Local interaction of prostaglandin $F_{2\alpha}$ with endothelin-1 and tumor necrosis factor- α on the release of progesterone and oxytocin in ovine corpora lutea *in vivo*: a possible implication for a luteolytic cascade

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Abstract

Endothelin-1 (ET-1) and tumor necrosis factor- α (TNF α) participate in the cascade of luteolysis. Thus, in the present study the interactions of ET-1 and TNF α with prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) on the release of progesterone and oxytocin (OT) within the corpus luteum (CL) were investigated. A microdialysis system (MDS) was surgically implanted in ovine CL (one MDS line/CL; 5–10 lines/ewe) formed after super-ovulation. A 4-h perfusion with PGF $_{2\alpha}$ (0.01–1 $\mu\text{mol l}^{-1}$) induced no clear effect on progesterone release, but acutely stimulated OT release in a dose-dependent manner. A perfusion of PGF $_{2\alpha}$ (1 $\mu\text{mol l}^{-1}$) increased ET-1 release over a period of 12 h. Two perfusions of ET-1 (0.1 $\mu\text{mol l}^{-1}$) or a perfusion of ET-1 followed by TNF α (200 ng ml $^{-1}$) decreased progesterone release (56–64% at 36–48 h). When the CL were pre-perfused with PGF $_{2\alpha}$ (1 $\mu\text{mol l}^{-1}$), two consecutive perfusions of ET-1 decreased progesterone release more rapidly. Similarly, a pre-perfusion with PGF $_{2\alpha}$ followed by consecutive perfusions of ET-1 and then TNF α rapidly decreased progesterone release, with the inhibition most pronounced (35%) at 36–48 h. The simultaneous infusion of ET-1 with PGF $_{2\alpha}$ induced a rapid decrease in progesterone release (36% at 36–48 h). In a further study, the possible second messenger systems involved in PGF $_{2\alpha}$ action on the release of progesterone, OT and ET-1 were investigated. A perfusion with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; 10 $\mu\text{mol l}^{-1}$), A23187 (10 $\mu\text{mol l}^{-1}$), or PGF $_{2\alpha}$ + A23187 increased progesterone release during infusion, but decreased it after perfusion. All treatments induced a massive release of OT during infusion, and increased ET-1 release after infusion. These results show that ET-1 is capable of suppressing progesterone release in the PGF $_{2\alpha}$ -primed ovine CL *in vivo* and thus ET-1 works as a local luteolysin together with PGF $_{2\alpha}$ during the process of functional luteolysis. During structural luteolysis, TNF α may interact with PGF $_{2\alpha}$ and ET-1 to cause a rapid drop in progesterone release and accelerate the process of luteolysis. This result supports the contention that ET-1 and TNF α interact with PGF $_{2\alpha}$ as local luteolytic mediators in the ewe as previously suggested.

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Introduction

It is well known that a local counter-current transfer of uterine prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) primes the luteolytic cascade of bovine corpora lutea (CL). Recent evidence in the cow suggests that endothelin-1 (ET-1), a vaso-constrictive 21-amino acid peptide, interacts with PGF $_{2\alpha}$ in the control of functional luteolysis (Girsh *et al.* 1996a,b, Miyamoto *et al.* 1997, Ohtani *et al.* 1998, Meidan *et al.* 1999). This hypothesis has been further supported by *in vivo* studies in the ewe. A single intra-luteal injection of

BQ123, a highly specific antagonist of the endothelin type A receptor (ETA-R), at the mid luteal phase mitigated the luteolytic effect of PGF $_{2\alpha}$ (Hinckley & Milvae 2001). This study also demonstrated that a systemic injection of ET-1 15 min after an injection of a sub-luteolytic dose of PGF $_{2\alpha}$ potentiated the decrease in plasma progesterone concentration, which resulted in the return of estrus (Hinckley & Milvae 2001). Moreover, the administration of a luteolytic dose of PGF $_{2\alpha}$ rapidly stimulated gene expression for ET-1 in ovine CL collected at mid-cycle (Hinckley & Milvae

2001). It is likely, therefore, that ET-1 released from microvascular endothelial cells in the CL and luteal cells (Levy *et al.* 2001, Schams *et al.* 2003) cooperates with PGF_{2α} to initiate luteolysis by both directly inhibiting progesterone release (Girsh *et al.* 1996a, Miyamoto *et al.* 1997) via ETA-R (Girsh *et al.* 1996a, Hinckley & Milvae 2001) and probably by vasoconstriction of arterioles that results in an acute drop in progesterone release.

