A Cooperative Action of Endothelin-1 with Prostaglandin $F_{2\alpha}$ on Luteal Function in the Cow

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Running title: Action of ET-1 with $PGF_{2\alpha}$ on luteolysis

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Abstract

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the primary luteolysin in the cow, and luteal endothelin-1 (ET-1) interacts with $PGF_{2\alpha}$ during the process of luteolysis. In contrast, a developing CL is refractory to exogenous administration of $PGF_{2\alpha}$. Thus, the present study was aimed to investigate the functional relationship between ET-1 and $PGF_{2\alpha}$ in the mid-CL ($PGF_{2\alpha}$-sensitive) and early-CL ($PGF_{2\alpha}$-refractory). In the mid-CL model, cows (n=6/treatment) were assigned to receive one of 5 types of treatments on Day 10 of the estrous cycle: (1) an injection of saline; control, (2) a 500 μg of $PGF_{2\alpha}$ analogue (sufficient dose to induce luteolytis); full-PG, (3) an intraluteal injection of 0.25 mg ET-1; ET-1, (4) a 125 μg of $PGF_{2\alpha}$ (insufficient dose to induce luteolytis); 1/4PG, or (5) an intraluteal injection of 0.25 mg ET-1 after administration of a insufficient dose of $PGF_{2\alpha}$ analogue; 1/4PG/ET. In the early-CL model, cows were assigned to receive one of 2 types of treatments on Day 5 of the estrous cycle: (1) a sufficient dose of $PGF_{2\alpha}$ analogue; PG (n=5), or (2) an intraluteal injection ET-1 after a sufficient dose of $PGF_{2\alpha}$; PG/ET (n=7). In the mid-CL model, 1/4PG/ET resulted in a rapid reduction of progesterone (P) concentrations similar to that in full-PG from the next day. However, the levels of P in 1/4PG/ET (1.5-2.5 ng/ml) kept significantly higher than that in full-PG (<0.5 ng/ml). ET-1 or 1/4PG did not decrease plasma P concentrations (4-6 ng/ml). The plasma ET-1 levels increased with the full PG administration. In the early-CL model, both treatments had no effect on plasma P increase and ET-1 levels. The overall results indicate that the intraluteal ET-1 injection after administration of insufficient dose of $PGF_{2\alpha}$ induces the depression of P secretion in vivo during the mid luteal phase in the cow, supporting the concept that ET-1 is one of a local mediator of functional luteolysis in the cow. The result further indicates that the early-CL is not only PG-refractory but also ET-1-refractory.

***Key words**:* Endothelin-1, Prostaglandin $F_{2\alpha}$, Luteolysis, Corpus Luteum, Cow

Introduction

It is well established that pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium is a physiological signal to induce luteolysis in ruminants [1]. However, in the early corpus luteum (CL) up to Day5 of the estrous cycle, a normal dose of $PGF_{2\alpha}$ can not induce luteolysis in the cow [2, 3]. Though administration of $PGF_{2\alpha}$ decreased mRNA expression of $PGF_{2\alpha}$ receptor and 3β-hydroxysteroid dehydrogenase in both Day 4 and Day 11 of the estrous cycle in the bovine CL [4]. Thus, the early CL does not typically result in luteal regression as is the case with the mid CL, so that early CL is not non-responsive but refractory to $PGF_{2\alpha}$. On the other hand, the direct exposure of the microenvironment within the bovine mid-CL to $PGF_{2\alpha}$ by using the in vitro [5, 6] and in vivo [7] microdialysis system (MDS) stimulated, but not inhibited progesterone (P) secretion. These findings suggest that the transfer of $PGF_{2\alpha}$ to the ovary via blood circulation plays a crucial role in initiating the rapid drop in P release. Our previous study revealed that a luteolytic dose of $PGF_{2\alpha}$ analogue during mid luteal phase induces a very clear and acute increase in blood flow surrounding the CL, but this increase in blood flow was not observed in the early-CL [8]. These findings indicate that this acute increase in blood flow may play one of the key roles to trigger the luteolytic cascade.

