (diameter: 2–8 mm) (Fig. 1A; $P<0.05$). BNIP3 protein expression in granulosa cells was also higher in the large follicles than in the small follicles at the follicular stage (Fig. 1B; $P<0.05$), based on the band intensities after normalization to ACTB-specific bands.

Expression of BNIP3 mRNA and protein in CL

The mRNA expression of BNIP3 was markedly higher at the early luteal stage than at the other stages (Fig. 2A; $P<0.05$). The BNIP3 protein expression was also highest at the early luteal stage (Fig. 2B; $P<0.05$), based on the band intensities after normalization to ACTB-specific bands.

Effect of hypoxia on BNIP3 mRNA expression in granulosa cells

The results of real-time PCR analysis showed that *BNIP3* mRNA expression increased significantly under hypoxia (3% O_2) at 6 hr of culture (Fig. 3; $P<0.05$).

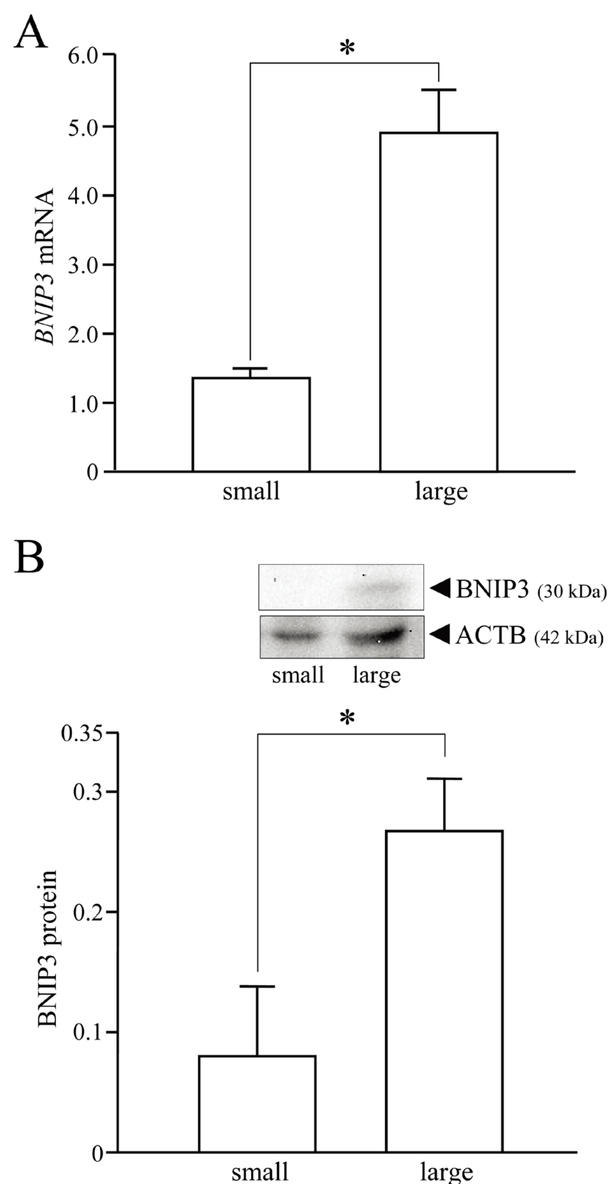

Fig. 1. Relative amounts of (A) BNIP3 mRNA and (B) protein in the bovine follicular granulosa cells from small (diameter: 2–8 mm) and large (diameter: >10 mm) follicles at the follicular stage. Data are expressed as relative ratios of *BNIP3* mRNA to *18SrRNA* (means ± SEM for 4 samples) (A), and as relative ratios of BNIP3 protein to ACTB protein (means ± SEM for 5 samples) (B). Representative samples of Western blot for BNIP3 (30 kDa) and ACTB (42 kDa) are shown in upper panels (B). Asterisks indicate significant differences ($P<0.05$), as determined by a Student's *t*-test.

