Production and localisation of angiotensin II in the bovine early corpus luteum: a possible interaction with luteal angiogenic factors and prostaglandin F2 α

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Abstract

The newly formed corpus luteum (CL) rapidly develops after ovulation and has the features of active vascularisation and mitosis of steroidogenic cells. These stage-specific mechanisms also may contribute to gain the function of prostaglandin F2 α (PGF2 α)-resistant CL at this stage. Recent studies suggest that the vasoactive peptide angiotensin II (Ang II) regulates luteal function. Thus, this study aimed to investigate (i) the expression of angiotensin-converting enzyme (ACE) mRNA by RT-PCR and the ACE protein expression by immunohistochemistry, (ii) the effects of angiogenic growth factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), on the secretion of Ang II, PGF2 α , progesterone and oxytocin (OT), and (iii) the effects of luteal vasoactive peptides (Ang II and endothelin-1 (ET-1)) or OT on the secretion of PGF2 α , progesterone and OT from bovine early CL (days 3–4 of the oestrous cycle), and evaluate a possible interaction of these substances with PGF2 α . The expression of mRNA for ACE was found in theca interna of mature follicle, early CL and endothelial cells from developing CL as well as pituitary and kidney, but granulosa cells of mature follicle were negative. The immunohistochemical analysis revealed that blood capil-

laries (endothelial cells) were stained for ACE, but luteal cells were negative in early CL. To examine the effects of substances on the secretory function of the CL, an *in vitro* microdialysis system was used as a model. The infusion of bFGF and VEGF stimulated Ang II and PGF2 α secretion as well as progesterone, but not OT secretion in early CL. The infusion of Ang II after PGF2 α infusion continued the stimulatory effect on progesterone and OT release within early CL until 3 h thereafter. However, the infusion of ET-1 alone had no effect on progesterone or OT release. The infusion of luteal peptides such as Ang II and OT stimulated PGF2 α secretion, whereas the infusion of ET-1 did not. In conclusion, the overall results of this study indicate that a functional angiotensin system exists on the endothelial cells of early CL, and that angiogenic factors bFGF and VEGF upregulate luteal Ang II and PGF2 α secretion, which fundamentally supports the mechanism of progesterone secretion in bovine early CL. This idea supports the concept that the local regulatory mechanism involved in active angiogenesis ensures the progesterone secretion in the developing CL *in vivo*.

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Introduction

The new corpus luteum (CL) formed after ovulation rapidly develops within a few days. This process consists of highly active vascularisation and mitosis of steroidogenic cells in parallel. Such local mechanisms must be strictly regulated, and probably affect each other. It is well known that basic fibroblast growth factor (bFGF) (Gospodarowicz *et al.* 1986) and vascular endothelial growth factor (VEGF) (Ferrara & Davis-Smyth 1997) stimulate the growth of new capillary vessels. Besides its angiogenic activity, bFGF stimulates progesterone secretion from bovine early

CL *in vitro* (Miyamoto *et al.* 1992). Also, the inhibition of VEGF bioactivity causes the suppression of CL angiogenesis and progesterone release (Ferrara *et al.* 1998, Fraser *et al.* 2000). Furthermore, bFGF increases prostaglandin F2 α (PGF2 α) production in bovine luteal cells from the early luteal phase (Schams *et al.* 1995). Taken together, these angiogenic growth factors appear to upregulate both structure (angiogenesis) and function (secretion) of developing CL *in vivo*. This raises the possibility that an interaction among angiogenic growth factors, PGF2 α and progesterone plays an essential role in CL development. These stage-specific mechanisms

also may contribute to gain the function of PGF2 α -resistant CL at this stage.

A vasoactive peptide, angiotensin II (Ang II) that is converted from Ang I by angiotensin-converting enzyme (ACE), regulates oocyte maturation (Kuo *et al.* 1991), ovulation (Kuo *et al.* 1991) and steroidogenesis (Yoshimura *et al.* 1993). The localisation of ACE in bovine pulmonary endothelial cells (Ideishi *et al.* 1993) and the existence of Ang II receptor in bovine aortic endothelial cells (Tallant *et al.* 1997) have been reported. Furthermore, we recently showed that Ang II was converted from Ang I in microvascular endothelial cells from bovine CL, and that these cells have Ang II receptors (Hayashi *et al.* 2000). Additionally, Ang II has an inhibitory effect on progesterone release from bovine midcycle CL, and this inhibitory effect was potentiated by PGF2 α (Hayashi & Miyamoto 1999).

