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—Original—

Relative Changes in mRNA Expression of Angiopoietins and Receptors Tie in Bovine Corpus Luteum during Estrous Cycle and Prostaglandin $F_{2\alpha}$-induced Luteolysis: A Possible Mechanism for the Initiation of Luteal Regression

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Abstract. Local angiogenesis and angiolysis in the corpus luteum (CL) relate to the luteal function. Recent studies indicate that angiopoietins (ANPT) and their receptors Tie regulate remodeling of microvasculature. We therefore examined 1) the relative changes in the expression of mRNA for ANPT-1, ANPT-2, Tie1 and Tie2 in bovine CL by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) during the estrous cycle and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis, and 2) the effect of ANPT-2 on progesterone (P_4) release from CL at the late stage of the estrous cycle by an *in vitro* microdialysis system (MDS). The CLs were classified into 4 stages (early: Day 2–5, n=7, mid: Day 8–12, n=15, late: Day 15–17, n=9, regressing: Day >18, n=19). The levels of ANPT-1 mRNA in early and regressing CL were lower than those in mid and late CL, whereas ANPT-2 mRNA expression did not change during the estrous cycle. The Tie2 mRNA expression decreased as the CL aged. During $PGF_{2\alpha}$-induced luteolysis, ANPT-2 mRNA expression was acutely and temporally increased at 2 h after $PGF_{2\alpha}$ injection. The expression of ANPT-1 mRNA was decreased from 4 h after $PGF_{2\alpha}$ injection and kept low levels. In the experiment with the *in vitro* MDS, an infusion of ANPT-2 (100 ng/ml) acutely inhibited P_4 release from late CL. Overall, results suggest that decrease of ANPT-1 mRNA is a basic mechanism of vascular remodeling in CL. In addition, ANPT-2 might play a role in regulation of P_4 secretion in CL during luteolysis.

Key words: Angiopoietin, Angiolysis, Luteolysis, Corpus luteum, Cow

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Physiological angiogenesis plays an essential role in the female reproductive system, occurring cyclically in the ovary and the uterus [1]. Cyclic changes of angiogenesis in the corpus luteum (CL) involve distinct phases of blood vessel growth, maturation and regression [2]. The vascular changes are well associated with progesterone (P_4) secretion during the estrous cycle in CL. The developing CL is characterized by highly active vascularization and mitosis of

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steroidogenic cells in parallel with a gradual increase in plasma P_4 levels. The majority of the steroidogenic cells of the mature CL are in contact with one or more capillaries [1]. In midcycle CL, vascular endothelial cells account for up to 50% of the bovine CL [3, 4], and a drastic regression of blood vessels (angiolysis) is observed during luteolysis [5]. Therefore, changes in the vascular network closely relate to the function and structure of CL during the estrous cycle.

Recent findings have suggested that angiopoietin (ANPT)-1, ANPT-2, and their endothelial cell receptor tyrosine kinases Tie1 and Tie2 may have important roles in the modulation of angiogenesis and angiolysis in CL during the estrous cycle [2, 6, 7]. Generally, ANPT-1 is necessary to maintain and stabilize blood vessels [8]. On the other hand, ANPT-2, which acts as a natural antagonist for ANPT-1, appears to cause endothelial cells to undergo active remodeling [8], thereby destabilizing the vascular structure. Since ANPT-1 and ANPT-2 bind to the same receptor Tie2, the balance of ANPT-2 and ANPT-1 binding to Tie2 appears to play a crucial role in vascular stability. A low relative level of ANPT-1 to ANPT-2 in the microenvironment induces destabilization of blood vessels, which is a prerequisite for vascular formation and regression, and the presence of an angiogenic factor such as vascular endothelial growth factor (VEGF) may determine the fate of destabilized blood vessels [9]. When VEGF is high, destabilization of blood vessels results in the formation of a new vascular network, whereas a lack of VEGF support results in regression of blood vessels. Therefore, the ANPT-Tie system acts on angiogenesis and angiolysis in concert with an angiogenic factor.

