Intraluteal Release of Progesterone and Prostaglandins during PGF₂ α -Induced Luteolysis in Ewes: Local Effects of Tumor Necrosis Factor- α

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Abstract. The role of tumor necrosis factor- α (TNF- α) in the local regulatory mechanisms of functional luteolysis in the ewe was examined. TNF- α was infused into a microdialysis system (MDS) implanted in the corpus luteum (CL) for 24 h from 2 h after the intramuscular injection of cloprostenol (defined as 0 h), and plasma as well as intraluteal changes of progesterone (P) and prostaglandins (PG) were observed. Three ewes were treated for superovulation, and multiple CL formed thereafter were surgically implanted with the MDS (1 line/CL) on Day 7 after GnRH injection. Serial fractions from the MDS were collected every 30 min for 40 h on Days 9–11 following a pre-perfusion period of 30 h. An intramuscular injection of cloprostenol stimulated an acute increase of plasma P concentration (an increase to 150%) within 1 h, followed by a rapid decrease within 6 h ($P < 0.05$), which directly correlates with functional luteolysis. Intraluteal P release into the MDS showed a profile similar to that in the plasma, but significantly decreased from 17 h ($P < 0.05$). Twenty-four-hour infusions of TNF- α at 20 ng/ml and 200 ng/ml into the MDS starting at 2 h after cloprostenol injection slightly hastened the time of onset of P decrease when they were compared with the time of the control. In contrast, the higher dose of TNF- α at 2000 ng/ml clearly delayed the time of onset of P decrease. Intraluteal release of PGF₂ α and PGE₂ in the control was slightly increased between 18–32 h ($P < 0.05$). A 26-h infusion with indomethacin partly blocked the increase in basal release of both PG ($P < 0.05$), whereas it had no effect on the P release. A 24-h infusion with different concentrations of TNF- α stimulated the release of PGE₂, but not the release of PGF₂ α ($P < 0.05$). The results support the idea that TNF- α may have a passive or secondary role in functional luteolysis. Furthermore, the intraluteal PG production appears to have no direct correlation with functional luteolysis.

Key words: TNF- α , Prostaglandins, Progesterone, Luteolysis, Ewes.

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During luteolysis, immune cells, especially white blood cell series such as macrophages [1–3], eosinophils [3, 4] and T lymphocytes [2], have been observed within the corpus luteum (CL). The major role of the immune cells within the CL at this stage is most likely to be a phagocytosis of the CL

cells, which directly correlates with structural luteolysis. In addition, recent observations suggest that cytokines including tumor necrosis factor- α (TNF- α) released from macrophages may influence the process of functional luteolysis [5]. However, most data on granulosa cells *in vitro* have described the inhibitory actions of TNF- α on steroidogenesis [6–8], while the data on luteal cells *in vitro* revealed little or no effect of TNF- α [5, 9, 10] on the proges-

terone (P) production. Therefore, the predominant action of cytokines on luteal cells may be to induce the release of prostaglandins (PG) rather than to suppress steroidogenesis [5]. Indeed, we have demonstrated that TNF- α slightly stimulated the release of P and PGF_{2 α} from the microenvironment within the CL at the mid-luteal stage when it was applied through a microdialysis system (MDS) that was implanted in the CL in cyclic ewes [11]. Additionally, the idea was proposed that luteal PGF_{2 α} may interact with uterine or exogenous PGF_{2 α} as a local luteolysin during luteolysis in the cow [5].

In this paper, we describe trials in which TNF- α was continuously applied into the MDS implanted in the CL from 2 h after an intramuscular PGF_{2 α} injection, and the local release of P and PG was observed in the regressing CL *in vivo*. We also applied indomethacin in this model to examine the possibility that an inhibition of intraluteal production of PG may affect the changing profile of P during luteolysis.

