Journal of Reproduction and Development, Vol. 50, No. 4, 2004

—Research Note—

Expression of mRNA for the Angiopoietin-Tie System in Granulosa Cells during Follicular Development in Cows

Ken-Go HAYASHI[1)], Bajram BERISHA[2)], Motozumi MATSUI[1)], Dieter SCHAMS[2)] and Akio MIYAMOTO[1)]

[1)]Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan, [2)]Institute of Physiology, Technical University of Munich, Weihenstphaner Berg 3, D-85350 Freising-Weihenstphan, Germany

Abstract. Recent findings indicate that the changing profile of angiopoietins (ANPT) and their receptor Tie2 are closely associated with development and regression of the vascular network in the cyclic ovary. We previously reported that mRNA expression for the ANPT-Tie system in theca interna changes during bovine follicular development and atresia, and both ANPTs affect steroidogenesis in the preovulatory follicle. The aim of this study was to investigate mRNA expression for ANPT1, ANPT-2 and Tie2 in granulosa cells (GC) during follicular development in the cow. Bovine follicles were classified according to the estradiol-17β (E_2) concentration in follicular fluid (FF) as follows: (1) E_2<0.5, (2) 0.5<E_2<5, (3) 5<E_2<20, (4) 20< E_2<180 and (5) E_2>180 ng/ml FF. Semi-quantitative RT-PCR analysis revealed that the expression of ANPT-1 mRNA was not detected in most of the follicle with E_2 <5 ng/ml (diameter of 5–10 mm), but clearly detected in all follicles with E_2>5 ng/ml (diameter of >10 mm). The mRNA expression for ANPT-2 was drastically decreased in the follicles with E_2>5 ng/ml. Tie2 mRNA expression remained unchanged at the different stages of follicular development. The present data show that ANPT-1 becomes predominant in the follicle producing high levels of E_2, indicating the possible switch-over from ANPT-2 (antagonist) to ANPT-1 (agonist). Thus, the result suggests that the ANPT-Tie system in bovine GC may stimulate E_2 secretion rather than angiogenesis in the late stages of follicular development.

Key words: Follicular development, Angiopoietin-Tie system, Granulosa cell, Cow

(J. Reprod. Dev. 50: 477–480, 2004)

The ovary is known as an adult tissue which continuously undergoes physiological angiogenesis. The process of ovarian follicular development is closely related to formation of the blood capillary network (angiogenesis) [1, 2]. It has gradually become evident that angiopoietins (ANPT)-1 and ANPT-2, which acts as a natural antagonist for ANPT-1, as well as their endothelial cell-specific tyrosine kinase receptor, Tie2, regulate angiogenesis as modulators of vascular stabilization [3]. Recent observations suggest that the ANPT-Tie system may have a critical role in the modulation of vascular growth and regression in the follicle of the rat, primate, pig and cow [4–7]. In the bovine follicle, we previously showed: 1) the change of mRNA expression for ANPT-1, ANPT-2 and Tie2 in theca interna (TI) is involved with follicular development and atresia; 2) both ANPT-1 and ANPT-2 affect steroidogenesis in the preovulatory follicle; and 3) mRNA expression for ANPTs is detected in granulosa cells (GC) of the mature follicle [7]. However, the changing profile of mRNA expression for the ANPT-Tie system has not yet been clarified in bovine GC during follicular

Accepted for publication: April 26, 2004
Correspondence: A. Miyamoto (e-mail: akiomiya@obihiro.ac.jp)

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

Target gene	Cycle number	Sequence of nucleotide	Fragment size (bp)
ANPT-1	35	For: 5′-cagaccagaaagctgacagatg-3′ Rev: 5′-ccagtgtgacccttcaaataca-3′	806
ANPT-2	30	For: gagaaagatcagctccaggtgt-3′ Rev: 5′-attcccttcccagtctcttagg-3′	507
Tie2	38	For: 5′-gctggggaactagaggttcttt-3′ Rev: 5′-atcatacacctcgtcgtcacag-3′	600
β-actin	26	For: 5′-ccaaggccaaccgtgagaagat-3′ Rev: 5′-ccacgttccgtgaggatcttca-3′	256

development. Thus, in this study, we aimed to determine the mRNA expression for the ANPT-Tie system in GC during antral follicular development in the cow.