Several studies have reported that other luteal peptides such as oxytocin (OT) and endothelin-1 (ET-1) influence progesterone secretion in bovine CL *in vitro* (Miyamoto & Schams 1991, Girsh *et al.* 1996a, Sakumoto *et al.* 1996, Miyamoto *et al.* 1997). OT increases the progesterone secretion from bovine CL throughout the luteal phase and this stimulatory effect is highest in early CL (Miyamoto & Schams 1991). Furthermore, OT increases PGF2 α release by luteal cells compared with control cultures irrespective of day of oestrous cycle (Grazul *et al.* 1989). In contrast, ET-1 has been shown to inhibit progesterone release from bovine CL from the mid luteal phase of the oestrous cycle (Girsh *et al.* 1996a, Miyamoto *et al.* 1997), and the inhibitory effect of ET-1 was potentiated by PGF2 α (Miyamoto *et al.* 1997) as in the case for Ang II (Hayashi & Miyamoto 1999). On the other hand, the tissue concentration of ET-1 in early CL is lower than that of other luteal phases (Girsh *et al.* 1996b). Thus, the local production and action of these peptides and PGF2 α in early CL appear to be quite different from those of the midcycle CL. This difference in local concentration of each product could be an important basis for the mechanisms for the regulation of PGF2 α -resistant CL in which angiogenesis is mostly active.

Thus, in this study, we aimed to investigate (i) the production and localisation of vasoactive peptide Ang II by using RT-PCR and immunohistochemistry, (ii) the effects of angiogenic growth factors, bFGF and VEGF, on the secretion of Ang II, PGF2 α , progesterone and OT, and (iii) the effects of luteal vasoactive peptides (Ang II and ET-1) or OT on the secretion of PGF2 α , progesterone and OT from bovine early CL, and evaluate a possible interaction of these substances with PGF2 α .

Materials and Methods

Collection of bovine tissues

Entire reproductive tracts, pituitary and kidney tissue from Holstein cows were collected at a local slaughterhouse

within 10–20 min of slaughter. The stage of the oestrous cycle was carefully defined by macroscopic observation of the uterus and ovaries (follicles and CL). Characteristics of the ovaries included size, colour, consistency, connective tissue and thickness of the endometrium, mucus, and absence of elongated early embryos. Ovaries from five cows with early CL (days 1–4 after oestrus), which were identified as stage I according to the criteria of Ireland *et al.* (1980), were collected. Follicles (>14 mm) were collected from six cows only after CL regression with signs of mucus production in the uterus and cervix, and were assumed to be preovulatory (follicular fluid oestradiol-17 β concentrations of >660 nmol/l) and healthy (all follicles with a progesterone >300 nmol/l follicular fluid were excluded). For the RNA extraction, the follicles were taken from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. After aspiration of follicular fluid, follicles were bisected and the inside wall was gently scraped and flushed with Ringer's solution (Fresenius AG, St Wendel, Germany) to remove the granulosa cells. All tissues were frozen rapidly in liquid nitrogen, and then stored at –80 °C until processed for studies of gene expression. Luteal tissue was immersion-fixed immediately after collection. For the microdialysis study, the ovaries were washed several times with sterile saline solution (0.9% NaCl) and transported to the laboratory in sterile saline solution containing 60 mg/l penicillin and 100 mg/l streptomycin at 37 °C.

Culture of microvascular endothelial cells

Cytokeratin-negative endothelial cells, type 3, derived from the microvascular bed of the developing bovine CL were used, and cultured as described (Okuda *et al.* 1999). These cytokeratin-negative cells are known to occur with the form of a cobblestone appearance. Only the cells at 11 passages were used in the present study.

RNA isolation and RT-PCR

Total RNA from bovine tissues (CL, theca interna, granulosa cells, pituitary, kidney and endothelial cells) was isolated by the single-step method of Chomczynski & Sacchi (1987) using Trizol reagent (Gibco-BRL, Eggenstein, Germany) 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. The RNA yield was then either immediately subjected to RT-PCR or stored at –80 °C until analysis.

Two micrograms total RNA were used to generate single-strand cDNA in a 60 μ l reaction mixture as described previously (Berisha *et al.* 2000a). The resulting cDNA templates were subjected to PCR amplification. Conditions for enzymatic amplification were optimised for each PCR as follows. The ACE contained 10 mmol/l

Tris-HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.1% Triton X-100, 0.6 μ mol/l of each primer, and 0.5 units of the thermostable polymerase PrimeZyme (Biomera, Göttingen, Germany) to 5 μ l cDNA (final volume 25 μ l). The ubiquitin PCR was performed under the same conditions as those for ACE, but a higher concentration of primer (1.5 μ mol/l) was used. On the basis of optimisation of temperature, amplification of ACE analysis consisted of one denaturing step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s and at 64 °C for 45 s. One additional elongation step was carried out at 72 °C for 2 min. Amplification of the housekeeping gene consisted of one denaturing step at 94 °C for 2 min, 22 cycles at 94 °C for 45 s, 55 °C for 45 s; 72 °C for 45 s and one additional elongation at 72 °C 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 amount of transcript used, varying quantities of transcript template were used in the PCR reaction process. The RT-PCR product from 3 μ l cDNA was within the exponential phase of PCR reaction (plateau phase was avoided) and produced a visible band. To exclude the possibility of amplification of genomic DNA, all experiments included reactions in which the RT enzyme or cDNA template was omitted. As a negative control, water was used instead of RNA for the RT-PCR to exclude any contamination from buffers and tubes. The primers were designed by using the European Molecular Biology Laboratory (EMBL) database, or were used as described elsewhere and were commercially synthesised (Amersham-Pharmacia, Freiburg, Germany). The primers were as follows: ACE forward 5'-ATCCCGGAATTA TCAGGACC-3' and reverse 5'-AGGGTGCCACCA AGTCATAG-3' (EMBL Ac. no. AJ309016) and ubiquitin forward 5'-ATGCAGATCTTTGTGAAGAC-3' and reverse 5'-TTCAGGAGCACACTGGAGTG-3'. The predicted sizes of the resulting RT-PCR products were 365 bp for ACE and 189 and 417 bp for ubiquitin respectively, corresponding to identical multimeric gene cassettes.

