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Nitric Oxide and Luteal Blood Flow in the Luteolytic Cascade in the Cow

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Abstract. The corpus luteum (CL) of the estrous cycle in the cow is a dynamic organ which has a lifespan of approximately 17–18 days. The main function of the CL is to produce progesterone (P) that requires for achievement and maintenance of pregnancy. If pregnancy does not occur successfully, the CL must regress within a few days to induce the next chance of ovulation. As the CL matures, the steroidogenic cells establish contact with many capillary vessels, consequently the CL is composed of a large number of vascular endothelial cells that can account for up to 50% of all cells. Therefore, blood vessels and blood flow within the CL have an essential role in luteal function. Nitric oxide (NO), a strong vasorelaxant, is now known to play key roles in a variety of physiological process. Indeed, NO has established itself as a polyvalent molecule which plays a decisive role in regulating multiple functions within female reproductive system. In the CL, NO is produced and regulates luteal blood flow, P secretion and apoptosis of luteal cells as well as endothelial cells. This review describes the current investigation for possible roles of NO in the luteolytic cascade within the bovine CL.
Key words: Corpus luteum, Luteal blood flow, Luteolysis, Nitric oxide

The corpus luteum (CL) is a transient organ in the ovary of mammals. The bovine CL rapidly develops within 2–3 days after ovulation, which is accompanied by an active angiogenesis and vascularization from the preovulatory follicle. The main function of the CL is to produce progesterone (P) that requires for achievement and maintenance of pregnancy [1]. If pregnancy does not occur successfully, the CL is only functional for 17–18 days and it must regress within a few days to induce the next chance of ovulation. In ruminants, it is well known that a pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus on days 17–18 of the estrous cycle is essential to induce regression of the CL [2]. Luteolytic $PGF_{2\alpha}$ induces a drastic decrease in P release from the CL as well as CL volume and blood flow to the CL in the non-pregnant cow [3, 4]. Thus, it is easily considered that the bovine CL has well designed mechanisms by which development and regression are effectively controlled.

In the cow, the CL is composed of a large number of vascular endothelial cells that can account for up to 50% of all the CL cells [5]. On the other hand, steroidogenic large luteal cells and small luteal cells constitute about 30% of all CL cells [5, 6]. The bovine CL also has various cell types such as smooth muscle cells, pericytes, fibrocytes and immune cells, indicating that the CL is a heterogeneous tissue [7, 8]. In addition, the CL is constituted by the condensed blood vasculature including large blood vessels and microcapillaries, and the majority of steroidogenic cells are adjacent to one or more capillaries [9]. It is known that a cell-to-cell

interaction and these adhesions are essentially important for the CL integrity and physiological functions. Additionally, the bovine CL produces many vasoactive factors such as nitric oxide (NO) [10], endothelin-1 (EDN1) [11–13], angiotensin II (Ang II) [14], and $PGF_{2\alpha}$ [15, 16]. In fact, these factors are involved in the regulation of CL blood flow and P secretion in the cow.

Nitric Oxide in the CL

NO is now known to play functional roles in a variety of physiological systems. Indeed, NO has established itself as a polyvalent molecule which plays a decisive role in regulating multiple functions within female as well as the male reproductive system. NO is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms. Neuronal NOS (nNOS; type I) and endothelial NOS (eNOS; type III) are responsible for the continuous basal release of NO and require calcium/calmodulin for activation [17]. On the other hand, inducible NOS (iNOS; type II) is calcium-independent enzyme induced in monocytes, macrophages and neutorphils [18].