The functional CL is highly vascularized and consists of about 50 % vascular endothelial cells [9, 10]. Endothelin-1 (ET-1), a vasoconstrictive 21-amino acid peptide secreted by vascular endothelial cells, interacts with $PGF_{2\alpha}$ in the control of functional luteolysis [6, 11, 12]. A luteolytic injection of $PGF_{2\alpha}$ to cows [13-16] and ewes [17, 18] results in elevated ET-1 synthesis within the CL, and this increase is partly reflected in plasma levels [13, 15]. Moreover, ET-1 inhibits P secretion in bovine [6, 12, 14], ovine [17] and human [19] CL in vitro. Thus, ET-1 released from microvascular endothelial cells in the CL is closely associated with the process of luteolysis. In the luteal tissue, the ET-1 levels of early luteal stage is significantly lower than that of late and regressing luteal stages [11, 16, 20, 21]. In addition, Levy et al. [15] demonstrated that administration of $PGF_{2\alpha}$ during mid luteal phase, but not early luteal phase, induced both ET-1 and ETA-R mRNA expression. Thus, low ET-1 synthesis and lack of response in early CL may one of reasons for being refractory to $PGF_{2\alpha}$. However, little is known about the mechanisms responsible for this refractoriness of the early-CL to the luteolytic action of $PGF_{2\alpha}$.

Thus, the present study was aimed to investigate in vivo the interaction between ET-1 and $PGF_{2\alpha}$ on

the function of mid- ($PGF_{2\alpha}$-sensitive) and early- ($PGF_{2\alpha}$-refractory) CL. For this purpose, we utilized the cow model [22] modified from sheep model [17] in which intraluteal injection of ET-1 is combined with or without $PGF_{2\alpha}$ administration in both mid- and early-CL.

Materials and methods

Animals

The experimental were carried out at the Field Center 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. Forty-two multiparous, lactating Holstein cows were used to observe the effect of ET-1 in the early- and mid-CL. They had at least 2 estrous cycles of normal length (20-23 days) before being used. The day of estrus was designated as Day 0.

Experimental design

In the mid-CL model, cows were randomly assigned to receive one of 5 types of treatments (n=6 for each group) on Day 10 of the estrous cycle: (1) a 500 μg single i.m. administration of saline; control, (2) a 500 μg single i.m. administration of a $PGF_{2\alpha}$ analogue (cloprostenol- Estrumate®; Sumitomo Pharm. Co., Osaka, Japan), sufficient dose to induce luteolysis (= luteolytic dose of $PGF_{2\alpha}$); full-PG, (3) a single intraluteal injection of 0.25 mg ET-1 (Peptide Institute Inc., Osaka, Japan) in 500 μl 0.1% acetic acid; ET-1, (4) a 125 μg single i.m. administration of $PGF_{2\alpha}$ analogue, insufficient dose to induce luteolysis (= subluteolytic dose of $PGF_{2\alpha}$); 1/4PG, (5) a single intraluteal injection of 0.25 mg of ET-1 in 500 μl 0.1% acetic acid 30 min after a 125 μg single i.m. administration of a $PGF_{2\alpha}$ analogue; 1/4PG/ET.

In the early-CL model, cows were randomly assigned to receive 2 types of intraluteal injection on Day 5 of the estrous cycle: (1) a single intraluteal injection of 500 μl 0.1 % acetic acid (vehicle) 30 min after a 500 μg single i.m. administration of a $PGF_{2\alpha}$ analogue; PG (n=5), (2) a single intraluteal injection of 0.25 mg ET-1 in 500 μl 0.1% acetic acid 30 min after a 500 μg single i.m. administration of a $PGF_{2\alpha}$ analogue; PG/ET

(n=7).

The intraluteal injection of ET-1 was described previously [22]. For intraluteal injections of ET-1 or 0.1% acetic acid, a 16-gauge customized needle, 23-cm-long was inserted through the thinnest abdominal wall ipsilateral to CL to peritoneal cavity. The ovary with CL was grasped with a hand inserted through the rectum. A 1 ml disposable syringe was filled with either 500μl saline or ET-1 according to the experimental design, and with 200 μl of air (a dead volume of the customized needle). The needle was inserted through the peritoneal cavity and the ovarian parenchyma to approach the CL, so that the injection of 500 μl fluid into the CL was conducted via the backside of the ovary. The time of the intraluteal injection or i.m. was designed as 0 h.