The presence of tumor necrosis factor-α (TNFα) and its receptors in the pig and bovine CL was observed by Okuda *et al.* (1999), Sakumoto *et al.* (2000) and Miyamoto *et al.* (2002) and suggested that TNFα plays a significant role in the process of luteolysis (Murdoch *et al.* 1988, Benyo & Pate 1992, Shaw & Britt 1995, Wuttke *et al.* 1998). Elevated local secretion of TNFα has been found in the regressing ovine (Ji *et al.* 1991) and bovine (Shaw & Britt 1995) CL. Moreover, it was reported that TNFα acts synergistically with PGF_{2α} during the process of luteolysis in pig (Wuttke *et al.* 1997, 1998). However, the possible synergism among PGF_{2α}, ET-1 and TNFα during luteolysis (Meidan *et al.* 1999) in the ewe is not well established. Thus, the present study was designed to examine the direct local effects and interaction of ET-1 and TNFα with PGF_{2α} on the release of progesterone and oxytocin (OT) within the CL, by using an *in vivo* microdialysis system (MDS) implanted into the CL of ewes (Miyamoto *et al.* 1998a,b). This system allows cells to maintain the integrity of CL structures, and thus enables observations to be made of real-time local changes of different substances in the CL that may play a role in cell-to-cell communication. The study was extended further to examine the possible second messenger systems involved in PGF_{2α} action on the release of progesterone, OT and ET-1 in the CL *in vivo*.

Materials and Methods

Experimental design and animals

The experiment was carried out at the Field Centre of Animal Science and Agriculture, Obihiro University and the experimental procedures complied with the Guide for Care and Use of Agriculture Animals of Obihiro University.

The study used a multiple CL model to implant the MDS, as the CL formed after super-ovulation were shown to regress in response to a luteolytic injection of PGF_{2α} in a way that was very similar to that of intact CL (Miyamoto *et al.* 1998a). This model enables us to examine in parallel several experimental infusions into the MDS implanted in the different CL (one MDS line/CL) within a ewe (Miyamoto *et al.* 1998a,b). The study comprised three experiments. The first experiment ($n = 3$ ewes) observed the effect of PGF_{2α} (Sigma Chemical Co., St Louis, MO, USA) infused at concentrations of 0.01 μmol l⁻¹, 0.1 μmol l⁻¹, or 1 μmol l⁻¹ into the MDS on the local release of progesterone, OT and ET-1. The second experiment ($n = 5$ ewes) examined the effects of infusions with PGF_{2α} (1 μmol l⁻¹), ET-1 (0.1 μmol l⁻¹; Peptide Institute Inc.,

Osaka, Japan), and/or TNFα (200 ng ml⁻¹, recombinant human TNFα; 2.55 × 10⁶ JRU mg⁻¹ protein, generously provided by Dainippon Pharm. Co. Ltd, Osaka, Japan) on the release of progesterone and OT. The third experiment ($n = 4$ ewes) compared the pharmacological effect PGF_{2α} (10 μmol l⁻¹), 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 10 μmol l⁻¹), ionophore A23187 (10 μmol l⁻¹), and PGF_{2α} + A23187 (all substances from Sigma Chemical Co.) on the release of progesterone, OT and ET-1.