Aliquots of the PCR reaction products (5 μ l) were added to 1 μ l bromophenol blue glycerine and fractionated by electrophoresis through a 1.5% agarose gel containing ethidium bromide in a constant 60 V field. To determine the length of the RT-PCR products, a mass ladder and 100 bp marker were used. The resultant band intensities were scanned by a video documentation system (Amersham-Pharmacia) and analysed with the Image Master ID program (Amersham-Pharmacia). To verify each PCR product, double-strand sequencing was performed directly or after subcloning (TopLab, Munich, Germany).

Fixation of CL tissue for immunohistochemistry

The CL were dissected into 0.5 cm thick tissue slices, immersion-fixed in Bouin's solution for 12 h, dehydrated

in a graded series of ethanol, cleared in xylene and embedded in paraffin wax using conventional procedures. Serial sections of 5 μ m thickness were cut on a microtome (Leitz, Wetzlar, Germany) and processed for immunohistochemistry.

Tissue preparation and immunohistochemistry

For the histological investigations, early CL from five cows, after collecting the small section for RNA extraction, were fixed via immersion with methanol/glacial acid (2/1 v/v) or Bouin's solution for ACE immunohistochemistry. After 12 h fixation, tissues were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax using conventional procedures. Serial sections of 5 μ m thickness were cut on a Leitz microtome and processed for immunohistochemistry. Localisation of ACE was performed according to the avidin-biotin-peroxidase complex method (Hsu *et al.* 1981). The sections were deparaffinised, rehydrated and treated for 30 min with 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. After three washing steps in PBS (pH 7.4), rabbit serum (DAKO, Hamburg, Germany) was used in 1:10 dilution for 30 min to prevent the non-specific binding of the antibodies. Overnight incubation with the primary antibody from chicken against rabbit lung ACE (Biotrend, Cologne, Germany; dilution 1:500) in a humid chamber at 4 °C followed. The antibody strongly cross-reacts with bovine, pig and human ACE. On the next day the slides were washed three times again with PBS and incubated for 30 min with a biotinylated goat anti-rabbit or rabbit anti-chicken serum IgG as the second antibody (1:400). A further 30 min later, after building the streptavidin-biotin-horseradish-peroxidase complex, peroxidase staining was performed with 3,3'-diaminobenzidine (DAB).

The specificity of the staining reaction was proved by serial dilution of the primary antigen, which resulted in a gradual decrease in intensity. Finally, all specimens were counterstained with haematoxylin and mounted with DePeX (Serva, Heidelberg, Germany) for light microscope examination.

The specificity of the immunocytochemical reactions was assessed by (i) replacement of the primary antibodies with buffer, (ii) their substitution with non-immune goat IgG (1:10 diluted), and (iii) incubation with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Lack of detected staining of tissue elements in the controls demonstrated the specificity of the reactions.

Microdialysis system (MDS) in vitro

The MDS of the bovine CL *in vitro* has been previously described in detail (Miyamoto & Schams 1991). Eight different CL were used for the experiment on angiogenic growth factors. In other experiments, four to seven

different CL in each experimental group were used. In brief, CL of the early luteal phase having diameters of 0.8–1.0 cm were isolated from the ovary, and divided into halves. The surrounding stromal tissue of each piece was then removed. Two capillary dialysis membranes (Fresenius SPS 900 Hollow Fibers, cut-off molecular mass 1000 kDa, 0.2 mm diameter, 5 mm long; Fresenius AG) with each end glued to a 4.5 cm long piece of Silastic tubing (internal diameter 0.3 mm) were implanted into each half of the CL at least a 5 mm distance between dialysis membranes. Thus, four membranes were implanted in each CL. One of the four dialysis membranes was used for a control and the other three were used for the infusion of stimulants. For infusion, one end of the tube was connected to a multiple-line peristaltic pump (IPC-24; Ismatec SA, Zurich, Switzerland) and the other was 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 filled with 50 ml M199 (Sigma Chemical Co., Deisenhofen, Germany) containing Eagle's salts, 10 mmol/l NaHCO₃, 365 mg/l L-glutamine, 25 mmol/l Hepes, 5 g/l BSA, 60 mg/l penicillin, 100 mg/l streptomycin, 56 mg/l ascorbic acid, and 2 mg/l amphotericin B at pH 7.4 maintained in a water bath at 38 °C. The luteal pieces were perfused with Ringer's solution at a flow rate of 3.0 ml/h throughout the experiments.

