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Abstract

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the uterus causes alterations in luteal blood flow, reduces progesterone secretion, and induces luteolysis in the bovine corpus luteum (CL). We have recently discovered that luteal blood flow in the periphery of the mature CL acutely increases coincidently with pulsatile increases in a metabolite of $PGF_{2\alpha}$ (PGFM). In this study, we characterized changes in regional luteal blood flow together with regional alterations in endothelial nitric oxide synthase (eNOS) expression during spontaneous luteolysis and in response to $PGF_{2\alpha}$. Smooth muscle actin-positive blood vessels larger than 20 μm were observed mainly in the periphery of mature CL. $PGF_{2\alpha}$ receptor was localized to luteal cells and large blood vessels in the periphery of mid-CL. $PGF_{2\alpha}$ acutely stimulated eNOS expression in the periphery but not in the center of mature CL. Injection of the NO donor S-nitroso-N-acetylpenicillamine into CL induced an acute increase in luteal blood flow and shortened the estrous cycle. In contrast, injection of the NOS inhibitor L-NAME into CL completely suppressed the acute increase in luteal blood flow induced by $PGF_{2\alpha}$ and delayed the onset of luteolysis. In conclusion, $PGF_{2\alpha}$ has a site-restricted action depending on not only luteal phase but also the region in the CL. $PGF_{2\alpha}$ stimulates eNOS expression, vasodilation of blood vessels, and increased luteal blood flow in periphery of mature CL. Furthermore, the increased blood flow is mediated by NO, suggesting that the acute increase in peripheral blood flow to CL is one of the first physiological indicators of NO action in response to $PGF_{2\alpha}$.
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Introduction

The corpus luteum (CL) is a transient organ established by follicular cells following ovulation in many mammals. The development of the CL is accompanied by angiogenesis. In the bovine CL, vascular endothelial cells account for up to 50% of the total cells whereas steroidogenic cells account for ∼30% of the cells (O'Shea *et al.* 1989, Lei *et al.* 1991). The formation of a competent vascular system is essential for proper development and function of the CL (Dickson *et al.* 2001). The steroidogenic cells, particularly large luteal cells, produce and secrete a large amount of progesterone (P) during the estrous cycle (Rodgers *et al.* 1988, Alila *et al.* 1989, Meidan *et al.* 1990). On the other hand, vascular endothelial cells secrete many vasoactive substances that directly regulate P secretion within the CL (Miyamoto *et al.* 1993, 1997a, 1997b,

Girsh *et al.* 1996a, 1996b, Hayashi *et al.* 2000, Milvae 2000, Skarzynski *et al.* 2000). Therefore, the vascular components and blood flow within the CL have an essential role in luteal function.

The CL of the bovine estrous cycle has a life span of ∼17–18 days. It is universally accepted that pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium initiates functional and structural regression of the CL (McCracken *et al.* 1972, Silvia *et al.* 1991). As such, $PGF_{2\alpha}$ is regarded as a physiological luteolysin in the cow. In general, the administration of $PGF_{2\alpha}$ during the mid-luteal phase (days 8–12 of the estrous cycle, mid-CL) drastically reduces plasma P concentrations and the volume of the CL. However, $PGF_{2\alpha}$ does not induce luteolysis during the early luteal phase (up to day 5 of the estrous cycle; Henricks *et al.* 1974, Schallenberger *et al.* 1984). We have previously reported that treatment of

mature CL (day10 of the estrous cycle, referred to as mid-CL), but not early CL, with a luteolytic dose of $PGF_{2\alpha}$ induced an acute increase (from 30 min to 2 h) in blood flow at the periphery of the CL, which was followed by a gradual decrease in luteal blood flow (Acosta *et al.* 2002). Thus, $PGF_{2\alpha}$-induced increase in luteal blood flow is one of the earliest physiological events observed during the luteolytic cascade in the cow. However, the increase in luteal blood flow might be a pharmacological response induced by injection of exogenous $PGF_{2\alpha}$ and physiological impact of this phenomenon is not well known.

Recent studies suggest that the CL of the cow produces many autocrine/paracrine factors that potentially regulate vasodilation, vasoconstriction, angiogenesis, and angiolysis (Miyamoto *et al.* 1993, 1997a, 1997b, Girsh *et al.* 1996a, 1996b, Hayashi *et al.* 2000, Milvae 2000, Skarzynski *et al.* 2000). Endothelium-derived nitric oxide (NO), a potent vasorelaxant, has been suggested to be an important mediator of luteolysis in the cow (Skarzynski *et al.* 2000, 2003, Korzekwa *et al.* 2004), rabbit (Boiti *et al.* 2003), rat (Motta *et al.* 1999, 2001), and human (Vega *et al.* 1998, 2000, Friden *et al.* 2000). The bovine CL has two types of NO synthase (NOS), endothelial NOS (eNOS), and inducible NOS (iNOS; Skarzynski *et al.* 2003). The eNOS binds to calmodulin in a reversible and Ca^{2+}-dependent manner and releases NO for short periods (Moncada *et al.* 1991). On the other hand, iNOS is calmodulin- and Ca^{2+}-independent (Sessa 1994). Treatment of bovine luteal cells with agents that increase NO directly inhibits P secretion (Skarzynski & Okuda 2000), and the inhibition of ovarian NOS *in vivo* prolongs the duration of the estrous cycle in the cow (Skarzynski *et al.* 2003). Therefore, NO appears to be an important mediator of the luteolytic cascade.

Endothelin-1 (EDN1), a vasoconstrictive peptide (Yanagisawa *et al.* 1988), may also play a role in $PGF_{2\alpha}$-induced luteolysis as well as spontaneous luteolysis in the cow. EDN1 is produced by luteal endothelial cells, and receptors for endothelin (EDNRA and EDNRB) are expressed in the bovine CL (Girsh *et al.* 1996a, 1996b, Berisha *et al.* 2000, 2002). $PGF_{2\alpha}$ stimulates *EDN1* mRNA expression and the release of EDN1 in the bovine CL both *in vivo* (Ohtani *et al.* 1998, Shirasuna *et al.* 2004a, Watanabe *et al.* 2006) and *in vitro* (Girsh *et al.* 1996a, 1996b, Hinckley & Milvae 2001, Wright *et al.* 2001). Importantly, EDN1 has been shown to inhibit P secretion by cultured bovine luteal cells or luteal explants (Girsh *et al.* 1996a, 1996b, Miyamoto *et al.* 1997a, 1997b, Hinckley & Milvae 2001). These findings strongly suggest that $PGF_{2\alpha}$ together with EDN1 suppresses P and decreases blood flow within the regressing CL (Shirasuna *et al.* 2006, Watanabe *et al.* 2006).

In the present study, we aimed to clarify the role of eNOS and NO in regulation of the increase in blood flow observed at periphery of bovine CL during the early stage of luteolysis using *in vivo* experimental models. The series of experiments in the present study were designed (1) to investigate and characterize the changes in local blood flow with the expression of eNOS, iNOS, and EDN1 mRNA during spontaneous CL regression, (2) to determine the acute effects of $PGF_{2\alpha}$ administration on eNOS expression at the early and mid-luteal phase (immature or early CL versus mature or mid-CL) and the site (periphery versus center) of the CL, and (3) to examine whether the $PGF_{2\alpha}$-induced blood flow increase is mediated by NO by determining whether the administration of an NO donor or an NOS inhibitor affects the occurrence of blood flow and luteolysis in the cow.

