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The Expression of Angiotensin and Endothelin System Members in Bovine Corpus Luteum During Estrous Cycle and Pregnancy

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The aim of this study was to determine the changing profiles of the mRNA expression of members of angiotensin and endothelin system in bovine corpus luteum (CL) from different stages of the estrous cycle and pregnancy. Corpora lutea were accordingly assigned to the following stages; d 1–2, 3–4, 5–7, 8–12, 13–18, >18 (after regression) of estrous cycle and of early and late pregnancy (<4 and >4 mo). The block RT-PCR analysis of CL showed a significantly higher angiotensin converting enzyme (ACE) mRNA expression during mid and late luteal phases as well as after regression, but lower levels during pregnancy. Full quantitative real-time RT-PCR (LightCycler) confirmed this pattern of ACE mRNA expression. The angiotensin receptor type 1 (AT1R) mRNA expression was relatively stable throughout the periods examined. In contrast, AT2R mRNA temporarily decreased on d 8–12, followed by an increase to the highest levels during late luteal phase, and it remained at high levels during regression and pregnancy. Concentration of angiotensin II (Ang II) peptide in luteal tissue was highest after ovulation (d 1–2), decreased afterward, increased again during late luteal phase, and decreased to lower levels during regression and pregnancy. The mRNA expression and peptide concentration of endothelin 1 (ET-1) was high after ovulation followed by a decrease during mid and late luteal phases and increased again to the highest level after regression. The endothelin receptor type B (ETR-B) mRNA expression increased during late luteal phase and further after regression. In contrast, ETR-A and endothelin converting enzyme 1 (ECE-1) mRNA expression were relatively constant during all stages examined. In conclusion, the regulatory changes of both angiotensin and endothelin family members during early luteal phase and again during late luteal phase suggest a possible modulatory role of these vasoactive peptide families for bovine CL formation and regression.

Key Words: Angiotensin II; endothelin-1; expression; corpus luteum; cow.

Introduction

It is indicated that the local angiotensin and endothelin systems may have important roles in ovarian physiology *(1–5)*. Angiotensin II (Ang II) and endothelin-1 (ET-1) may regulate reproductive phenomena such as oocyte maturation, ovulation, and corpus luteum (CL) function *(6–12)*.

Most Ang II is generated in two sequential steps: renin catalyzes the conversion of angiotensinogen to the decapeptide Ang I, which is subsequently hydrolyzed by angiotensin converting enzyme (ACE) to form Ang II *(13)*. Two main types of Ang II receptor (AT1R and AT2R) located on the cell membrane have been characterized *(14)*.

The three types of endothelin family members (ET-1, ET-2, and ET-3) are derived from three different precursors, the pre-proendothelins, which are proteolytically processed by endopeptidases, to generate the inactive big ET. Big ET-1 is cleaved by endothelin converting enzyme-1 (ECE-1) to generate the 21-amino acid active vasopeptide. The ETs are produced in various tissues, but ET-1 is the only isoform synthesized and secreted by vascular endothelial cells. Two endothelin receptor subtypes (ETR-A and ETR-B) have been identified *(15)*.

Both Ang II and ET-1 stimulate vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2), the two most important angiogenic factors. Ang II induces VEGF expression in cardiac microvascular endothelial cells *(16)* and potentiates VEGF-mediated angiogenic activity through up-regulation of VEGFR-2 in retinal microvascular endothelial cells *(17)*. Ang II and ET-1 regulate FGF2 production in endothelial cells *(18)*. Moreover, it has been reported that both Ang II and ET-1 are most potent vasoconstrictors. There is now clear evidence that the vasoactive peptides Ang II and ET-1 may play an important role during physiological and induced luteolysis in cows. Ang II and ET-1 may be responsible for the observed decrease of blood flow in mid-cycle corpus luteum (CL) after induced luteal regression and suggest an important role to trigger the luteolytic cascade *(19)*. Ang II and ET-1 may have also direct effects on luteal cells by inhibiting progesterone production