Materials and Methods

Collection and classification of follicles

Collection and classification of the bovine follicles was performed as previously described [8]. Briefly, ovaries from German Fleckvieh cows were collected at a local slaughterhouse in Germany within 10–20 min after slaughter and transported on ice to the laboratory. Only follicles which appeared healthy (i.e., well vascularized and having transparent follicular wall and fluid) and whose diameters were >5 mm were used. The follicles were classified according to the estradiol-17β (E_2) concentration in follicular fluid (FF) as follows: (1) $E_2<0.5$, (2) $0.5<E_2<5$, (3) $5<E_2<20$, (4) $20<E_2<180$ and (5) $E_2>180$ ng/ml FF. The corresponding sizes of follicles were in the ranges of (1) 5–7, (2) 8–10, (3) 10–13, (4) 12–14, and (5) >14 mm. To exclude atretic follicles, only follicles with progesterone (P_4) below 100 ng/ml FF were used for evaluation. Follicles were isolated with forceps under a stereomicroscope. After aspiration of FF, the follicles were bisected and GC were gently scraped and flushed with PBS. Follicular tissues were frozen and stored at –80 C until RNA isolation.

Hormone determinations

Concentrations of E_2 and P_4 were determined directly in FF with enzyme immunoassays using the second antibody technique described previously [8]. The ED_{50} value of the assay was 6 ng/ml for P_4 and 3.5 pg/ml for E_2. The FF was diluted accordingly with assay buffer. The intra- and inter-assay coefficients of variation were 4–5% and 8–9% for P_4 and 6–7% and 9–10% for E_2, respectively.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as previously described [8]. Briefly, total RNA from GC was extracted using the NucleoSPin RNA kit (Macherey-Nagel, Duren, Germany), and then extracted total RNA was reverse transcribed with hexanucleotides as primers using the M-MLV Reverse Transcriptase Kit (Promega, Madison, WI, USA). The PCR reaction was performed using thermostable polymerase PrimeZyme (Biometra, Götingen, Germany). The primers for ANPT-1, ANPT-2, Tie2 and β-actin were as described in the literature [7]. The number of amplification cycles, primer sequences and resulting fragment sizes for all examined genes are shown in Table 1. All amplifications were done as follows: an initial denaturation step at 94 C for 2 min, each cycle at 94 C for 30 sec, 55 C for 1 min, 68 C for 2 min and afterwards one additional elongation step at 68 C for 2 min. Aliquots of the PCR products (5 μl) were fractionated by electrophoresis using a 1.5% agarose gel containing ethidium bromide and visualized under UV. The gels were evaluated using a video documentation system (Amarsham-Pharmacia, Freiburg, Germany). Band intensities (relative) were analyzed by computerized densitometry (arbitrary units).

Statistical analyses

The statistical significance of differences in mRNA expression of examined genes was assessed by ANOVA, followed by the Scheffe F test as a multiple comparison test.

Results

The expression of ANPT-1 mRNA was not significantly different among the different stages of follicular development (Fig. 1A). However, ANPT-1 mRNA expression was undetectable in five of eight follicles with E_2<5 ng/ml (diameter of 5–10 mm) but clearly detected in all follicles with E_2>5 ng/ml (diameter of >10 mm). The expression of ANPT-2 mRNA was detected in small follicles with E_2<5 ng/ml, but was drastically decreased in follicles with E_2>5 ng/ml (Fig. 1B). Tie2 mRNA expression in the follicles was not different among the different stages of follicular development (Fig. 1C).

Discussion

This is the first report to show the changing profile of mRNA expression for ANPT-1, ANPT-2 and Tie2 in bovine GC at different stages of follicular development. It was surprising to find that bovine GC express mRNA for Tie2, which is known to be selectively expressed by endothelial cells [3]. However, more amplification cycles were required to assess the changes of mRNA expression in GC (ANPT-1: 35 cycles, ANPT-2: 30 cycles, and Tie2: 38 cycles) as compared to those in TI (26 cycles for each). Thus, mRNA expression for ANPTs and Tie2 was very weak in bovine GC as compared to that in TI. It is well known that the blood capillary network and its active angiogenesis are observed only in the theca layer of the ovarian follicle, while the granulosa layer remains avascular area until the LH surge [9, 10]. Therefore, the low level expression of mRNA of the ANPT-Tie system in bovine GC does not appear to contribute to regulation of angiogenesis in the theca layer.