Materials and Methods

Animals and Treatment

The experiments were conducted in October–December, 1994 during the breeding season. Three mature Suffolk ewes were kept in a paddock under natural daylength and temperature, and were fed a diet of concentrates and hay with water *ad libitum*. All animals had at least 2 normal estrous cycles (16 or 17 days) before being used. The estrous cycles were based on P concentrations in plasma taken every 3 days. In order to induce multiple CL using superovulation, ewes were pretreated with MAP sponges for 11 days. The ewes were superovulated according to a regimen involving combination of PMSG, FSH and GnRH [12]. The time of the injection of GnRH was defined as Day 0.

Implantation of MDS into the CL

On Day 7 after GnRH injection, the MDS was implanted into CL in both ovaries of three animals as described in detail previously [12]. The animals were treated according to the university guiding principles for the care and use of research animals. The CL formed after superovulation and were selected to have similar macroscopic characteristics (size, color and consistency). Each CL was implanted with one capillary dialysis membrane

(Fresenius SPS 900 Hollow Fibers, cut-off Mr 1000 kDa, 0.2 mm diameter, 3 mm long; Fresenius AG, St Wendel, Germany) via a mid-ventral abdominal incision under anaesthesia. The MDS of each animal had 12 lines that were used for perfusion with TNF- α (recombinant human TNF- α ; 2.55×10^6 JRU/mg protein, a generously provided by Dainippon Pharm. Co., Ltd., Osaka, Japan) or indomethacin (Sigma, St. Louis, MO, USA) on Days 9–11. Seven–8 lines were used for TNF- α (20, 200 and 2000 pg/ml), 2–3 lines were used for indomethacin (10^{-5} M) and 2–3 lines were used for Ringer's solution only (control). 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 throughout the experiments.

After 30 h perfusion, fractions of the perfusate were collected every 30 min for 40 h from Day 9. After a period of 8 h to observe spontaneous release of P (baseline), 250 μ g of a PGF_{2 α} analogue (cloprostenol; Estrumate, Sumitomo Pharm. Co., Osaka, Japan) was injected intramuscularly (designated as 0 h) to induce luteolysis. Further fractions were collected for 32 h in order to examine the effects of infusion with different concentrations of TNF- α (2–26 h) and indomethacin (0–26 h) on the release of P and PG within the regressing CL.

The transfer capacity of the microdialysis membrane was about 0.1% of the concentration infused as determined for TNF- α , and about 1% for P and PG under the conditions used.

Blood samples (5 ml) were collected through a jugular venous catheter at - 8, - 6, - 4, - 2, 0, 0.17, 0.33, 0.5, 1 h, and then every 2 h from 2 to 32 h.

Hormone determination

P concentrations were determined directly in the perfusate fractions from the MDS with a second antibody enzyme immunoassay [13]. Plasma samples were extracted by diethylether and were applied to the EIA. The standard curve ranged from 0.16 to 160 nM and the ED₅₀ of the assay was 10 nM. The intra- and interassay coefficients of variation were on average 4.8% and 7.2%, respectively.

Because the concentrations of PGF_{2 α} and PGE₂ were expected to be very low in the MDS samples, every 16 fractions (8 h) were pooled and concentrated by the method by Meyer *et al.* [14], which

used acidic extraction and purification by octadecyl (C_{18}) silicagel columns (7020-01; J.T. Baker Inc., Phillipsburg, NJ, USA). The recovery rates in the MDS perfusates based on 4 different control samples were 88.2% for PGF $_{2\alpha}$ and 81.7% for PGE $_2$. Likewise, the recovery rates of plasma samples were 75.8% for PGF $_{2\alpha}$ and 72.1% for PGE $_2$. The final samples dissolved in assay buffer (0.04M PBS, 0.1% BSA, pH=7.2) were processed to PGF $_{2\alpha}$ - and PGE $_2$ -EIA [11, 15]. The standard curve ranged from 20 to 20,000 pM for PGF $_{2\alpha}$ and 80 to 40000 pM for PGE $_2$. The intra- and interassay CVs were on average 7.8% and 11.5% for PGF $_{2\alpha}$ and 9.4% and 12.3% for PGE $_2$, respectively.