NO, a potent vasorelaxant, has been suggested to be an important mediator of luteolysis in the cow [19–21], rabbit [22], rat [23, 24] and human [25–27]. The bovine CL has two types of NOS, eNOS and iNOS, and immunostaining of eNOS and iNOS are observed in endothelial cells and luteal cells [20]. Moreover, intensity of reaction of eNOS and iNOS are increased from the early to late luteal phase of the estrous cycle and then decreased in regressed luteal phase in the cow [20]. On the other hand, in the recent our study, many eNOS-positive areas were observed in the early and mid CL, where many arteriolovenous vessels were

observed [28]. Additionally, Rosiansky *et al*. indicated that eNOS mRNA and protein expression were higher in the early and mid luteal phase than in the late and regressed luteal phase during the estrous cycle in the cow [29]. In other species, the area of positive staining and mRNA expression for eNOS in luteal tissues was the greatest in the early luteal phase (Day 4) than in other phases (Day 10 and 15) of the estrous cycle in the ovine [30]. In the human CL, eNOS is the most abundant with highest values during the late luteal phase, but immunoreactive iNOS did not display apparent phase-specific changes [27]. In the pseudopregnant rabbits, although eNOS mRNA expression was dropped almost to half, luteal eNOS protein was increased 2-fold from the early to late luteal phase [31]. On the other hand, iNOS mRNA and protein expression gradually declined from the early to late luteal phase in the pseudopregnant rabbit CL [31].

In the effect of NO on luteal function, treatment of luteal cells with agents that increase NO directly inhibits P secretion in the bovine [21, 32, 33], human [27, 34], rat [23] and rabbit [35]. Moreover, the inhibition of ovarian NOS *in vivo* prolongs the duration of the estrous cycle in the cow [20]. Additionally, NO donor significantly increased production of $PGF_{2\alpha}$ in mixture of luteal cells culture, and NOS inhibitor (L-NAME) stimulated P secretion in the bovine [21] and rat CL [23]. Interestingly, NO has dual actions for luteal function. In the bovine luteal cells, although NO donor (SNAP, 10^{-5}M) drastically decreased P secretion from mid cycle CL, this NO donor did not affect in the early cycle CL [32]. Also, NOS inhibitors stimulated P secretion only in mid luteal cells, although they did not affect the cells from the early CL [32]. In the human [27], rabbit [35] and rat [24] CL, NO donor significantly decreased the production of P in luteal cells of the late luteal phase, but not in the mid luteal phase. However, Weems *et al*. [36] reported that NOS inhibitor infused chronically during the ovine estrous cycle did not delay the decline in P secretion and luteal weight compared with NO donor treated group, suggesting that NO is not luteolytic in vivo in the ewe but instead may be antiluteolytic factor. Therefore, these above findings suggest that the production and role of NO in the CL is dependent on the stage of the estrous cycle and species.

Nitric Oxide in the Luteal Blood Flow and Initiation of Luteolysis

In the CL, blood flow regulates luteal function. It has been proposed that a rapid decrease in luteal blood flow is one of the essential impacts of exogenous and endogenous (uterine) $PGF_{2\alpha}$ [37]. On the other hand, the administration 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 in the mid cycle CL (Day 10 of the estrous cycle), but not in the early cycle CL (Day 4 of the estrous cycle) (Fig. 1) [4]. Additionally, an increase in luteal blood flow in the periphery of the CL on Day 17–18 was associated with increase of eNOS mRNA expression (but not iNOS mRNA) and peak levels of plasma 13, 14 dihydro 15 keto $PGF_{2\alpha}$ (PGFM; a product of the metabolism of the PGF2?), just prior to the decline in P secretion during spontaneous luteolysis in the cow [38, 39]. A recent report provided additional
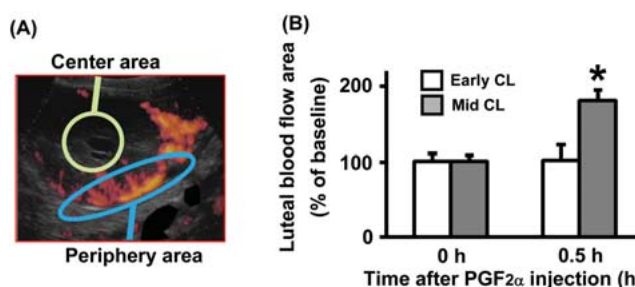