The experiments were conducted in October to December, during the breeding season. Mature Suffolk ewes were kept in a paddock under natural day length and temperature, and were fed a diet of concentrates and hay with water available *ad libitum*. All animals had at least two normal estrous cycles (16 or 17 days) before being used. The estrous cycles were based on progesterone concentrations in plasma taken every 3 days. In order to induce super-ovulation, ewes were pretreated according to the regimen of Miyamoto *et al.* (1998a,b) with an intra-vaginal sponge containing 60 mg medroxyprogesterone acetate (MPA, Repromap: The Upjohn Co. International Ltd, NSW, Australia) for 12 days. Pregnant mare's serum gonadotropin (200 IU) (Serotropin; Teikoku-zoki Co., Tokyo, Japan) was injected intramuscularly on the evening of the 9th day after the insertion of sponges, together with the first follicle-stimulating hormone (FSH) (6 mg Antorin 10; FSH of porcine origin, Denka Chemical Co., Kawasaki, Japan) injection. The FSH treatment comprised six intramuscular injections given at approximately 12-h intervals in a decreasing dose regimen (6, 6, 4, 4, 2, 2 mg) to give a total dose of 24 mg. The MPA sponges were withdrawn on the morning of the 12th day after the insertion. An injection of 100 μg synthetic gonadotropin releasing hormone (GnRH) (Conceral; Takeda Chemical Co., Osaka, Japan) was intramuscularly administered 24 h after the removal of the MPA sponges. The administration time of the GnRH injection was considered to be day 0. Daily blood samples (5 ml) for progesterone analysis in all experiments were collected through a jugular venous catheter over the whole period of the experiment, and the plasma samples were stored at -30 °C until assay.

Implantation of MDS into the CL

On day 7 after GnRH injection, several lines of MDS were surgically implanted into CL (one MDS line/CL) in both ovaries of animals as detailed earlier (Miyamoto *et al.* 1998a,b). The CL formed after super-ovulation (5–10 CL/ewe) were selected so as to have similar macroscopic characteristics (size, color and consistency). Each CL was penetrated with one capillary dialysis membrane (Fresenius SPS 900 Hollow Fibers, cut-off M_r 1000 kDa, 0.2 mm diameter, 3 mm long; Fresenius AG, St Wendel, Germany). The capillary was affixed to the surface of the CL by Histoacryl Blue (B Braun-Dexon GmbH, Spangenberg, Germany). Both ends of the capillary were glued to 20-cm-long pieces of silastic tubing (i.d. 0.3 mm) that

were connected to pieces of Teflon tubing leading to the outside of the abdomen. The exteriorized bundle of afferent and efferent Teflon tubes was taped to the body from the abdomen to the back with an adhesive bandage (Benefix; Japan Sigmax, Tokyo, Japan). The animals were brought to the pen where they were chained immediately after surgery. For perfusion, one end of the tube was connected to a peristaltic pump and the other was routed to a fraction collector. The CL were perfused with Ringer's solution at a flow rate of 1.5-ml/30 min/fraction throughout the experiments. The MDS lines of each animal were used for perfusion with substances on days 9 to 10. At least one line was used for Ringer's solution only (control) in each animal.

After 30-h perfusion, fractions of the perfusate were collected every 30 min for 32 to 48 h on days 9–11. After a period of 8 h for observing the spontaneous release of progesterone (baseline), further fractions were collected according to the experimental design. The transfer capacity of the microdialysis membrane was about 0.1% of the concentration infused as determined for OT, ET-1 and $\text{TNF}\alpha$, and about 1% for progesterone and $\text{PGF}_{2\alpha}$ under the conditions used (Miyamoto *et al.* 1997, 1998a). At the end of the experiments, the ewes were ovariectomized, and the CL were fixed in Bouin's solution, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax, and then processed for histology after hematoxylin–eosin staining. In most cases, little damage and no connective tissues such as fibroblasts were observed around the microdialysis capillary membrane.

Hormone determination

Progesterone, OT and ET-1 concentrations in the perfusate fractions from the MDS, and progesterone concentrations in plasma were determined with second-antibody enzyme immunoassays (EIAs) that were based on a competitive assay using a horseradish peroxidase-labeled progesterone (Miyamoto *et al.* 1992) or biotin-labeled peptides as tracers (Miyamoto *et al.* 1997). Progesterone concentrations in plasma samples were assayed after extraction by diethyl ether, but those in the MDS fractions were assayed directly. The standard curve ranged from 0.05 to 50 ng ml⁻¹ and the ED₅₀ of the assay was 1.8 ng ml⁻¹. The intra- and interassay coefficients of variation were on average 6.2% and 9.3% respectively.

