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Luteolysis in the cow: a novel concept of vasoactive molecules

A. Miyamoto, K. Shirasuna

Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Japan.

Abstract

The corpus luteum (CL) undergoes drastic changes in its function and structure during the estrous cycle. To secrete a sufficient amount of progesterone (P4) to ensure the occurrence of pregnancy in a cow with a body weight greater than 500 kg, the bovine CL weighs 5-8 g which is 2-3 thousand times heavier than rat CL. If pregnancy does not occur successfully, rapid luteolysis is caused by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that is released from the endometrium around days 17-19 of the estrous cycle in the cow. Thus, it is clear that the bovine CL lifespan is controlled by well-coordinated mechanisms. As the CL matures, the steroidogenic cells establish contact with many capillary vessels, so that the CL is composed of a large number of vascular endothelial cells that can account for up to 50% of all cells in the bovine CL. Also, luteal endothelial cells secrete several vasoactive substances such as $PGF_{2\alpha}$, nitric oxide, endothelin-1 and angiotensin II that regulate blood flow as well as P4 secretion in an autocrine/paracrine manner within the CL. Therefore, blood vessels and endothelial cells within the CL have an essential role in luteal function in the cow, suggesting that the study of vasoactive molecules from the CL is of great importance to give an insight into systems which regulate luteolysis locally. In the present review, we describe novel concepts on the luteolytic mechanisms in the cow, with emphasis on luteal blood flow and vasoactive molecules.

Keywords: endothelin-1, luteal blood flow, luteolysis, nitric oxide, prostaglandin $F_{2\alpha}$.

Introduction

In mammalian species, the ovary plays essential roles, both as the site of oocyte production and as an endocrine gland. In the ovary, the corpus luteum (CL) derived from the ovulated follicle is a unique hormone-regulated, transient reproductive gland that produces and secretes progesterone (P4). In cattle and other species, the main function of the CL is to produce P4 that is a prerequisite for implantation and maintenance of pregnancy (Rodgers *et al*., 1988). The bovine CL develops rapidly within 2-3 days after ovulation, supported by active angiogenesis and vascularization, and is functional for 17-18 days in the non-pregnant cow. If pregnancy does not occur successfully, the CL must regress within a few days to allow the opportunity of a new ovulation. In non-pregnant cows, luteolysis is caused by pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that are secreted by the endometrium around days 17-19 of the estrous cycle. $PGF_{2\alpha}$ induces a decrease in P4 release from the CL as well as a decrease in the CL volume and blood flow to the CL (Niswender *et al*., 1976; Acosta *et al*., 2002).

The bovine CL is composed of heterogeneous cell types that consist of not only steroidogenic cells (small and large luteal cells) but also non-steroidogenic cells (endothelial cells, smooth muscle cells, pericytes, fibrocytes and immune cells; Farin *et al*., 1986; Penny, 2000). The steroidogenic cells, particularly large luteal cells, produce and secrete a large amount of P4 during the estrous cycle in the cow (Rodgers *et al*., 1988; Meidan *et al*., 1990; Girsh *et al*., 1995). Vascular endothelial cells represent more than 50% of the total number of cells in the CL (O'Shea *et al*., 1989; Lei *et al*., 1991) and luteal endothelial cells secrete various vasoactive substances, such as nitric oxide (NO), endothelin-1 (EDN1), angiotensin II (Ang II) and prostaglandins (PGs), that directly regulate P4 secretion within the CL (Miyamoto *et al*., 1993, 1997; Girsh *et al*., 1996a, b; Hayashi *et al*., 2000; Skarzynski *et al*., 2000). Therefore, blood vessels and endothelial cells within the CL have an essential role in luteal function in the cow. Thus, the study of vasoactive molecules from the CL is of great importance to provide an insight into a local regulatory system for luteolysis.

The site-restricted action of $PGF_{2\alpha}$: the earliest physiological sign of luteolytic cascade in the cow

Blood flow has a crucial role in the physiology of reproductive organs. During the past 30 years, it has been proposed that a rapid decrease in luteal blood flow is one of the essential impacts of exogenous and endogenous (uterine) $PGF_{2\alpha}$ (Nett *et al*., 1976). 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 P4 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 reported previously that treatment of mature CL (Day 10 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