In the experiment of angiogenic growth factors, fractions of the perfusate after a 3 h pre-perfusion were collected every 2 h (6 ml per fraction) up to 10 h (0 h corresponds to the end of the pre-perfusion). Bovine bFGF (7.5 nmol/l; Boehringer Mannheim GmbH, Mannheim, Germany) or human VEGF (5 nmol/l; Pepro Tech Ltd, London, UK) were infused during 2–4 h.

In other experiments, fractions of the perfusate after a 3 h pre-perfusion were collected every 30 min (1.5 ml per fraction) up to 5 h. The collected samples were stored at –20 °C until hormone determination. After progesterone concentration was directly measured, the fractions were pooled (among 0–1 h, 1–1.5 h, 1.5–2 h, 2–3.5 h and 3.5–5 h) depending on the experimental design for the purpose of desalting and concentrating the peptides, in order to reduce the number of samples. This step was needed to extract and concentrate OT in the samples that enabled us to determine the concentration by enzyme immunoassay (EIA). Consequently, for statistical analysis ten samples (every hour) were processed for progesterone secretion, while five samples (pooled fractions) were processed for OT, as described below.

Four substances were infused: PGF2 α (Sigma), ET-1, Ang II, and OT (all purchased from Peptide Institute Inc., Osaka, Japan). All were diluted in Ringer's solution to obtain the required final concentrations (10 μ mol/l for PGF2 α and OT, 0.1 μ mol/l for ET-1, and 10 or 1 μ mol/l for Ang II). PGF2 α was infused between 1 and 1.5 h, and

ET-1 or Ang II was infused between 1.5 and 2 h. To determine whether an interaction between PGF2 α and OT had an effect on progesterone release, PGF2 α was first infused for 30 min (1–1.5 h), and then OT was infused for the next 30 min (1.5–2 h). Then the experiment was repeated but with the order of infusion reversed (OT infusion from 1 to 1.5 h followed by PGF2 α infusion from 1.5 to 2 h). The transfer capacity of the microdialysis membrane was about 0.1% for bFGF, VEGF, OT, ET-1, and Ang II, and about 1% for progesterone and PGF2 α . These values of transfer capacity were determined according to the method of Jarry *et al.* (1990). The concentrations of the infused substances were chosen based on both of the above transfer capacities, the preliminary experiment, the previous results for bFGF (Miyamoto *et al.* 1992), OT (Miyamoto & Schams 1991), ET-1 (Miyamoto *et al.* 1997) and Ang II (Hayashi & Miyamoto 1999), and basal release of each substance in this study.

PGF2 α extraction

The volumes of perfusate samples were adjusted with Ringer's solution to 6 ml in the experiment on growth factors and to 5 ml in the other experiments. These pooled fractions were adjusted to pH 3.5 with acetic acid and extracted using diethyl ether as described previously (Acosta *et al.* 1998). The residue was dissolved in 200 μ l assay buffer for the steroid and PGF2 α EIA (40 mmol/l PBS, 0.1% BSA, pH 7.2). The samples were concentrated 15- to 30-fold as a result of the process. To estimate the recovery rate in the MDS perfusate, 4 pmol/l PGF2 α were added to Ringer's solution, and the obtained value was 61%.

Extraction of peptides

After the diethyl ether extraction, the remaining Ringer's solution (5 or 6 ml) was used for the extraction of peptides. BSA was added to the samples to a final concentration of 1 mg/ml and then the samples were adjusted to pH 2.5 with acetic acid. The samples were then applied to a Sep-Pak C18 Cartridge (Waters, Millford, MA, USA) as described previously (Miyamoto *et al.* 1997). The residue was dissolved in 300 μ l assay buffer for peptide EIA (42 mmol/l Na₂HPO₄, 8 mmol/l KH₂PO₄, 20 mmol/l NaCl, 4.8 mmol/l EDTA, 0.05% BSA, pH 7.5). The samples were concentrated 7.5- to 20-fold as a result of the process. To estimate the recovery rate in the MDS perfusate, OT, ET-1 and Ang II were added to Ringer's solution (50, 100, and 10 pmol/l respectively), and the obtained values were 73, 62 and 88% respectively. However, since the ET-1 concentration was too low to detect with an enrichment rate of 7.5- to 20-fold, to estimate the baseline ET-1 concentration (0–1 h), the fractions between 0–1 h in another experiment from four lines implanted in single CL were pooled to 10 ml for six CL

and then extracted, and the samples for ET-1 determination were concentrated 100-fold as a result of the process.

Hormone determination

Progesterone, PGF2 α , OT, ET-1 and Ang II concentrations were determined in the perfusate fractions from the MDS with second-antibody EIAs that were based on a competitive assay using horseradish peroxidase-labelled progesterone and PGF2 α or biotin-labelled peptides as tracers.