For peptide extraction, 1-ml samples were taken from every fraction, and every 8 consecutive 1-ml samples (corresponding to a 4-h period) were pooled. BSA was added to each of the pooled samples to a final concentration of 1 mg ml⁻¹, and the solution was adjusted to pH 2.5 with 1 M acetic acid. The samples were then applied to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) according to the established method (Miyamoto *et al.* 1997). The MDS fractions were concentrated 40-fold as a result of the process. The recoveries of synthetic OT and

ET-1 added to Ringer's solution were 73% and 62% respectively ($n = 60$). The EIAs for OT and ET-1 were conducted as described previously (Miyamoto *et al.* 1997). The standard curve for OT ranged from 1.6 to 200 pg ml⁻¹ and the ED₅₀ of the assay was 21 pg ml⁻¹. The intra- and interassay coefficients of variation of the OT assay were on average 6.2% and 8.6% respectively. The standard curve for ET-1 ranged from 9.7 to 5000 pg ml⁻¹ and the ED₅₀ of the assay was 450 pg ml⁻¹. The intra- and interassay coefficients of variation of the ET-1 assay were on average 8.7% and 12.6% respectively.

Statistical analyses

The mean hormone concentrations in the first 8 h (progesterone was based on each 0.5-h fraction, and OT and ET-1 were based on each 4-h pooled fraction) were used to calculate the individual baseline, because of the large variation among individuals in the basal concentrations of each hormone released into the MDS. All hormone concentrations in the fractions were then expressed as a proportion of this individual baseline. This treatment enables an evaluation of the relative changes of hormonal values between the CL of different animals. The change in hormonal release after substance infusions was tested based on individual time points throughout the experiment as compared with the baseline. Means were analyzed by time-dependent repeated measures ANOVA followed by Student's *t*-test. Where a comparison among several treatment groups during the same time period was needed, means were analyzed by ANOVA followed by Tukey–Kramer test. For the figures showing MDS data, all hormonal concentrations were expressed as a percentage of the baseline with an 8 h basis. The absolute concentrations of the hormones in the MDS fractions (means $\pm$ S.E.M.) are given in the figure legends.

Results

The super-ovulation treatment induced multiple CL (16.6 ± 1.8 ; mean $\pm$ S.E.M., $n = 12$). There was no significant change in plasma progesterone levels during the MDS experiments, but the concentrations depended on the number of CL formed in each animal (8.2 – 27.8 ng ml⁻¹).

Intra-luteal release of progesterone, OT and ET-1 in response to $\text{PGF}_{2\alpha}$ infusion

The release of hormones into the MDS from days 9 to 10 after GnRH was relatively constant over the experimental period. A 4-h perfusion with $\text{PGF}_{2\alpha}$ at 0.01 $\mu\text{mol l}^{-1}$, 0.1 $\mu\text{mol l}^{-1}$, or 1 $\mu\text{mol l}^{-1}$ via the MDS induced no clear effect on progesterone release, but acutely stimulated OT release in a dose-dependent manner ($P < 0.05$; Fig. 1). The increase in OT was followed by a sustained drop after the stimulation. A 4-h perfusion with $\text{PGF}_{2\alpha}$ at 1 $\mu\text{mol l}^{-1}$

Figure 1 Effects of 4-h intraluteal application of $\text{PGF}_{2\alpha}$ ($0.01 \mu\text{mol l}^{-1}$, $0.1 \mu\text{mol l}^{-1}$ and $1 \mu\text{mol l}^{-1}$) at 8–12 h on the local release of progesterone and OT from microdialyzed CL in the ewe ($n = 3$). The data are expressed as a percentage of the baseline (0–8 h) value, and are the means $\pm$ S.E.M. of 6–7 CL/group. The baseline (100%) values of each hormone were $2.2 \pm 0.2 \text{ ng ml}^{-1}$ for progesterone and $0.74 \pm 0.19 \text{ pg ml}^{-1}$ for OT. * $P < 0.05$, *** $P < 0.001$ compared with baseline.

increased ET release over a period of 12 h ($P < 0.05$; Fig. 2), but the lower doses of $\text{PGF}_{2\alpha}$ at $0.01 \mu\text{mol l}^{-1}$ or $0.1 \mu\text{mol l}^{-1}$ did not affect ET-1 release.