[1]Corresponding author: akiomiya@obihiro.ac.jp
Fax: +81(155)49-5593

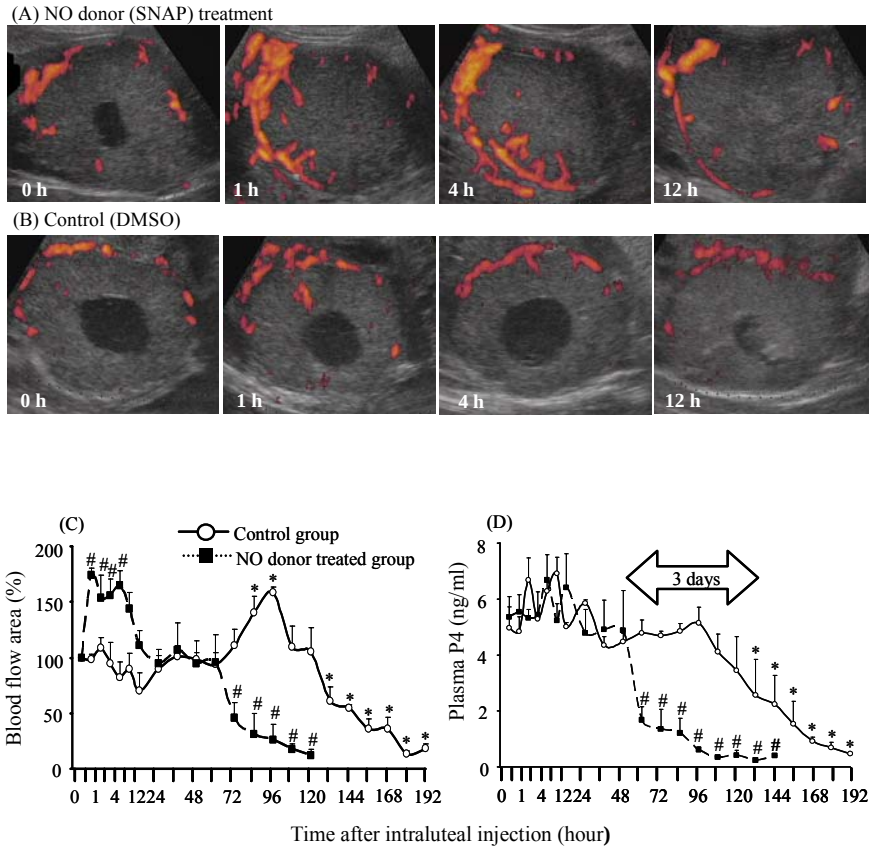
the periphery of the CL, which was followed by a gradual decrease in luteal blood flow (Acosta *et al*., 2002). Moreover, during spontaneous CL regression in the cow, an increase in luteal blood flow in the periphery of the CL on Day 17-18 was associated to peak levels of plasma 13,14 dihydro 15 keto $PGF_{2\alpha}$ (PGFM; a product of the metabolism of the $PGF_{2\alpha}$), just prior to the decline in luteal P4 secretion (Miyamoto *et al*., 2005; Shirasuna *et al*., 2008c). 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 heifer. Thus, we demonstrated that $PGF_{2\alpha}$-induced increases in luteal blood flow are one of the earliest physiological events observed during the luteolytic cascade in the cow.

Nitric oxide (NO) is a potent vasorelaxant which could mediate the increases 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). We examined if 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 (Shirasuna *et al*., 2008b). As a result, a direct injection of the NO donor into the CL clearly induced an increase in blood flow (Fig. 1-A, B and C) and anticipated the fall in P4 by 3 days (Fig. 1-D). This was associated with reduced CL volume and a shortening of the estrous cycle length. Support for a role of NO in luteal blood flow was also obtained by the injection of the NOS inhibitor (L-NAME) that completely suppressed the acute increase in luteal blood flow induced by $PGF_{2\alpha}$ (Fig. 1-E to G). Moreover, administration of the NOS inhibitor also delayed the decreases in P4 secretion for 23 hours (Fig. 1-H) and maintained the CL volume. These findings strongly suggest that NO has a potential to regulate luteal blood flow and a luteal NO is a crucial factor in the initiation of luteolysis by inducing a drastic increase of luteal blood flow in the cow (Shirasuna *et al*., 2008b). Our results on functional (plasma P4) and structural regression (CL volume) are consistent with previous reports in which the NOS inhibitor L-NAME prevented the occurrence of spontaneous and $PGF_{2\alpha}$-induced luteolysis and extended the functional life of the CL in the cow (Jaroszewski and Hansel, 2000; Skarzynski *et al*., 2003). Furthermore, treatment of bovine luteal cells with NO donors in vitro directly inhibited P4 secretion (Skarzynski and Okuda, 2000) and induced apoptosis as increased DNA fragmentation and expression of Fas, Bax and caspase-3 mRNA were observed (Korzekwa *et al*., 2006).