Concentrations of progesterone were assayed directly as described in detail earlier (Miyamoto *et al.* 1992). The standard curve of progesterone ranged from 0.16 to 160 nmol/l, and the ED₅₀ of the assay was 5.7 nmol/l. The intra- and interassay coefficient of variation (CV) values were on average 6.2 and 9.3% respectively. The EIA for PGF2 α was carried out as described previously (Miyamoto *et al.* 1995). The standard curve of PGF2 α ranged from 55 to 5650 pmol/l, and the ED₅₀ of the assay was 1000 pmol/l. The intra- and interassay CV values were on average 7.8 and 11.5% respectively. The EIAs for OT and ET-1 were conducted as described previously (Miyamoto *et al.* 1997). The standard curve of OT ranged from 1.6 to 200 pmol/l, and the ED₅₀ of the assay was 21 pmol/l. The intra- and interassay CV values of the OT assay were on average 6.2 and 8.6% respectively. The standard curve of ET-1 ranged from 3.9 to 2000 pmol/l, and the ED₅₀ of the assay was 180 pmol/l. The intra- and interassay CV values of the ET-1 assay were on average 8.7 and 12.6% respectively. The EIA for Ang II used rabbit antiserum raised against human Ang II as described in detail earlier (Hayashi & Miyamoto 1999). The standard curve of Ang II ranged from 2.3 to 9560 pmol/l and the ED₅₀ of the assay was 105 pmol/l. The intra- and interassay CV values were on average 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.

Statistical analysis

The mean hormone (progesterone, PGF2 α , OT, ET-1 and Ang II) concentrations in the first 2 h (growth factors) or 1 h (others) fraction were used to calculate the individual baseline, because of a large variation in the basal concentrations of each hormone released into the MDS lines implanted in the different CL (progesterone 4.8–39.4 nmol/l, OT 0.8–30.2 pmol/l, PGF2 α 13.6–85.9 pmol/l, ET-1 0.03–0.06 pmol/l and Ang II 4.5–38.4 pmol/l). All hormone concentrations were expressed as a proportion of this individual baseline. This treatment enables an evaluation of relative changes of hormonal values between the different CL. The change in hormonal release after substance infusions was tested based on

individual time points throughout the experiment as compared with the value at the same time point in the control. The change in hormone release by treatment was tested as compared with the value during the same time period in the control throughout the experiment (1–1.5 h, 1.5–2 h, 2–3.5 h, and 3.5–5 h). Means were analysed by ANOVA followed by the Tukey–Kramer test as a multiple comparison test. For the figures of MDS data, all hormone concentrations in the fractions were then expressed as a percentage of this individual baseline. The significance of the difference is shown only for the experimental groups where the values were significantly higher than those in the control group at the same time period. The absolute concentrations of each hormone during the first 1 or 2 h (baseline) of an experiment are given in the figure legends.

Results

Expression of mRNA for ACE

The RT-PCR product of ACE (partial sequence) showed 82% homology to the known human genes (EMBL Accession no. P12821) after sequencing. A specific transcript for ACE was detected in pituitary and kidney as well as the theca interna, early CL and endothelial cells from developing CL. However, the expression of ACE mRNA was not observed in granulosa cells (Fig. 1a). To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping gene ubiquitin was examined in all samples (Fig. 1b).

Immunohistochemical localisation of ACE in bovine early CL

The histological evaluation revealed that blood vessels selectively show a strong positive reaction for ACE. At the cellular level, ACE immunostaining is thereby restricted to the endothelial cells (Fig. 2A). Higher magnification demonstrates particular intense staining of the cell membrane (Fig. 2B). Specificity of the ACE immunostaining was confirmed by replacement of the primary antibody by non-immune serum (Fig. 2C).

Basal substance release of microdialysed early CL in vitro

Absolute concentrations of each substance in the MDS perfusate during basal release were 17.1 ± 0.6 nmol/l (S.E.M.) for progesterone, 8.8 ± 0.7 pmol/l for Ang II, 0.04 ± 0.004 pmol/l for ET-1, 29.4 ± 4.0 pmol/l for PGF2 α and 7.1 ± 0.8 pmol/l for OT.

Effects of growth factors on hormone release

The infusion of bFGF and VEGF for 2 h stimulated progesterone, PGF2 α and Ang II secretion during infusion but had no effect on OT secretion (Fig. 3).

Figure 1 Specific RT-PCR products for (a) ACE (365 bp) and (b) ubiquitin (189+417 bp) from bovine pituitary, kidney, theca interna tissue, granulosa cells, early luteal tissue and endothelial cells from developing CL, and a DNA mass ladder (1200, 800, 400 and 200 bp), separated by agarose gel electrophoresis.

Interaction of Ang II with PGF2 α on progesterone and OT release

The infusion of Ang II at both 1 and 10 $\mu\text{mol/l}$ significantly increased progesterone release ($P < 0.05$; Fig. 4). Ang II (1 $\mu\text{mol/l}$) stimulated progesterone release to 140% during infusion only (Fig. 4), while Ang II (10 $\mu\text{mol/l}$) stimulated progesterone release during and after infusion (Fig. 4). The infusion of Ang II (10 $\mu\text{mol/l}$) after PGF2 α infusion maintained the increased progesterone release between 160 and 250% during the experimental period ($P < 0.05$; Fig. 4). In contrast, an interaction of Ang II (1 $\mu\text{mol/l}$) and PGF2 α was not observed on the progesterone release (Fig. 4). Although Ang II (1 $\mu\text{mol/l}$) did not stimulate OT release from the CL (Fig. 5), Ang II (10 $\mu\text{mol/l}$) significantly stimulated it ($P < 0.05$; Fig. 5). Furthermore, the infusion of Ang II (10 $\mu\text{mol/l}$) after PGF2 α infusion maintained the increased OT release between 400 and 600% during the experimental period ($P < 0.05$; Fig. 5).