Effect of pre- and simultaneous exposure with $\text{PGF}_{2\alpha}$ on the suppressing activity of ET-1 and $\text{TNF}\alpha$

Two 4-h perfusions of ET-1 ($0.1 \mu\text{mol l}^{-1}$) between 12 and 16 h and 24 and 28 h decreased progesterone release between 24 and 48 h ($78.2 \pm 2.8\%$; $P < 0.05$; Fig. 3 upper panel). Likewise, a 4-h perfusion of ET-1 between 12 and 16 h and of $\text{TNF}\alpha$ (200 ng ml^{-1}) between 24 and 28 h decreased progesterone release between 24 and 48 h ($76.2 \pm 8.4\%$; $P < 0.05$; Fig. 3 upper panel). A 4-h perfusion with $\text{PGF}_{2\alpha}$ ($1 \mu\text{mol l}^{-1}$) between 8 and 12 h slightly decreased progesterone release at 36 to 48 h ($79.5 \pm 6.4\%$; $P < 0.05$; Fig. 3 lower panel). When the CL were pre-perfused with $\text{PGF}_{2\alpha}$ for 4 h between 8 and 12 h, two consecutive perfusions of ET-1 between 12 and 16 h and 24 and 28 h rapidly decreased progesterone

release between 16 and 48 h ($74.6 \pm 5.9\%$; $P < 0.05$; Fig. 3 lower panel); this decrease occurred 8 h earlier than in the CL treated with ET-1 alone. Similarly, a pre-perfusion with $\text{PGF}_{2\alpha}$ for 4 h between 8 and 12 h, followed by consecutive perfusions of ET-1 between 12 and 16 h and of $\text{TNF}\alpha$ between 24 and 28 h, rapidly decreased progesterone release at 16 to 48 h ($67.5 \pm 7.0\%$; $P < 0.05$; Fig. 3 lower panel), but the inhibitory effect between 36 and 48 h was more pronounced than it was in the above-described treatments ($35.1 \pm 3.3\%$; $P < 0.05$; Fig. 3 lower panel). The simultaneous infusion of ET-1 and $\text{PGF}_{2\alpha}$ between 8 and 12 h induced a rapid and pronounced decrease in progesterone release ($65.0 \pm 6.5\%$; $P < 0.05$; Fig. 3 lower panel). ET-1 alone slightly stimulated OT release ($403.7 \pm 72.6\%$; $P < 0.05$; Fig. 4 upper panel). An infusion of $\text{PGF}_{2\alpha}$ between 8 and 12 h induced an acute release of OT ($628.2 \pm 100.4\%$; $P < 0.05$; Fig. 4 lower panel). The simultaneous infusion of ET-1 and $\text{PGF}_{2\alpha}$ induced the highest stimulation in OT release ($897.7 \pm 70.7\%$; $P < 0.05$; Fig. 4 lower panel), followed by a drop for the next 12 h ($52.9 \pm 16.0\%$; $P < 0.05$). ET-1 or $\text{TNF}\alpha$ after $\text{PGF}_{2\alpha}$ also stimulated OT release (666.9 ± 123.4 ; $P < 0.05$; Fig. 4 lower panel).

Figure 2 Effects of 4-h intra-luteal application of $\text{PGF}_{2\alpha}$ ($1 \mu\text{mol l}^{-1}$) at 8–12 h on the local release of ET-1 from microdialyzed CL in the ewe ($n = 3$). The data are expressed as a percentage of the baseline (0–8 h) value, and are the means $\pm$ S.E.M. of 5 CL/group. The baseline (100%) value of ET-1 release was $3.8 \pm 0.8 \text{ pg ml}^{-1}$. * $P < 0.05$ compared with baseline.

Comparison of the effects of $\text{PGF}_{2\alpha}$, TPA and A23187

A 6-h perfusion with $\text{PGF}_{2\alpha}$ ($10 \mu\text{mol l}^{-1}$) between 8 and 14 h slightly increased progesterone release during infusion ($P < 0.05$), but did not affect it during the 16-h period after infusion (14–30 h). A 6-h perfusion with TPA ($10 \mu\text{mol l}^{-1}$), A23187 ($10 \mu\text{mol l}^{-1}$), or $\text{PGF}_{2\alpha}$ together with A23187 between 8 and 14 h increased progesterone release during infusion more than with $\text{PGF}_{2\alpha}$ alone ($P < 0.05$), but induced a decrease in progesterone release after infusion, in the order A23187 (89%), TPA (67%), and $\text{PGF}_{2\alpha}$ + A23187 (52%; Table 1). All three values were significantly different from each other ($P < 0.05$). All treatments induced a massive release of OT during infusion