In the next step, we went on to investigate the relationships between $PGF_{2\alpha}$, endothelial NO synthase (eNOS) and luteal blood vessels in the cow (Shirasuna *et al*., 2008b). The localization of blood vessels and microcapillary vessels in the periphery and center of the CL revealed large blood vessels (>20 μm) in the periphery of the mid CL that were not seen in the early CL (Fig. 2A and C). On the other hand, no differences were noted in the numbers of microvascular vessels (<10 μm) in the peripheral and central regions of early and mid CL (Fig. 2B and C). Moreover, we compared the effect of $PGF_{2\alpha}$ on eNOS expression in the peripheral and the central regions of the early CL (resistant to $PGF_{2\alpha}$-induced blood flow increases and luteolysis) and in the mid CL (sensitive to $PGF_{2\alpha}$). 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 or immunostaining in the Early CL (Fig. 2D and E; Shirasuna *et al*., 2008b). In contrast, $PGF_{2\alpha}$ acutely stimulated the expression of eNOS mRNA and protein in the periphery, but not in center of the mid CL 30 min after $PGF_{2\alpha}$ administration (Fig. 2D and E; Shirasuna *et al*., 2008b). Consistently, an injection of $PGF_{2\alpha}$ stimulated eNOS mRNA expression within the CL of the sheep (Vonnahme *et al*., 2006) and of the rabbit (Boiti *et al*., 2003). These results indicate that $PGF_{2\alpha}$-stimulated eNOS expression is positively correlated with the luteal blood flow increase in periphery of the mid CL.

To investigate additional candidate regulators of the increase in luteal blood flow in the periphery of the mid CL, we examined the apelin-APJ system. APJ (putative receptor protein related to angiotensin type 1 receptor) is a G-protein-coupled receptor (O'Dowd *et al*., 1993; Tatemoto *et al*., 1998), and its ligand, apelin, has been identified as a novel regulator of blood pressure through NO-dependent vasodilation (Tatemoto *et al*., 2001; Ishida *et al*., 2004). The $PGF_{2\alpha}$ increased apelin mRNA expression at 0.5-2 h, which coincided completely with the timing of luteal blood flow increase (Shirasuna *et al*., 2008a). Moreover, both apelin mRNA and protein expression were stimulated by $PGF_{2\alpha}$ injection at 30 min in the peripheral area of the mid CL, but not in the early CL (Shirasuna *et al*., 2008a). These results suggest that the apelin-APJ system is involved in the acute increase of luteal blood flow due to regulated NO-vasodilation mechanism and this is the earliest physiological event in the luteolytic cascade in the cow (Shirasuna *et al*., 2008a).

Thus, we hypothesize that $PGF_{2\alpha}$ has a site-restricted action depending not only on the luteal phase (i.e., early vs. mid) but also on the region of the CL. $PGF_{2\alpha}$ stimulates the eNOS-NO system, apelin-APJ system, and vasodilation of blood vessels, such that the net result is an increase in luteal blood flow in the periphery of the mature CL. It is evident that this increased blood flow is mediated by NO. In summary, we demonstrated that the acute increase in peripheral blood flow to the CL is one of the first physiological indicators of NO action in response to $PGF_{2\alpha}$.



