Journal of Reproduction and Development, Vol. 53, No. 3, 2007

—Full Paper—

Changes in the Messenger RNA Expressions of the Endothelin-1 and Angiotensin Systems in Mature Follicles of the Superovulated Bovine Ovary

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Abstract. The aim of the present study was to examine the messenger RNA expressions of the endothelin and angiotensin systems during the periovulatory phase in gonadotrophin releasing hormone (GnRH)-treated cows. Ovaries were collected by transvaginal ovariectomy (n=5 cows/group), and the follicles (n=5, one follicle/cow) were classified into the following groups: before GnRH administration (control, before LH surge), 3–5 h after GnRH (during LH surge), 10 h after GnRH; 20 h after GnRH, 25 h after GnRH (peri-ovulation), and early corpus luteum (CL) (Days 2–3). Expression of mRNA was investigated using quantitative real-time PCR. The expression of angiotensin converting enzyme (ACE) mRNA significantly decreased immediately after onset of the LH surge and remained at low levels. The levels of angiotensin II receptor type 1 (AT1R) and type 2 (AT2R) expression during the periovulatory period significantly decreased compared with other periods. The concentration of angiotensin II in follicular fluid began to increase 10 h after GnRH treatment and further increased as ovulation approached. The level of *ET-1* mRNA significantly decreased 10 h after GnRH treatment compared with the levels before GnRH treatment and those of the early CL period. The expression of *ETR-A* and *ETR-B* mRNA during the periovulatory period were lower than in other periods. The expression of *ECE-1* mRNA began to decrease in the LH surge period and significantly decrease in the periovulatory period compared with other periods. These results suggest that the vasoactive peptides angiotensin and endothelin may be associated with final maturation of follicles.

Key words: Angiotensin, Bovine, Endothelin-1, Gonadotrophin releasing hormone (GnRH), Ovulation

(J. Reprod. Dev. 53: 655–662, 2007)

Angiotensin II (Ang II) is generated in two sequential steps in which rennin catalyzes the conversion of angiotensinogen to the decapeptide Ang I, which is subsequently hydrolyzed by angiotensin converting enzyme (ACE) to form Ang II [1]. In the bovine ovary, theca cells have been identified as a major source of ovarian prorenin [2, 3]. Ang II stimulates estradiol production by rat follicles [4] and rabbit ovaries perfused *in vitro* [5], indicating a role in follicular development [6]. Ang II administration at 2-h intervals induces oocyte maturation and ovulation in perfused rabbit ovaries in the absence of gonadotropin, while gonadotropin exposure enhanced the ovarian Ang II secretion rate and intrafollicular Ang II content

Accepted for publication: February 4, 2007
Published online: March 14, 2007
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during the ovulatory process [7, 8]. These findings demonstrate that ovarian Ang II may play an important role in the preovulatory cascade.

Ang II receptor is located in the granulosa cells and theca cell interna of rat and bovine follicles [9–11]. So far, two types of Ang II (AT) receptors, AT1R and AT2R, have been identified [12, 13]. The granulosa layer and theca interna of ovarian follicles contain ANG II receptors that are predominantly comprised of AT2R [9, 14].

Endothelin (ET) is a potent vasoconstrictive peptide initially isolated from the conditioned medium of cultured endothelial cells [15]. The ET family members (ET-1, ET-2 and ET-3) are initially synthesized as 203-amino acid precursor proteins, called preproET, that are first proteolytically cleaved to generate big ET and then processed to the active peptides via an endothelin-converting enzyme (ECE) [16, 17]. In humans, *ET-1* mRNA and the mature peptide are expressed in granulosa cells [18]. ET-1 has been reported to have several direct actions on bovine follicular [19, 20] and luteal cells [21, 22]. ECE-1 null mice exhibit a phenotype similar to that of ET-1-deficient mice, demonstrating the significance of ECE-1 in generating bioavailable ET-1 [23]. The ovarian ECE-1 levels vary throughout the estrous cycle [24, 25].