We found a drastic reduction of mRNA expression for ANPT-2 accompanied by increase of E_2 concentration in FF, whereas ANPT-1 mRNA became clearly detectable in all follicles producing high E_2. This suggests that ANPT-1 is predominant during the late stage of follicular development. Moreover, expression of Tie2 mRNA was detected in GC at all stages of follicular development. The bovine developing follicle is characterized by an increase of E_2 secretion synthesized in GC, resulting in high concentrations of E_2 in FF [11]. We previously reported that the infusion of ANPT-1, but not ANPT-2, increased E_2 secretion in the bovine preovulatory follicle [7]. Taken together with the present data, the present data show that ANPT-1 becomes predominant in the follicle

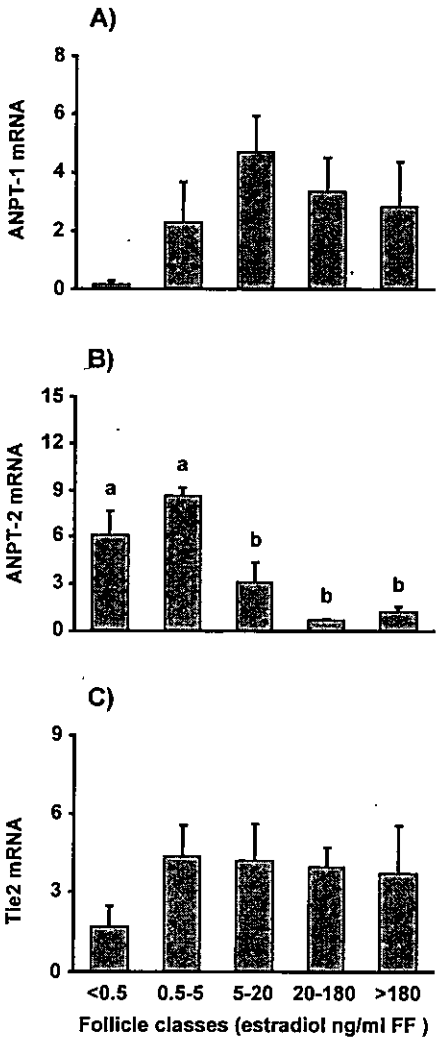

Fig. 1. Expression of mRNA for ANPT-1, ANPT-2 and Tie2 in GC of bovine follicles collected at different developmental stages. Data of mRNA expression are presented as arbitrary units based on the ratio of examined genes/β-actin mRNA. A) ANPT-1 mRNA; B) ANPT-2 mRNA; C) Tie2 mRNA. Results are the means ± SEM from 4 follicles per stage. Different superscripts denote statistically different values ($p<0.05$). The values of mRNA expression for ANPT-1 and Tie2 were not statistically different at different stages of follicular development due to large variations in the results.

producing high levels of E_2, indicating the possible switch-over from ANPT-2 (antagonist) to ANPT-1 (agonist). Thus, the result suggests that ANPT-Tie system in bovine GC may stimulate E_2 secretion rather than angiogenesis in the late stages of follicular development.

Acknowledgments

This study was supported by the Grant-in-Aid for Scientific Research of the Japan Society for the Promotion of Science (JSPS), the 21st Century COE Program (A-1) of Ministry of Education, Culture, Sports, Science and Technology, Japan, and German Research Foundation. M.M was a postdoctoral fellow supported by the COE program.

References

1. **Kranzfelder D, Maurer-Schultze B.** Development of the perifollicular capillary network. Autoradiographic and morphometric studies in the rabbit ovary. *Eur J Obstet Gynecol Reprod Biol* 1989; 30: 163–171.
2. **Jiang JY, Macchiarelli G, Tsang BK, Sato E.** Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction* 2003; 125: 211–223.
3. **Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J.** Vascular-specific growth factors and blood vessel formation. *Nature* 2000; 407: 242–248.
4. **Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD.** Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 1997; 277: 55–60.
5. **Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM.** Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). *Endocrinology* 2001; 142: 3244–3254.
6. **Shimizu T, Iijima K, Sasada H, Sato E.** Messenger ribonucleic acid expressions of hepatocyte growth factor, angiopoietins and their receptors during follicular development in gilts. *J Reprod Dev* 2003; 49: 203–211.
7. **Hayashi KG, Acosta TJ, Tetsuka M, Berisha B, Matsui M, Schams D, Ohtani M, Miyamoto A.** Involvement of angiopoietin-tie system in bovine follicular development and atresia: messenger RNA expression in theca interna and effect on steroid secretion. *Biol Reprod* 2003; 69: 2078–2084.
8. **Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R.** Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *J Endocrinol* 2000; 167: 371–382.
9. **Smith MF, McIntush EW, Smith GW.** Mechanisms associated with corpus luteum development. *J Anim Sci* 1994; 72: 1857–1872.
10. **Stouffer RL, Martinez-Chequer JC, Mblskness TA, Xu F, Hazzard TM.** Regulation and action of angiogenic factors in the primate ovary. *Arch Med Res* 2001; 32: 567–575.
11. **Henderson KM, McNeilly AS, Swanston IA.** Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. *J Reprod Fertil* 1982; 65: 467–473.