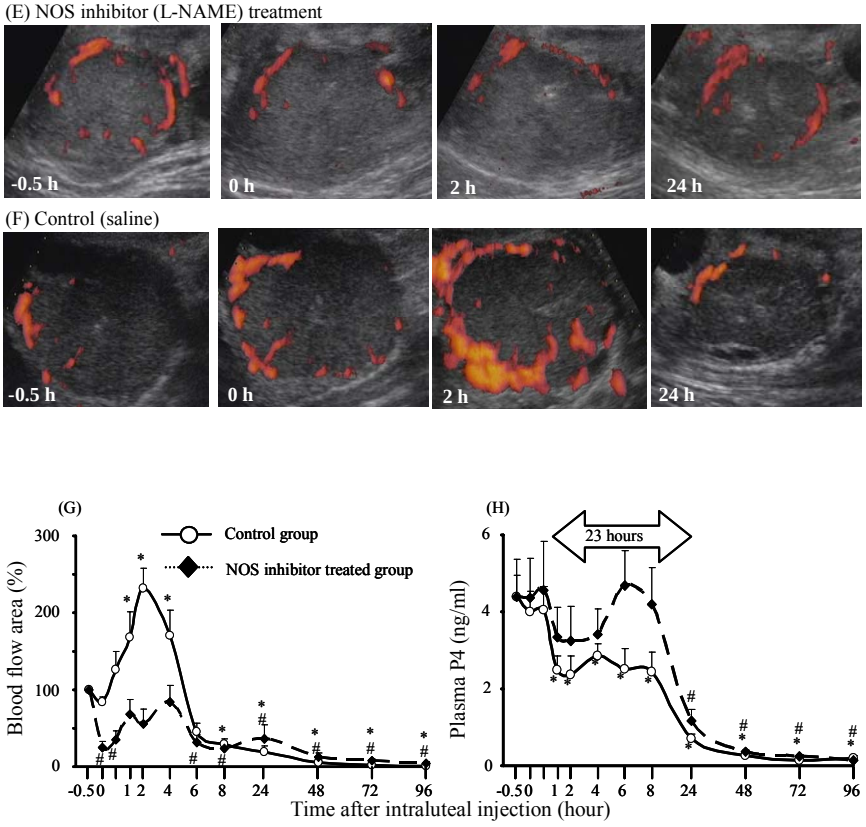


Figure 1. Effect of NO donor (SNAP) or control (DMSO) treatment on luteal blood flow area and plasma P4 (Fig. 1A-D) and effect of the NOS inhibitor (L-NAME) or control (saline) treatment during $PGF_{2\alpha}$–induced luteolysis in the cow on luteal blood flow area and plasma P4 (Fig. 1E-H). Fig. 1A-D: 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 dimethylsulfoxide (DMSO) as control (n = 4) was injected directly into the CL twice at 0 h (first injection) and 4 h. Fig. 1A and Fig. 1B show typical image of luteal blood flow treated by NO donor (SNAP) and DMSO, respectively. Fig. 1C shows change of luteal blood flow area, and Fig. 1D shows change of plasma P4 concentration. A direct injection of the NO donor into the CL clearly induced blood flow increase (Fig. 1-A and C) only after the first injection, and shortened the start of decrease of P4 for 3 days (Fig. 1-D) resulting in a shortening of the estrous cycle. Fig. 1E-H: 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 was directly injected within the CL of cows in the control group, n = 5). Fig. 1E and Fig. 1F show typical image of luteal blood flow treated by NOS inhibitor (L-NAME) and saline as control, respectively. Fig. 1G shows change of luteal blood flow area. Change of plasma P4 concentration is shown in Fig. 1H. The injection of the NOS inhibitor (L-NAME) completely suppressed the acute increase in luteal blood flow induced by $PGF_{2\alpha}$ (Fig. 1-E and G). Moreover, administration of the NOS inhibitor delayed the decreases in P4 secretion for 23 hours (Fig. 1-H). In luteal blood flow, the mean values of 0 h (Fig. 1C and D) or -0.5 h (Fig. 1G and 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 pound key symbols indicate statistically different values ($P < 0.05$) from baseline for control and treated groups, respectively. These figures were modified from Shirasuna *et al.,* (2008b).