There is increasing evidence that the ANPT-Tie system is an important regulator of angiogenesis in CL of the human [6], primate [10] and cow [2]. However, the expression profile of mRNA for the ANPT-Tie system has not been fully clarified in the bovine CL. This study aimed to investigate the temporal mRNA expression of ANPT-1, ANPT-2, and their receptors, Tie1 and Tie2, in the bovine CL throughout the estrous cycle as well as during prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis. Our recent report demonstrated that ANPTs altered steroid hormone release from mature bovine follicles [11]. Therefore, we also examined the possibility that ANPT-2 directly influences P_4 secretion by using a microdialysis system (MDS) implanted within late CL *in vitro* where the cells maintain intact cell-to-cell communication.

Materials and Methods

Collection of bovine corpus luteum

The CLs were collected at local slaughterhouse, and the luteal stage was carefully determined by macroscopic examination of the ovaries (follicles and CL), as described in previous studies [12]. The uterine characteristics (size, color, consistency, and connective tissue, thickness of endometrium, mucus, and absence of elongated early embryo) were also considered. To determine the mRNA expression for ANPT-1, ANPT-2, Tie1 and Tie2 in CL during the estrous cycle the CLs were divided into four groups: early (Day 2–5), mid (Day 8–12), late (Day 15–17) and regressing (after Day 18).

For the determination of the mRNA expression for ANPT-1, ANPT-2, Tie1 and Tie2 during $PGF_{2\alpha}$-induced luteolysis, thirty Simmental cows at midcycle luteal phase (Days 8–12) received an injection of 500 μg $PGF_{2\alpha}$ analogue (cloprostenol: Estrumate®; Takeda. Co., Osaka, Japan) intramuscularly. To collect CLs, transvaginal ovariectomy was performed at 2, 4, 12, 24, 48 and 64 h after $PGF_{2\alpha}$ injection (n=5 per each time point). Corpora lutea from untreated cows at midcycle luteal phase (Days 8–12, n=5) were collected as control CL. All CL samples were quickly frozen in liquid nitrogen and stored at –80 C until RNA extraction. Since we found in the PCR study that an intramuscular injection of $PGF_{2\alpha}$ in the cow induced a rapid increase in ANPT-2 mRNA within 2 h (see Fig. 4), we decided to examine the direct effect of ANPT-2 on P_4 secretion by a MDS implanted in CL. For the MDS study *in vitro*, CL from the late luteal phase (Day 15–17) were washed several times with sterile saline solution (0.9 % NaCl) and transported at 38 C to the laboratory in saline containing 100,000 IU/L penicillin and 100 mg/L streptomycin. The late CL (Days 15–17) received surgical implants of microcapillary dialysis membranes of the MDS as described below.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Semi-quantitative analyses of mRNA expression

Table 1. Gene transcript, number of cycles used, primer sequences and resulting fragmant size

Target gene	Cycle number	Sequence of nucleotide	Accession No.*	Position	Fragment size (bp)
ANPT-1	26	Forward; 5′-cagaccagaaagctgacagatg-3′ Reverse; 5′ -ccagtgtgacccttcaaataca-3′	AF093573	564–585 1369–1348	806
ANPT-2	26	Forward; 5′ -gagaaagatcagctccaggtgt-3′ Reverse; 5′ -attcccttcccagtctcttagg-3′	AF094699	276–297 782–761	507
Tie1	26	Forward; 5′ -ccatgatcaagaaggatggact-3′ Reverse; 5′ -aacagcgacatgttcacatagg-3′	X71423	2604–2625 3405–3384	802
Tie2	26	Forward; 5′ -gctggggaactagaggttcttt-3′ Reverse; 5′ -atcatacacctcgtcgtcacag-3′	X71424	2934–2955 3533–3512	600
β-actin	20	Forward; 5′ -ccaaggccaaccgtgagaagat-3′ Reverse; 5′ -ccacgttccgtgaggatcttca-3′	K00622	107–128 362–341	256

* Entrez Nucleotides database accession number.