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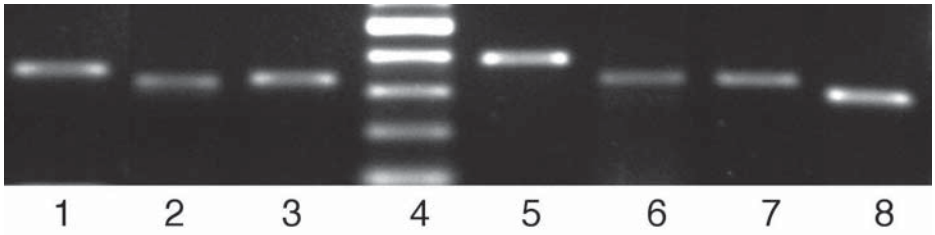

Fig. 1. Specific RT-PCR products for *(1)* ACE (365 bp), *(2)* AT1R (324 bp), *(3)* AT2R (335 bp), *(4)* 100 bp DNA marker (from bottom row 100, 200, 300, 400, 500 pb), *(5)* ECE-1 (395 bp), *(6)* ET-1 (333 bp), *(7)* ETR-A (329 bp), and ETR-B (296 bp) in bovine corpus luteum separated by agarosegel electrophoresis.

as shown for bovine CL *(20–22)*. This inhibitory effect of Ang II and ET-1 was potentiated by prostaglandin (PG) $F_{2\alpha}$ *(21,22)*.

However, there is not enough information available for an overview of the local expression of both vasoacive peptide system members in bovine CL during different stages of estrous cycle and pregnancy. Therefore, the objective of this study was to determine the changing profiles of the mRNA expression and protein concentration for components of angiotensin system (ACE, Ang II, AT1R, AT2R) and endothelin system (ECE-1, ET-1, ETR-A, ETR-B) in bovine CL during estrous cycle and pregnancy

Results

Specificity and Validation of RT-PCR Data

Initial experiments verified specific RT-PCR transcripts for all examined factors (Fig. 1) in bovine CL. Each PCR product showed 100% homology to the known bovine genes after sequencing. To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping genes ubiquitin (UBQ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined in all samples. The relative signal intensities for PCR products specific for all examined factors were assessed after correction based on the ubiquitin PCR signal intensities. The mRNA expression data obtained by block RT-PCR were confirmed for ACE by quantitative real-time RT-PCR (LightCycler).

Expression of mRNA for Angiotensin System Members in CL

The results of the densitometric analysis of mRNA expression by RT-PCR (ratio of examined factor/ubiquitin mRNA; arbitrary units) for ACE, AT1R, and AT2R examined in CL during estrous cycle and pregnancy are presented in Fig. 2. The RT-PCR analysis of CL showed a significantly higher ACE mRNA expression during mid and late luteal phases as well as after regression, but lower expression during pregnancy (Fig. 2A). Full quantitative real-time RT-PCR (Light Cycler) further confirmed this pattern of ACE mRNA expression (Fig. 2B). The AT1R mRNA expression (Fig. 2D) increased weakly to the mid, late, and regression phases, but remained low during pregnancy. In contrast, AT2R mRNA (Fig. 2E) temporarily decreased during d 8–12, followed by an increase to the highest levels during late luteal phase, after regression and during pregnancy.

Expression of mRNA for Endothelin System Members in CL

The results of the densitometric analysis of mRNA expression by RT-PCR (ratio of examined factor/ubiquitin mRNA; arbitrary units) for ECE-1, ET-1, ETR-A, and ETR-B examined in CL during estrous cycle and pregnancy are presented in Fig. 3. The expression intensity of ECE-1 transcripts in bovine CL (Fig. 3A) was without any clear regulatory change during estrous cycle and pregnancy. In contrast, the mRNA expression of ET-1 in CL was high during the early luteal phase (d 1–4) followed by a significant decrease during mid luteal phase and significant increase again to a higher plateau after regression (Fig. 3B). In contrast, the expression intensity of ET-1 transcripts during pregnancy was very low. The ETR-B mRNA expression (Fig. 3D) increased during late luteal phase and after regression. Low mRNA expression was observed during pregnancy. No significant changes in ETR-A mRNA expression between examined groups during estrous cycle and pregnancy were found (Fig. 3E).