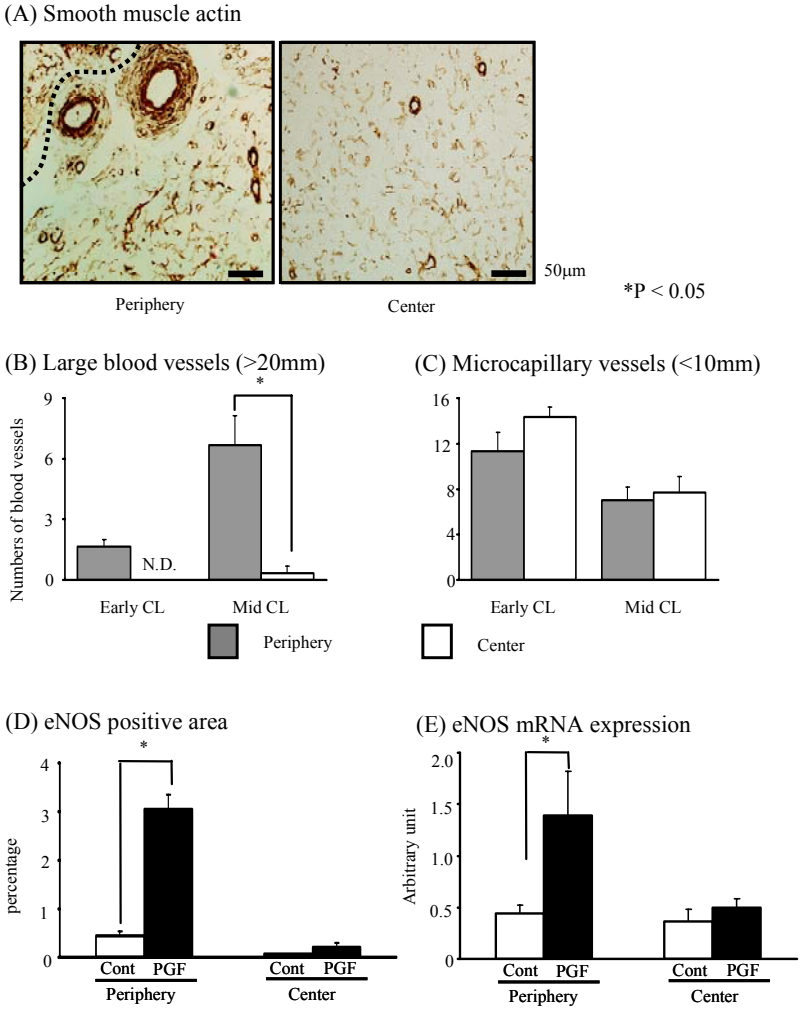


Figure 2. Localization of smooth muscle actin, numbers of large blood vessels and microvascular vessels in the early- and mid CL, and image analysis of eNOS immunostaining and expression of eNOS mRNA in the mid CL. In Fig. 2A, immunohistochemistry was performed to detect smooth muscle actin (SMA) in luteal tissue sections. Area within the CL were designated as the periphery of the CL (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 center section of the CL). Blood vessels were classified depending on their diameter; large blood vessels as >20 μm and microvascular vessels <10 μm. The number of large blood vessels (Fig. 2B) and microvascular (Fig. 2C) vessels was determined as indicated in the methods of Shirasuna *et al.* 2008b. Mean ± SEM are presented, n = 4-5. In Fig. 2D and 2E the experiments were conducted on Day 10-12 in mid CL; $PGF_{2\alpha}$ or saline (as control) was injected (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 a color Doppler ultrasound. After ultrasonography, cows were ovariectomized, 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. 2D and 2E 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 symbol indicates statistically different values ($P < 0.05$). These figures were modified from Shirasuna *et al.* (2008b).

Luteolysis accelerators/mediators within the CL

Endothelin-1

The EDN1 is a 21-amino acid peptide produced by endothelial cells that was first isolated from porcine vascular endothelial cells (Yanagisawa *et al.*, 1988). This peptide binds to two distinct subtypes of G protein-coupled receptors, termed endothelin type A receptor (ETR-A) and endothelin type B receptor (ETR-B) (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Recent studies have demonstrated that EDN1 is involved in the process of luteal regression (Girsh *et al.*, 1996a, b; Miyamoto *et al.*, 1997; Ohtani *et al.*, 1998; Levy *et al.*,

2000; Hinckley and Milvae, 2001). Components of the ET system such as prepro EDN1, endothelin converting enzymes (ECE), ETR-A and ETR-B are present in the bovine CL throughout the estrous cycle (Berisha *et al*., 2002). In fact, using in vivo microdialysis system (MDS), the intraluteal EDN1 secretion started to increase from the onset of luteolysis (start of intraluteal P4 decrease; Fig. 3A) and remained at high levels during spontaneous luteolysis (Fig. 3B). Also, the prepro EDN1 mRNA and its peptide levels are rapidly upregulated during spontaneous and $PGF_{2\alpha}$-induced luteolysis in the cow (Girsh *et al*., 1996b; Ohtani *et al*., 1998; Milvae, 2000; Berisha *et al*., 2002; Schams *et al*., 2003). Moreover, the intraluteal release of $PGF_{2\alpha}$ and EDN1 into MDS medium was positively and highly correlated during spontaneous luteolysis in the cow (Shirasuna *et al*., 2004b). Therefore, it is suggested that $PGF_{2\alpha}$ and EDN1 may act as a positive local feedback system during luteolysis.

In support of the impact of EDN1 on luteolysis in the cow, we further indicated that an intraluteal EDN1 injection 30 min after administration of a sub-luteolytic dose of $PGF_{2\alpha}$ (1/4 dose of $PGF_{2\alpha}$) induced a depression of P4 secretion in vivo during the mid luteal phase (Shirasuna *et al*., 2006). Moreover, the intraluteal administration of an ETR-A antagonist during the mid-luteal phase blocked the luteolytic effect of $PGF_{2\alpha}$ in ewes (Hinckley and Milvae, 2001). In addition, EDN1 and/or $PGF_{2\alpha}$ inhibited P4 secretion from the CL, and this inhibition was blocked by an ETR-A antagonist in vitro (Girsh *et al*., 1996a). Thus, we evaluated the physiological impact of EDN1 via ETR-A in vivo during $PGF_{2\alpha}$-induced luteolysis in the cow. Although there were no differences in the timing and profile of P4 decrease, the start of the decline in CL volume and in the blood flow area surrounding the CL was delayed in the ETR-A antagonist-treated cows during $PGF_{2\alpha}$-induced luteolysis (Watanabe *et al*., 2006). Overall, EDN1 stimulated by $PGF_{2\alpha}$ in the mid CL appears to play a key role in regulation of not only functional luteolysis but also structural luteolysis in the bovine CL.