were carried out as described previously [11]. Total RNA was extracted from luteal tissues by guanidium acid-isothiocyanate-phenol-chloroform methods [13]. RNA was dissolved in water and spectroscopically quantified at 260 nm and its integrity confirmed by gel electrophoresis. Approximately 250 ng of total RNA was subjected to semi-quantitative RT-PCR using the Access RT-PCR System (Promega Co., Madison, WI, USA). This system employs a one-tube, two-enzyme method and uses AMV reverse transcriptase for the first strand cDNA synthesis and the thermostable *Tfl* DNA polymerase for the subsequent DNA amplification. The primers for ANPT-1, ANPT-2, Tie1, Tie2 and β-actin were reported in our previous study [11], and primer sequences and resulting fragment sizes are shown in Table 1. The reverse transcription reaction was carried out for 45 min at 48 C followed by inactivation of the reverse transcriptase for 2 min at 94 C. In the following PCR step, the resultant cDNA was amplified for 26 cycles for ANPT-1, ANPT-2, Tie1 and Tie2, or 20 cycles for β-actin. Each amplification cycle comprised denaturation at 94 C for 30 sec, annealing at 55 C for 1 min and extension at 68 C for 2 min with final extension for 7 min. To determine the optimal quantity of reverse transcripts needed for PCR and to verify that cDNA product was dependent on the number of cycles in the PCR used, various quantities of transcript template and different numbers of cycles were tested in the PCR assay. To exclude any contaminating genomic DNA, all experiments included controls lacking the Reverse Transcriptase. As a negative control, water was used instead of RNA for the RT-PCR to exclude any contamination from buffers and tubes. The authenticity of the PCR products was verified by the restriction enzyme analysis.

Aliquots of the PCR products (10 μl) were fractionated by electrophoresis through a 2% agarose gel containing ethidium bromide, and bands were visualized under the UV. The signal intensity was analyzed by computerized densitometry using the Image Master Program (Luminous Imager version 2.0 G, Aisin Cosmos RD Co. Ltd., Aichi, Japan). A set of serially diluted RNA samples (1–1000 ng per tube) obtained from CL tissues was included in each quantification to construct a standard curve. Relative abundance of target mRNA was estimated using the standard curve. The value was normalized using β-actin as the internal standard.

MDS in vitro

The MDS of the bovine CL *in vitro* has been previously described in detail [12]. In brief, each CL was cut into a 15 mm cube and divided into four pieces through the face of the apex. Three pieces were used to infuse different concentrations of ANPT-2 (1, 10 and 100 ng/ml, respectively), and the remaining piece was used as control (Ringer's solution only). Each experimental group and control group consisted of 7 luteal pieces derived from different CL. Each piece was separately penetrated by a 10-mm-long dialysis capillary (Fresenius SPS 900 Hollow Fibres; Fresenius AG, St. Wendel, Germany; cut-off M_r 1000) with each end glued to a 5-cm-long piece of silicone elastomer tubing (i.d. 0.3 mm). For perfusion, the four inlet

tubes were connected to a multiple-line peristaltic pump, and the four outlet tubes were routed to a multiple-line fraction collector. The prepared luteal pieces were then placed in organ culture chambers (modified 2070 tube; Falcon, Franklin Lakes, NJ, USA) that were maintained in a water bath at 38 C. The chambers were filled with 50 ml M199 (Sigma, St. Louis, MO, USA) containing 10 mM $NaHCO_3$, Earle's salts, 365 mg/L L-glutamine, 25 mM Hepes, 5 g/L BSA, 60 mg/L penicillin, 100 mg/L streptomycin, 56 mg/L ascorbic acid 2 mg/L, and amphotericin B at pH 7.4. The medium was continuously exchanged at a flow rate of 15 ml/h. During incubation the luteal pieces were perfused with Ringer's solution at a flow rate of 3.0 ml/h throughout the experiments. After 3 h pre-perfusion, fractions of the perfusate were collected every 2 h (6 ml per fraction) up to 12 h (0 h corresponds to the end of the pre-perfusion). These conditions were selected because it had previously been shown that CL tissues produce a constant release of P_4 under these conditions. Collected samples were stored at –30 C until hormone determination.