Figure 3 Effects of intra-luteal application of ET-1 ($0.1 \mu\text{mol l}^{-1}$) and $\text{TNF}\alpha$ (200 ng ml^{-1}) with (lower panel) or without (upper panel) $\text{PGF}_{2\alpha}$ ($0.1 \mu\text{mol l}^{-1}$) on the local release of progesterone from microdialyzed CL in the ewe ($n = 5$). The data are expressed as a percentage of the baseline (0–8 h) value, and are the means $\pm$ S.E.M. of 5–8 CL/group. The baseline (100%) value of progesterone release was $2.8 \pm 0.3 \text{ ng ml}^{-1}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baseline.

($P < 0.05$), whereas only A23187 and $\text{PGF}_{2\alpha} + \text{A23187}$ depressed OT release after infusion ($P < 0.05$). All treatments similarly increased ET-1 release after infusion ($P < 0.05$; Table 1).

Discussion

The results of the present study indicate that $\text{PGF}_{2\alpha}$ acts cooperatively with ET-1 and $\text{TNF}\alpha$ to suppress progesterone secretion in the ovine CL *in vivo*. Substances infused through the MDS act in the local microenvironment around the capillary membrane. Thus, the model used here does not have any major effect on the constriction of blood vessels in the whole CL, and hence excludes a direct effect on the vasoconstriction that was shown to be

a crucial process for luteolysis (Niswender *et al.* 1976, Azmi & O'Shea 1984, Knickerbocker *et al.* 1988, Acosta *et al.* 2002). In this context, the present *in vivo* data may give basic and important information on the interaction of $\text{PGF}_{2\alpha}$ with ET-1 and $\text{TNF}\alpha$ in the cascade of functional luteolysis.

It was observed in the present study that a single infusion of $\text{PGF}_{2\alpha}$ for 4 h did not change progesterone release in the CL up to 20 h after the infusion; notwithstanding the results of our similar *in vivo* investigation that a $\text{PGF}_{2\alpha}$ infusion to the bovine mid-cycle CL via MDS stimulated, but did not inhibit progesterone secretion (Ohtani *et al.* 1999). These findings suggest that the countercurrent transfer of uterine or exogenous $\text{PGF}_{2\alpha}$ to the ovary plays a crucial role in inducing a rapid drop in progesterone release from the CL during luteolysis. On the other hand,

Figure 4 Effects of intra-luteal application of ET-1 ($0.1 \mu\text{mol l}^{-1}$) and $\text{TNF}\alpha$ (200 ng ml^{-1}) with (lower panel) or without (upper panel) $\text{PGF}_{2\alpha}$ ($0.1 \mu\text{mol l}^{-1}$) on the local release of OT from microdialyzed CL in the ewe ($n = 5$). The data are expressed as a percentage of the baseline (0–8 h) value, and are the means $\pm$ S.E.M. of 5–7 CL/group. The baseline (100%) value of OT release was $0.67 \pm 0.08 \text{ pg ml}^{-1}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baseline.

Table 1 Comparison of secretion rates of progesterone, OT, and ET-1 into the MDS implanted in the CL formed after super-ovulation among 5 treatment groups during (8–14 h) and after (14–30 h) stimulant infusion at $10 \mu\text{mol l}^{-1}$ in the ewe ($n = 4$). Values are expressed as a percentage (means $\pm$ S.E.M.) of the respective baseline (0–8 h). The baseline (100%) of progesterone release was $5.13 \pm 0.14 \text{ ng ml}^{-1}$, of OT release was $0.89 \pm 0.17 \text{ pg ml}^{-1}$, and of ET-1 release was $3.1 \pm 0.9 \text{ pg ml}^{-1}$.