Angiotensin II

This component of local rennin-angiotensin system has been identified in the ovary of many species (Yoshimura 1997; Hayashi and Miyamoto 1999; Speth *et al*., 1999). The Ang II is a strong vasoactive peptide which is converted from Ang I by angiotensin converting enzyme (ACE). The two major types of receptors for Ang II have been designated as angiotensin type 1 and 2 receptor (AT1R and AT2R) (Yoshimura, 1997). Ang II inhibited LH-stimulated P4 release in bovine luteal cells (Stirling *et al*., 1990). An in vitro study using MDS implanted in the bovine CL showed that $PGF_{2\alpha}$ directly stimulated the release of Ang II and that Ang II also stimulated the release of $PGF_{2\alpha}$ (Hayashi and Miyamoto, 1999; Kobayashi *et al*., 2001). Using in vivo MDS, the intraluteal Ang II secretion increased just after the onset of luteolysis and remained at high levels during spontaneous luteolysis (Fig. 3C). Consistent with the positive correlation between $PGF_{2\alpha}$ and EDN1, the intraluteal release of $PGF_{2\alpha}$ and Ang II into MDS medium was also highly and positively correlated during spontaneous luteolysis in the cow, suggesting that $PGF_{2\alpha}$ and Ang II also have established a positive feedback communication during luteolysis (Shirasuna *et al*., 2004b). In fact, the intraluteal injection of Ang II at 30 min after a subluteolytic dose of $PGF_{2\alpha}$ i.m. (1/4 dose of $PGF_{2\alpha}$) drastically decreased the plasma P4 concentration, leading to complete luteolysis and estrus in the cow (Hayashi *et al*., 2002). These findings suggest that Ang II and $PGF_{2\alpha}$ systems directly interact to induce the process of luteal regression in the cow.

Prostaglandins

PGs are local mediators produced by a variety of tissues and play important roles in many physiological processes (Smith *et al*., 1996). It is well known that $PGF_{2\alpha}$ pulses released from the uterus on days 17-18 of the estrous cycle are the primary luteolytic factor in the cow (McCracken *et al*., 1984; Wolfenson *et al*., 1985). In addition to the uterine-derived $PGF_{2\alpha}$, the functional CL of the cow produces and secretes at least three kinds of PGs, notably $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$, the stable inactive metabolite of prostacyclin (PGI_2; Shemesh and Hansel, 1975; Milvae and Hansel, 1983). Many studies have concluded that PG metabolizing enzymes such as cyclooxygenase (COX)-1, COX-2, PGF synthase, PGE synthase, PG dehydrogenase (PGDH) and receptors for $PGF_{2\alpha}$ and PGE_2 are expressed and regulated in the CL during the estrous cycle (Sakamoto *et al*., 1995; Tsai and Wiltbank, 1998; Silva *et al*., 2000; Arosh *et al*., 2004). In fact, levels of mRNA for COX-2 within the CL were elevated in response to an injection of $PGF_{2\alpha}$ on Day 10 of the estrous cycle ($PGF_{2\alpha}$-responsive; Tsai and Wiltbank, 1998). However, on Day 4 of the estrous cycle ($PGF_{2\alpha}$-resistant), $PGF_{2\alpha}$ does not stimulate COX-2 mRNA within the CL (Tsai and Wiltbank, 1998). It is possible that intraluteal $PGF_{2\alpha}$ has a key role in the mechanism for attaining $PGF_{2\alpha}$-resistance. During spontaneous luteolysis, intraluteal $PGF_{2\alpha}$ secretion increased slightly immediately after the onset of luteolysis and drastically increased from 24 h after the onset of luteolysis in the cow (Fig. 3D; Shirasuna *et al*., 2004a). Furthermore, in the mid CL, a luteolytic injection of $PGF_{2\alpha}$ induced a rapid and transient increase of intraluteal $PGF_{2\alpha}$ secretion during luteolysis as observed by a MDS in the cow (Hayashi *et al*., 2003). These data suggest that intraluteal $PGF_{2\alpha}$ may mediate structural rather than functional luteolysis. To support

this concept, intraluteal implants of indomethacin, a potent PG synthase inhibitor, on Day 11 of the estrous cycle in ewes resulted in heavier CL on Day 18 than that in untreated control ewes (Griffeth *et al*., 2002), suggesting that intraluteal production of $PGF_{2\alpha}$ is required for structural luteolysis. Furthermore, the systemic administration of PG synthesis inhibitors delayed structural luteolysis in rats (Kurusu *et al*., 2001).