Fig. 1. Investigation of luteal blood flow using color Doppler ultrasound in the bovine CL. Fig. 1A indicates example of luteal blood flow picture. In the periphery area (blue circle), intensive luteal blood flow can observe because large blood vessels (>20 μm) concentrates in this area. On the other hand, luteal blood flow cannot be observed in the center area (green circle) by reason of closely-spaced microcapillary vessels (<20μm). Fig. 1B indicates the change of luteal blood flow area after $PGF_{2\alpha}$ administration during the early and mid luteal phase in the cow. During the mid luteal phase, $PGF_{2\alpha}$ induces the acute increase of luteal blood flow at 0.5 h after administration in the periphery of the CL, whereas the early CL has no effect on luteal blood flow by $PGF_{2\alpha}$ administration. White column indicates the early CL, and gray column indicates the mid CL. All values are shown as the mean ± SEM (n=5). Symbol (✱) indicates significant differences (P<0.05) between 0 and 0.5 h after $PGF_{2\alpha}$-administration as determined by ANOVA followed by the Bonferroni multiple comparison test. Modified from Acosta *et al*. (2002) [4].

evidence that during luteolysis, the CL blood flow increases in response to each peak of uterine $PGF_{2\alpha}$ in the heifer [40]. Thus, the increases in luteal blood flow induced by $PGF_{2\alpha}$ are one of the earliest physiological events observed during the luteolytic cascade in the cow.

NO is a potent vasorelaxant and appears to be a good candidate to mediate the increases in luteal blood flow. NO may be an important mediator of luteolysis in the cow [19, 20], and in the rat the evidence was provided for the existence a positive feedback mechanism between $PGF_{2\alpha}$ and NOS in the CL [23]. To investigate the relationships between increase of luteal blood flow by $PGF_{2\alpha}$ injection and NO (especially eNOS), we collected the CL both from the early and mid luteal phase at 30 min after $PGF_{2\alpha}$ administration in the cow [41]. Consistent with a lack of effect of $PGF_{2\alpha}$ on luteal blood flow in the early CL, $PGF_{2\alpha}$ did not increase eNOS mRNA and immunostaining in the early CL (Fig. 2). In contrast, we observed that $PGF_{2\alpha}$ acutely stimulated the expression of eNOS mRNA and protein in the periphery, but not in center of the mid CL (Fig. 2). In other species, injection of $PGF_{2\alpha}$ consistently stimulated eNOS mRNA expression and/or NOS activity within the CL of sheep [42], rat [23], and rabbit [22]. Moreover, $PGF_{2\alpha}$ upregulates NOS activity in mature CL (Day 9) but not in immature CL (Day 4) of pseudopregnant rabbits [35]. These results indicate that $PGF_{2\alpha}$-stimulated eNOS expression is positively correlated with the luteal blood flow increase in the periphery of the mid cycle CL.

Why does $PGF_{2\alpha}$ stimulate eNOS expression in the periphery of the mid CL but not of the early CL? To fine the answer to this mechanism, we investigated eNOS and vascular distribution according to our hypothesis that more arteriolovenous vessels,
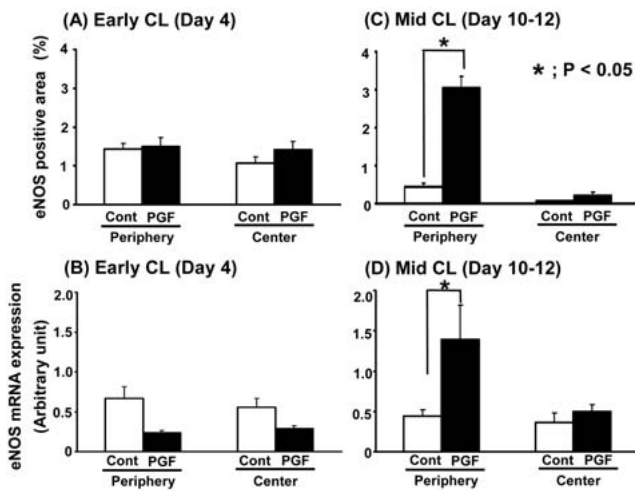
Fig. 2. Image analysis of eNOS immunostaining and expression of eNOS mRNA in the early and mid CL. The experiments were conducted on Day 4 as early CL and Day 10–12 as mid CL, and PGF$_{2\alpha}$ or saline as control was injected respectively (early CL control; n=5, early CL PGF$_{2\alpha}$ treat; n=5, mid CL control; n=4, mid CL PGF$_{2\alpha}$ treat; 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 0.5 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 periphery and center of the CL. Fig. 4A and 4B indicate eNOS positive staining area and eNOS mRNA expression in the early CL, respectively. Fig. 4C and 4D indicate eNOS positive staining area and eNOS mRNA expression in the mid CL, respectively. White bar indicates control group, and black bar indicates PGF$_{2\alpha}$ treated group in each figure. Mean ± SEM are presented. Asterisk indicates statistically different values (P<0.05). Modified from Shirasuna *et al.* (2008) [41].