Statistical Analyses

For analysis of changes in P, PGF $_{2\alpha}$ and PGE $_2$ concentrations in the MDS fractions, the mean concentrations of the fractions during the first 8 h before PGF $_{2\alpha}$ injection were used to establish the baseline for each line of the MDS, because absolute concentrations of each hormone in the perfusate fractions greatly changed between animals. All hormone concentrations in the fractions collected were then expressed as a proportion of this individual baseline. The change in hormonal release after PGF $_{2\alpha}$ injection was tested based on individual time points throughout the experiment as compared with the baseline. The absolute concentrations of PG in plasma were directly analyzed for change after PGF $_{2\alpha}$ injection, and PG concentrations in the MDS fractions between control and TNF- α or indomethacin perfusions were also compared. Means were analyzed by ANOVA followed by Student's *t*-test. To plot the MDS data, all hormonal concentrations were expressed as a percentage of the baseline. The absolute concentrations of each hormone in the MDS fractions (mean $\pm$ SEM) are given in the legends of the figures.

Results

Effect of cloprostenol injection on the plasma profiles of P and PG, and on intraluteal P release

Superovulation treatment induced multiple CL (18.0 ± 1.7 , mean $\pm$ SEM, $n=3$). Changes in P, PGF $_{2\alpha}$ and PGE $_2$ concentrations in plasma and the intraluteal release of P into the MDS during PGF $_{2\alpha}$ -induced luteolysis are shown in Fig. 1. A

cloprostenol injection stimulated an acute increase of plasma P concentration (an increase to 150%) within 1 h, followed by a rapid decrease within 6 h ($P<0.05$), which directly correlates with functional luteolysis. Plasma P concentration decreased to 20% of the baseline after 16 h. Intraluteal P release into the MDS showed a profile similar to that in plasma, but significantly decreased from 17.0 h after cloprostenol ($P<0.05$).

There were no clear changes in plasma PGF $_{2\alpha}$ and PGE $_2$ concentrations after cloprostenol injection (Fig. 1).

Fig. 1. Changes in plasma PG and P, and intraluteal P concentrations during PGF $_{2\alpha}$ -induced luteolysis in ewes whose CL had been implanted with the MDS (mean $\pm$ SEM; $n=3$ ewes/group). The ewes were superovulated, and the newly formed CL were used for the MDS study. Each CL was implanted with one capillary dialysis membrane on Day 7 after GnRH injection via a mid-ventral abdominal incision under anaesthesia (12–13 CL/ewe). The experiment was conducted from Days 9–11. The basal levels of P in plasma and in the MDS perfusates before PGF $_{2\alpha}$ injection were 38.4 ± 9.8 nM and 5.9 ± 2.2 nM, respectively.

Fig. 2. Effect of intraluteal application of TNF- α (20, 200, and 2000 ng/ml) or indomethacin (10^{-5} M) on the P release into the MDS during PGF $_{2\alpha}$ -induced luteolysis in ewes (mean $\pm$ SEM; $n=7-8$ CL/group). Each CL was implanted with one capillary dialysis membrane on Day 7 after GnRH injection *via* a mid-ventral abdominal incision under anaesthesia. One line of the MDS was implanted into individual CL formed after superovulation, and 2–3 lines implanted within the same ewe were assigned to the control, 3 doses of TNF- α or indomethacin, respectively (total 12–13 CL/ewe). The solid columns indicate the infusion of substance. The solid flags indicate the time-point of onset of P decrease in the control. Control lines were perfused with Ringer's solution only.