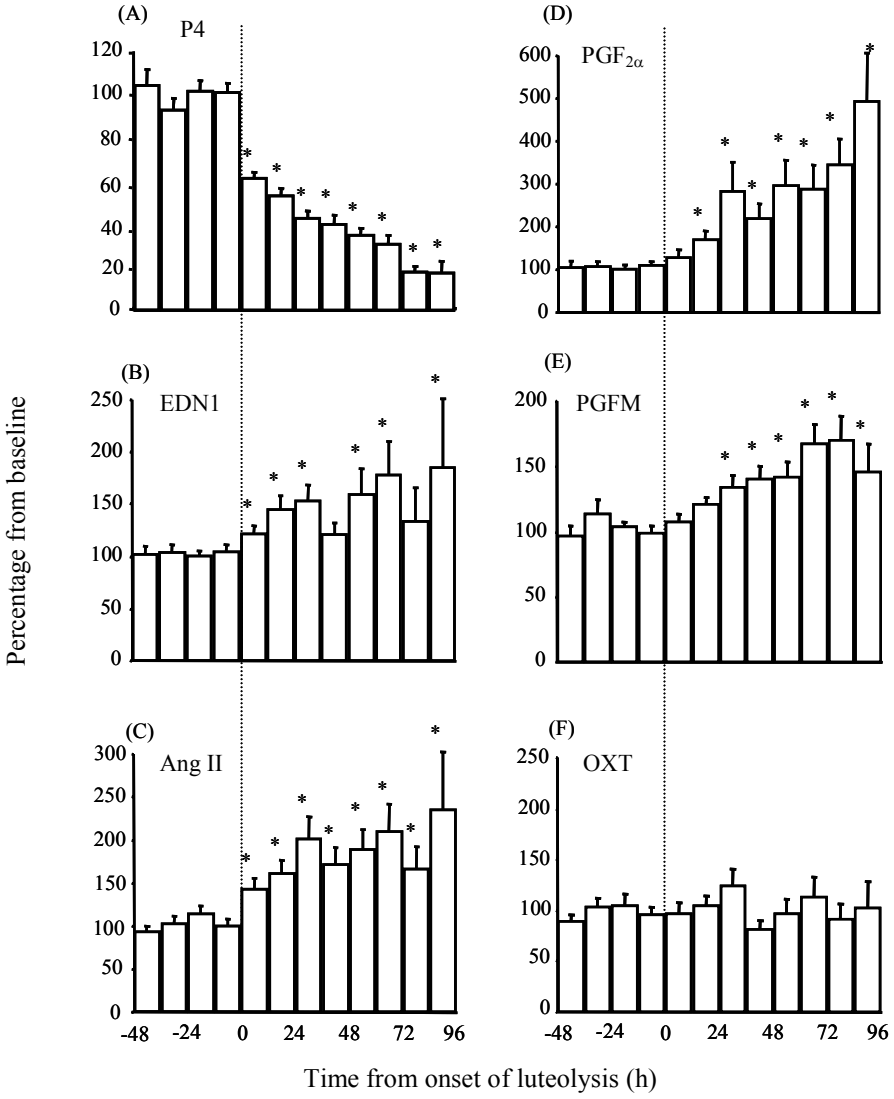


Figure 3. Local release within the corpus luteum of P4 (A), EDN1 (B), Ang II (C), $PGF_{2\alpha}$ (D), PGFM (E) and OXT (F) into MDS (bars; 18 lines from 6 cows) during spontaneous luteolysis in the cow. For statistical analysis, the experimental period was divided into 12 stages. Each stage contains measurements from samples collected within a 12 h period (3 fractions). The MDS data are expressed as a percentage of basal release (baseline) for first 24 h. Mean ± SEM are presented. Asterisk symbol indicates statistically different values ($P < 0.05$) from baseline (-24-0 h). These figures were modified from Shirasuna *et al.,* (2004a, b, 2007a).

Oxytocin

In the bovine CL, the expression of oxytocin (OXT) mRNA is high during the early luteal phase (Ivell *et al*., 1985; Furuya *et al*., 1990; Wathes and Denning-Kendall, 1992), and the OXT peptide is expressed at a higher level during the mid luteal phase (Parkinson *et al*., 1992; Wathes and Denning-Kendall, 1992). $PGF_{2\alpha}$ stimulates OXT secretion from the CL (Flint and Sheldrick, 1982), and OXT in turn stimulates uterine secretion of $PGF_{2\alpha}$ (Sharma and Fitzpatrick, 1974; Roberts and McCracken, 1976). Thus, endometrial $PGF_{2\alpha}$ and luteal OXT comprise a positive feedback mechanism which acts between the uterus and the CL to induce luteal regression (Schallenberger *et al*., 1984). Although the mean release of intraluteal OXT was maintained at the same levels during spontaneous luteolysis in the cow (Fig. 3F), the pulsatile release of

OXT within CL was highly positively associated with intraluteal $PGF_{2\alpha}$ and EDN1, but not with intraluteal Ang II (Shirasuna *et al*., 2007a). In fact, OXT stimulates the release of $PGF_{2\alpha}$ in luteal cells (Grazul *et al*., 1989) and EDN1 in luteal endothelial cells (Girsh *et al*., 1996b). Thus, we propose that luteal OXT, $PGF_{2\alpha}$ and EDN1 may establish a local positive feedback loop within the microenvironment, and OXT may amplify the frequency of vasoactive substance secretion after the onset of luteal regression within the CL (Fig. 4).