Fig. 3. Effects of PGF$_{2\alpha}$ treatment on eNOS mRNA expression in co-culture experiment. A) Center model of the early CL, co-cultures of endothelial cells (EC) with luteinizing granulosa cell (GC); B) Center model of the mid CL, co-cultures of EC with fully-luteinized GC; C) peripheral model of the early CL, mix-cultures of EC and smooth muscle cells (SMC) with luteinizing GC; D) peripheral model of the mid CL, mix-cultures of EC and SMC with fully-luteinized GC. Data are shown as the mean ± SEM for each time point (n=3). The data are expressed as the percentage from individual pretreatment levels. The white bar indicates control group and black bar indicates PGF$_{2\alpha}$ treated group (10^{-6}M). Symbols (✱) indicate significantly different values compared to control of each time point as determined by ANOVA followed by the Bonferroni multiple comparison test (P<0.05). Modified from Shirasuna *et al.* (2008) [39].

which are capable of vasodilation, should exist in the periphery of the mid CL and that therefore PGF$_{2\alpha}$ could induce an increase in luteal blood flow in the arteriolovenous vessels, where eNOS is dominantly expressed compared with the center of the mid CL; they are thereby more sensitive to PGF$_{2\alpha}$ in vasodilating response [28]. The bovine CL has two types of blood vessels, arteriolovenous vessels and capillary vessels. Indeed, non-capillary vessels having smooth muscle layers increases markedly in the regressing CL compared with the late CL in the cow [43, 44]. During PGF$_{2\alpha}$-induced luteolysis in the cow and sheep, the endothelial component of the vascular bed decreases, whereas high numbers of smooth muscle cells are maintained during luteal regression, suggesting that pericytes and vascular smooth muscle cells have a role in luteolysis [42, 44]. Importantly, the arteriolovenous vessels existed more in the periphery of the matured CL (mid, late and regressing CL) than in the center region [28]. Indeed, in the early CL, there were as many arteriolovenous vessels in the periphery as in the center, while more capillaries existed in the center than in the periphery of the mid and late CL [28]. Also, eNOS protein existed in the periphery more than in the center of the matured CL. These results indicate that the early CL has a homogeneous, but the
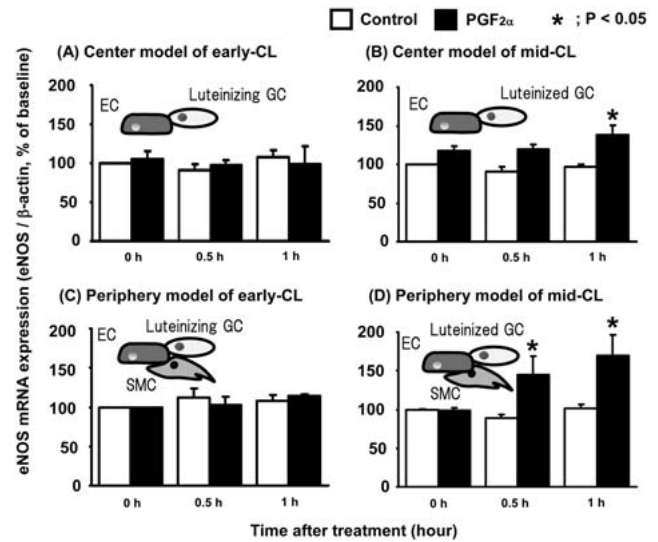
matured CL is a heterogeneous organ having a higher vascular and eNOS distribution in the periphery than in the center [28]. Thus, we suggest that this structural change from the early (homogeneous) to the mid (heterogeneous) luteal phase is related to the difference in the CL response of blood flow increase due to PGF$_{2\alpha}$, which is only observed in the mature CL.