	Control ($n = 5$)	PGF _{2α} ($n = 7$)	TPA ($n = 7$)	A23187 ($n = 6$)	PGF _{2α} + A23187 ($n = 7$)
Progesterone					
During infusion (8–14 h)	95 ± 5^a	124 ± 4^b	160 ± 10^c	169 ± 7^c	154 ± 8^c
After infusion (14–30 h)	98 ± 5^a	99 ± 5^a	67 ± 4^c	89 ± 4^b	52 ± 2^d
Oxytocin					
During infusion	130 ± 29^a	763 ± 235^{bc}	322 ± 53^b	923 ± 160^c	760 ± 83^c
After infusion	89 ± 23^b	121 ± 39^{ab}	117 ± 32^b	41 ± 11^a	58 ± 24^{ab}
Endothelin-1					
During infusion	86 ± 8	149 ± 59	98 ± 3	145 ± 29	189 ± 66
After infusion	110 ± 16^a	202 ± 36^b	165 ± 16^b	217 ± 41^b	206 ± 34^b

a,b,c,d: $P < 0.05$ at the same period.

the present study showed that a high dose of PGF_{2 α} ($1 \mu\text{mol l}^{-1}$) could increase ET-1 release from the CL. This fact further supports the concept that a vasoconstriction of arterioles in the CL (Niswender *et al.* 1976, Azmi & O'Shea 1984, Knickerbocker *et al.* 1988) at the beginning of luteolysis may be attributed to the strong effect of ET-1 as well as of PGF_{2 α} . It was observed in the present study that the simultaneous exposure of CL to PGF_{2 α} and ET-1 was much more effective in reducing progesterone release than was the sequential exposure to these two compounds. Indeed, PGF_{2 α} stimulates ET-1 release from ovine luteal cells (Hinckley & Milvae 2001) and it is this elevated local ET-1, stimulated by PGF_{2 α} , together with the infused ET-1 which collectively act on the luteal cells, rather than a direct action of PGF_{2 α} . This might result in a greater progesterone suppression when CL are exposed simultaneously to PGF_{2 α} and ET-1. Our recent report revealed that PGF_{2 α} and ET-1 cooperatively induce functional luteolysis of mid-cycle CL in the cow (Miyamoto *et al.* 2001). We recently proposed that another vasoconstrictive peptide, angiotensin II (Ang II), might have a similar role. As in the case for ET-1, PGF_{2 α} increases luteal Ang II release, and Ang II acts with PGF_{2 α} as a suppressor of progesterone release in the bovine CL (Hayashi & Miyamoto 1999, Hayashi *et al.* 2002). In both vasoactive peptides, a concomitant perfusion of CL with PGF_{2 α} had the greatest effect on progesterone suppression as observed in the present *in vivo* study, suggesting that these peptides are important luteal factors in inducing functional luteolysis.

Interestingly, PGF_{2 α} did not suppress progesterone release in the present MDS model, but TPA, a stimulator of protein kinase C (PKC) clearly suppressed it. Moreover, TPA but not PGF_{2 α} inhibited basal and low density lipoprotein-supported steroidogenesis in cultured regressing porcine luteal cells (Brannian *et al.* 1995). Thus, TPA and PGF_{2 α} have different effects on the CL. Stimulation of PKC in ovine large luteal cells was shown to be a dominant intracellular mechanism involved in progesterone suppression (Wiltbank *et al.* 1990). This was confirmed later using the *in vivo* model in which an infusion of TPA into the

ovarian artery also decreased plasma progesterone concentrations (McGuire *et al.* 1994). Our present *in vivo* data further support this concept. It appears that TPA infusion into the CL affects luteal progesterone release but differently in cows and ewes: TPA inhibited progesterone release in the ewe (the present result), while it did not affect progesterone release in the cow (Ohtani *et al.* 1999). On the other hand, an infusion of A23187 slightly inhibited progesterone release in the ewe (the present result), but strongly inhibited it in the cow (Ohtani *et al.* 1999). PGF_{2 α} has been shown to increase the intracellular concentration of Ca^{2+} in the luteal cells in both species (Davis *et al.* 1987, Wiltbank *et al.* 1989, Wegner *et al.* 1991). Thus, there might be some difference between ewes and cows in cell sensitivity to PGF_{2 α} at the second messenger level. This may be especially true in large luteal cells, in which a PKC pathway is dominant in the ewe, whereas an increase in Ca^{2+} is a crucial function in the cow.