Human ANPT-2 (R&D Systems, Inc. MN, USA) was diluted in Ringer's solution to obtain the required final concentrations of 1, 10 and 100 ng/ml, respectively. Only Ringer's solution was infused into the control lines. The solutions were then infused into the MDS during the middle 4 h of the 12 h culture period. The doses were determined based on the result of a preliminary experiment and on the transfer capacity of the membrane, which was previously estimated to be 0.1% for peptides and LH [12].

Hormone determination

P_4 concentration was determined directly in the perfusate fraction from the MDS with second antibody enzyme immunoassay that based on the competitive assay using horseradish peroxidase-labeled P_4 [14]. Concentrations of P_4 were assayed directly for all fractions by using a polyclonal P_4 antibody provided by Dr. K. Okuda, Okayama University. The standard curve of P_4 ranged from 0.05 to 50 ng/ml, and the ED_{50} of the assay was 1.8 ng/ml. The intra- and interassay coefficients of variation (CVs) were on average 6.2% and 9.3%, respectively.

Statistical analysis

The expression of various genes was quantified using densitometric analysis (optical densities) relative to an internal standard (β-actin). Changes in the mRNA expressions during the estrous cycle were analyzed by ANOVA followed by Scheffe's test. Changes in the mRNA expression before and after $PGF_{2\alpha}$ analogue injection were analyzed by ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) test.

The mean P_4 concentrations in the MDS perfusate in the first 4 h were used to calculate the individual baseline. Because of a large variation in the basal concentrations of P_4 released into the MDS lines implanted in the different CL. P_4 concentration was expressed as a proportion of the individual baselines. This transformation enables an evaluation of relative changes of hormonal values between different CL. Means were analyzed by repeated measures ANOVA followed by t-test with the Bonferroni method. The effects of the different concentrations of ANPT-2 on P_4 release were compared with the baseline values.

Results

Expression of mRNA for ANPT-1, ANPT-2, Tie1 and Tie2 in the CL during the estrous cycle

The signals for mRNA encoding ANPT-1, ANPT-2, Tie1 and Tie2 were detected in CL (Fig. 1). The expression of ANPT-1 mRNA in early and regressing CL were lower than those in mid and late CL ($P<0.05$, Fig. 2A), whereas ANPT-2 mRNA expression did not change during the estrous cycle

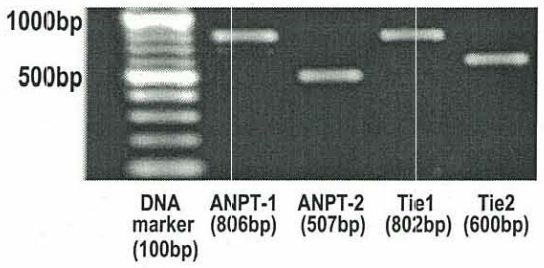

Fig. 1. Expression of mRNA for ANPT-1 (806 base pairs [bp]), ANPT-2 (507 bp), Tie1 (802 bp), and Tie2 (600 bp) in the bovine CL. Specific RT-PCR products were separated by agarose gel electrophoresis.

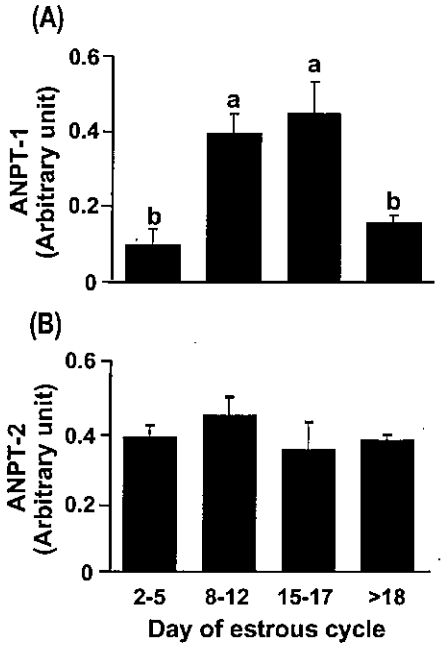

Fig. 2. Expression of mRNA of ANPT-1 and ANPT-2 in the early (Day 2–5), mid (Day 8–12), late (Day 15–17) and regressing CL (>Day 18). Data of mRNA expression are presented as arbitrary units based on the ratio of examined genes: β-actin mRNA. ANPT-1 mRNA (A) and ANPT-2 mRNA (B). Results are the mean ± SEM from 7 to 19 CL per stage. Different superscripts denote statistically different values (P<0.05).