The effect of ET-1 appears through the ET receptor. ETs act on two distinct ET receptor subtypes of the seven transmembrane G-protein-coupled receptors, the ET type A (ETR-A) and type B (ETR-B) receptors. Both ET-1 and ET-2 bind to ETR-A with higher affinity than ET-3 [26]. ETR-B binds all three isopeptides with equal affinity [27]. In the ovary, ETR-A has been observed in CL during the midluteal phase in the cow [21]. Expression of *ETR-A* mRNA has been observed in small and large luteal cells and in endothelial cells of the bovine corpus luteum (CL) [28].

The manner of expression of endothelin, angiotensin II and their receptors during the periovulatory phase, however, is still unknown. Thus, the present study used superovulated cows to determine the manner expression of these factors that would be associated with the ovulatory process.

Materials and Methods

Animals and superovulation

The experimental protocol was approved by the institutional care (AZ 211-2531.3-33/96) and use committee. This study was conducted on 30 non-lactating German Fleckvieh cows. The cows were induced to have multiple follicles (for different experimental purposes) by administration of a reduced dose (8.4 mg in total) of follicle stimulating hormone (FSH, Ovagen; ImmunoChemical Products, Auckland, New Zealand). FSH injections (a total of seven) were given i.m. at 12 h intervals in gradually decreasing doses for 3.5 days starting between days 8 and 11 of the estrous cycle, and the day heat was detected considered to be day 0. After the sixth FSH injection, a luteolytic dose of 500 μg of prostaglandin (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue, Estrumate (Cloprostenol; Berna Veterinärprodukte, Bern, Switzerland), was injected i.m., and then 40 h after injection, 100 μg of GnRH (Receptal; Berna Veterinärprodukte AG), was injected to induce a luteinizing hormone (LH) surge [29]. The ovaries were collected by transvaginal ovariectomy (n=5 cows/group).

Collection, classification, and preparation of follicles and CL

Follicles (n=5, one follicle/cow) collected by transvaginal ovariectomy (5 cows/group) were classified into the following groups: (I) control (40 h after $PGF_{2\alpha}$, before LH surge), (II) 3–5 h after GnRH administration (during LH surge), (III) 10 h after GnRH, (IV) 20 h after GnRH, (V) 25 h after GnRH (peri-ovulation), and (VI) early CL (Days 2–3). An LH surge was induced 3–5 h after GnRH administration.

Only follicles that appeared to be healthy (i.e., well vascularised and having a transparent follicular wall and fluid) and that had a diameter of >10 mm were collected. The number of follicles per ovary varied between 8–20. Follicular fluid (FF) was aspirated from the follicles and stored at –20 C until assayed. Follicles were dissected from the ovaries for RNA extraction. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. All follicles were aliquoted, quickly frozen in liquid nitrogen, and stored at –80 C until RNA extraction.

Table 1. Oligonucleotide sequences of the primers used for real-time PCR

Genes	Sequences	Product size (bp)	Reference/GenBank[1]
ACE	FWD: 5′-ATC CCG GAA TTA TCA GGA CC-3′ REV: 5′-AGG GTG CCA CCA AGT CAT AG-3′	365	[41]
AT1R	FWD: 5′-GAA GCT GGA AGA CAA CCA-3′ REV: 5′-TCC CAA AGT AGA CCT GCC-3′	324	[40]
AT2R	FWD: 5′-CAC CAC CAC CAT CTG CTT-3′ REV: 5′-TCT GAA CTG GGG TGC AGA-3′	335	[40]
ET-1	FWD: 5′-AAG CCC TTC TAG GTC CAA GC-3′ REV: 5′-CGA GCT GCT GAT GAA GAC AC-3′	227	NM181010
ETR-A	FWD: 5′-TGC AGA AGT CCT CAG TGG G-3′ REV: 5′-GAT CGC AGT GCA CAC CAG-3′	329	[33]
ETR-B	FWD: 5′-AAA CTG AGA ATC TGC TTG CTC C-3′ REV: 5′-AGA GTG AGC TTC AAA ATC CTG C-3′	296	[33]
ECE-1	FWD: 5′- GAG AAT GAG AAG GTG CTG ACG-3′ REV: 5′-GAG CTC GTC TTC CGT ACC AG-3′	395	[32]
Ubiqutin	FWD: 5′-ATG CAG ATC TTT GTG AAG AC-3′ REV: 5′-CTT CTG GAT GTT GTA GTC-3′	189	[33]