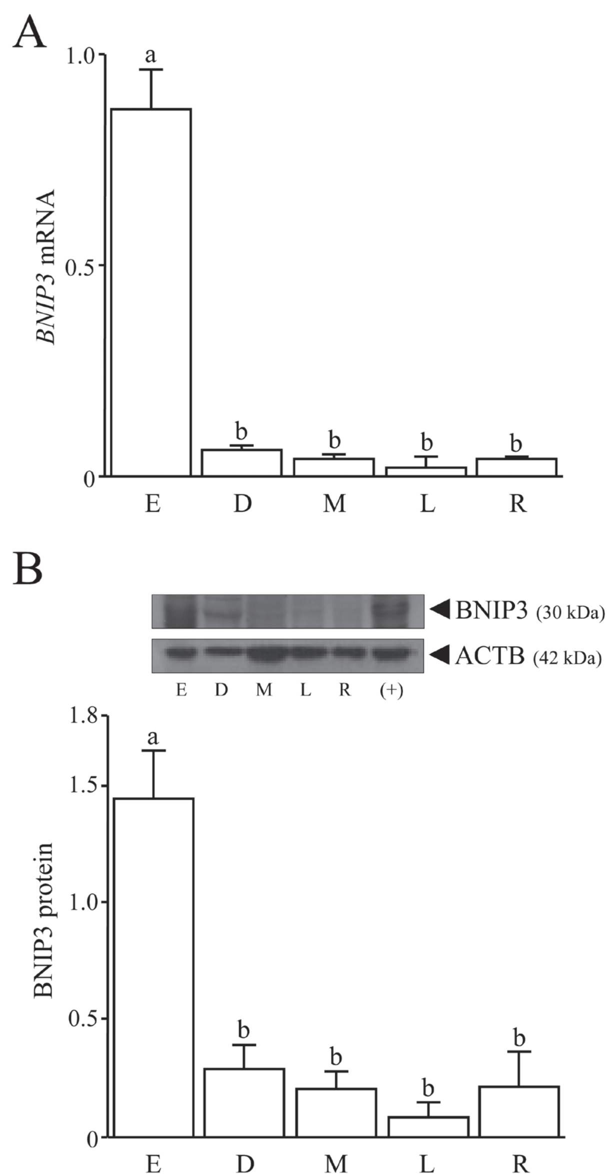

Fig. 2. Changes in the relative amounts of (A) BNIP3 mRNA and (B) protein in the bovine CL throughout the estrous cycle (E: early, Days 2–3; D: developing, Days 5–6; M: mid, Days 8–12; L: late, Days 15–17; R: regressed luteal stages, Days 19–21). Data are the means ± SEM for 5 samples/stage and are expressed as relative ratios of *BNIP3* mRNA to *18SrRNA* (A), and as relative ratios of BNIP3 protein to ACTB protein (B). Representative samples of Western blot for BNIP3 (30 kDa) and ACTB (42 kDa) are shown in upper panels ((+): positive control) (B). Different letters indicate significant differences ($P<0.05$), as determined by ANOVA followed by a multiple comparison with Bonferroni correction.

DISCUSSION

The present study demonstrated for the first time that the expression of BNIP3 in bovine follicular granulosa cells was much higher in the large follicles than in the small follicles at the follicular stage (Fig. 1). We also found that BNIP3 mRNA and protein expressions in CL (Fig. 2A and 2B, respectively) were very high just after ovulation (early luteal stage), and quickly decreased thereafter. Ovarian blood flow decreases during luteal regression, and after ovulation it increases with luteal formation [41]. Thus, the intraovarian environment during a few days around the time of ovulation is under low oxygen conditions. In

fact, HIF1A protein, one of the markers of hypoxic conditions, highly expresses in the early CL after ovulation [26]. Under hypoxic conditions, HIF1 transcriptionally up-regulates BNIP3, which then participate in various cellular processes such as apoptosis induction and mitochondrial autophagy [4, 43, 44]. These observation and finding that BNIP3 is induced by hypoxic conditions around the time of ovulation, suggest that BNIP3 is associated with the regulation of ovulation and luteal development.