Blood samples were collected by caudal venopuncture once a day (7:00 a.m.) from 3 days before until 5 days after treatments into sterile glass tubes containing 200 μl of a stabilizer solution (0.3 M EDTA, 1% acetyl salicylic acid, pH 7.4). The blood samples were immediately chilled in ice water for 10 min and plasma was obtained by centrifugation at 900 g for 20 min at 4 ºC, then was stored at –30 ºC until assayed. The time schedule of the present study is shown in Figure 1.

Hormone determination

Concentrations of P and ET-1 in plasma were determined in duplicate by second antibody enzyme immunoassays (EIAs) after extraction using 96-well ELISA plates (NUNC-Immuno Plate, NUNC^{TM} Brand Products, Denmark).

The 200 μl blood samples were taken for P extraction. The P was extracted using diethyl ether as described previously [23]. The residue was dissolved in 200 μl assay buffer for steroid EIA (40 mM PBS, 0.1 % BSA, pH 7.2) and thus concentrated 1-fold. The recovery rates obtained were to 88 % for P.

To extract ET-1, the 5 ml plasma samples were diluted with 5 ml of distilled water, and the pH was adjusted to 2.5 with 5N HCl. All samples were then applied to a Sep-Pak C_{18} Cartridge (Waters, Milford, MA) as described previously [6]. The residue was evaporated and then dissolved in 300 μl assay buffer (42 mM Na_2HPO_4, 8 mM KH_2PO_4, 20 mM NaCl, 4.8 mM EDTA, 0.05 % BSA, pH 7.5) for the peptide EIA. Thus, the samples were concentrated 16.7 fold for plasma as a result of this process that enabled us to determine peptide

levels in EIA within the range of the standard curve. The recovery rates of ET-1 were 50 %.

The EIAs for P [24] and ET-1 [6] were performed according to previous reports. Within-assay and between-assay coefficients of variation (CV) were 4.7 % and 6.5% respectively, for P, and 7.5 % and 12.5 % for ET-1. The effective dose $(ED)_{50}$ were 2.4 ng/ml for P and 60 pg/ml for ET-1, respectively. The ranges of the standard curves for these assays were 0.05-50 ng/ml for P and 10-500 pg/ml for ET-1, respectively.

Statistical analysis

For data analysis, the experimental period was divided into 3 time periods; Day 8-10, Day 11-12, and Day 13-15 in the mid-CL model, and Day 3-5, Day 6-7 and Day 8-10 in the early-CL model. For analysis of changes in concentrations of peptides in plasma, the mean concentrations of Day 8-10 (mid-CL model) and Day 3-5 (early-CL model) were used for calculation of an individual baseline. All concentrations in the plasma collected were then expressed as a proportion of this individual baseline. This transformation enables an evaluation of the relative changes of substance between different treatments. The absolute levels of each hormone during each period were averaged and the mean levels of each hormone in plasma before and after treatments were compared using ANOVA followed by Fisher's Protected Least Significant Difference Test (PLSD). Probabilities less than 5 % (P <0.05) were considered significant.

Results

Effect of different treatments on plasma P concentrations in mid luteal phase

The change in plasma P concentrations with different treatments in mid-CL is shown in Figure 2. Plasma P levels in all groups before treatments (on Day 8-10) were similar and ranged from 4-6 ng/ml. In control group, plasma P concentration gradually increased with the time. Treatment with full-PG induced a decrease in plasma P concentrations (< 0.5 ng/ml) and P concentration remained low during the experimental period. In contrast, the treatment with ET-1 or 1/4PG did not decrease plasma P concentrations (4-6 ng/ml). 1/4PG/ET resulted in a rapid reduction of P concentrations similar to that in full-PG from the next day. However, the levels of P in 1/4PG/ET (1.5-2.5 ng/ml) kept significantly higher than that in full PG (< 0.5

ng/ml).

Effect of different treatments on plasma ET-1 levels in mid luteal phase

The change in plasma ET-1 concentrations with different treatments in mid-CL is shown in Table 1 and 2. The basal concentrations (100 %) of ET-1 in plasma were 18.82 ± 0.91 pg/ml (mean ± SEM). Treatment with full-PG increased plasma levels of ET-1 (P <0.05) on Day 11-12 and Day 13-15 (P <0.01). ET-1 treatment gradually decreased plasma ET-1 levels on Day 13-15 (P <0.05). The plasma ET-1 levels in 1/4PG/ET groups showed a tendency to increase on Day 11-12 (P =0.067). In contrast, there was no effect on plasma ET-1 by 1/4PG throughout the experiment period (Table 1).