[1]Reference of the published sequence or GenBank accession number.
FWD: Forward, REV: Reverse.

Ang II concentration in follicular fluid

The concentration of Ang II in follicular fluid was determined in duplicate by enzyme immunoassay (EIA) after extraction using 96-well ELISA plates (Nunc-Immuno Plate, Nunc, Roskilde, Denmark) as described previously [30]. The follicular fluid was transferred to a small Sep-Pak C18 Cartridge (Waters, Milford, MA, USA). Ang II was eluted with 3 ml acetonitrile and 0.1% trifluoroacetic acid (60/40 v/v). The eluate was evaporated and diluted for Ang II with EIA assay buffer. The standard curve for Ang II ranged from 2.4 to 10,000 pg/ml, and the ED_{50} of the assay was 110 pg/ml. The average intra- and interassay coefficients of variation were 6.4 and 8.7%, respectively. The cross-reactivities of Ang II antibody with Ang I, Ang II, Ang III and renin substrate were 10, 100, 50 and 5%, respectively.

Isolation of RNA

Total RNA was prepared from follicular and CL tissues according to the method of Chomczynski and Sacchi [31] using TriPure® isolation reagent (Roche Diagnostics, Mannheim, Germany) as described previously [32]. Possible DNA contamination was eliminated by an additional DNase digestion (Promega, Madison, WI, USA), according to the manufacturer's protocol. Total RNA was purified using NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany), with the concentration and purity determined spectroscopically at an absorbance of 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). Aliquots (1 μg) were subjected to 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of the total RNA.

Reverse transcription

Total RNA was reverse transcribed to cDNA in a volume of 60 μl containing 1 μg RNA, 2.5 μmol/l random hexamers (Gibco BRL, Grand Island, NY, USA), and M-MLV Reverse Transcriptase (200 U/μl, Promega, Madison, WI, USA) according to Berisha *et al.* [32]. A minus-RT reaction (the RT-enzyme was replaced with water) was performed to detect residual DNA contamination.

Real-time RT-PCR

Quantitative fluorescence real-time RT-PCR analysis was performed using a Rotor-Gene 3000™ system (Corbett Research, Sydney, Australia). Online PCR reactions were carried out using a LightCycler® DNA Master SYBR Green I Kit (Roche Diagnostics) with 1 μl of each cDNA (16.66 ng) in a 10 μl reaction mixture (3 mM $MgCl_2$, 0.4 μM of each forward and reverse primer, 1 × LightCycler® DNA Master SYBR Green I). The primers are indicated in Table 1. After initial incubation at 95 C for 10 min to activate the Taq DNA polymerase, templates of

