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

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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 ANPT-1, ANPT-2 and Tie2 in granulosa cells (GC) during follicular development in the cow. Bovine follicles were classified according to the estradiol-17β (E2) concentration in follicular fluid (FF) as follows: (1) E2<0.5, (2) 0.5<E2<5, (3) 5<E2<20, (4) 20<E2<180 and (5) E2>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 E2 <5 ng/ml (diameter of 5–10 mm), but clearly detected in all follicles with E2>5 ng/ml (diameter of >10 mm). The mRNA expression for ANPT-2 was drastically decreased in the follicles with E2>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 E2, 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 E2 secretion rather than angiogenesis in the late stages of follicular development.

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

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 (E2) concentration in follicular fluid (FF) as follows: (1) E2<0.5, (2) 0.5<E2<5, (3) 5<E2<20, (4) 20<E2<180 and (5) E2 >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 (P4) 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 E2 and P4 were determined directly in FF with enzyme immunoassays using the second antibody technique described previously [8]. The ED50 value of the assay was 6 ng/ml for P4 and 3.5 pg/ml for E2. The FF was diluted accordingly with assay buffer. The intra- and inter-assay coefficients of variation were 4–5% and 8–9% for P4 and 6–7% and 9–10% for E2, 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, Göttingen, 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 (Amasham-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 Scheffé 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 E2<5 ng/ml (diameter of 5–10 mm) but clearly detected in all follicles with E2>5 ng/ml (diameter of >10 mm). The expression of ANPT-2 mRNA was detected in small follicles with E2<5 ng/ml, but was drastically decreased in follicles with E2>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 E2 concentration in FF, whereas ANPT-1 mRNA became clearly detectable in all follicles producing high E2. 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 E2 secretion synthesized in GC, resulting in high concentrations of E2 in FF [11]. We previously reported that the infusion of ANPT-1, but not ANPT-2, increased E2 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.
producing high levels of E₂, 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 E₂ secretion rather than angiogenesis in the late stages of follicular development.

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References