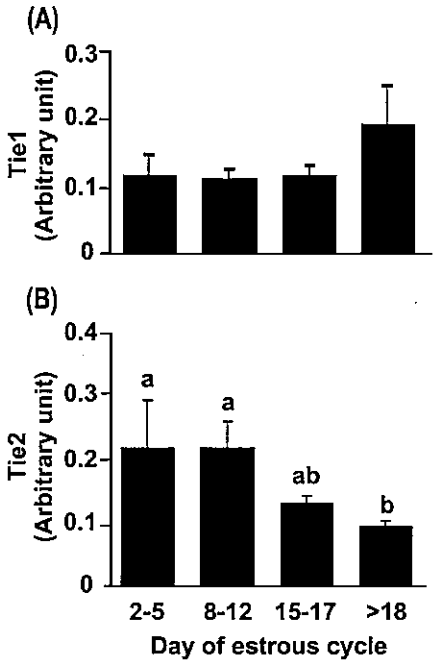

Fig. 3. Expression of mRNA for Tie1 and Tie2 in the early, mid, late and regressing CL. Data of mRNA expression are presented as arbitrary units based on the ratio of examined genes: β-actin mRNA. Tie1 mRNA expression (A) and Tie2 mRNA expression (B). Results are the mean ± SEM from 7 to 19 CL per stage. Different superscripts denote statistically different values (P<0.05).

(Fig. 2B). The expression of Tie1 mRNA did not change during the estrous cycle (Fig. 3A), whereas the expression of Tie2 mRNA decreased in the regressing CL (P<0.05, Fig. 3B).

Expression of mRNA for ANPT-1, ANPT-2, Tie1 and Tie2 in the CL during the $PGF_{2\alpha}$-induced luteolysis

During $PGF_{2\alpha}$-induced luteolysis, the expression of ANPT-1 mRNA in the CL decreased from 4 h after the $PGF_{2\alpha}$ injection, and then maintained low levels throughout the remainder of the experimental period (P<0.05, Fig. 4A). The ANPT-2 mRNA expression acutely increased at 2 h (P<0.01), but it returned to the basal level after 4 h and kept this low level thereafter (Fig. 4B). The mRNA expression for Tie1 and Tie2 was not changed by $PGF_{2\alpha}$ treatment (Fig. 5A,B).

Effect of ANPT-2 on the P_4 release from bovine CL in vitro

The release of P_4 into the MDS from the late CL was relatively constant in control lines throughout the experimental period (Fig. 6). The basal release of P_4 (100%) was 4.3 ± 0.37 ng/ml. An infusion of lower doses of ANPT-2 (1 and 10 ng/ml) between 4 and 8 h did not change P_4 release into the MDS, but an infusion of a higher dose of ANPT-2 (100 ng/ml) between 4 and 8 h resulted in an acute decrease of P_4 release during the infusion period (P<0.01). After infusion of ANPT-2, the release of P_4 from the CL recovered to the basal level.