Table 1. Time-lag (h) of onset of P decrease in microdialyzed perfusates (control, TNF- α and indomethacin) during cloprostenol-induced luteolysis in ewes ($n=7-8$ MDS lines/group using 3 ewes: multiple CL model)

Control (Ringer's)	Indomethacin (10^{-5} M)	TNF- α (20 ng/ml)	TNF- α (200 ng/ml)	TNF- α (2000 ng/ml)
0 (+11.0 vs plasma)	+ 0.5	- 4.5	- 3.5	+ 9.0

Effect of TNF- α and indomethacin on the intraluteal release of P and PG within the regressing CL

Changes in the intraluteal release of P into the MDS infused with different concentrations of TNF- α and indomethacin during PGF $_{2\alpha}$ -induced luteolysis are shown in Fig. 2. On the basis of consecutive time points that were significantly decreased from the baseline level, a 24-h infusion of TNF- α at 20 ng/ml and 200 ng/ml starting from 2 h after cloprostenol injection slightly hastened the time of onset of P decrease by 4.5 h and 3.5 h, respectively, compared with the time of the control (17.0 h after cloprostenol). In contrast, the higher dose of TNF- α at 2000 ng/ml clearly de-

layed the time of onset of P decrease for 9.0 h. Indomethacin had no effect on the P release when compared with the values of the control (Table 1).

Changes in concentrations of PG in the pooled fractions from the MDS perfusates are shown in Fig. 3. Intraluteal release of PGF $_{2\alpha}$ and PGE $_2$ in the control was slightly increased between 18–32 h after cloprostenol injection ($P<0.05$). A 26-h infusion with indomethacin from just after cloprostenol injection partly blocked the increase in basal release of both PG (10–26 h for PGF $_{2\alpha}$ and 26–32 h for PGE $_2$, $P<0.05$). A 24-h infusion with different concentrations of TNF- α from 2 h after cloprostenol injection stimulated the release of PGE $_2$, but not the release of PGF $_{2\alpha}$ ($P<0.05$).

Fig. 3. Effect of intraluteal application of TNF- α (20, 200, and 2000 ng/ml) or indomethacin (10^{-5} M) on the PG release into the MDS during PGF_{2 α} -induced luteolysis in ewes (mean $\pm$ SEM; $n=7-8$ CL/group). The basal release of PG into the MDS before PGF_{2 α} injection was 17.8 ± 3.4 pM for PGF_{2 α} and 35.1 ± 5.8 pM, respectively. For further details, see the legend of Fig. 2.

Discussion

The present results demonstrate that the infusion of TNF- α into the MDS implanted in the CL affects the intraluteal release of P and PGE₂ during PGF_{2 α} -induced luteolysis in the ewe. Twenty-four-hour infusions of TNF- α at 20 ng/ml and 200 ng/ml slightly hastened the time of onset to P decrease by 4.5 h and 3.5 h respectively, compared with the time of the control (17.0 h after PGF_{2 α} injection), while the higher dose of TNF- α at 2000 ng/ml clearly delayed the time to onset of P decrease for 9.0 h. In addition, this high dose greatly stimulated the release of PGE₂ between 2–10 h after PGF_{2 α} injection. However, the stimulative effects of this high dose of TNF- α appear to not be physiological, but to be pharmacological. The lower doses of TNF- α at 20 ng/ml and 200 ng/ml could not suppress the P release from the beginning period of infusion. Thus, the present

observations suggest that TNF- α may take part in the local mechanisms involved in the last period of functional luteolysis (a decrease of P release), but is unlikely to play an active role in the suppression of P release during the first period of functional luteolysis.

We have previously shown that the infusion of TNF- α at 20 ng/ml into the MDS implanted in the mid-CL stimulated rather than inhibited the release of P [11]. Thus, the weak inhibitory effect of TNF- α at the same dose at luteolysis suggest that this cytokine may be bifunctional throughout the life of cyclic CL (from CL development to CL regression), as has been proposed in the porcine CL [16–18].