Results

Experiment 1: luteal blood flow and mRNA expression during spontaneous luteolysis
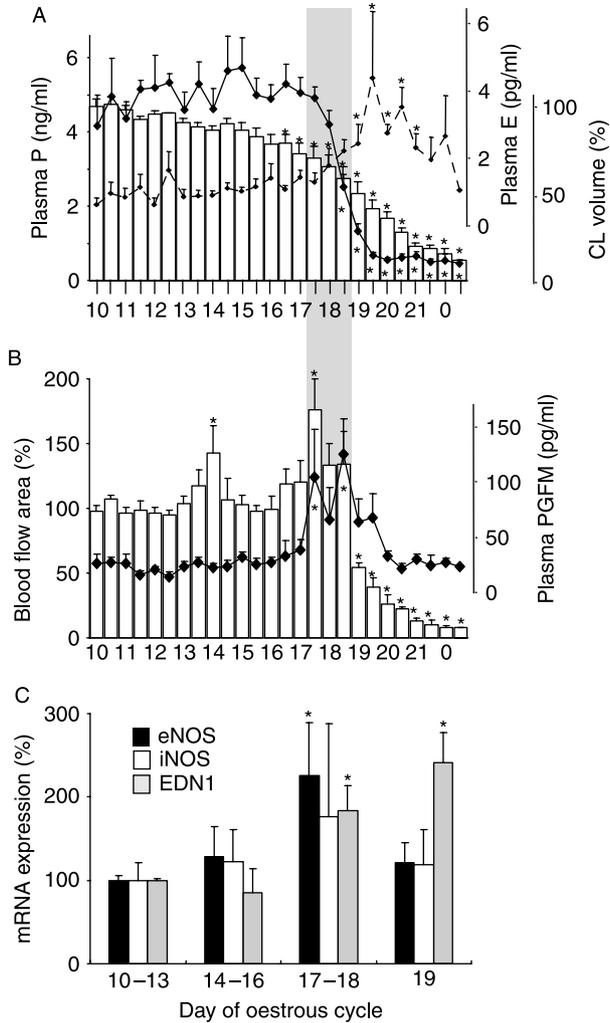
We determined the changes in luteal blood flow together with the changes in mRNA expression within the CL during spontaneous luteolysis in the cow (Fig. 1). The serum concentrations of P were maintained at high levels from days 10 to 17 and rapidly decreased during afternoon on day 18 (Fig. 1A). The serum concentrations of estradiol 17 β (E) started to gradually increase during afternoon on day 18 as plasma P decreased, and remained elevated until estrus (Fig. 1A). The basal volume of the CL on days 10–13 was 7.88 ± 1.03 cm^3 (mean $\pm$ s.e.m., $n=8$). The volume of the CL started to decrease slowly on day 16, and further decreased until the onset of estrus (Fig. 1A).

The luteal blood flow area was increased during morning on day 14 (by about 40%, seven out of eight cows), and on days 17–18 (by about 80%; Fig. 1B). Thereafter, luteal blood flow area decreased to about 10–50% on days 19–21 of the estrous cycle (Fig. 1B). The increase in luteal blood flow area on days 17–18 was temporally associated with peaks in plasma PGFM. The increase in blood flow on day 14 did not have a corresponding peak in PGFM (Fig. 1B).

The expression of *eNOS* mRNA was greater on days 17–18 compared with on days 10–13 (Fig. 1C). The expression of *iNOS* mRNA did not change during the estrous cycle (Fig. 1C). The expression of *EDN1* mRNA was greater on days 17–18 and day 19 than on days 10–13 (Fig. 1C).

Experiment 2: luteal phase (early CL versus mid-CL)- and site (periphery versus center of the CL)-dependent effect of $PGF_{2\alpha}$

We investigated the localization of blood vessels and microcapillary vessels in the periphery and center of the CL using immunohistochemical detection of smooth muscle actin (a marker of smooth muscle cells) and von Willebrand factor (a marker of endothelial cells; Figs 2–5). Morphological evidence revealed relatively
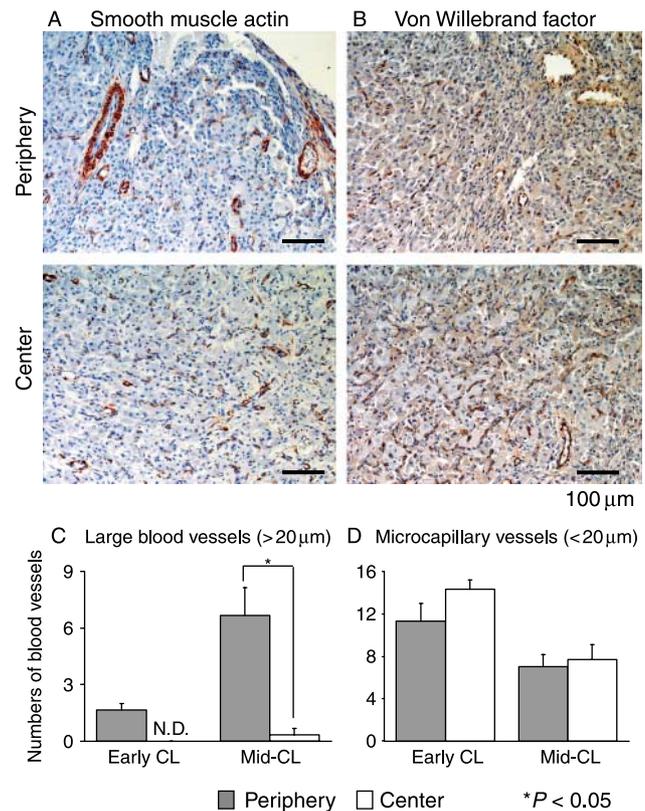
in serial section. On the other hand, there was a little evidence of large blood vessels with smooth muscle layers in the center of the mid-CL (Fig. 2). In order to make quantitative determinations blood vessels were grouped into two types depending on their diameter, large blood vessels (> 20 µm) and microvascular vessels (< 20 µm) and the number of blood vessels was determined as described in Materials and Methods. We observed that the mid-CL contained more large blood vessels in the periphery than did the early CL (Fig. 2C). Although a few large blood vessels were observed in the center of the mid-CL, none were observed in the center of the early CL. No differences were noted in the numbers of microvascular vessels in the peripheral and center regions of early and mid-CL (Fig. 2D).

Immunostaining for eNOS is shown in Fig. 3. Although eNOS immunostaining was technically very difficult and

Figure 1 Changes in plasma progesterone (P), plasma estradiol-17β (E), corpus luteum (CL) volume, luteal blood flow area, plasma PGFM, *eNOS* mRNA, *iNOS* mRNA, and *EDN1* mRNA during spontaneous luteolysis. (A) P concentrations are marked with a solid line, E concentrations are marked with a dashed line, and CL volume is denoted with bars. (B) PGFM concentrations are marked with a solid line, and luteal blood flow area is denoted with bars. The gray area in (A) and (B) indicates time period of the increase in luteal blood flow. (C) Black bars indicate *eNOS* mRNA expression, white bars indicate *iNOS* mRNA expression, and gray bars indicate *EDN1* mRNA expression. The mean values of luteal blood flow area and volume of CL during days 10–13 were used to calculate the baseline for each measurement (defined as 100%) and all values were expressed as a percentage of the corresponding baseline. For data analysis on mRNA levels, the experimental period was divided into four time periods (days 10–13, 14–16, 17–18, and 19) and the data during days 10–13 was used to calculate the baseline for each measurement (defined as 100%). The mRNA levels for *eNOS, iNOS,* and *EDN1* were normalized using β-actin mRNA and expressed as a percentage of this individual baseline values. Mean ± S.E.M. are presented, $n = 5$–8. The asterisk (*) indicates values statistically different compared with baseline ($P < 0.05$).

large blood vessels (> 20 µm) with smooth muscle layers in the periphery of the mid-CL (Fig. 2). In the periphery of the mid-CL, large blood vessels were detected with immunoreactive staining for both vWF and SMA