We indicated that PGF$_{2\alpha}$ has a site-restricted action depending on not only luteal phase but also the region in the CL, thus we tried to examine the possible cell-to-cell communication to regulate eNOS expression in response to PGF$_{2\alpha}$. To mimic the local luteal region both of the periphery and center of the CL, we utilized co-cultures system (Fig. 3) using endothelial cells (EC), smooth muscle cells (SMC) and "luteinizing" granulosa cells (GC) or "fully-luteinized" GC [39]. In the *in vitro* system, PGF$_{2\alpha}$ rapidly stimulated the expression of eNOS mRNA at 0.5 h in the periphery model or the mid CL (co-cultures of EC, SMC and "fully-luteinized" GC). On the other hand, the expression of eNOS mRNA was increased by PGF$_{2\alpha}$ at 1 h only in the center model of the mid CL (co-cultures of EC and "fully-luteinized" GC). Interestingly, PGF$_{2\alpha}$ could not affect eNOS mRNA expression both in the periphery and center model of the early CL (co-cultures of "luteinizing"
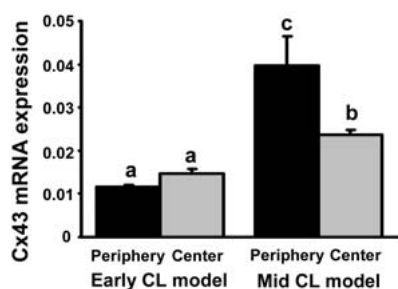
Fig. 4. Relative mRNA levels of Cx43 using co-culture models. In the early CL model (left part), the periphery model is mix-cultures of endothelial cells (EC) and smooth muscle cells (SMC) with luteinizing GC, and the center model is co-cultures of EC with luteinizing granulosa cell (GC). In the mid CL model (right part), the periphery model is mix-cultures of EC and SMC with fully-luteinized GC, and the center model is co-cultures of EC with fully-luteinized GC. Data are shown as the mean ± SEM for each time point (n=3). Different superscript letters indicate significant differences as determined by ANOVA followed by the Bonferroni multiple comparison test (P<0.05).

GC with EC and/or SMC).

In the above experimental model, $PGF_{2\alpha}$ had no effect on the change of mRNA expressions of $PGF_{2\alpha}$ receptor (FPr) and Cx43 during the experimental period in all culture models. One interesting question of the CL function is the refractoriness of the CL to the $PGF_{2\alpha}$ in luteolytic action at the early luteal phase. The FPr mRNA is expressed in the CL at high levels throughout the estrous cycle, and this receptor in the early CL can induce specific response [45]. Although little is known of the mechanisms for refractory in the early CL to the action of luteolytic $PGF_{2\alpha}$, it is clear that it is not due to lack of high affinity FPr in the cow [46]. In the border within the cells, the gap junction is formed with a tunnel-like structure and enables regulatory molecules, nutrients and ions of less than about 1 kDa (i. e., calcium ions, cAMP and inositol 1,4,5-triphosphate) to be exchanged between adjacent cells [47]. Gap junctions are formed by connexin (Cx) proteins such as Cx26, Cx32, Cx43 and Cx45 [48, 49], and Cx43 is the main member of the gap junctional protein family. In the mixed culture model to mimic the CL, the mRNA expression of Cx43 was higher in the model of mid-CL using fully-lutenized GC than in the model of early-CL using lutenizing GC, particularly in the periphery model of mid-CL (Fig. 4). Thus, there is the possibility that gap junction might be formed between EC and fully-lutenized GC/SMC to acquire the responsiveness to $PGF_{2\alpha}$ in the mixed cells and have a role to transfer the luteolytic signal after binding of $PGF_{2\alpha}$ to FPr including up-regulation of eNOS expression. In fact, Cx43 localizes on the borders between luteal cells and endothelial cells in the ovine CL [50]. Thus, the three dimensional structure of luteal tissue and cell-cell interactions appear to be required for maximal responsiveness to $PGF_{2\alpha}$.