Peptide Concentration of Ang II and ET-1 in CL Tissue During Estrous Cycle and Pregnancy

Concentration of Ang II peptide in luteal tissue (Fig. 2C) was highest after ovulation (d 1–2), decreased afterward during mid luteal phase, increased during late luteal phase, and decreased again to lower levels during regression. The peptide concentration of ET-1 in CL (Fig. 3C) was high during the very early luteal phase (d 1–2) followed by a significant decrease afterward and significant increase again during regression (Fig. 3B). In contrast, the peptide concentration of ET-1 during pregnancy was low.

Discussion

We have demonstrated in this study clear regulatory changes in mRNA expression and peptide concentration of components of the angiotensin and endothelin systems in bovine CL during estrous cycle and pregnancy. A basal

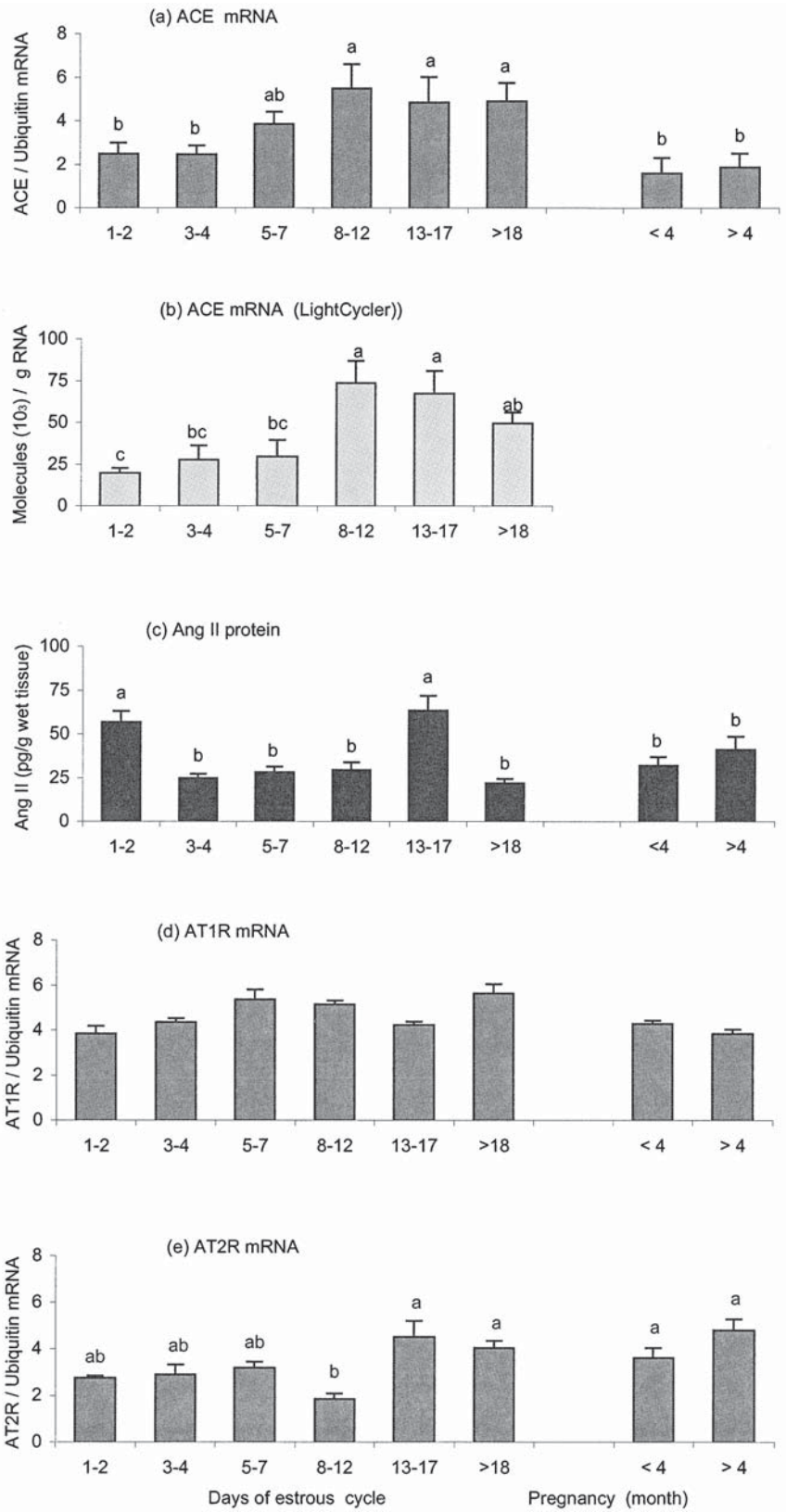

Fig. 2. The RT-PCR mRNA expression (block-PCR; ratio of examined factor/ubiquitin mRNA; arbitrary units and real-time LightCycler PCR; molecules/µg total RNA) and protein concentration (pg/g wet tissue) of angiotensin family members in bovine corpus luteum during estrous cycle and pregnancy: (**A**) ACE mRNA expression (block-PCR; 35 cycles); (**B**) ACE mRNA expression (real-time LightCycler PCR); (**C**) Ang II protein concentration; (**D**) AT1R mRNA expression (block-PCR; 35 cycles); and (**E**) AT2R mRNA expression (block-PCR; 35 cycles). Data are expressed as a means ± SEM. (n = 4–6 CL/stage for RT-PCR and 6–18 for EIA). Different superscripts denote statistically different values ($p < 0.05$).