Figure 2 Localization of smooth muscle actin and von Willebrand factor, and a number of large blood vessels and microvascular vessels in the early and mid-CL. Immunohistochemistry was performed to detect (A) smooth muscle actin (SMA) and (B) von Willebrand factor (vWF) in luteal tissue sections. Areas within the CL were designated as the periphery (in the range of 1 mm from the boundary between luteal tissue and ovarian parenchyma) and the center of the CL (in the range of 1.5 mm from the center of the CL). Blood vessels were classified depending on their diameter, large blood vessels as > 20 µm and microvascular vessels < 20 µm. The number of blood of (C) large and (D) microvascular vessels was determined as indicated in the Materials and Methods. Mean ± S.E.M. are presented, $n = 4$–5. Asterisk (*) indicates statistically different values ($P < 0.05$).
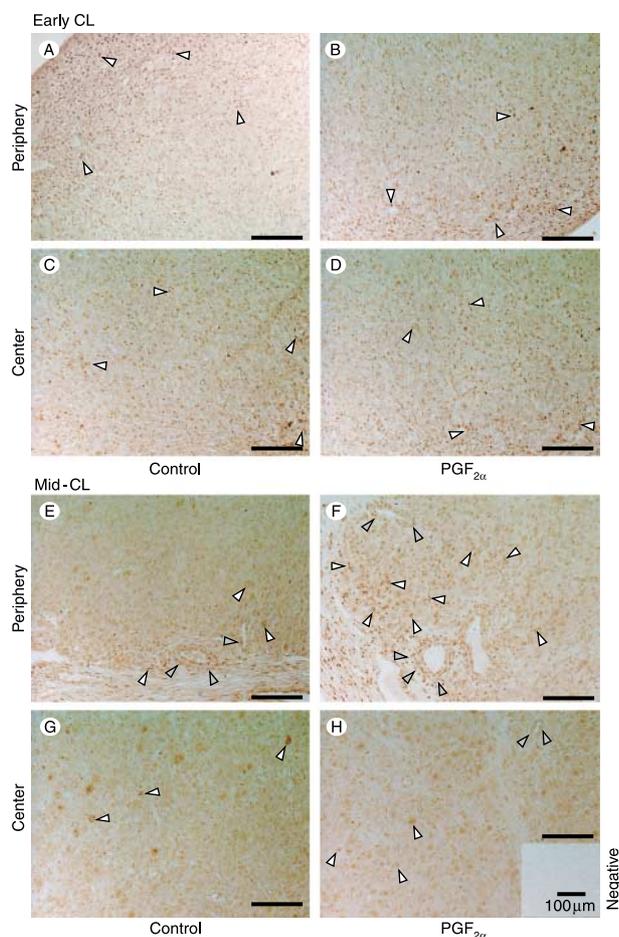
Figure 3 Representative micrographs of eNOS immunostaining in the early and mid-CL. The experiments were conducted on day 4 (early CL) and on days 10–12 (mid-CL). Cows were either injected with $PGF_{2\alpha}$ or saline as control (early CL control, $n=5$; early CL $PGF_{2\alpha}$ treated, $n=5$; mid-CL control, $n=4$; and mid-CL $PGF_{2\alpha}$ treated, $n=4$). At 30 min after injection of $PGF_{2\alpha}$ or saline, luteal blood flow was observed using color Doppler ultrasound; and thereafter the cows were ovariectomized and the CL were fixed for immunohistochemistry. Slides were stained using a specific eNOS antibody as described in Materials and Methods. Representative areas within the CL were designated as the periphery and the center of the CL. White arrowheads indicate eNOS immunostaining and gray arrowheads indicate eNOS immunostaining on large blood vessels of the CL.

the expression of eNOS protein was low as described in a previous study (Skarzynski *et al*. 2003), we observed that eNOS protein was localized mainly in the endothelial cells on blood vessels in the present study. eNOS protein expression was localized to endothelial cells of blood vessels and capillaries both in the early and mid-CL. In saline-treated control animals, staining for eNOS was observed in the periphery of the mid-CL (Fig. 3E) and occasionally observed in the center of the mid-CL (Fig. 3G). On the other hand, in $PGF_{2\alpha}$-treated animals, greater staining for eNOS was observed in the periphery (Fig. 3F) compared with the center (Fig. 3H) of the mid-CL. Quantitative analysis of eNOS

immunostaining and mRNA expression is shown in Fig. 4. In the early CL, $PGF_{2\alpha}$ did not increase the eNOS immunostaining area (Fig. 4A) or eNOS mRNA (Fig. 4B) in the periphery and center of the CL. In contrast, $PGF_{2\alpha}$ administration significantly increased eNOS immunostaining and *eNOS* mRNA in the periphery, but not in the center, of the mid-CL (Fig. 4C and D).

$PGF_{2\alpha}$ receptor (FPr) immunostaining was localized to luteal cells and blood vessels within the mid-CL (Fig. 5). FPr immunostaining was present in some large blood vessels, mainly in endothelial cells (Fig. 5A, C, E, and G; indicated by arrows) in the peripheral region and some small vessels in the center of the CL (Fig. 5B, D, and F; indicated by gray arrowheads). Interestingly, vascular interspace and vascular diameter clearly expanded in the periphery (Fig. 5E and G; indicated by white arrowheads) of the CL after $PGF_{2\alpha}$ injection compared with the periphery of the control CL (Fig. 5C).

Experiment 3: the effect of intraluteal injection of an NO donor on day 14 of the estrous cycle

Injection of the NO donor S-nitroso-*N*-acetylpenicillamine (SNAP) significantly increased blood flow in the periphery of the CL (Fig. 6A). The increase in blood flow was observed within 30 min of injection and was
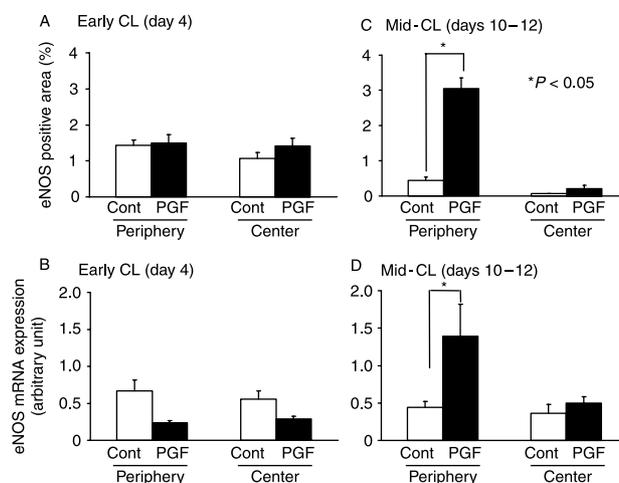
Figure 4 Image analysis of *eNOS* immunostaining and expression of *eNOS* mRNA in the early and mid-CL. The experiments were conducted on day 4 as early CL and days 10–12 as mid-CL, and $PGF_{2\alpha}$ or saline as control was injected respectively (early CL control, $n=5$; early CL $PGF_{2\alpha}$ treated, $n=5$; mid-CL control, $n=4$; and mid-CL $PGF_{2\alpha}$ treated, $n=4$). At 30 min after injection of $PGF_{2\alpha}$ or saline, luteal blood flow was observed using color Doppler ultrasound. After observing, the cows were ovariectomized at 1 h after treatment, and portions of the CL were fixed for immunohistochemistry and processed for mRNA analysis. Sampling areas within the CL were designated as the periphery and the center of the CL. (A and B) eNOS-positive staining area and *eNOS* mRNA expression in the early CL. (C and D) eNOS-positive staining area and *eNOS* mRNA expression in the mid-CL. White bar indicates control group, and black bar indicates $PGF_{2\alpha}$-treated group in each figure. Mean±s.e.m. are presented. Asterisk (*) indicates statistically different values ($P<0.05$).