Interaction of ET-1 with PGF2 α on progesterone and OT release

The infusion of ET-1 had no effect on progesterone or OT release (Figs 4 and 5). When ET-1 was infused after PGF2 α infusion, progesterone release was slightly stimulated ($P < 0.05$; Fig. 4), but this stimulatory effect was not different from that of PGF2 α alone. In contrast to progesterone release, OT release was increased to around 400% by the infusion of ET-1 after PGF2 α , and this concentration was maintained throughout the experimental period ($P < 0.05$; Fig. 5).

Interaction of OT with PGF2 α on progesterone release

The infusion of OT stimulated progesterone release between 170 and 195% only during the infusion period ($P < 0.05$; Fig. 6). There was no clear interaction of OT with PGF2 α on the progesterone release (Fig. 6).

Effect of luteal peptides on PGF2 α release

The infusion of ET-1 had no effect on PGF2 α release (Fig. 7). However, the infusion of Ang II stimulated PGF2 α secretion ($P < 0.05$ for 1 $\mu\text{mol/l}$ and $P < 0.01$ for 10 $\mu\text{mol/l}$; Fig. 7). The infusion of OT weakly stimulated PGF2 α release ($P < 0.05$; Fig. 7).

Discussion

The present study provides direct evidence that the endothelial cell is the only site of Ang II production in the bovine early CL. The MDS study also showed that angiogenic growth factors bFGF and VEGF stimulate Ang II, PGF2 α and progesterone secretion from bovine early CL *in vitro*. Furthermore, luteal peptides such as Ang II and OT also stimulated PGF2 α secretion, and Ang II together with PGF2 α highly stimulated progesterone secretion. The results of this study, therefore, suggest that the interaction of PGF2 α and Ang II, the productions of which are upregulated by angiogenic factors, ensures the mechanisms of progesterone production in the developing CL.

It is well known that bFGF and VEGF stimulate neovascularisation (Gospodarowicz *et al.* 1986, Ferrara & Davis-Smyth 1997). The present MDS study shows that these growth factors are also capable of stimulating PGF2 α and Ang II secretion as well as progesterone, but not OT production in the bovine early CL (Fig. 8). The expression of bFGF mRNA (Stirling *et al.* 1991) and the production of bFGF peptide have been shown in bovine (Gospodarowicz *et al.* 1985) and ovine (Grazul-Bilska *et al.* 1992) CL. The specific binding sites for bFGF have also been observed in cultured bovine luteal cells throughout the oestrous cycle (Schams *et al.* 1993). Importantly, the

infusion of bFGF stimulated progesterone secretion from bovine CL in the MDS (Miyamoto *et al.* 1992, the present study), whereas bFGF had no effect on the progesterone secretion in bovine luteal cell culture (Miyamoto *et al.* 1992). Under the culture condition, cells are isolated and without almost any cell-to-cell contact, while cell-to-cell contact exists in the experiment with MDS. This raises the

question as whether a cell-to-cell contact may be the requisite for a luteal response to bFGF. In this context, the present MDS study indicates Ang II and PGF2 α as the possible mediators of bFGF action on the progesterone secretion in early CL (Fig. 8).

The expression of mRNA for VEGF and the VEGF production in ovine (Redmer *et al.* 1996) and bovine (Schams *et al.* 1994, Berisha *et al.* 2000b) CL and in bovine luteinised granulosa cells (Garrido *et al.* 1993, Schams *et al.* 1999) have been described. On the other hand, the receptors for VEGF are expressed within CL not only during active angiogenesis, but also at similar levels in the midcycle CL (Goede *et al.* 1998). The VEGF secretion is stimulated by luteinising hormone (LH) and insulin-like growth factor-I as well as forskolin in bovine luteinised granulosa cells (Schams *et al.* 1999), suggesting that the main pathway depends on the cAMP activation. Thus, this study with granulosa cell culture indicated that the most potent stimulators for differentiation/luteinisation are also stimulators of VEGF secretion. This was also confirmed in another study, in which LH upregulates VEGF mRNA expression by ovine luteal cells in culture (Toutges *et al.* 1999). Consequently, it is most likely that luteinising granulosa cells secrete VEGF that accelerates early angiogenesis in the newly formed CL. In support of this idea, the treatment of marmoset monkey with gonadotrophin-releasing hormone antagonist (LH suppression) or anti-VEGF severely suppressed both endothelial cell proliferation in the CL and plasma progesterone levels (Fraser *et al.* 2000). Similarly, the treatment of rats with truncated soluble Flt-1 receptors, which inhibits VEGF bioactivity, resulted in virtually complete suppression of CL angiogenesis (Ferrara *et al.* 1998).