Discussion

The present study demonstrates that ANPTs and Tie mRNAs are expressed in bovine CL throughout

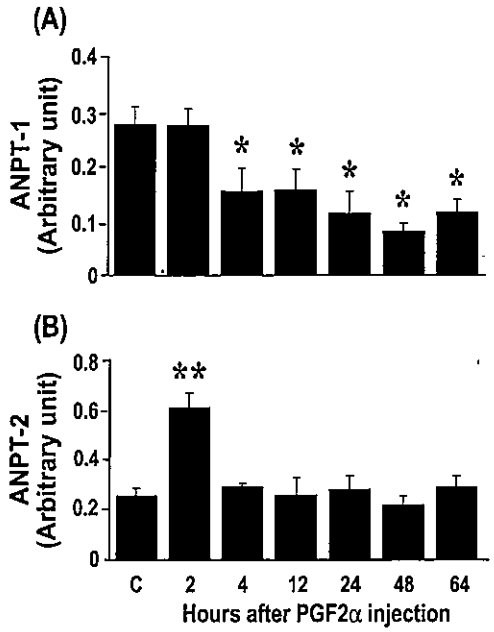

Fig. 4. Changes in the expression of mRNA for ANPT-1 and ANPT-2 in bovine CL collected at different times after $PGF_{2\alpha}$ treatment. Data of mRNA expression are presented as arbitrary units based on the ratio of examined genes: β-actin mRNA. ANPT-1 mRNA (A) and ANPT-2 mRNA (B). Results are the mean ± SEM from 5 CL per stage. * $P<0.05$ and ** $P<0.01$ vs. control.

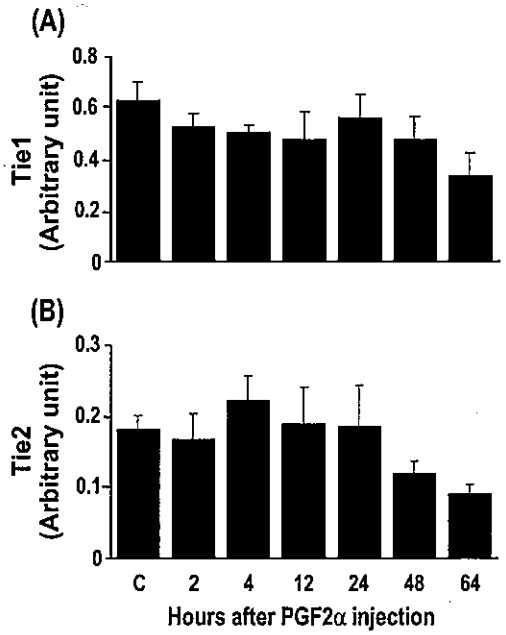

Fig. 5. Expression of mRNA for Tie1 and Tie2 in bovine CL collected at different times after $PGF_{2\alpha}$ treatment. Data of mRNA expression are presented as arbitrary units based on the ratio of examined genes: β-actin mRNA. Tie1 mRNA expression (A) and Tie2 mRNA expression (B). Results are the mean ± SEM from 5 CL per stage.

the estrous cycle and during $PGF_{2\alpha}$-induced luteolysis, in which the expression of ligands ANPT-1 and ANPT-2 change in a manner independent from each other. This study also provides the first observation that ANPT-2 acutely inhibits P_4 secretion in bovine CL *in vitro*.

In the present study, the expression of mRNA for ANPT-1, but not for ANPT-2, changed with the stage of the estrous cycle. The ANPT-1 mRNA expression is low in the early and regressing stages. It is known that active angiogenesis and angiolysis are associated with CL formation and regression, respectively [1, 15]. Generally, destabilization of blood vessels is a prerequisite for angiogenesis and angiolysis, and ANPT-1 is known as a factor which maintains and stabilizes blood vessels [8]. Goede *et al.* [2] proposed that the ratio of ANPT-2 to ANPT-1 (ANPT-2/ANPT-1 ratio) reflects the degree of instability of vessels. In fact, they showed that a