Effect of different treatments on plasma P concentrations in early luteal phase

Plasma P concentrations significantly increase as indicated by P value (P <0.01 on Day 6-7 and P <0.001 on Day 8-10) after treatment (Figure 3). There was no significant difference between treated groups (full-PG vs. PG/ET).

Effect of different treatments on plasma ET-1 levels in early luteal phase

The basal concentrations (100 %) of ET-1 in the plasma were 6.06 ± 0.75 pg/ml (mean ± SEM). Both PG and PG/ET treatment had no effect on plasma ET-1 levels (Table 2).

Discussion

The present study indicates that functional luteolysis in the cow is at least partly associated with the cooperative action of $PGF_{2\alpha}$ and ET-1 in the mid-CL. Moreover, the result that direct injection of ET-1 into early-CL did not affect luteal function may contradict the possibility that low levels of ET-1 in early-CL contribute to PG-refractory capacity at this stage.

Although 1/4PG or ET-1 treatment alone could not induce plasma P decrease, the intraluteal ET-1 injection after 1/4 dose of $PGF_{2\alpha}$ administration resulted in a reduction of CL function. These data are well consistent with our previous study that perfusion of mid-CL with ET-1 following $PGF_{2\alpha}$ by MDS in vitro greatly promotes the inhibitory action of ET-1 on P secretion [6]. Therefore, the present observation further supports the concept that $PGF_{2\alpha}$ and ET-1 act cooperatively to initiate the functional luteolysis in the cow [6, 12, 15, 17, 20, 25]. However, it has been reported that an ET-1 administration following pretreatment with a subluteolytic dose of $PGF_{2\alpha}$ injection caused a rapid decline in plasma P levels and shortened the length of the estrous cycle in ewes [17]. On the other hand, luteal regression is induced by ET-1 alone in pseudopregnant rabbit [26]. The contribution of importance of ET-1 for luteolysis may therefore differ between in species each other.

Previously, we have shown in the cow treated with a luteolytic injection of $PGF_{2\alpha}$ that the blood flow surrounding the mid-CL increased rapidly at 0.5-2 h, decreased at 4 h to the basal value, and further decreased from 8 to 48 h [8]. There is evidence that increased shear stress induces ET-1 expression in human endothelial cells from umbilical vein and glomerular microvessels [27, 28]. We hypothesize, therefore, that the shear stress induced by the first acute increase in blood flow may stimulate ET-1 release from endothelial cell of microcapillary within the CL, in addition to direct induction of ET-1 by $PGF_{2\alpha}$. On the other hand, a treatment with the subluteolytic dose of $PGF_{2\alpha}$ also induced an acute increase in blood flow within the mid-CL, but the degree of the increase was less than that observed with full-dose $PGF_{2\alpha}$ treatment [22]. In the present study, an injection of ET-1 after i.m. administration of a 1/4 dose of $PGF_{2\alpha}$ suppressed P release, but not induced the structural luteolysis and estrus. This failure inducing complete luteolysis due to lack of the impact on luteal function to elevate plasma ET-1 levels by injection of ET-1 after i.m. administration of a 1/4 dose of $PGF_{2\alpha}$ as full dose of $PGF_{2\alpha}$ did. Furthermore, it is speculated that the weaker shear stress induced by lower dose of $PGF_{2\alpha}$ may not enough to stimulate unknown mechanisms to make luteal cells sensitive to ET-1, or just simply the amount of injected ET-1 was not enough to progress further steps of the cascade of luteolysis.