FPr expression in the bovine mid-CL
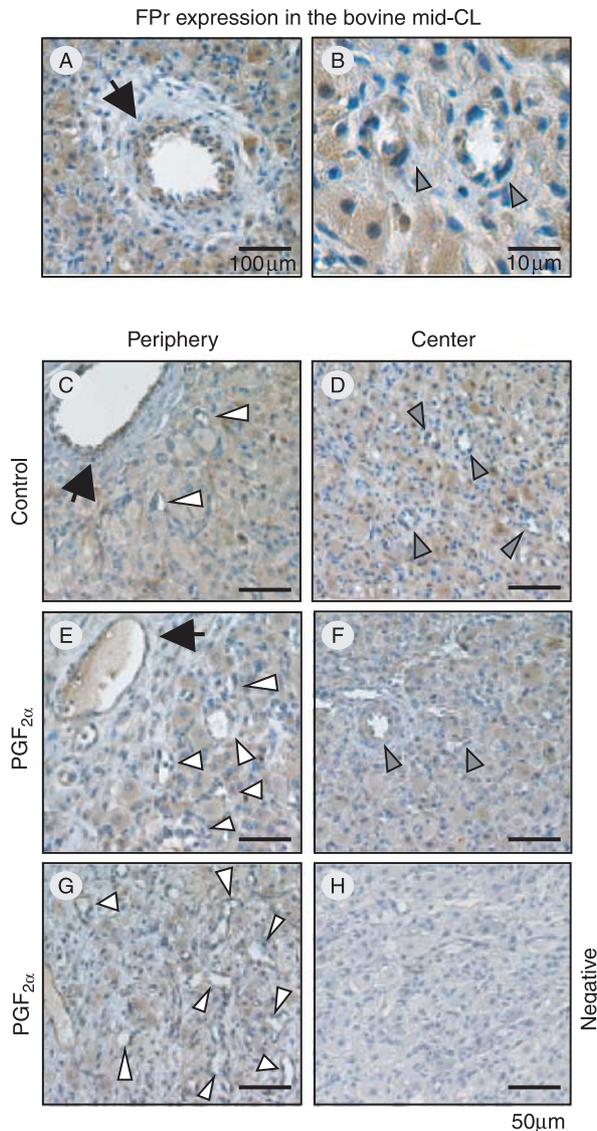
Figure 5 Representative micrographs of FPr immunostaining in the mid-CL. The experiments were conducted on days 10–12 as mid-CL, and (A–E) saline or (F–H) PGF$_{2\alpha}$ as control was injected respectively (mid-CL control, $n=4$ and mid-CL PGF$_{2\alpha}$ treated, $n=4$). At 30 min after injection of PGF$_{2\alpha}$ or saline, luteal blood flow was observed using color Doppler ultrasound; and thereafter the cows were ovariectomized and portions of the CL were fixed for immunohistochemistry. Large blood vessels in the periphery of the CL are shown in (A), and capillary vessels in the center of the CL in (B). The observation areas within the CL were designated as (C, E and G) the periphery and (D and F) the center of the CL. (H) The negative control of the CL. Black arrows indicate the FPr immunostaining in vascular elements of large blood vessels, gray arrowheads indicate capillary vessels, and white arrowheads indicate vasodilated blood vessels following PGF$_{2\alpha}$ injection in the periphery of the CL.

maintained at high levels for up to 8 h, compared with the control group (Fig. 6A). Color Doppler images of blood flow in SNAP and vehicle injected CL are shown in Fig. 6B and C respectively.

Plasma P concentrations decreased 60 h following the NO donor injection (Fig. 6D). In the control group, plasma P decreased at 120 h after vehicle injection which corresponded to the increase in luteal blood flow observed after 72–96 h (equivalent to days 17–18). Compared with the vehicle-treated control group, treatment with the NO donor also resulted in a more rapid reduction in the volume of the CL (Fig. 6E). As a result, the NO donor significantly shortened the estrous cycle by about 3 days (17.5 ± 0.6 days in the NO donor-treated group versus 21.3 ± 0.7 days in the control group).

In the NO donor-treated group, plasma PGFM was increased at 36 and 60 h after NO donor injection, thereafter returned to basal levels (Fig. 6F). The changes in PGFM were followed by decrease in plasma P secretion (Fig. 6D). In the control group, plasma PGFM increased at 96 h together with an increase in luteal blood flow (Fig. 6A).

The expression of *eNOS* mRNA tended ($P<0.1$) to increase at 4 and 12 h after treatment after NO donor injection (Fig. 6G). On the other hand, *EDN1* mRNA expression increased within 12 h and remained elevated for 72 h after NO donor injection (Fig. 6H).

Experiment 4: the effect of intraluteal injection of the NOS inhibitor L-NAME on PGF$_{2\alpha}$-induced luteolysis

PGF$_{2\alpha}$ administration induced an acute increase in luteal blood flow in the control group (Fig. 7A). The injection of the NOS inhibitor suppressed the acute increase in luteal blood flow induced by PGF$_{2\alpha}$ administration (Fig. 7A). Images of luteal blood flow are shown in Fig. 7B and C. In the control group, plasma P concentrations started to decrease in the first hours after PGF$_{2\alpha}$ administration, however, in the NOS inhibitor group the PGF$_{2\alpha}$-induced decline in plasma P was delayed by at least 8 h (Fig. 7D). The volume of the CL was 6.43 ± 0.43 cm^3 in the control group and 5.92 ± 0.69 cm^3 in the NOS inhibitor group. In the control group, the CL volume started to decrease 4 h after PGF$_{2\alpha}$ administration. Treatment with the NOS inhibitor delayed the decrease in the CL volume by 8 h (Fig. 7E). In the NOS inhibitor-treated group, CL volumes were larger than in the control group throughout the 96-h period (Fig. 7E).

Discussion

The present study demonstrates that during spontaneous CL regression in the cow an acute increase in luteal blood flow in periphery of the mature CL is correlated with pulsatile increase in plasma PGFM (a metabolite of PGF$_{2\alpha}$ produced by the uterus). We also demonstrate that PGF$_{2\alpha}$ acutely stimulates the expression of eNOS in the periphery, but not in the center, of the mature CL. Moreover, the administration of the NO donor SNAP induced an acute increase in luteal blood flow and
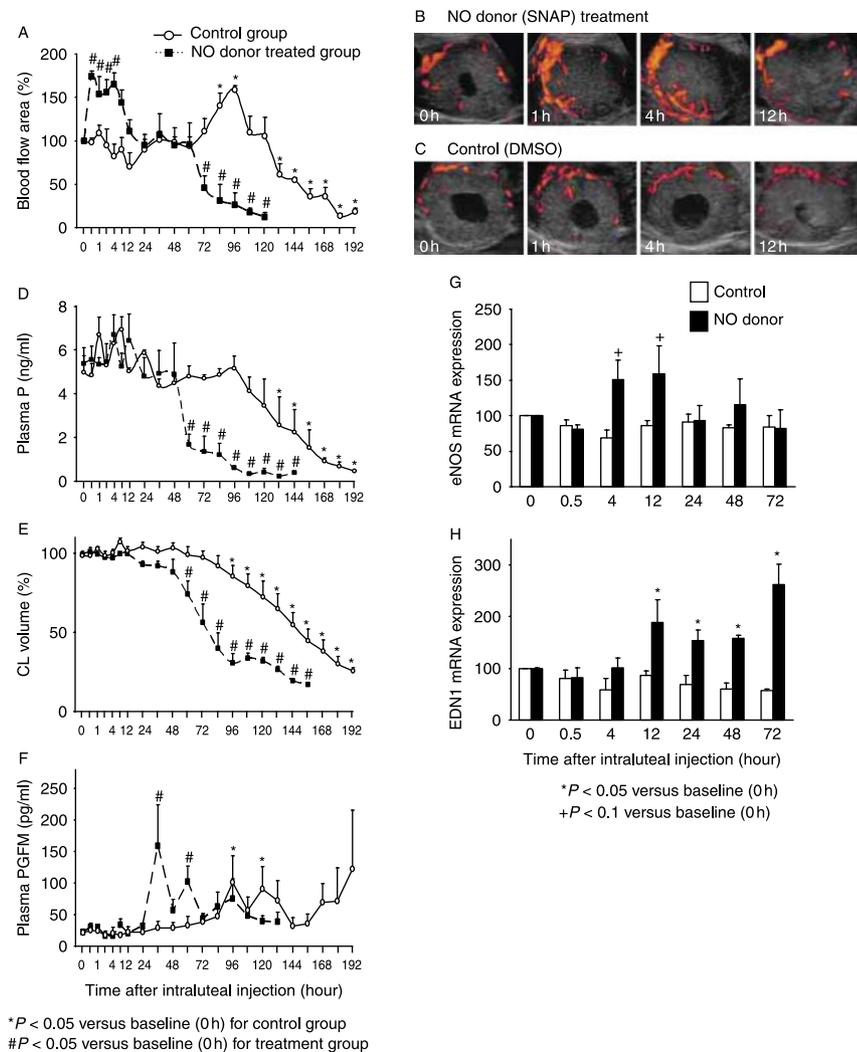
Figure 6 Effect of NO donor (SNAP) or control (DMSO) treatment on plasma P, luteal blood flow area, plasma PGFM, CL volume, luteal blood flow images, *eNOS* mRNA, and *EDN1* mRNA. The experiments were started on day 14 of the estrous cycle in the cow. The NO donor (S-nitroso-*N*-acetylpenicillamine, SNAP; 10 mg/ml and 500 µl, $n = 5$) or dimethyl sulfoxide (DMSO) as control ($n = 4$) was injected directly into the CL twice at 0 h (first injection) and 4 h. (A) Change of luteal blood flow area. (B and C) Typical image of luteal blood flow treated by NO donor (SNAP) and DMSO. (D–F) Change of plasma P concentration, CL volume, and plasma PGFM concentration. Control group is marked with a solid line and NO donor-treated group is marked with a dashed line (A and D–F). (G and H) The mRNA expression of *eNOS* and *EDN1*; white bar indicates control group, and black bar indicates NO donor-treated group. The mean values of luteal blood flow area, volume of CL and mRNA expressions, the mean values of 0 h were used to calculate the baseline for each measurement (defined as 100%) and all values were expressed as a percentage of the corresponding baseline. Mean ± S.E.M. are presented. Asterisk (*) and sharp (#) indicate statistically different values ($P < 0.05$). Cross (+) indicates tendency towards significant difference ($P < 0.1$).