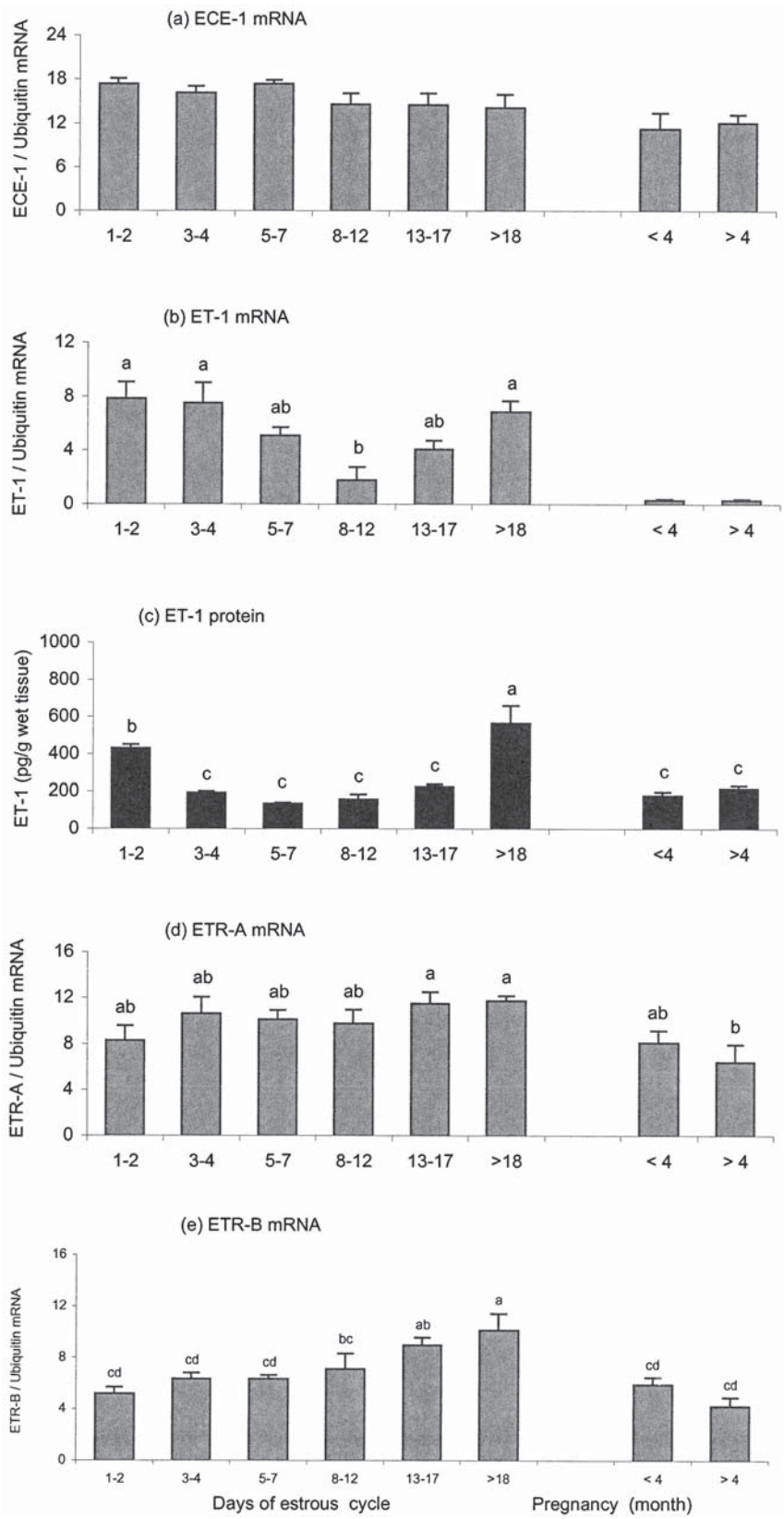

Fig. 3. The RTPCR mRNA expression (block-PCR; ratio of examined factor/ubiquitin mRNA; arbitary units and real-time LightCycler PCR; molecules/µg total RNA) and protein concentration (pg/g wet tissue) of endothelin family members in bovine corpus luteum during estrous cycle and pregnancy: (**A**) ECE-1 mRNA expression (block-PCR; 30 cycles); (**B**) ET-1 mRNA expression (block-PCR; 38 cycles); (**C**) ET-1 protein concentration; (**D**) ETR-A mRNA expression (block-PCR; 30 cycles); and (**E**) ETR-B mRNA mRNA expression (block-PCR; 30 cycles). Data are expressed as a means ± SEM (n = 4–6 CL/stage for RT-PCR and 6–18 for RIA). Different superscripts denote statistically different values ($p < 0.05$).

expression of mRNA and peptide concentration for all system members examined is obvious during all physiological phases tested. The results agree with the local secretion of Ang II and ET-1 in other in vitro CL studies *(23,24)*.

The clear up-regulation of angiotensin and endothelin system members during the early luteal phase and again during the late regression phase suggest participation of these peptides for ovulation/early CL formation and luteolysis. For the angiotensin system only Ang II peptide concentration was high during d 1–2. In the literature there is evidence for direct involvement of Ang II in ovulation and oocyte maturation in the rabbit *(25)*. ACE activity in bovine follicular fluid is present but did not differ in pre-ovulatory, luteal, or cystic follicles *(26)*. In contrast, pro-renin and renin activity in bovine follicular fluid increased in superovulated heifers after the LH peak *(27)*.

Another process is going on in the early CL, namely, angiogenesis. The newly formed CL after ovulation rapidly develops within a few days. This process consists of highly active vascularization and mitosis of steroidogenic cells in parallel. The expression and localization of the most important factors for angiogenesis FGF2 and VEGF have been described in bovine CL *(28–30)*. Ang II up-regulates dose dependently the expression of FGF2 in bovine luteal cells *(7)* and VEGF mRNA in endothelial cells *(16)* and may therefore actively participate in the process of early CL formation. On the other hand, FGF2 and VEGF enhance Ang II production in vitro in CL tissue or luteal endothelial cells *(12,31)*. The highest levels of ECE-1 and ET-1 mRNA and ET-1 peptide during the very early luteal phase suggest an involvement of the endothelin system information of new CL. ET-1 stimulates proliferation and migration of endothelial cells through ETR-B *(32,33)* and is a potent mitogen for vascular smooth muscle cells *(34)*