It is considered that there are several other pathways in bovine CL to initiate luteolysis such as Ang II [22], nitric oxide [29, 30] and $PGF_{2\alpha}$ of luteal origin [4, 31]. Moreover, $PGF_{2\alpha}$ stimulates ET-1 [6, 11, 13], Ang II [32, 33] and $PGF_{2\alpha}$[31] secretion in bovine CL. Accordingly, we suggested that a local positive feedback system among ET-1, Ang II and $PGF_{2\alpha}$ in the CL is established after onset of luteolysis, and ensures

the decrease in P secretion and promotes the complete luteolysis [34]. In the present study, administration of full-dose of $PGF_{2\alpha}$ in the mid-CL increased plasma ET-1 levels. This observation further confirms the previous studies that both synthesis and secretion of ET-1 increase during $PGF_{2\alpha}$-induced luteolysis [11, 13, 15-17, 20, 25]. In addition, 1/4PG/ET treatment slightly increased the ET-1 release ($P<0.07$), followed by P depression, supporting further the concept that ET-1 act on functional luteolysis together with $PGF_{2\alpha}$ in the cow. Therefore, ET-1 may be one of the early steps of cascade for luteolysis, and uterine or exogenous $PGF_{2\alpha}$ must stimulate all necessary pathways above including ET-1 to initiate the cascade.

In contrast, in the early-CL, the plasma P continuously increased in spite of full-dose $PGF_{2\alpha}$ administration or in combination with intraluteal ET-1 injection, and plasma ET-1 levels did not change during the experimental period. The data indicate that the early-CL is not only PG-refractory but also ET-1-refractory, suggesting that early-CL does not have an efficient positive feedback system among ET-1, Ang II and $PGF_{2\alpha}$ as observed in mid-CL that is PG-sensitive. Indeed, the early-CL produces minimum amount of ET-1 compared to those of later stages [11, 16, 20, 21]. Furthermore, it has been reported that local production of PGs and Ang II is high within newly developing CL, and $PGF_{2\alpha}$ and Ang II interact and enhance the capacity of luteal cell to produce P whereas ET-1 has no effect [35]. Namely, early-CL has a functional Ang II-$PGF_{2\alpha}$ system that is luteotropic but not luteolytic [36]. In contrast, mid-CL acquires the functional system of ET-1-Ang II-$PGF_{2\alpha}$ to initiate luteolysis in the cow [36]. Thus, we propose that bovine CL has a possible switchover of the vasoactive peptides-$PGF_{2\alpha}$ system from early-CL (PG- and ET-1-refractory) to mid-CL (PG- and ET-1-sensitive). It is likely that $PGF_{2\alpha}$ activates this mechanism first [37]. In addition, $PGF_{2\alpha}$ and ET-1 can evoke increases in intracellular calcium^{2+} not only mid-CL but also early-CL in the cow, but the degree of increase in mid-CL was greater than that elicited in early-CL [38]. Therefore, these and our findings in early-CL suggest that ET-1 as well as $PGF_{2\alpha}$ stimulates intracellular calcium^{2+}, but the degree of calcium^{2+} increase (30 % of that in mid-CL) may not reach the threshold to initiate luteolysis [38]. Alternatively, the downstream of intracellular calcium^{2+} might not be functional in early-CL. Contrary to the present result, Choudhary et al. reported that ET-1 can suppress P secretion in early- as well as mid-luteal cells in vitro [38], indicating that the lower efficiency of $PGF_{2\alpha}$ in the early-CL is likely related more to the transduction differences associated sensitivity to $PGF_{2\alpha}$ than to the absence of ET-1 actions to mediate the luteolytic action of $PGF_{2\alpha}$. The findings of the present study suggest that ET-1 alone is insufficient to depress P secretion in early-CL in vivo, and active angiogenesis might

correlate to ET-1-refractory capacity at this stage.

The overall results indicate that the intraluteal ET-1 injection 30 min after i.m. administration of subluteolytic dose of $PGF_{2\alpha}$ induces the depression of P secretion in vivo in the mid-CL in the cow, supporting the concept that the ET-1 is one of a local mediator of functional luteolysis in the cow. The result further indicates that early-CL is not only PG-refractory but also ET-1-refractory.