shortened the estrous cycle. In contrast, injection of the NOS inhibitor L-NAME completely suppressed the increase in luteal blood flow and delayed the onset of luteolysis in response to $PGF_{2\alpha}$ administration. These findings suggest that the acute increase in luteal blood flow during luteolysis may be mediated by the local action of NO on the arterioles in the periphery of the CL.

It is well known that the pulsatile release of $PGF_{2\alpha}$ from the uterus on days 17–18 of the estrous cycle initiates luteolysis in the cow (Knickerbocker *et al.* 1988). In the present study, increase in luteal blood flow in the periphery of the CL on days 17–18 correlated with the peak levels of plasma PGFM, prior to the decline in luteal P secretion (Fig. 1). These observations suggest that pulsatile release of $PGF_{2\alpha}$ from the uterus stimulates the increase in luteal blood flow. A recent report by Ginther *et al.* (2007) provided additional evidence showing that CL blood flow increased with each PGFM pulse during spontaneous luteolysis in the cow. In a previous study using an electromagnetic flow transducer probe

surgically placed around the largest ovarian artery ipsilateral to the CL, we demonstrated an acute increase in blood flow volume into the ovary 30 min to 2 h after $PGF_{2\alpha}$ injection (Miyamoto *et al.* 1997a, 1997b). Taken together, these observations indicate that the increase in luteal blood flow is a common phenomenon in both spontaneous and $PGF_{2\alpha}$-induced luteolysis in the cow.

NO is a potent vasorelaxant and appears to be a good candidate to mediate the increase in luteal blood flow. Previous studies have suggested that NO may be an important mediator of luteolysis in the cow (Skarzynski *et al.* 2000, 2003). Other studies in the rabbit provide evidence for the existence a positive feedback mechanism between $PGF_{2\alpha}$ and NOS in the CL (Motta *et al.* 1999). In the present study, we did not observe changes in *iNOS* mRNA expression during the estrous cycle. However, the expression of *eNOS* mRNA was increased on days 17–18 coincident with the increase in blood flow observed in the periphery of the CL during natural CL regression. We also provided evidence for the
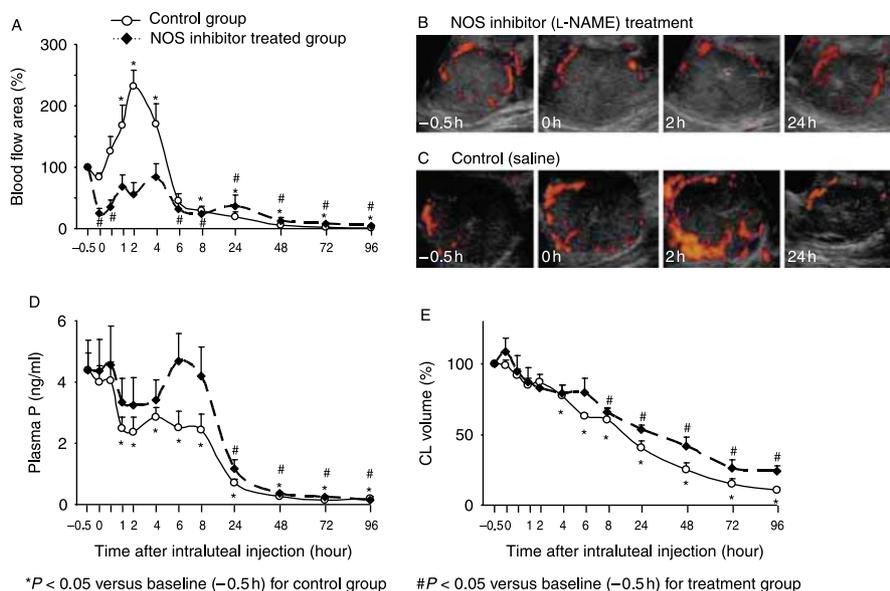
Figure 7 Effect of the NOS inhibitor (L-NAME) or control (saline) treatment during PGF$_{2\alpha}$-induced luteolysis in the cow on plasma P, luteal blood flow area, CL volume, and luteal blood flow images. NOS inhibitor (L-NAME, 50 mg/ml and 1 ml, $n=5$) was directly injected into the CL four times at -0.5 h, 0 h (PGF$_{2\alpha}$ administration), 2 h, and 4 h together with PGF$_{2\alpha}$ administration on day 14 of the estrous cycle in the cow (saline directly was injected within the CL as control group, $n=5$). (A) Change of luteal blood flow area. (B and C) Typical image of luteal blood flow treated by NOS inhibitor (L-NAME) and saline as control. (D and E) Change of plasma P concentration and CL volume. Control group are marked with a solid line, and NOS inhibitor-treated group are marked with a dashed line (A, D, and E). The mean values of luteal blood flow area and volume of CL, the mean values of -0.5 h were used to calculate the baseline for each measurement (defined as 100%), and all values were expressed as a percentage of the corresponding baseline. Mean $\pm$ S.E.M. are presented. Asterisk (*) and sharp (#) indicate statistically different values ($P<0.05$).

presence of large blood vessels possessing smooth muscle layers in the periphery of the CL, which should be capable of regulating blood flow to the CL. In the rabbit CL, the majority of large blood vessels with smooth muscle layers also exist in the peripheral area, but not in the central, of the CL (Wiltbank *et al.* 1990). The results of this study provide morphological and functional evidence for differential regulation of blood flow in the periphery and center of the CL. Thus, we suggested that the acute increase in luteal blood flow occurs in the peripheral arterioles of the CL and this phenomenon is induced by NO in response to uterine-derived PGF$_{2\alpha}$.