It is interesting to note that a basal release of PG within the regressing CL slightly increased from 18 h after PGF_{2 α} injection, when the intraluteal P release totally decreased to 25% of the intact level before PGF_{2 α} injection. The data suggest that the increase in intraluteal PG production has no direct correlation with functional luteolysis. Indeed, the

infusion of indomethacin had no effect on the decreasing profile of P release, although it partly blocked the increase in the release of PG (between 10–26 h for PGF_{2α}, and between 26–32 h for PGE₂). Consequently, we speculate that some local factor(s) other than luteal PGF_{2α} should react to uterine or exogenous PGF_{2α} as an intermediary(s) of the cascade of functional luteolysis.

Several studies have been accumulating evidence that the influx of macrophages into the porcine CL [3] and the increase of TNF-α in the ovine [19] and bovine [20] CL during luteolysis were detected only during structural luteolysis. These findings strongly suggest that the invasion of immune cells into the regressing CL is the major reason for the increase of TNF-α concentration in the CL at this period. Recently, we and others have shown that the production and release of endothelin-1, a vasoconstrictive peptide originated from endothelial cells, increased in the CL and ovarian venous plasma ipsilateral to the CL from 2 h after PGF_{2α} injection in the cow, and proposed that endothelin-1 interacts with PGF_{2α} as a local luteolytic factor [21–24]. It is likely that such vasoconstrictive peptides are responsible for an acute response to luteolytic PGF_{2α} in both 1) direct inhibition of P release and 2) a vasoconstriction of arterioles, and thereby they can start an acute depletion of P re-

lease followed by a degeneration of CL tissue in which TNF-α plays a major role. During functional luteolysis, TNF-α might further stimulate a local release of endothelin-1, since endothelial cells are also well known as target cells of cytokines and at the same time, cytokine-producing cells [25].

Collectively, the present results support the idea that TNF-α may have a passive or secondary role in functional luteolysis. Furthermore, the intraluteal PG production appears to have no direct correlation with functional luteolysis.

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References

1. McClellan MC, Abel JH, Niswender GD. Function of lysosomes during luteal regression in normally cycling and PGF_{2α}-treated ewes. *Biol Reprod* 1977; 16: 499–512.
2. Bagavandoss P, Wiggins RC, Kunkel SL, Remick DG, Keyes PL. Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. *Biol Reprod* 1990; 42: 367–376.
3. Hehnke KE, Christenson LK, Ford SP, Taylor M. Macrophage infiltration into the porcine corpus luteum during prostaglandin F_{2α}-induced luteolysis. *Biol Reprod* 1994; 50: 10–15.
4. Murdoch WJ, Steadman LE, Belden EL. Immunoregulation of luteolysis. *Med Hypotheses* 1988; 27: 197–199.
5. Benyo DF, Pate JL. Tumor necrosis factor-α alters bovine luteal cell synthetic capacity and viability. *Endocrinology* 1992; 130: 854–860.
6. Emoto N, Baird A. The effect of tumor necrosis factor/cachectin on follicle-stimulating hormone-induced aromatase activity in cultured rat granulosa cells. *Biochem Biophys Res Comm* 1988; 153: 792–798.
7. Adashi EY, Resnick CE, Croft CS, Payne DW. Tumor necrosis factor α inhibits gonadotropin hormonal action in nontransformed ovarian granulosa cells. *J Biol Chem* 1989; 264: 11591–11597.
8. Darbon JM, Oury F, Laredo J, Bayard F. Tumor necrosis factor-α inhibits follicle-stimulating hormone-induced differentiation in cultured rat granulosa cells. *Biochem Biophys Res Comm* 1989; 163: 1038–1046.
9. Tekpetey FR, Engelhardt H, Armstrong DT. Differential modulation of porcine theca, granulosa, and luteal cell steroidogenesis *in vitro* by tumor necrosis factor. *Biol Reprod* 1993; 48: 936–943.
10. Richards RG, Almond GW. Tumor necrosis factor-α differentially alters progesterone and prostaglandin F_{2α} production by porcine luteal cells. *J Endocrinol* 1994; 143: 75–83.
11. Miyamoto A, Tashiro Y, Nakatsuka T, Meyer