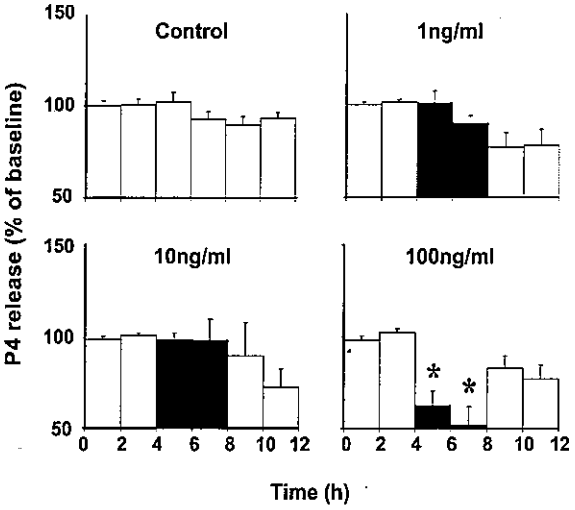

Fig. 6. Effects of ANPT-2 (1, 10 and 100 ng/ml) on P_4 release from microdialyzed bovine CL *in vitro*. ANPT-2 was infused between 4 to 8 h (solid columns). P_4 concentrations in each 2 h fraction are presented. Data are expressed as a percentage of the basal release, between 0 to 4 h, of P_4 (n=7; mean ± SEM). The baseline 100% = 4.3 ± 0.37 ng/ml. * $P<0.01$ vs. values of the baseline.

dramatic shift was observed in the ANPT-2/ANPT-1 ratio from low in midcycle CL to high in regressing CL in the cow [2]. In support of their findings, our data clearly showed that mRNA expression for ANPT-1 was low in the early and regressing stages when active angiogenesis or angiolysis is occurring under the high ANPT-2/ANPT-1 ratio.

During $PGF_{2\alpha}$-induced luteolysis, an acute and temporal increase in ANPT-2 mRNA expression was observed at 2 h after $PGF_{2\alpha}$ injection, preceding the decrease in ANPT-1 mRNA expression from 4 h. The data suggest that $PGF_{2\alpha}$ stimulates ANPT-2 secretion first and the destabilization of blood vessels may be started by this acute increase in ANPT-2, followed by the CL maintaining a low concentration of ANPT-1 that ensures instability of microvessels. This mechanism might contribute to create the microenvironment that leads to destruction of the CL.

The expression of Tie2 mRNA significantly decreased in the regressing stage of the estrous cycle. Since ANPT-1 and ANPT-2 act on endothelial cells through the receptor Tie2 [16], the decrease of Tie2 mRNA expression may reduce the sensitivity of endothelial cells to ANPTs in regressing CL. The decrease of both ANPT-1 and Tie2 mRNA in the regressing stage indicate that the ANPT-Tie system was down-regulated in the later stage of luteolysis. We also detected the mRNA expression of Tie1, but it did not change throughout the luteal phase. The physiological role of Tie1 in the CL has not been clarified yet.

Interestingly, the perfusion of CL tissue with ANPT-2 decreased P_4 release in the MDS within 2 h. This is the first observation that ANPT-2 affects P_4 release in the bovine CL. It should be noted that ANPT-2 mRNA expression increased at 2 h after $PGF_{2\alpha}$ injection when plasma P_4 levels had already started to decrease [17]. Taken together, the increased ANPT-2 induced by $PGF_{2\alpha}$ may partly suppress P_4 release from luteal cells directly or indirectly. Our recent study showed that ANPT-1 increases P_4 release from bovine preovulatory follicles [11]. Since ANPT-2 acts as a natural antagonist for ANPT-1, the results from the MDS experiments suggest that ANPT-2 may inhibit the stimulating effect of ANPT-1 on P_4 release from CL by antagonizing ANPT-1 binding to Tie2. Consequently, we hypothesize that ANPT-2 induced by $PGF_{2\alpha}$ may relate to the initiation of functional luteolysis before the start of vascular degeneration during luteal regression. Further analysis for cell distribution of Tie2 expression in luteal tissues as well as the binding and action of ANPT-2 on luteal cells *in vitro* will be required.

In conclusion, the present results demonstrate that the expression of ligands ANPT-1 and ANPT-2 in bovine CL changes throughout the estrous cycle and during $PGF_{2\alpha}$-induced luteolysis. It is likely that the decrease of ANPT-1 mRNA is a basic mechanism of vascular remodeling in CL. In addition, ANPT-2 might play a role in regulation of P_4 secretion in CL during luteolysis.

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