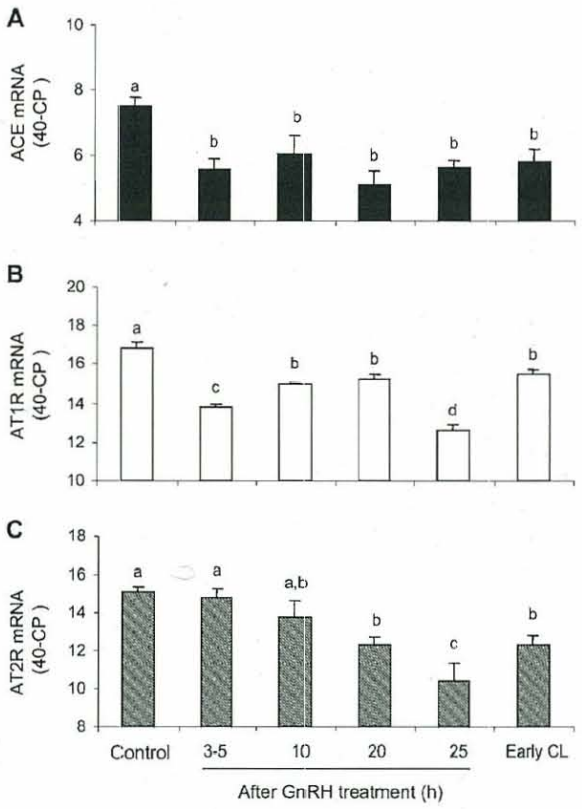

Fig. 1. Expressions of mRNA for (a) ACE, (b) AT1R and (c) AT2R in bovine follicular tissue and early CLs before GnRH administration (control, before LH surge), 3–5 h after GnRH administration (during LH surge), 10 h after GnRH, 20 h after GnRH and 25 h after GnRH (peri-ovulation). Results are presented as 40-CP (CP ± SEM from 5 follicles or CLs per group) in the target gene expression. Different superscripts denote statistically different values ($P<0.05$).

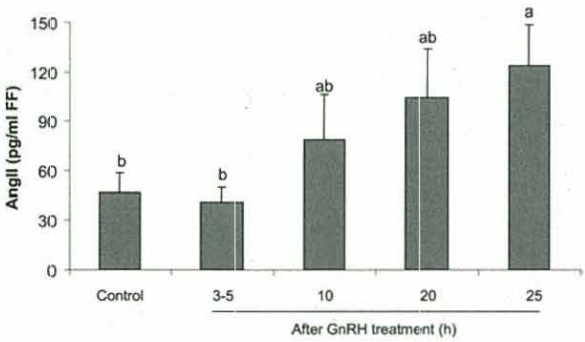

Fig. 2. Concentration of Ang II in follicular fluid before GnRH administration (control, before LH surge), 3–5 h after GnRH administration (during LH surge), 10 h after GnRH, 20 h after GnRH and 25 h after GnRH (peri-ovulation). Different superscripts denote significantly different values ($P<0.05$).

all the specific transcripts were amplified for 40 cycles at 95 C for 10 sec and this was followed by annealing at 60 C for 10 sec for all primers used and elongation at 72 C for 15 sec. Fluorescence data used for quantitation was acquired for 5 sec at the end of each 72 C elongation step by SYBR Green binding to the amplified dsDNA. Single product formation was verified using melting curve (Rotor-Gene 3000™) and agarose gel analyses after completion of PCR; melting curve analysis was conducted by heating at 95 C for 5 sec, cooling to 65 C 5 sec, and then continuous heating to 99 C at 5 C/sec under permanent fluorescence detection.

Data were analyzed using the Rotor-Gene 3000™ software (version 5.03). The relative expressions of each target gene were calculated using the "comparative quantification" method ("Takeoff" points). Amplification of all target genes in our experiment was completed after 40 cycles. This cycle number is the basis of the presented results. Generally, a high cycle number (CP; crossing point) indicates a low level of gene expression. The results for the mRNA expressions of the target genes in this paper are presented as 40-CP (CP mean ± SEM). The value of CP corresponds to the expression level of mRNA.

Statistical analyses

The statistical significance of differences in the examined factors was analyzed by ANOVA followed by Fisher's protected least significant difference test. All experimental data are shown as the mean ± SEM. Follicles and CLs (n=5) were obtained from 5 cows per group.