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Fig. 1

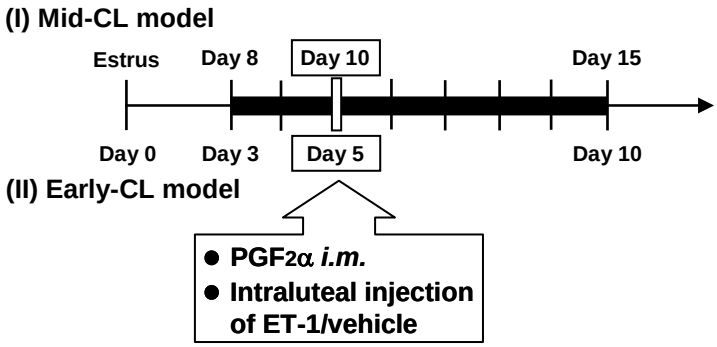
(I) Mid-CL model
Estrus
Day 8
Day 10
Day 15
Day 0
Day 3
Day 5
Day 10
(II) Early-CL model
● PGF2α *i.m.*
● Intraluteal injection of ET-1/vehicle

Fig. 2

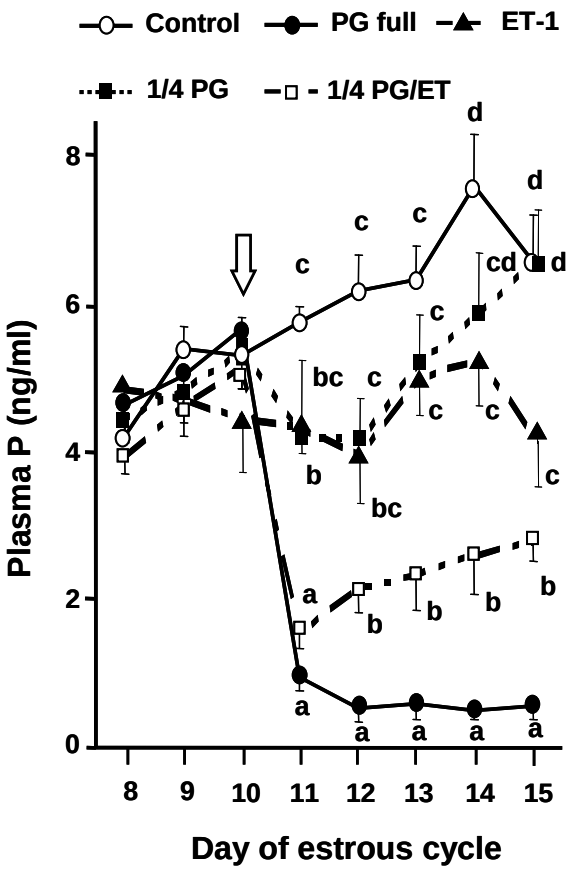
Control
PG full
ET-1
1/4 PG
1/4 PG/ET
Plasma P (ng/ml)
Day of estrous cycle
0
2
4
6
8
8
9
10
11
12
13
14
15

Fig. 3

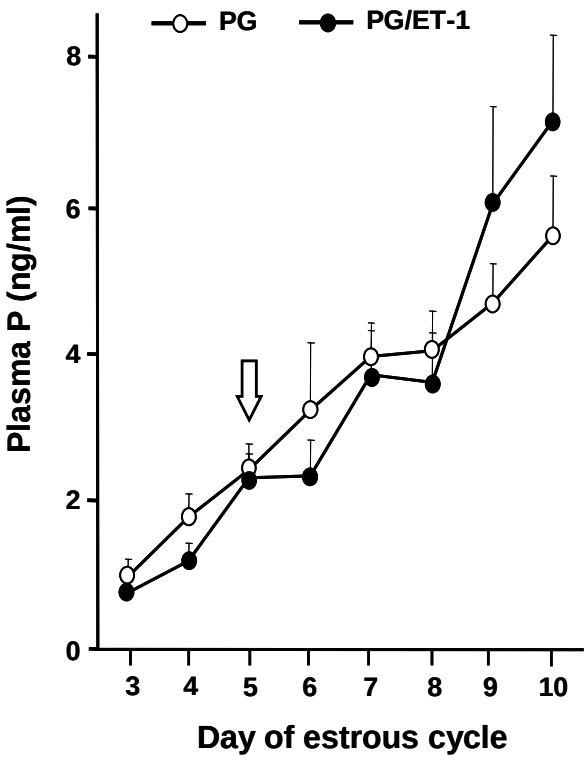

Table 1. Effect of different treatments on plasma ET-1 levels during mid luteal phase in the cow. The data are expressed as a percentage of basal release before treatment (Day 8-10).