More obvious was the up-regulation of mRNA and peptide concentration for both systems during the late luteal and regression phases. It has been widely documented in recent years that Ang II and ET-1 may act as essential mediators of luteal regression *(20–22,35–39)*. Both peptides are most active vasoconstrictors and may be responsible for functional luteolysis by decrease of blood flow about 8–24 h after a luteolytic dose of $PGF_{2\alpha}$ *(19)*. Both peptides (Ang II and ET-1) additionally exhibit intraluteal effects inducing apoptosis or necrosis of luteal cells (structural luteolysis). Ang II either alone or together with $PGF_{2\alpha}$ can suppress the release of progesterone *(22)* or decrease LH-stimulated expression of the mRNA encoding cytochrome P450 side chain cleavage enzyme (P450scc), and Ang II promoted the association of cholesterol with cytochrome P450scc in bovine luteal cells *(7)*. The progesterone inhibitory effect of Ang II was blocked by the AT1R and AT2R antagonist Saralasin [Sar^1, Val^5, Ala^8]-Ang II *(22)*. $PGF_{2\alpha}$ stimulated the biosynthesis of ET-1 in a number of systems studied, including cultured luteal slices *(20)*, bovine luteal endothelial cells *(35)*, microdialyzed luteal tissue *(21)*, and bovine CL in vivo *(36)*. ET-1 in vitro is able to inhibit basal or LH-stimulated progesterone production by dispersed ovine luteal cells *(4)* or bovine luteal cells *(20)* in a dose-dependent manner. Normally the ETR-A was found in smooth muscle cells, but also in large and small luteal cells and endothelial cells *(20,40,41)*. The ETR-B was found normally in endothelial cells in blood vessels *(40)*.

In conclusion, it is obvious from our results that distinct regulatory changes of expression and peptide concentration for some members of the angiotensin and endothelin systems occur in bovine CL during different stages of estrous cycle and pregnancy. Particularly, a significant increase in the expression of both angiotensin and endothelin family members during early luteal phase (angiogenesis) and again during late luteal phase (CL regression) suggests a possible modulatory role of these vasoactive peptide families for bovine corpus luteum formation and regression.

Material and Methods

Collection of Bovine Ovaries

Entire reproductive tracts from German Simmental (Fleckvieh) cows were collected at a local slaughterhouse within 10–20 min after slaughter and were transported to the laboratory on ice. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (color, consistency, corpus luteum stage, number and size of follicles) and the uterus (color, consistency and mucus) *(42)*. The CL were accordingly assigned to the following stages: d 1–2, 3–4, 5–7, 8–12, 13–17, >18 of estrous cycle and of early (<4 month) and late (>5 month) pregnancy (the crown-rump length of the fetus was measured to evaluate the stage of pregnancy) *(43)*. All CL were aliquoted, quickly frozen in liquid nitrogen, and stored at –80ºC until RNA extraction and tissue extraction for Ang II and ET-1.

Ang II and ET-1 Peptide Determinations in CL Tissue

The concentration of Ang II in the CL tissue was determined in duplicate by second antibody enzyme immunoassays (EIA) after extraction using 96-well ELISA plates (NUNC-Immuno Plate, NUNC™ Brand Products, Denmark) as described previously *(22)*. The standard curve for Ang II ranged from 1.2 to 10000 pg/mL, and the ED50 of the assay was 150 pg/mL. The intra- and interassay coefficients of variation were on average 6.2% and 7.9%. The peptide concentration of ET-1 in the CL tissue was measured by RIA. The antiserum used was prepared in our laboratory by immunizing rabbits with a conjugate of bovine ET-1 and Limped Key Hole protein. Cross-reactivity of this antiserum with ET-1, ET-2, ET-3, and big ET were 100%, 51%, 20%, and 3%, respectively. Iodine-125-labeled ET-1 was purchased from Amersham-Pharmacia, Freiburg, Germany. ET-1 standard (200 µL) or samples (100 µL assay buffer) were incubated for 24 h at 4ºC with 100 µL of antiserum (final dilution 1/20 000). One hundred microliters of iodine-

labeled ET-1 was added afterward and incubation was continued for an additional 24 h. Bound and free ET was separated by adding 100 µL of second antibody from sheep against rabbit IgG followed by further incubation for 60 min and addition of 2 mL 6% polyethylenglycol and centrifugation at 4ºC. The precipitate was counted. The sensitivity of the assay was 3 pg/mL and the standard curve ranged from 3 to 2000 pg/mL. The intra- and interassay coefficients of variation were an average 5.8% and 10.4%, respectively.