Previous reports indicate that an injection of PGF$_{2\alpha}$ stimulated *eNOS* mRNA expression within the CL in the sheep (Vonnahme *et al.* 2006) and in the rabbit (Boiti *et al.* 2003). Moreover, plasma nitrite/nitrate concentrations increased immediately during the first 2 h after an injection of PGF$_{2\alpha}$ analog in the cow (Skarzynski *et al.* 2003). To extend these studies, we investigated the effect of PGF$_{2\alpha}$ on the early CL (resistant to PGF$_{2\alpha}$-induced blood flow increases and luteolysis) and mid-CL (sensitive to PGF$_{2\alpha}$). In addition, we analyzed the effect of PGF$_{2\alpha}$ on *eNOS* expression in the periphery and center regions of the CL. Consistent with a lack of effect of PGF$_{2\alpha}$ on luteal blood flow in early CL, PGF$_{2\alpha}$ did not increase *eNOS* mRNA and immunostaining in the early CL. In contrast, we observed that PGF$_{2\alpha}$ acutely

stimulated the expression of *eNOS* mRNA and protein in the periphery, but not in the center, of the mid-CL. These results indicate that PGF$_{2\alpha}$ stimulated *eNOS* expression is correlated with the luteal blood flow increase in periphery of the mid-CL. Thus, the present study clearly shows that PGF$_{2\alpha}$ has a site-restricted action depending on not only luteal phase but also the region in the CL. Indeed, we recently reported that to mimic the local luteal region both of the periphery and the center of the CL, we utilized co-cultures using endothelial cells, smooth muscle cells and luteinized granulosa cells (Shirasuna *et al.* 2008). Therefore, PGF$_{2\alpha}$ rapidly stimulated the expression of *eNOS* mRNA in the periphery model (co-cultures of endothelial cells, smooth muscle cells, and luteinized granulosa cells) than in the center model (co-cultures of endothelial cells and luteinized granulosa cells). Thus, the three-dimensional structure of luteal tissue and cell–cell interactions appears to be required for maximal responsiveness to PGF$_{2\alpha}$.

The present series of experiments suggest that luteal NO has multiple roles during luteolysis. First, we observed that the administration of the NO donor (SNAP) into the CL mimicked the actions of PGF$_{2\alpha}$ on the acute increase in luteal blood flow and expression of *EDN1* and *eNOS* mRNA. Also, direct injection of the NO donor into the CL clearly reduced in plasma P and CL volume resulting in a shortening of the estrous cycle.

In further support for a role for NO in luteal blood flow, we found that the injection of the NOS inhibitor (L-NAME) completely suppressed the acute increase in luteal blood flow induced by $PGF_{2\alpha}$. Moreover, administration of the NOS inhibitor also delayed the decrease in P secretion and CL volume. These findings strongly suggest that NO has a potential to regulate luteal blood flow and a luteal NO is a crucial factor to initiate luteolysis due to induce a drastic increase in luteal blood flow in the cow. Our results on functional (plasma P) and structural regression (CL volume) are consistent with previous reports that the NOS inhibitor L-NAME prevents the occurrence of spontaneous and $PGF_{2\alpha}$-induced luteolysis and extends the functional life of the CL in the cow (Jaroszewski & Hansel 2000, Skarzynski *et al.* 2003). Furthermore, treatment of bovine luteal cells with NO donors *in vitro* directly inhibits P secretion (Skarzynski & Okuda 2000) and induces apoptosis as observed by increased DNA fragmentation and expression of *FAS*, *BAX*, and caspase-3 mRNA (Korzekwa *et al.* 2006). The present series of experiments indicate that luteal NO has multiple roles during luteolysis that involve an increase in luteal blood flow, functional luteolysis as observed by a decrease in P secretion, and structural luteolysis as observed by a reduction in luteal volume.

The present study provides the first evidence that $PGF_{2\alpha}$ exerts different effects on blood flow and gene expression at the periphery and the center of the CL. The actions of $PGF_{2\alpha}$ are thought to be mediated by a plasma membrane receptor termed FPr (Anderson *et al.* 2001), although indirect actions of $PGF_{2\alpha}$ cannot be ruled out since our studies were conducted in a physiological context *in vivo*. To determine the site of action of FPr, immunohistochemistry was performed in sections of the peripheral and central regions of the mature bovine CL. As previously reported (Rao *et al.* 1979, Wiltbank *et al.* 1995), we observed that the steroidogenic luteal cells express FPr. We also provide new evidence that FPr staining is expressed in the larger blood vessels in the peripheral region of the mature CL. Interestingly, vascular interspace and vascular diameter expanded in the periphery of the CL after $PGF_{2\alpha}$ injection compared with values obtained from saline-injected control animals. This observation supports the idea that $PGF_{2\alpha}$ induces vasodilation in the periphery but not in the center of the mature CL. There are conflicting reports on the presence (Mamluk *et al.* 1998, Meidan *et al.* 2005) or absence of FPr (Liptak *et al.* 2005) in bovine luteal microvascular endothelial cells. The differences observed in FPr may be due to the documented presence of multiple endothelial cell types in the CL (Spanel-Borowski 1991). However, based on our current results it seems very likely that resistance blood vessels in the CL also express FPr. Detailed analysis of FPr staining patterns to specific cell types will require additional investigation. It seems reasonable to propose that $PGF_{2\alpha}$

may act on blood vessels and luteal cells in the periphery of the CL to stimulate the expression of eNOS and vasodilation, thus inducing an acute increase in luteal blood flow during the early stages of luteolysis. Future studies will focus on the localization and function of FPr in blood vessel types and their position within the CL.

There is a basic concept that shear stress is generated when blood flow is increased (Lie *et al.* 1970). Shear stress is the frictional tangential force imposed on the vessel wall when blood flows through a vessel. Shear stress modulates many physiological processes and is a potent stimulation for NO production and vasodilation (Davies 1995). In fact, NO production and expression of *eNOS* mRNA and protein were elevated by shear stress in a graded fashion in the ovine fetoplacental artery endothelial cells (Li *et al.* 2003, 2004). The acute burst of NO production may relate to an increase in eNOS activity, probably via increase in intracellular Ca^{2+} and phosphorylation of eNOS (Li *et al.* 2004). Indeed, the upregulation of *eNOS* mRNA by shear stress is suppressed by Ca^{2+} chelation in the bovine aorta endothelial cells (Xiao *et al.* 1997). Moreover, $PGF_{2\alpha}$ acutely increases intracellular-free Ca^{2+} in bovine luteal cells (Davis *et al.* 1987). It is possible that luteolytic pulses of $PGF_{2\alpha}$ stimulate eNOS activity by inducing intracellular Ca^{2+} and simultaneously increasing luteal blood flow/shear stress in periphery of the CL. Of interest is a report that shear stress also rapidly upregulates *EDN1* mRNA in human microvascular endothelial cells (Morawietz *et al.* 2000), which could also increase eNOS activity via increase in intracellular Ca^{2+}. In the present study, injection of the NO donor stimulated *EDN1* mRNA expression. Therefore, luteal blood flow and shear stress generated by NO action may have the potential to regulate luteolysis by stimulating *EDN1*, another potential luteolytic mediator in the cow. However, because of difficulty to experimentally control luteal blood flow independent of NO action *in vivo*, the exact evaluation of physiological impact of the blood flow increase awaits further investigation.

In conclusion, the present study clearly shows that $PGF_{2\alpha}$ has a site-restricted action depending on not only luteal phase but also the region in the CL. Our results demonstrate that $PGF_{2\alpha}$ stimulates eNOS expression, vasodilation of blood vessels, and increased luteal blood flow in the periphery of the mature CL. Furthermore, pharmacologic studies indicate that the increased blood flow is mediated by NO. Therefore, we suggest that the acute increase in peripheral blood flow to the CL is one of the first physiologic indicators of NO action in response to $PGF_{2\alpha}$.

Materials and Methods

All experiments were conducted at the Field Center of Animal Science and Agriculture, Obihiro University, and all the experimental procedures complied with the Guidelines for

the Care and Use of Agricultural Animals of Obihiro University. Multiparous non-lactating Holstein cows were used for this study; each had at least two estrous cycles of normal length (21–23 days) before being used. Luteolysis was induced by i.m. injection of 500 μg $PGF_{2\alpha}$ analog (cloprostenol: Estrumate; Takeda Co., Osaka, Japan); and 100 μg GnRH (Conceral; Takeda Co.) were injected intramuscularly 48 h after the $PGF_{2\alpha}$ injection to ensure ovulation. The day of estrus was designated as day 0.