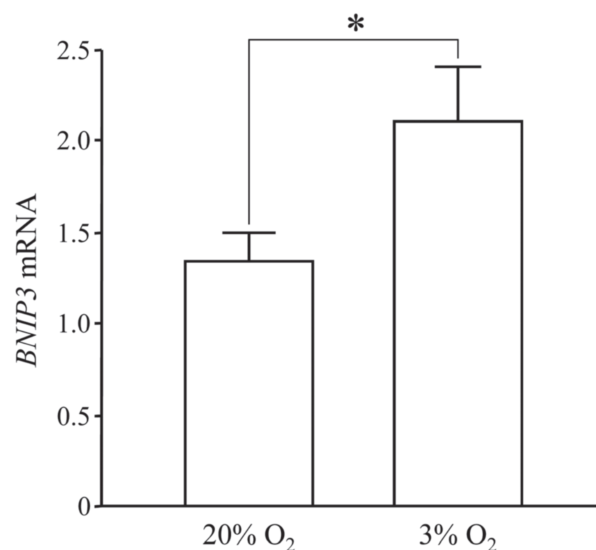

Fig. 3. Effect of hypoxia on the amounts of *BNIP3* mRNA in cultured bovine granulosa cells. The cells were cultured under 20% O_2 or 3% O_2 for 6 hr. The amounts of *BNIP3* mRNA are expressed relative to the amounts of *18SrRNA* (means ± SEM; n=8). Asterisk indicates a significant difference between oxygen tensions (P<0.05), as determined by a Student's t-test.

BNIP3 has been known to facilitate apoptosis under hypoxic conditions [3]. Apoptosis is modulated by many intracellular regulators, such as BCL2 family proteins [13, 30, 38, 40] and caspases [13, 30, 37]. BCL2 family proteins include both pro- and anti-apoptotic members. For example, BCL2 is known to protect cells from apoptosis, while BAX accelerates cell death [13, 30, 35, 38, 40]. BNIP3 is a pro-apoptotic member of the BCL2 family, and promotes apoptosis through heterodimerization with some anti-apoptotic proteins including BCL2 and BCL2L1 [44]. We previously showed that hypoxic conditions (3% O_2) increased BNIP3 expression and apoptosis in cultured bovine luteal cells, which suggests that BNIP3 takes part in the luteal cell apoptosis during luteal regression [25]. In the present study, BNIP3 expression in CL was much higher at the early luteal stage than the other stages. HIF1A protein expression was also highest at the early luteal stage [26]. These results suggest that BNIP3, which is increased by hypoxic conditions and HIF1, has a more important role in luteal formation than in luteal regression. BNIP3 has been suggested to promote mitochondrial autophagy to reduce intracellular reactive oxygen species, in order to adopt cells to hypoxic conditions [34, 43]. Recently, Zhou *et al.* [46] demonstrated in mouse granulosa cells that the autophagy induced by HIF1 and BNIP3 is related to follicle development, by showing that both blocking of HIF1A and knockdown of BNIP3 suppressed autophagy signaling. Since we found that BNIP3 expression was higher in the bovine granulosa cells from large follicle at the follicular stage, BNIP3 in follicular granulosa cells may act to protect granulosa cells by mitochondrial autophagy for follicle development. Furthermore, we also found that the expression of BNIP3 was also higher in the early stage CL, which is only 2–3 days after ovulation. In human cultured endothelial cells, vascular endothelial growth factor (VEGF) has been shown to antagonize BNIP3-mediated apoptosis under hypoxic conditions [14]. These results imply that BNIP3-mediated autophagy may also function for the development of CL. However, further studies are needed to clarify this point.