- HHD, Taguchi K, Abe N, Fukui Y. Effects of tumor necrosis factor- α and interleukin-1 on local release of progesterone, prostaglandin $F_{2\alpha}$ and oxytocin in microdialyzed ovine corpus luteum in vivo. *Assisted Reprod Tech/Andrology* 1995; 8: 21–31.
12. Miyamoto A, Takase S, Tashiro Y, Konari A, Tomino M, Abe N, Taguchi K, Fukui Y. Local release of progesterone and oxytocin from microdialyzed corpus luteum in superovulated ewes: characterization during the non-breeding season. *J Reprod Dev* 1995; 41: 321–329.
 13. Miyamoto A, Okuda K, Schweigert FJ, Schams D. Effects of basic fibroblast growth factor, transforming growth factor- β and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *J Endocrinol* 1992; 135: 103–114.
 14. Meyer HHD, Enzenhöfer G, Feck H. Improvement of radioimmunoassays for prostaglandins in bovine blood plasma and their application to monitor reproductive functions. *Theriogenology* 1989; 31: 701–720.
 15. Wijayagunawardane MPB, Miyamoto A, Cerbito WA, Acosta TJ, Takagi M, Sato K. Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* 1998; 49: 607–618.
 16. Pitzel L, Jarry H, Wuttke W. Effects and interactions of prostaglandin $F_{2\alpha}$, oxytocin, and cytokines on steroidogenesis of porcine luteal cells. *Endocrinology* 1993; 132: 751–756.
 17. Richards RG, Almond GW. Identification and distribution of tumor necrosis factor α receptors in pig corpora lutea. *Biol Reprod* 1994; 51: 1285–1291.
 18. Hehnke KE, Clark CL, Taylor MJ, Ford SP. Presence and localization of tumor necrosis factor α in the corpus luteum of nonpregnant and pregnant pigs. *Biol Reprod* 1995; 53: 1339–1344.
 19. Ji I, Slaughter RG, Ellis JA, Ji TH, Murdoch WJ. Analyses of ovine corpora lutea for tumor necrosis factor mRNA and bioactivity during prostaglandin-induced luteolysis. *Mol Cell Endocrinol* 1991; 81: 77–80.
 20. Shaw DW, Britt JH. Concentrations of tumor necrosis factor α and progesterone within the bovine corpus luteum sampled by continuous-flow microdialysis during luteolysis in vivo. *Biol Reprod* 1995; 53: 847–854.
 21. Girsh E, Milvae RA, Wang W, Meidan R. Effect of endothelin-1 on bovine luteal cell function: role in prostaglandin $F_{2\alpha}$ -induced antisteroidogenic action. *Endocrinology* 1996; 137: 1306–1312.
 22. Girsh E, Wang W, Mamluk R, Arditi F, Friedman A, Milvae RA, Meidan R. Regulation of endothelin-1 expression in the bovine corpus luteum: elevation by prostaglandin $F_{2\alpha}$. *Endocrinology* 1996; 137: 5191–5196.
 23. Miyamoto A, Kobayashi S, Arata S, Ohtani M, Fukui Y, Schams D. Prostaglandin $F_{2\alpha}$ promotes the inhibitory action of endothelin-1 on the bovine luteal function *in vitro*. *J Endocrinol* 1997; 152: R7–R11.
 24. Ohtani M, Kobayashi S, Miyamoto A, Hayashi K, Fukui Y. Real-time relationships between intraluteal and plasma concentrations of endothelin, oxytocin and progesterone during prostaglandin $F_{2\alpha}$ -induced luteolysis in the cow. *Biol Reprod* 1998; 58: 103–108.
 25. Martin M, Resch K. Interleukin 1: more than a mediator between leukocytes. *Trends in Pharm Sci* 1988; 9: 171–177.