	Day of the estrous cycle		
Treatment♦	8 - 10	11 - 12	13 - 15
Control	99.9 ± 7.9	101.9 ± 19.1	93.6 ± 8.3
full-PG	100.0 ± 8.7	191.6 ± 21.1 *	190.1 ± 18.7 **
ET-1	99.9 ± 7.4	78.7 ± 12.3	68.1 ± 11.3 *
1/4PG	100.1 ± 7.2	109.1 ± 21.3	100.4 ± 14.7
1/4PG/ET	100.0 ± 9.4	139.3 ± 17.6	109.9 ± 14.6

♦Control: an injection of saline, full-PG: a 500 µg of $PGF_{2\alpha}$ analogue (sufficient dose to induce the luteolytis), ET-1: an intraluteal injection of 0.25mg ET-1, 1/4PG: a 125 µg of $PGF_{2\alpha}$ (insufficient dose to induce the luteolytis), and 1/4PG/ET: an intraluteal injection of 0.25 mg ET-1 after administration of a insufficient dose of $PGF_{2\alpha}$ analogue. The basal release (100 %) of ET-1 into plasma was 18.82 ± 0.91 pg/ml (mean ± SEM).

1 *P <0.05, **P <0.01 before (Day8-10) vs. after treatment (Day11-12 or Day 13-15).

Table 2. Effect of different treatments on of blood plasma ET-1 levels during early luteal phase. The data are expressed as a percentage of basal release before treatment (Day 3-5).*

	Day of the estrous cycle		
Treatment♦	3 - 5	6 - 7	8 - 10
PG	100.1 ± 12.2	120.5 ± 28.5	77.9 ± 13.3
PG/ET	100.0 ± 10.8	127.3 ± 10.5	100.6 ± 15.7

♦PG: a 500 µg of $PGF_{2\alpha}$ analogue (sufficient dose to induce the luteolytis). PG/ET: an intraluteal injection of 0.25 mg ET-1 after administration of a 500 µg of $PGF_{2\alpha}$ analogue. The basal release (100 %) of ET-1 into plasma were 6.06 ± 0.75 pg/ml (mean ± SEM).

*There is no significant difference in ET-1 levels between the periods before (Day 3-5) and after (Day 6-7 , 8-10) treatment.

1

Figure Legends

Fig. 1. Time schedule of the present experiment in vivo.

Fig. 2. Effects of endothelin-1 (ET-1) and $PGF_{2\alpha}$ on mean plasma progesterone concentrations in mid-CL in the cow. (n=6 for each group): (1) a 500 µg single i.m. administration of saline (control, ○); (2) a 500 µg single i.m. administration of a $PGF_{2\alpha}$ analogue, sufficient dose to induce the luteolysis (= luteolytic dose of $PGF_{2\alpha}$) (full-PG, ●); (3) a single intraluteal injection of 0.25 mg ET-1 in 500 µl 0.1% acetic acid (ET-1, ▲); (4) a 125 µg single i.m. administration of $PGF_{2\alpha}$ analogue, insufficient dose to induce the luteolysis (= subluteolytic dose of $PGF_{2\alpha}$) (1/4PG, ■); (5) a single intraluteal injection of 0.25 mg of ET-1 in 500 µl 0.1% acetic acid 30 min after a 125 µg single i.m. administration of a $PGF_{2\alpha}$ analogue (1/4PG/ET, □). Data shows mean ± SEM for each time point. Values with different letters (a, b, c, d) are significantly different from other treatments at the same time point ($P < 0.05$).

Fig. 3. Effects of endothelin-1 (ET-1) and $PGF_{2\alpha}$ on mean plasma progesterone concentrations in early-CL in the cow. Cows received: (1) a single intraluteal injection of 500 µl 0.1 % acetic acid (vehicle) 30 min after a 500 µg single i.m. administration of a $PGF_{2\alpha}$ analogue (PG, n=5, ○); (2) a single intraluteal injection of 0.25 mg ET-1 in 500 µl 0.1% acetic acid 30 min after a 500 µg single i.m. administration of a $PGF_{2\alpha}$ analogue (PG/ET, n=7, ●). Data shows mean ± SEM for each time point.