Tissue Extraction

Tissue (1 g wet weight) was transferred into 10 mL of acidic buffer (pH 2.8). This buffer contains 205 µL orthophosphoric acid, 2.264 g $NaH_2PO_4 \cdot H_2O$, 1.86 g EDTA, 7.019 NaCl, 0.2 NaN_3, 2.0 g and one complete mini® tablet BSA, 1 mL Trition X-100 per liter (Boehringer, Mannheim, Germany). These tablets contain both reversible and irreversible protease inhibitors, and inhibit a broad spectrum of serine, cysteine, and metalloproteases. The mixture was homogenized in an ice bath with Ultra Turrax equipment (Janke and Kunkel, Staufen, Germany) with 15-s bursts of shredding at maximum speed, separated by 45-s intervals of cooling. The homogenate was subsequently centrifuged at 2000*g* for 15 min at 4°C. The supernatant (an aliquot of 4 mL) was transferred to a small Sep-Peak C18 Cartridge (Waters, Millford, MA, USA). The column was calibrated with 2 mL methanol (MeOH) and 5 mL distilled water. Ang II and ET-1 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 or for ET-1 in acidic buffer. The recovery rate of Ang II and ET-1 with this process was 42% and 67%, respectively. Values presented are corrected.

RNA Isolation

Total RNA from CL tissue was isolated by the single-step method *(44)* using TRIzol reagent (Gibco BRL, MD, USA). RNA was dissolved in water and spectroscopically quantified at 260 nm. Aliquots were subjected to 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of RNA.

Semiquantitative RT-PCR

Two micrograms of total RNA was used to generate single-strand cDNA in a 60 µL reaction mixture as described previously *(45)*. The primers were designed using the EMBL database or were used as described elsewhere (Table 1) and were commercially synthesized (Amersham-Pharmacia, Freiburg, Germany). Conditions for enzymatic amplification were established on a gradient cycler (Eppendorf, Hamburg, Germany) for all the factors studied. For each set of primers, the reaction was optimized for the amount of primers, cDNA, and number of cycles. The PCR for all examined factors contained 10 m*M* Tris-HCl (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, 0.6 µ*M* of each primer, and 0.5 units of thermostable polymerase PrimeZyme (Biometra, Göttingen, Germany) to 2.5 µL cDNA (final volume 25 µL). UBQ PCR was performed under the same conditions, but a higher concentration of primer (1.0 µ*M*) was used. The number of amplification cycles for each examined factor was individually optimized. All amplifications were done as follows: an initial denaturation step 94ºC for 2 min, each cycle 94ºC for 45 s, 60ºC (ACE, AT1R, 64ºC and AT2R, 67ºC) for 45 s, and afterward one additional elongation step 72ºC for 2 min. Samples for the UBQ were amplified by 22 cycles: a single denaturation step 94ºC for 2 min, each cycle 94ºC for 45 s, 55ºC for 45 s, 72ºC for 45 s and afterward one additional elongation step 72ºC for 2 min.

To determine the optimal quantity of reverse transcript needed for PCR and to verify that the cDNA product was dependent on the input of transcript, varying quantities of transcript were used in the PCR reaction. To exclude any contaminating genomic DNA, all experiments included controls lacking the RT enzyme. To confirm the integrity of the mRNA templates and the RT-PCR protocol, the housekeeping gene *UBQ* as internal standard was examined in all samples. As a negative control water was used instead of RNA for the RT-PCR to exclude any contamination from buffers and tubes. Aliquots of the PCR reaction products (5 µL) were fractionated by electrophoresis through a 1.5% agarose gel containing ethidium bromide in a constant 60 V field. To determine the length of the products, a 100-bp marker (Gibco BRL, MD, USA) was used. All RT-PCR reactions were performed twice for each RNA preparation and were run on a single gel. The ethidium bromide-stained gels were evaluated by a video documentation system (Amersham-Pharmacia, Freiburg, Germany). Band intensities (relative) were analyzed by computerized densitometry (arbitrary units) using the Image Master program (Amersham-Pharmacia, Freiburg, Germany). Confirmation of the PCR product identity was obtained by cDNA subcloning into a transcription vector (pCR-Script, Stratagene, La Jolla, CA), and subjecting them to commercial DNA sequencing (TopLab, Munich, Germany).