Experiment 1: luteal blood flow and mRNA expression of *eNOS, iNOS, and* EDN1 *during spontaneous luteolysis in the cow*

In experiment 1, to investigate whether luteal blood flow increases prior to starting luteolytic cascade during spontaneous luteolysis, we characterized the changes in local blood flow together with the expression of *eNOS, iNOS*, and *EDN1* mRNA in luteal tissue during spontaneous CL regression. Eight multiparous non-lactating Holstein cows were used for this study as described previously. The CL were examined by transrectal ultrasonography using an ultrasound scanner (Aloka SSD-5500; Mitaka, Tokyo, Japan) equipped with a 7.5 MHz convex transducer. Ultrasonographic examinations of the CL were carried out twice a day from day 10 of the estrous cycle until the observation of next estrus. During each ultrasonographic examination, the volume of CL and the blood flow area within the CL were estimated as described previously (Acosta *et al.* 2002). Areas of color represent regions with a flow velocity higher than 2 mm/s. Simultaneous blood samples were collected by caudal venipuncture for P, estradiol-17β (E), and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) measurements and were immediately frozen at −30 °C until further analysis.

Tissue biopsy samples were obtained from five of the eight cows in this study. Transvaginal ultrasound-guided biopsy of the CL was conducted at four intervals: days 10–13, 14–16, 17–18 (after observation of increased luteal blood flow), and 19. Biopsies were performed as described by Tsai *et al.* (2001) using an 18-guage biopsy needle (US Biopsy, Division of Promex Inc., Indianapolis, IN, USA) mounted on an 18G stainless steel needle guide attached to a 7.5 MHz transvaginal convex transducer (UST-M15-21079; Aloka Co, Tokyo, Japan) and an ultrasound scanner (SSD-5500, Aloka Co.). The biopsy needle was inserted in to the needle channel of the transvaginal probe and 3–5 mg CL tissue were collected. The CL tissue sample was then immediately placed into a 1.5 ml microcentrifuge tube containing 0.4 ml TRIzol reagent (Gibco BRL), immediately homogenized, and stored at −80 °C until being analyzed.

center regions of the CL at the early and mid-luteal phases (immature or early CL versus mature or mid-CL). Eighteen multiparous non-lactating Holstein cows were used for this study. The experiments were conducted on day 4 (early CL) and on days 10–12 (mid-CL). Cows were either injected with $PGF_{2\alpha}$ or saline as control (early CL control, $n=5$; early CL $PGF_{2\alpha}$ treated, $n=5$; mid-CL control, $n=4$; and mid-CL $PGF_{2\alpha}$ treated, $n=4$). After 30 min of the injection of $PGF_{2\alpha}$ or saline, luteal blood flow was determined using color Doppler ultrasound; and thereafter the cows were immediately transvaginal ovariectomized as described previously (Berisha *et al.* 2000). To examine the local effect of $PGF_{2\alpha}$ in the CL, tissue samples were collected from regions designated as the periphery (in the range of 1 mm from boundary between luteal tissue and ovarian parenchyma) and the center of the CL (in the range of 1.5 mm from the center of the CL). Immediately after collection, samples were placed into a 1.5 ml microcentrifuge tube containing 0.4 ml TRIzol reagent, homogenized, and stored at −80 °C until analysis. For immunohistochemistry, the CL was enucleated from the ovary and dissected free of connective tissue. Tissue samples from the periphery and center of the CL were fixed in Bouin's fixatives.

Experiment 3: effects of the NO donor SNAP on blood flow and the life span of the CL during spontaneous luteolysis

In experiment 3, we hypothesized that NO stimulated by $PGF_{2\alpha}$ increases luteal blood flow in the periphery of the CL, therefore, we investigated the effect of NO on the luteal blood flow during spontaneous luteolysis. Nine multiparous non-lactating Holstein cows were used for this study. The experiment was initiated on day 14 of the estrous cycle. The NO donor (S-nitroso-*N*-acetylpenicillamine, SNAP; 10 mg/ml and 500 μl, $n=5$) or an identical amount of vehicle (dimethyl sulfoxide, DMSO) as control ($n=4$) was directly injected to the CL at 0 h (first injection) and 4 h after the first injection. The transvaginal injections to the CL were performed as described in our previous study (Watanabe *et al.* 2006). To determine the changes in mRNA expression within the CL, luteal mini-biopsies were conducted at 0, 0.5, 4, 12, 24, 48, and 72 h after the first injection as described previously (Watanabe *et al.* 2006). Ultrasonographic examinations and blood sampling were carried out at 0, 0.5, 1, 2, 4, 6, 8, and 12 h; and thereafter once a day until estrus. The CL tissue samples were immediately placed into a 1.5 ml microcentrifuge tube containing 0.4 ml TRIzol reagent, immediately homogenized, and stored at −80 °C until being analyzed. Plasma samples were immediately frozen at −30 °C until further analysis.

Experiment 2: luteal phase (early CL versus mid-CL)-and site (periphery versus center of the CL)-dependent effects of $PGF_{2\alpha}$

We hypothesized that luteolytic $PGF_{2\alpha}$ induces acute increase in luteal blood flow in the periphery of the mature CL stimulating NO production (eNOS stimulation), therefore, the objective of experiment 2 was to determine the acute effects of $PGF_{2\alpha}$ administration on eNOS expression in the periphery and

Experiment 4: the effect of NOS inhibitor L-NAME on blood flow and the luteolytic cascade during $PGF_{2\alpha}$-induced luteolysis

In experiment 4, to investigate whether $PGF_{2\alpha}$-induced acute increase in luteal blood flow is occurred by mediating NOS–NO system, we determined whether the administration of an NOS inhibitor affected the occurrence of the acute increase in luteal blood flow and CL regression during $PGF_{2\alpha}$-induced

luteolysis. Ten multiparous non-lactating Holstein cows were used for this study. An NOS inhibitor (L-NAME; 50 mg/ml and 1 ml, $n = 5$) was directly injected to the CL at -0.5 h prior to $PGF_{2\alpha}$ administration, concomitant with $PGF_{2\alpha}$ administration (time 0), and 2 and 4 h after $PGF_{2\alpha}$ administration on day 14 of the estrous cycle. The control group ($n = 5$) received saline injections directly to the CL. Ultrasonographic examinations of blood flow and CL size, and blood sampling were carried out at -0.5, 0, 0.5, 1, 2, 4, 6, and 8 h following $PGF_{2\alpha}$ treatment; and thereafter once a day until estrus. Plasma samples were immediately frozen at $-30\,^\circ$C until further analysis.

Hormone determination

After extraction, the concentrations of P, E, and PGFM in plasma samples were determined in duplicate by second-antibody enzyme immunoassays (EIAs) using 96-well ELISA plates (NUNC-Immuno Plate, NUNC, Kampstrup, Denmark).

The extraction and EIA for P in plasma were performed as described previously (Miyamoto *et al*. 1992). The recovery of P from plasma was 85%. The standard curve ranged from 0.05 to 50 ng/ml, and the ED50 of assay was 2.4 ng/ml. The intra- and inter-assay coefficients of variation (CVs) averaged 6.2 and 9.3% respectively.

The extraction and EIA for E in plasma were performed as described previously Acosta *et al*. (2000). The recovery of E from plasma was 85%. The standard curve ranged from 1.95 to 2000 pg/ml, and the ED50 of assay was 5.2 pg/ml. The intra- and inter-assay CVs averaged 6.7 and 8.7% respectively.

The extraction and EIA for PGFM in plasma were performed as described previously (Shirasuna *et al*. 2004*b*). The recovery of PGFM from serum was 70%. The standard curve ranged from 2.5 to 2500 pg/ml, and the ED50 of assay was 78 pg/ml. The intra- and inter-assay CVs averaged 7.7 and 12.5% respectively.