In mammals, a surge of luteinizing hormone (LH), acting as a luteotropic signal from the adenohypophysis, triggers the ovulation of the preovulatory follicle and formation of the CL [32]. During the peri-ovulatory period, the most rapid physiological angiogenesis in the body occurs in the luteinizing granulosa layer and in the developing CL [5, 33]. VEGF has a fundamental role in luteal vascularization because inhibition of VEGF *in vivo* during luteal phase prevents luteal angiogenesis and progesterone secretion [8, 11, 12, 42, 47]. Human chorionic gonadotropin (hCG), which activates the LH/hCG receptor, induces VEGF synthesis in human luteinized granulosa cells [6, 10, 18–20, 24, 31]. Moreover, hCG has been found to up-regulate HIF1A expression, resulting in the induction of VEGF [39]. hCG also increases VEGF expression in bovine luteinized granulosa cells [45]. Our recent study showed that hypoxic conditions increased HIF1A and VEGF expression in cultured bovine luteal cells [26]. Based on these previous studies, hypoxic conditions and LH have been suggested to up-regulate the transcription factor HIF1, leading to VEGF-induced angiogenesis essential for CL formation in the cow. In the present study, BNIP3, which is also induced by hypoxic conditions and HIF1 [3], was also strongly expressed in the granulosa layer of preovulatory large follicles and in CL at the early luteal stage. These results imply that hypoxia and HIF-induced BNIP3, in addition to hypoxia and HIF-induced VEGF, is related to luteal formation in the cow.

Hormonal signals (hCG, progesterone), in association with low oxygen levels, mediate the periovulatory increase in HIF1A [15, 39, 45]. hCG increased HIF1A mRNA expression in cultured human granulosa cells under normoxia and hypoxia [39]. hCG has a similar effect on HIF1A mRNA expression in bovine granulosa cells [45]. The stimulatory effect of superovulation by hCG on HIF1A expression in granulosa cells was diminished in progesterone receptor null mice [15]. Therefore, BNIP3 expression is suggested to be induced by these hormonal signals via HIF1. Indeed, in the present study BNIP3 mRNA expression in cultured granulosa cells was only 1.6-fold higher under hypoxia than under normoxia, while the expression in granulosa cells of large follicles is 3.6-fold higher than that in small follicles. *BNIP3* mRNA expression is also more than ten times higher in the early CL than in the other luteal stages. The hormone-regulated HIF1 stabilization could also contribute to the high expression of BNIP3 in large follicles, in association with the stimulation by hypoxic conditions.

The importance of the hypoxic signal is also demonstrated in follicular differentiation and ovulation [16, 22, 36]. In bovine preovulatory follicles, endothelin-2 (EDN2), which has been suggested to facilitate ovulation in mouse [17], is expressed in

granulosa cells, not in the vascular theca interna [16]. EDN2 expression was regulated by HIF1, and induced by hypoxia and hCG in cultured bovine granulosa cells [16]. Therefore, hypoxia and hCG/LH regulate ovulation by activating HIF1 and EDN2. Because follicular granulosa layer is avascular, oxygen concentration in the granulosa layer is thought to decrease, when the ovarian blood flow decreases during the periovulatory period [41]. Indeed, in human [9] and swine [2], oxygen concentration in the follicular fluid decreased during follicular development. In the present study, BNIP3 expression in the bovine granulosa layer was the higher in the preovulatory large follicles than in small follicles at the follicular stage. Furthermore, *BNIP3* mRNA expression in the cultured bovine granulosa cells increased under hypoxic conditions in the present study. Thus, BNIP3 increased under the hypoxic conditions due to the decreased blood supply during periovulatory period, and this increase appears to have some roles in preovulatory follicles.

The overall results indicate that BNIP3 is activated in bovine early CL and in follicular granulosa cells at the follicular stage, especially in the preovulatory large follicles, and suggest that hypoxic conditions and other endocrine factors including gonadotropins induce BNIP3 which may enhance luteal formation in the cow.

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