The present MDS study suggests that PGF2 α produced in early CL acts as a stimulator of progesterone and OT release within bovine early CL, by cooperating with the vasoactive peptide Ang II (Fig. 8). Since PGF2 α was shown to affect progesterone secretion as a suppressor together with Ang II in bovine midcycle CL (Hayashi & Miyamoto 1999), the opposite effects strongly suggest that the mechanism for interaction of PGF2 α with Ang II in early CL is quite different from that of the aged CL after the end of active angiogenesis. A higher production of PGF2 α in early CL than in other luteal phases has been reported (Milvae & Hansel 1983, Rodgers *et al.* 1988). The present result also showed that concentrations of PGF2 α and Ang II in early CL in the MDS fractions were significantly higher than those of midcycle CL using the

Figure 2 Immunohistochemical localisation of ACE in bovine luteal tissue (2–3 days old). Positive staining for ACE was restricted to newly formed blood vessels (A). The immunostaining is localised to endothelial cells and higher magnification (B) demonstrates particular intense staining of the cell membrane. (C) Replacement of the primary antibody with non-immune serum. Bar=100 μ m for (A), 20 μ m for (B) and 100 μ m for (C).

Figure 3 Effects of bFGF (7.5 nmol/l) and VEGF (5 nmol/l) on progesterone, PGF2 α , Ang II and OT release from microdialysed bovine early CL *in vitro*. Data are expressed as means $\pm$ S.E.M. of the percentage of the basal release of each hormone during the infusion of growth factors for 2 h ($n=8$ CL/group). Baseline concentrations (100%) were 9.5 ± 1.0 nmol/l for progesterone, 14.1 ± 3.1 pmol/l for PGF2 α , 5.8 ± 1.9 pmol/l for Ang II and 5.2 ± 0.6 pmol/l for OT.

Figure 4 Interaction of Ang II (10 and 1 μ mol/l) or ET-1 (0.1 μ mol/l) and PGF2 α (10 μ mol/l) on progesterone release from microdialysed bovine early CL *in vitro*. Data are expressed as means $\pm$ S.E.M. of the percentage of the basal release of each hormone ($n=5-7$ CL/group). Baseline concentration (100%) was 25.7 ± 4.4 nmol/l for progesterone.

same MDS model (early vs mid (Hayashi & Miyamoto 1999): PGF2 α 29.4 vs 4.2 pmol/l, Ang II 8.8 vs 3.7 pmol/l). On the other hand, the ET-1 concentration in early CL was extremely low (0.04 vs 0.84 pmol/l). These differences may be related to the fact that a luteolytic injection of PGF2 α is not capable of inducing a full regression of the CL during the early luteal phase of the oestrous cycle.

The extracellular concentration of Ang II in the present result was calculated to be around 9.6 nmol/l if the concentration of Ang II in the perfusate and the transfer capacity of the MDS membrane are considered. This concentration is clearly much higher than that of plasma, which has been recently published (Acosta *et al.* 2000). The present result shows the existence of ACE in endothelial cells in bovine early CL. This implies that Ang II is converted from Ang I by ACE on the surface of endothelial cells in CL. This finding supports the concept that

bovine early CL is a site of Ang II production. Recently, we found that mRNAs for angiotensin type 1 and 2 receptors were expressed in bovine CL, and the level of type 2 receptor mRNA in early CL was higher than that of midcycle CL (Hayashi *et al.* 2000). Thus, Ang II may act as a paracrine factor in luteal secretory function, in addition to its vasoactive action. Importantly, an infusion of Ang II stimulated progesterone and PGF2 α secretion from early CL, and PGF2 α also stimulated progesterone secretion in the present study (Fig. 8). PGF2 α has been repeatedly shown to stimulate progesterone secretion of bovine CL *in vitro* (Hixon & Hansel 1979, Benhaim *et al.* 1987, Alila *et al.* 1988, Meidan *et al.* 1992, Miyamoto *et al.* 1993). A 30 min infusion of Ang II at 10 μ mol/l following PGF2 α continued to stimulate progesterone release. This result in early CL was completely opposite to what we observed in midcycle CL using the same MDS model,

Figure 5 Interaction of Ang II (10 and 1 $\mu\text{mol/l}$) or ET-1 (0.1 $\mu\text{mol/l}$) and PGF2 α (10 $\mu\text{mol/l}$) on OT release from microdialysed bovine early CL *in vitro*. Data are expressed as means $\pm$ S.E.M. of the percentage of the basal release of each hormone ($n=5-7$ CL/group). Baseline concentration (100%) was 4.9 ± 0.7 pmol/l for OT.

Figure 6 Interaction of OT (10 $\mu\text{mol/l}$) and PGF2 α (10 $\mu\text{mol/l}$) on progesterone release from microdialysed bovine early CL *in vitro*. Data are expressed as means $\pm$ S.E.M. of the percentage of the basal release of each hormone ($n=4-5$ CL/group). Baseline concentration (100%) was 15.2 ± 1.0 nmol/l for progesterone.

where Ang II fundamentally inhibited progesterone release and the inhibitory effect of Ang II was potentiated by the PGF2 α infusion (Hayashi & Miyamoto 1999). As described above, the local Ang II concentration was also higher in early CL than in midcycle CL. Thus, these data emphasise that Ang II in high concentration acts predominantly via type 2 receptors in early CL, by which it supports the mechanism that regulates progesterone secretion.