RNA extraction

Total RNA was extracted from CL biopsy samples following the protocol of Chomczynski & Sacchi (1987) using TRIzol reagent. The yield of extracted total luteal RNA for each sample was determined by u.v. spectroscopy (optical density, 260 nm). The RNA concentration was measured using Bio-Tech Photometer (WPA, Cambridge, UK) at 260 and 280 nm absorbance. The extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at $-80\,^\circ$C until used for cDNA production.

cDNA production

RNA samples were treated with DNase using the RQ1 RNase-free DNase kit (Promega Co). RNA (2 μl of 1 μg/μl) was incubated for 30 min at 37 °C with 1 μl RQ1 RNase-free DNase 10× reaction buffer and 2 μl of 1 μg/μl RNase-free DNase. Then, 1 μl RQ1 DNase stop solution (20 mM EGTA) was added to terminate the reaction and incubated again for 10 min at 65 °C. First-strand cDNA synthesis was conducted according to a commercial protocol described in SuperScript II Reverse Transcriptase (Invitrogen Corp). The synthesized cDNA was stored at $-30\,^\circ$C.

Real-time reverse transcription-PCR (real-time RT-PCR)

The levels of mRNA for *eNOS*, *iNOS*, *EDN1*, and β-actin were quantified by real-time PCR with a LightCycler (Roche Diagnostics Co.) using a commercial kit (LightCycler FastStart DNA Master SYBR Green I; Roche Diagnostics Co). The primers were designed using primer 3, based on bovine sequences. The amplification program consisted of 15-min activation at 95 °C followed by 40 cycles of PCR steps (15-s denaturation at 94 °C, 30-s annealing at 58 °C, and a 20-s extension at 72 °C). For the quantification of the target genes, a series of standards were constructed by amplifying a fragment of DNA (150–250 bp) that contains the target sequence for real-time PCR. The primers used for real-time PCR were as follows: *eNOS* (219 bp) forward 5′-GGAAATCGGGGGTCTG-GAGT-3′ and reverse 5′-TTGGCGAGCTGAAAGCTGTG-3′; *iNOS* (167 bp) forward 5′-TCATCTTCGCCACCAAGCAG-3′ and reverse 5′-CAGTGATGGCCGACCTGATG-3′; *EDN1* (160 bp) forward 5′-CAAATGCATCCTGCCTGGTC-3′ and reverse 5′-ATTGCCACCCCCATAGAGGA-3′; and β-actin (256 bp) forward 5′-CCAAGGCCAACCGTGAGAAAAT-3′ and reverse 5′-CCACATTCCGTGAGGATCTTCA-3′. The PCR products were subjected to electrophoresis, and the target band cut out and purified using a DNA purification kit; SUPRECTM-01 (TaKaRa Bio. Inc., Otsu, Japan). Three to five stepwise-diluted DNA standards were included in every PCR run. The quantification of mRNA expression was done using LightCycler Software (Version 3.5; Roche). Primer sets were tested in luteal tissue samples to confirm amplification of single bands, amplified products were cloned and sequenced to confirm their identity, prior to use of primers in analysis of samples. The values were normalized using β-actin as the internal standard.

Immunohistochemistry

The ovaries were transported to the laboratory within 10 min on ice immediately after surgical removal. The CL was enucleated from the ovary and dissected free of connective tissue. Tissue samples were fixed in Bouin's fixatives for 24 h at room temperature and then embedded in paraffin wax. Serial sections (5 μm) were mounted on to APS-coated glass microscope slides. The sections were stained with hematoxylin–eosin (H–E) for general histological observations.

Light microscopic immunohistochemical staining employing the avidin–biotin peroxidase complex (ABC) method (Hsu *et al*. 1981) was used in the present study. The sections were deparaffinized in xylene, rehydrated in graded series of ethanol, and washed in distilled water (DW). Subsequently, endogenous peroxidase was inactivated by treatment with 0.3% H_2O_2 in methanol for 10 min at room temperature. The slides were then washed in 0.01 M PBS (pH 7.4). After treatment with normal goat serum (2%) for 30 min at room temperature, the sections were incubated with polyclonal antibodies for von Willebrand factor (vWF; Dako, diluted 1:200), which is a marker of endothelial cells, smooth muscle actin (SMA; Dako, M0851, diluted 1:200), eNOS (PA1-037, diluted 1:50; Affinity BioReagents, Golden, CO, USA), and FPr (Cat no. 101802, diluted 1:100; Cayman Chemical Corp.,

Ann Arbor, MI, USA) overnight at 4 °C. As a negative control, the sections were incubated with goat anti-rabbit IgG overnight at 4 °C. After the incubation, the sections were washed in PBS, incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (1:200, BA-1000; Vector Laboratories Inc; Burlingame, CA, USA) for vWF, eNOS, FPr, and negative control and with biotinylated goat anti-mouse IgG (1:200, BA-9200, Vector Laboratories Inc.) for SMA, and then washed in PBS. Horseradish peroxidase (HRP)-conjugated ABC (1:2, PK-6100, Vectastain *Elite* ABC kit; Vector Laboratories Inc.) combined with secondary antibody was applied to tissue slides at room temperature for 30 min. The binding sites were visualized with 0.02% 3,3′-diaminobebzidine tetrahydrochloride (DAB) in 50 mM Tris–HCl (pH 7.4) containing 0.02% H_2O_2. After immunohistochemical staining, the sections were lightly counterstained with Mayer's hematoxylin. The sections were washed in DW, dehydrated in graded series of ethanol, and cleared in xylene and coverslipped.

Quantification methods

Number of blood vessels

The number of blood vessels was determined by counting the number of SMA-positive vessels per unit area in the histological section at 200× as described in a previous study (Sugino *et al*. 2005). Due to the regional variations in size, blood vessels were grouped depending on their diameter, one group consisted of large blood vessels (>20 μm) and another group consisted of microvascular vessels (<20 μm). Histological sections were obtained from three animals in each group. The number of blood vessels in each group was determined by counting three randomly chosen areas both at the periphery and the center of the CL. Quantification was performed independently by three observers. Average values obtained from each tissue section were used to determine the means ± s.e.m. from multiple sections from different animals. The results were expressed as number means ± s.e.m. per unit area.

Percentage area of eNOS immunostaining

The positive staining areas were extracted using PopImaging (Version 3.01; Digital Being Kids, Yokohama, Japan) to calculate the percentage area of the immunostaining (area of the immunostaining divided by the total area measured × 100) as described previously (Al-zi'abi *et al*. 2003). Areas were analyzed at 200× magnification using one section from each animal and five fields per section. The results were expressed as percentage means ± s.e.m. per unit area (early CL control, $n=5$; early CL PGF$_{2\alpha}$ treated, $n=5$; mid-CL control, $n=4$; and mid-CL PGF$_{2\alpha}$ treated, $n=4$).

Statistical analysis

All data are presented as means ± s.e.m. In experiment 1, for data analysis the mean values of luteal blood flow area and volume of CL during days 10–13 were used to calculate the baseline for each measurement (defined as 100%), and all values are expressed as a percentage of the corresponding baseline. For data analysis on mRNA levels in experiment 1, the

experimental period was divided into four periods (days 10–13, 14–16, 17–18, and 19), and the data during days 10–13 were used to calculate the baseline for each measurement (defined as 100%). The mRNA levels for each ligand were normalized using β-actin mRNA (ratio of ligand:β-actin) and expressed as a percentage of this individual baseline values. In experiment 2, the positive areas of eNOS staining were presented as a percentage of total area. The number of blood vessels was determined by counting, and the results were expressed as number means ± s.e.m. per unit area. In experiments 3 and 4, the mean values of luteal blood flow area and volume of CL at 0 h (experiment 3) or −0.5 h (experiment 4) were used to calculate the baseline for each measurement (defined as 100%), and all values were expressed as a percentage of the corresponding baseline. The statistical significance of differences was assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities <5% ($P<0.05$) were considered significant.

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