As a result of pharmacological stimulation of PKC and a calcium influx in the CL cells *in vivo*, a local release of OT was acutely stimulated during substance infusion while the ET-1 release was gradually stimulated after infusion. These data support the concept that Ca^{2+} and PKC are responsible for the release of ET-1 and OT (Cosola-Smith *et al.* 1990, Masaki 1993, Miyamoto *et al.* 1993). These data also support the concept that OT is released from large luteal cells by granule exocytosis (Cosola-Smith *et al.* 1990, Wathes & Denning-Kendall 1992), while ET-1 secretion is a result of transcriptional activation of prepro-ET-1 followed by cleavage steps from big-ET-1 to ET-1 in endothelial cells (Masaki 1993).

Our previous observation of a direct local effect of TNF α using the MDS implanted in the intact mid-cycle CL in the ewe showed that an infusion of TNF α alone induced a slight increase in the release of progesterone and PGF_{2 α} , but it did not inhibit progesterone release (Miyamoto *et al.* 1995). The effect of TNF α infusion was more or less similar when TNF α was perfused into the MDS implanted in the regressing CL in the same model (multiple CL) as the present study (Miyamoto *et al.*

1998a). However, the present results clearly indicated that TNFα is capable of decreasing progesterone release in the CL *in vivo*, if the CL is pre-treated by PGF_{2α} and ET-1. As an increase in local secretion of TNFα has been found in the regressing CL only after the completion of functional luteolysis in the ovine CL (Ji *et al.* 1991) and bovine CL (Shaw & Britt 1995), the timing of the exposure of CL to TNFα may have a physiological meaning; TNFα may indeed be an effective suppressor of progesterone release when the luteal cells are stimulated by PGF_{2α} and ET-1 *in vivo*. In fact, a similar phenomenon was observed in an MDS study in pigs in which TNFα and PGF_{2α} synergistically inhibited steroidogenesis (Wuttke *et al.* 1997, 1998). Therefore, a lack of up-regulation of TNFα mRNA in the regressing CL (Penny *et al.* 1999, Petroff *et al.* 1999) may not exclude the possibility of such a synergism among PGF_{2α}, ET-1 and TNFα. Consequently, we speculate that during structural luteolysis, TNFα inhibits progesterone release, and at the same time it further stimulates a local release of ET-1, since endothelial cells are identified as target cells of cytokines and as cytokine-producing cells (Martin & Resch 1988). ET-1, in turn, may stimulate TNFα secretion (Cunningham *et al.* 1997, Ruetten & Thiernemann 1997), thereby establishing a local positive feedback, which would accelerate the cascade of luteolysis. Indeed, the intra-luteal ET-1 concentration was shown to be maintained at a high level over the whole period of luteolysis in the cow (Ohtani *et al.* 1998). In this regard, Meidan *et al.* (1999) proposed the same hypothesis on the basis of their results showing that ET-1 induces secretion of TNFα by bovine macrophages, and that luteal cells express the p55 type receptor for TNFα. Moreover, mRNA for TNFα was detected in luteal tissue around natural and induced luteolysis (Penny *et al.* 1999) and this increase was associated with an influx of macrophages into the CL around the time of the second half of luteolysis, possibly in response to monocyte chemoattractant protein (Webb *et al.* 2002). Thus, the TNFα secreted by luteal cells and macrophages during structural luteolysis may interact with PGF_{2α} and ET-1, resulting in a rapid drop in progesterone release and accelerating the process of luteolysis.

Collectively, the present results provide further evidence that ET-1 is capable of suppressing progesterone release in the PGF_{2α}-primed ovine CL *in vivo*. This observation supports the previous finding that the systemic administration of ET-1 potentiates the progesterone-suppressing activity of PGF_{2α} in the ewe (Hinckley & Milvae 2001). The *in vivo* data obtained in the ewe (Hinckley & Milvae 2001, present study), as well as the *in vitro* data obtained from the cow (Miyamoto *et al.* 1997) suggest that ET-1 alone cannot promote functional luteolysis. ET-1 works as a local luteolysin together with PGF_{2α}, and the CL needs to be triggered by PGF_{2α} to start the cascade of luteolysis in which ET-1 acts in response to the first stimuli of PGF_{2α}, causing a rapid drop in progesterone release. In conclusion, ET-1 and TNFα together with PGF_{2α} are capable

of directly suppressing the local progesterone release in the ovine CL *in vivo*.

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