Results

Expression of AT1R, AT2R and ACE and concentration of Angiotensin II in follicular fluid

The expression of *ACE* mRNA significantly decreased during the LH surge compared with other periods (Fig. 1A). Expression of the *AT1R* gene decreased once during the LH surge and subsequently decreased further during the periovulatory period (Fig. 1B). The level of *AT2R* mRNA began to decrease 20 h (phase IV) after

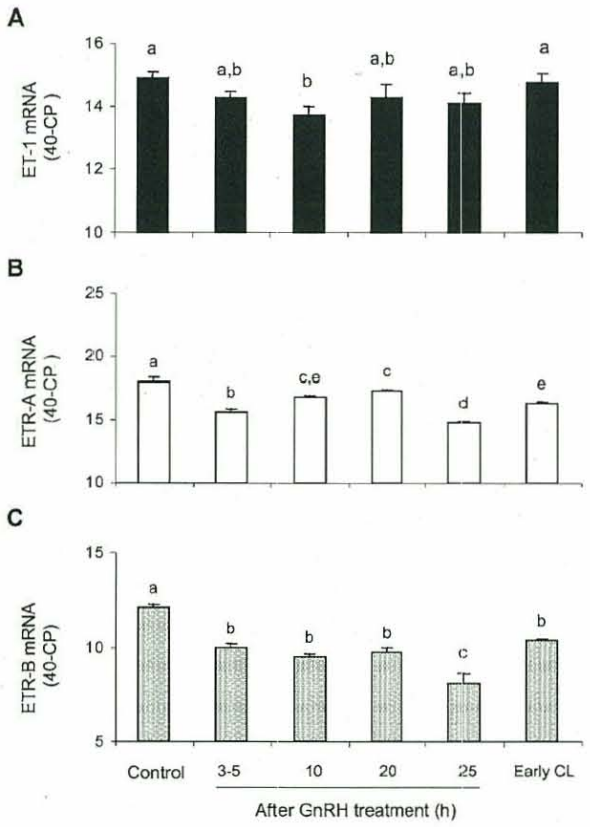

Fig. 3. Expressions of mRNA for (A) ET-1, (B) ETR-A and (C) ETR-B in bovine follicular tissue and early CLs before GnRH administration (control, before LH surge), 3–5 h after GnRH administration (during LH surge), 10 h after GnRH, 20 h after GnRH and 25 h after GnRH (peri-ovulation). Results are presented as 40-CP (CP ± SEM from 5 follicles or CL per group) in the target gene expression. Different superscripts denote statistically different values ($P<0.05$).

GnRH treatment and significantly decreased during the periovulatory period compared with the other phases. The concentration of Angiotensin II in follicular fluid significantly increased 25 h after GnRH compared with the control groups (Fig. 2).

Expression of ET-1, ETR-A, ETR-B, and ECE-1 mRNA

The level of *ET-1* mRNA significantly decreased 10 h after GnRH treatment compared with before GnRH treatment and during the early CL period (Fig. 3A). The expressions of *ETR-A* and *ETR-B* mRNA during the periovulatory period were significantly lower than in other periods (Fig. 3B and C). The expression of ECE-1 mRNA began to decrease during the LH surge period and significantly decreased during periovulatory period compared with the other periods (Fig. 4).

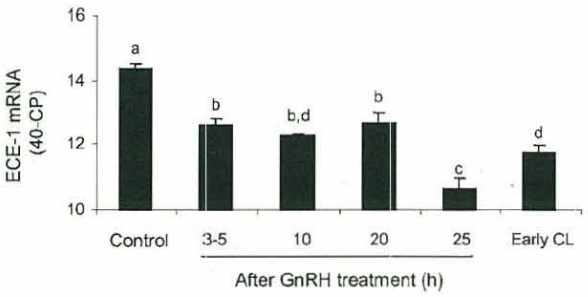

Fig. 4. Expression of mRNA for ECE-1 in bovine follicular tissue and early CLs before GnRH administration (control, before LH surge), 3–5 h after GnRH administration (during LH surge), 10 h after GnRH, 20 h after GnRH, 25 h after GnRH (peri-ovulation) and during the early CL period (Days 2–3). Results are presented as 40-CP (CP ± SEM from 5 follicles or CLs per group) in the target gene expression. Different superscripts denote statistically different values ($P<0.05$).