Full Quantitative LightCycler PCR for ACE

For the LightCycler reactions *(46,47)* a master mix of the following reaction components was prepared to the indicated end-concentration: 6.4 µL water, 1.2 µL $MgCl_2$ (4 m*M*), 0.2 µL forward primer (0.2 µ*M*), 0.2 µL reverse primer (0.2 µ*M*), and 1.0 µL LightCycler Fast Start DNA Master SYBR Green I (Roche). Nine microliters of LightCycler master mix was filled in the LightCycler glass capillaries and 33 ng reverse transcribed total RNA in 1 µL was added as PCR template. The following LightCycler protocol was used for ACE real-time PCR: denaturation program (95ºC for 10 min), a four-segment amplification and quantification program repeated 50 times (95ºC for 15 s; 64ºC for 10 s; 72ºC for 20 s; 82ºC for 5 s with a single fluorescence

Table 1
Primer Sequences, Resulting Fragment Size, and Number of Cycles Used[a]

Target	Sequence of nucleotide[a,b]	Fragment size (bp)	Cycle number	EMBL/ Reference[c]
ACE	For 5'-ATCCCGGAATTATCAGGACC-3' Rev 5'-AGGGTGCCACCAAGTCATAG-3'	365	35	*12*
ATlR	For 5'-AAATACATTCCCCCAAAGGC-3' Rev 5'-TGTGGCTTTGCTTTGTTGAG-3'	324	35	*2*
AT2R	For 5'-TTTGGCTACTCTTCCTCTCTGG-3' Rev 5'-CATACTTCTCAGGTGGGAAAGC-3'	335	35	*2*
ECE-1	For 5'-GAGAATGAGAAGGTGCTGACG-3' Rev 5'-GAGCTCGTCTTCCGTACCAG-3'	395	30	Z35306
ET-1	For 5-TCCCCAGAATGGATTATTTCC-3' Rev 5'-TCTTCCTGTGGACTGTCGC-3'	333	38	S37093
ETR-A	For 5'-TGCAGAAGTCCTCAGTGGG-3' Rev 5'-GATCGCAGTGCACACCAG-3'	329	30	*2*
ETR-B	For 5'-AAACTGAGAATCTGCTTGCTCC-3' Rev 5'-AGAGTGAGCTTCAAAATCCTGC-3'	296	30	*2*
UBQ	For 5'-ATGCAGATCTTTGTGAAGAC-3' Rev 5'-CTTCTGGATGTTGTAGTC-3'	189	22	*2*
GAPDH	For 5'- GTCTTCACTACCATGGAGAAGG -3' Rev 5'- TCATGGATGACCTTGGCCAG -3'	197	25	*46*

[a]For angiotensin converting enzyme (ACE), angiotensin receptor 1 (AT1R), angiotensin receptor 2 (AT2R), endothelin converting enzyme-1 (ECE-1), endothelin-1 (ET-1), endothelin receptor A (ETR-A), endothelin receptor B (ETR-B), ubiquitin (UBQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR mRNA expression in bovine corpus luteum.

[b]For, forwards; Rev, reverse.

[c]EMBL accession number or reference of published sequence.

acquisition point), melting curve program (60ºC to 95ºC with a heating rate of 0.1ºC/s and a continuous fluorescence acquisition), and a final cooling program down to 40ºC. To confirm a constant housekeeping gene expression level in the investigated total RNA extractions, a GAPDH real-time RT-PCR was performed. Real-time RT-PCR was quantified in the LightCycler with the following GAPDH-specific settings (95ºC for 15 s; 58ºC for 10 s; 72ºC for 20 s; 85ºC for 5 s with a single fluorescence acquisition point) as described above. For all quantitative assays an external calibration curve was used, based on a single stranded DNA (ssDNA) molecule calculation. ACE products (RT-PCR) from *Bos taurus* were cloned separately in pCR4.0 (Invitrogen, Leek, The Netherlands), linearized by a unique restriction digest and dilutions of each plasmid preparations from single copies ssDNA (<10 molecules) up to 10^{10} ssDNA molecules were used in calibration curve *(47)*.

Statistical Analyses

The statistical significance of differences in mRNA expressions of examined factors, was assessed by ANOVA followed by Fisher's LSD as a multiple comparison test. All experimental data are shown as the mean ± SEM. Follicles per class were obtained from at least $n = 4$ cows.

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