Recently, Acosta *et al*. [51] reported that $PGF_{2\alpha}$ induces an acute increase in NO (within 15 min) in the ovarian circulation. From these above investigation, $PGF_{2\alpha}$ clearly stimulates eNOS/NO system to induce luteolysis in the cow. Therefore, we examined the
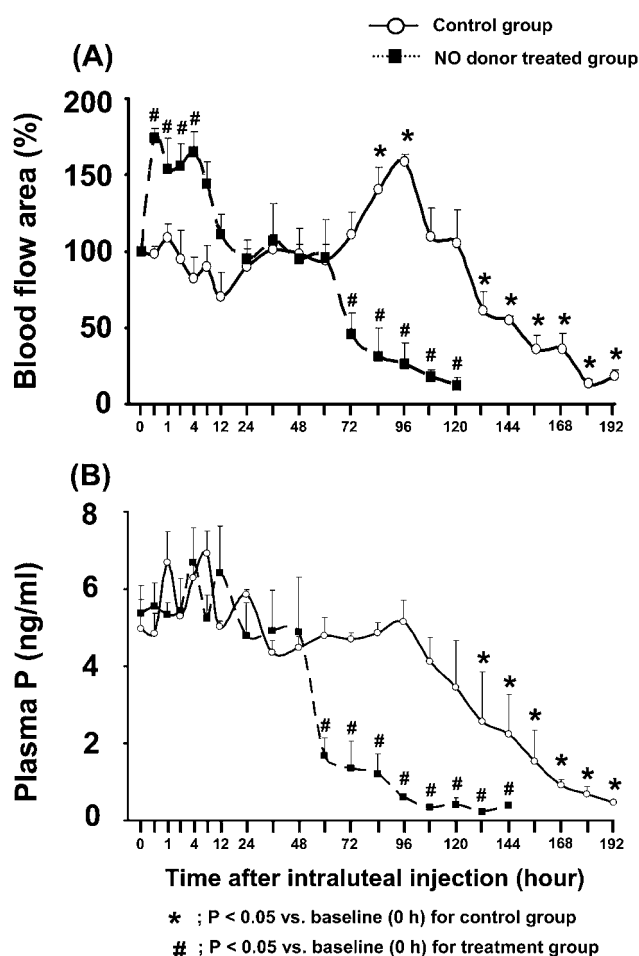

Fig. 5. Effect of NO donor (SNAP) or control (DMSO) treatment on plasma P and luteal blood flow area. The experiments were started on Day 14 of the estrous cycle in the cow. The NO donor (S-nitroso-N-acetylpenicillamine, SNAP; 10 mg/ml; 500 µl, n=5) or dimethylsulfowide (DMSO) as control (n=4) was injected directly into the CL twice at 0 h (first injection) and 4 h. Fig. 5A shows change of luteal blood flow area, and Fig. 5B shows change of plasma P concentration. Control group are marked with a solid line and NO donor treated group are marked with a dashed line. The mean values of luteal blood flow area, 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 ± SEM are presented. Asterisk and sharp indicate statistically different values (P<0.05). Modified from Shirasuna *et al*. (2008) [41].