Discussion

Although considerable information exists on the involvement of angiogenesis from the final stages of follicular development to early corpus luteum formation, the factors involved in angiogenesis have not been fully elucidated. Herein, we report characterization of gene expression for endothelin, angiotensins, and related receptors and the protein levels of angiotensin II in follicular fluid during the periovulatory phase in GnRH-treated cows.

We found that expression of the ACE gene starts to decrease immediately after onset of the LH surge and that the expression remains at low levels. The Ang II, a component converted by ACE, in follicular fluid showed a tendency to increase towards ovulation, and the maximum levels were observed during the ovulation phase. On the other hand, AT1R gene expression decreased once during the LH surge and subsequently decreased further during the periovulatory period. In addition, expression of the AT2R gene drastically decreased during the periovulatory period compared with the other periods. A previous study using hypertensive transgenic (mRen-2)27

homozygous and heterozygous rats, which overexpress renin and angiotensin in extrarenal tissues, reported that AT1R expression is reduced in (mRen-2)27 homozygous rats, and this decrease may be a result of elevated levels of Ang II in this strain compared with (mRen-2)27 heterozygous rats [34]. In addition, high levels of Ang II downregulate Ang II receptors in rat cultured granulosa cells [35] and aortic vascular smooth muscle cells [36]. The present study indicated high concentrations of Ang II in follicular fluid during periovulatory period. Thus, our findings suggest that high levels of Ang II may suppress expression of the AT1R and AT2R genes.

The present study also indicated that the expression of *ET-1* mRNA decreased after the LH surge. In our previous study, we reported that release of ET-1 from bovine preovulatory follicles into a microdialysis system implanted in mature follicles during the periovulatory period decreased after the LH surge compared with the levels before the LH surge [37]. Therefore, our present and previous data suggest that the LH surge transiently inhibits expression and release of ET-1 during the periovulatory period in bovine ovaries. Heparin suppressed *ET-1* gene expression at the transcription level via inhibition of the GATA- and AP-1-binding activities, which are ET-1 promoters [38]. Generally, cumulus-oocyte complexes synthesize and secrete large amounts of glycosaminoglycans, which is a general term for polysaccharides including heparin [39]. Therefore, LH may act as an inhibitory factor in the expression mechanism of ET-1 within the bovine ovary, in part, through heparin and may suppress ET-1 expression during the periovulatory period.

Sequence analysis of the cDNA encoding ET revealed that ET is produced from a precursor named preproendothelin. After removal of a signal peptide, the precursor is selectively processed by an enzyme to yield a biologically inactive intermediate called big endothelin (big-ET). Big-ET is further converted into active ET by ECE. We observed that expression of *ECE-1* mRNA drastically decreased during the periovulatory period and in the early CL. This result indicates that the ovary may have a small amount of active ET during this period. ET-induced vasoconstriction is mediated mostly through ET receptors (ETR). Two cDNAs encoding these receptors have been cloned: ETR-A and ETR-B [26, 27]. The present study indicated that expression of the *ETR-A* and *ETR-B* genes drastically decreased during the periovulatory period compared with the other periods. In addition, our data demonstrates that ECE-1 expression also decreased; thus, the concentration of active ET-1 should also be lower during this period. Thus, the decrease in the ETR-A and ETR-B expressions may be associated with the decline in ET-1 by suppression of ECE-1 expression, suggesting a limited or passive role for the ET-system during the periovulatory period.

In conclusion, the present data is the first indication of changes in Ang II, endothelin and their receptors in the process of follicular maturation and ovulation in the cow. The vasoactive peptides angiotensin and endothelin may become important for downregulation of vasoconstrictor activity by differentiated follicles during the periovulatory period.

Acknowledgements

We greatly acknowledge support of this work by the German Research Foundation (DFG, Scha 257/14-2 and BE 3189/1-3), the 21st Century COE program (A-1) of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Grant-in-Aid for Scientific Research (16380183) from the Japan Society for the Promotion of Science.

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