Ang II induces neovascularisation in the eye of rabbit (Fernandez *et al.* 1985) and in endometrium of rat and human (Hu *et al.* 1996, Li & Ahmed 1996, Walsh *et al.*

1997). Therefore, our study shows that Ang II not only stimulated angiogenesis itself, but also had an ability to augment progesterone secretion (Fig. 8). Several lines of evidence support this concept. Namely, Ang II upregulates the expression of mRNA for an angiogenic factor bFGF in bovine luteal cells (Stirling *et al.* 1990), and bFGF and VEGF enhance PGF2 α and Ang II production of bovine early CL in the present study. In fact, we recently found that bFGF and VEGF together with oestradiol-17 β augment the Ang II production in endothelial cells derived from developing bovine CL (Hayashi *et al.* 2000). Likewise, bFGF increases prostaglandin H synthase activity

Figure 7 Effects of the luteal peptides on PGF2 α release from microdialysed bovine early CL during the infusion of substances. Data are expressed as percentages (means $\pm$ S.E.M.) of the basal release of each hormone ($n=5$ CL/group). The baseline (100%) of PGF2 α was 7.1 ± 0.8 pg/ml.

Figure 8 Schematic interrelationship among angiogenic growth factors, Ang II, PGF2 α , OT and progesterone in bovine early CL, based on the results obtained in microdialysed early CL *in vitro*.

(Rosenstock *et al.* 1997) and VEGF induces constitutive cyclooxygenase-1 expression (Bryant *et al.* 1998) in bovine endothelial cells. Indeed, the infusion of Ang II stimulated PGF2 α production in our MDS study. Therefore, the cause for the very high concentration of PGF2 α in early CL appears to be the result of active angiogenesis involving the action of Ang II, bFGF and VEGF (Fig. 8).

ET-1 is one of the major vasoactive 21-amino acid peptides that is synthesised and secreted from endothelial cells (Yanagisawa *et al.* 1988). Recent studies by our laboratory and by others have indicated that luteal ET-1 interacts with PGF2 α and have suggested that ET-1 plays an essential role in luteolysis in the cow (Girsh *et al.* 1996a, Miyamoto *et al.* 1997, Ohtani *et al.* 1998). In the present study, however, the infusion of ET-1 had no effect on progesterone release from early CL and also did not stimulate PGF2 α release. Importantly, the ET-1 concentration in the MDS perfusate of early CL in which neovascularisation is mostly active was about one-twentieth of the value in the midcycle CL as described above. This difference in the ET-1 concentrations in CL between the two luteal phases was similar to the result of

Girsh *et al.* (1996b). In fact, ET-1 was shown to have no effect on the stimulation of angiogenesis in the rat while Ang II stimulated neovascularisation (Hu *et al.* 1996). However, another study has shown the stimulatory role of ET-1 in angiogenesis in rat kidney (Goligorsky *et al.* 1999). Thus, we can not exclude the possibility that ET-1 also has angiogenic activity in the early CL. Collectively, these findings suggest that ET-1 does not take part in the local mechanisms involved in angiogenesis and secretory function in the developing CL. Also, this may be one of the reasons why early CL before day 5 of the oestrous cycle are resistant to an injection of PGF2 α .

It was suggested that a positive feedback of uterine PGF2 α and luteal OT is established during CL regression (Flint & Scheldrick 1983, Schams *et al.* 1985). In the present study, the infusion of PGF2 α stimulated OT release in early CL, and likewise the infusion of OT stimulated PGF2 α release. This indicates that a local positive feedback between PGF2 α and OT may exist within bovine early CL. OT stimulates secretion of PGF2 α (Grazul *et al.* 1989, the present study) and progesterone (Miyamoto & Schams 1991), and bovine luteal cells possess binding sites for OT (Okuda *et al.* 1992). In addition, a recent study has revealed that an OT antagonist, atosiban, inhibits PGF2 α and progesterone secretion from bovine luteal cells on days 3–5 of the oestrous cycle (Skarzynski & Okuda 1999). These lines of evidence suggest that OT supports the mechanism that is responsible for PGF2 α and progesterone production within the developing CL. In the present study, interactions between PGF2 α and Ang II appeared to stimulate OT secretion, whereas the interaction of PGF2 α with ET-1 was very weak. The infusion of Ang II stimulated both PGF2 α and OT release from bovine early CL. Consequently, we hypothesise that Ang II further activates the positive feedback of PGF2 α and OT within the developing CL, and this effect ensures the mechanism of progesterone synthesis and secretion (Fig. 8).

In conclusion, the overall results of this study indicate that the functional angiotensin system exists on the endothelial cells of early CL, and that angiogenic factors bFGF and VEGF upregulate luteal Ang II and PGF2 α secretion, which fundamentally supports the mechanism of progesterone production in bovine early CL. This idea supports the concept that the local regulatory mechanism involved in active angiogenesis ensures the progesterone secretion in the developing CL *in vivo*.

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