effect of direct administration into the CL of a NO donor (SNAP) on luteal blood flow (Fig. 5) [41]. As a result, a direct injection of the NO donor into the CL clearly induced an increase in blood flow similar to the response to $PGF_{2\alpha}$ administration. This was followed by an earlier fall in plasma P concentrations by 3 days, a reduced CL volume, and a shortened estrous cycle length. In contrast, the injection of a NOS inhibitor (L-NAME) completely suppressed the acute increase in luteal blood flow induced by $PGF_{2\alpha}$, and also delayed the decreases in P secretion (Fig. 6). Indeed, the NOS inhibitor L-NAME prevented the occurrence of spontaneous and
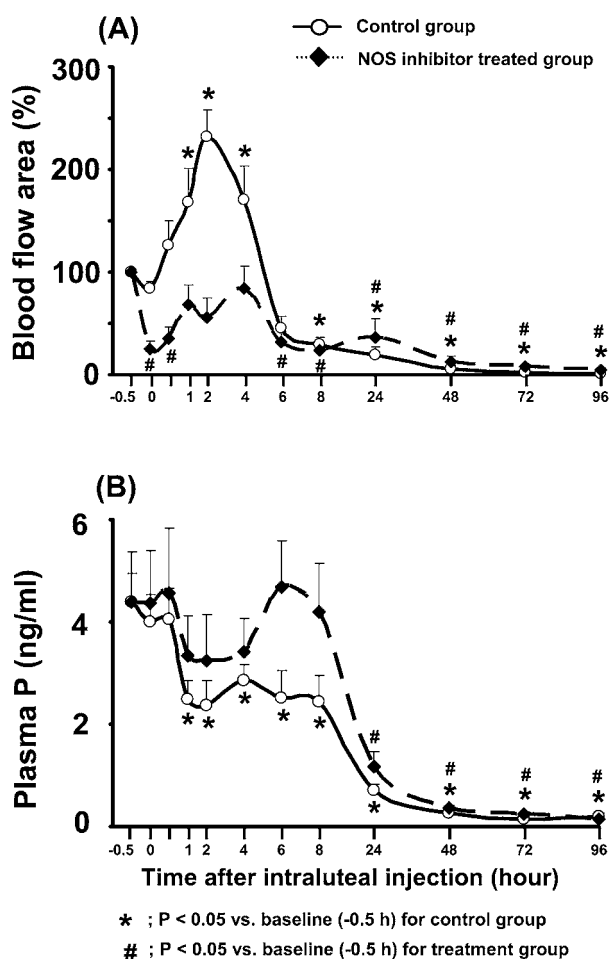
(A)

—O— Control group
···◆··· NOS inhibitor treated group

(B)

Time after intraluteal injection (hour)

* ; $P < 0.05$ vs. baseline (-0.5 h) for control group

; $P < 0.05$ vs. baseline (-0.5 h) for treatment group

Fig. 6. Effect of the NOS inhibitor (L-NAME) or control (saline) treatment during $PGF_{2\alpha}$ -induced luteolysis in the cow on luteal blood flow area and plasma P. NOS inhibitor (L-NAME; 50 mg/ml; 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). Fig. 6A shows change of luteal blood flow area, and Fig. 6B shows change of plasma P concentration. Control group are marked with a solid line and NOS inhibitor treated group are marked with a dashed line. The mean values of luteal blood flow area, 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 ± SEM are presented. Asterisk and sharp indicate statistically different values (P<0.05). Modified from Shirasuna *et al*. (2008) [41].

$PGF_{2\alpha}$-induced luteolysis and extended the functional life of the CL in the cow [20, 52]. Additionally, treatment of bovine luteal cells with NO donors *in vitro* directly not only inhibits P secretion [32] but also induces apoptosis as observed by increased DNA fragmentation and expression of Fas, Bax and caspase-3 mRNA [53]. These findings 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 apoptosis of luteal cells and a reduction in luteal volume. Thus, NO can regulate luteal blood flow and this molecule is one of the main factors to control the luteolytic cascade in the cow.

Conclusion

The acute increase of luteal blood flow is one of the earliest physiological signals for the luteolytic cascade. Luteolytic $PGF_{2\alpha}$ stimulates the eNOS-NO system, then, luteal NO regulates luteal blood flow and is a crucial factor in the initiation of luteolysis by inducing a drastic increase of luteal blood flow in the cow. Additionally, the bovine CL is a large and heterogeneous endocrine organ, in which $PGF_{2\alpha}$ has a site-restricted action depending not only on the luteal phase but also the region within the CL. A three dimensional regulation of vasoacitive factors, vascular structure in the mature CL and interactions among several different cell types appear to be required for maximal responsiveness to $PGF_{2\alpha}$ to achieve a rapid luteolysis in the cow.

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