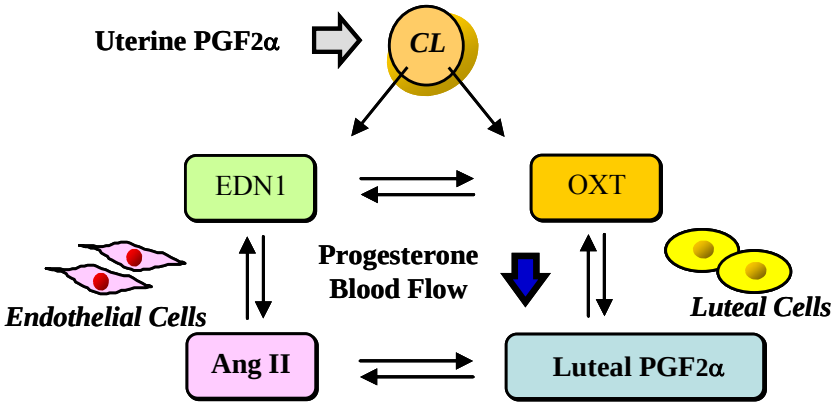


Figure 4. Proposed model for local positive feedback system among vasoactive molecules established during luteolysis in the cow. In the CL, following an increase in luteal blood flow, $PGF_{2\alpha}$ directly stimulates the production of EDN1 and Ang II from luteal endothelial cells and luteal $PGF_{2\alpha}$ from luteal cells. It is suggested that $PGF_{2\alpha}$, EDN1 and Ang II may act as a positive local feedback system during luteolysis. Moreover, luteal OXT, $PGF_{2\alpha}$ and EDN1 may establish a local positive feedback loop within the microenvironment, and OXT may amplify the frequency of vasoactive substance secretion after the onset of luteal regression within the CL. Overall, these mechanisms in the CL appear to play a key role in regulation of not only functional luteolysis (P4 decrease) but also the decrease of luteal blood flow in the bovine CL.

Involvement of cell adhesion systems within the CL

It is likely that cell-to-cell interactions are important for the maintenance and regulation of CL integrity and physiological function. Therefore, it has been suggested that there are intercellular communications via a contact-dependent pathway among various cell types within the CL.

Gap junction are formed with tunnel-like structures that enable regulatory molecules, nutrients and ions of less than about 1 kDa (e.g., calcium ions, cAMP and inositol 1,4,5-triphosphate) to be exchanged between adjacent cells (Yamasaki and Naus, 1996). Gap junctions are formed by connexin (Cx) proteins such as Cx43 (Beyer *et al*., 1990), and these have been suggested to be predominantly important for regulation of growth, differentiation and regression of the CL (Grazul-Bilska *et al*., 1997). On the other hand, the adherence junction is another cell adhesion type, and cadherins have key roles in these junctions. The cadherin family, includes vascular endothelial cell cadherin (VE-cadherin), epithelial cadherin (E-cadherin) and neuronal cadherin (N-cadherin). Cadherins act as major factors in tissue development and in the differentiation of many organs, including the CL of humans (Khan-Dawood *et al*., 1996), baboons (Khan-Dawood *et al*., 1996), mice (Nakhuda *et al*., 2005) and rats (Trolice *et al*., 1997; Sundfeldt *et al*., 2000).

In the bovine CL, Cx43 mRNA expression was detected in luteal endothelial cells and in luteinized granulosa cells (GCs). Moreover, Cx43 mRNA expression tended to increase 24 h after $PGF_{2\alpha}$ administration and thereafter it was significantly low during the regressing luteal stage. In fact, expression of Cx43 mRNA remained at a relatively high level during $PGF_{2\alpha}$-induced luteolysis in sheep (Borowczyk *et al*., 2006), and $PGF_{2\alpha}$ enhanced gap junctional intercellular communication of bovine luteal cells from the late luteal phase (Grazul-Bilska *et al*., 1997). In addition, the mRNA expressions of VE-cadherin and E-cadherin increased at 24 h during $PGF_{2\alpha}$-induced luteolysis. These observations suggest that maintenance of Cx43, VE-cadherin and E-cadherin mRNA expression during regression may contribute to ensure the cell adhesion and gap junction necessary for transferring the luteolytic signal between luteal cells and endothelial cells (Grazul-Bilska *et al*., 1997; Borowczyk *et al*., 2006).

To test the hypothesis that cell adhesion is important for luteolysis, we recently established a co-culture system using bovine aorta endothelial cells (BAEC) and "fully-luteinized" GCs, and investigated the effect of $PGF_{2\alpha}$ on the expression of eNOS mRNA and EDN1 system (Shirasuna *et al*., 2007b, 2008c). $PGF_{2\alpha}$ stimulated the expression of eNOS, EDN1 and ECE-1 mRNA only in the co-cultures of endothelial cells with "fully-luteinized GCs", but not in BAEC or

"luteinized GCs" alone (Fig. 5; Shirasuna *et al*., 2007b, 2008c). Interestingly, levels of Cx43 mRNA expression in the co-cultures of BAEC with fully-luteinized GCs were higher than in BAEC or luteinized GCs alone (unpublished data). These data suggest that interactions between BAEC and "fully-luteinized GCs" enhance the capability of BAEC to produce NO by eNOS and EDN1 by ECE-1 in response to $PGF_{2\alpha}$. Thus, cell-cell interactions appear to be essentially required for maximal responsiveness to $PGF_{2\alpha}$.

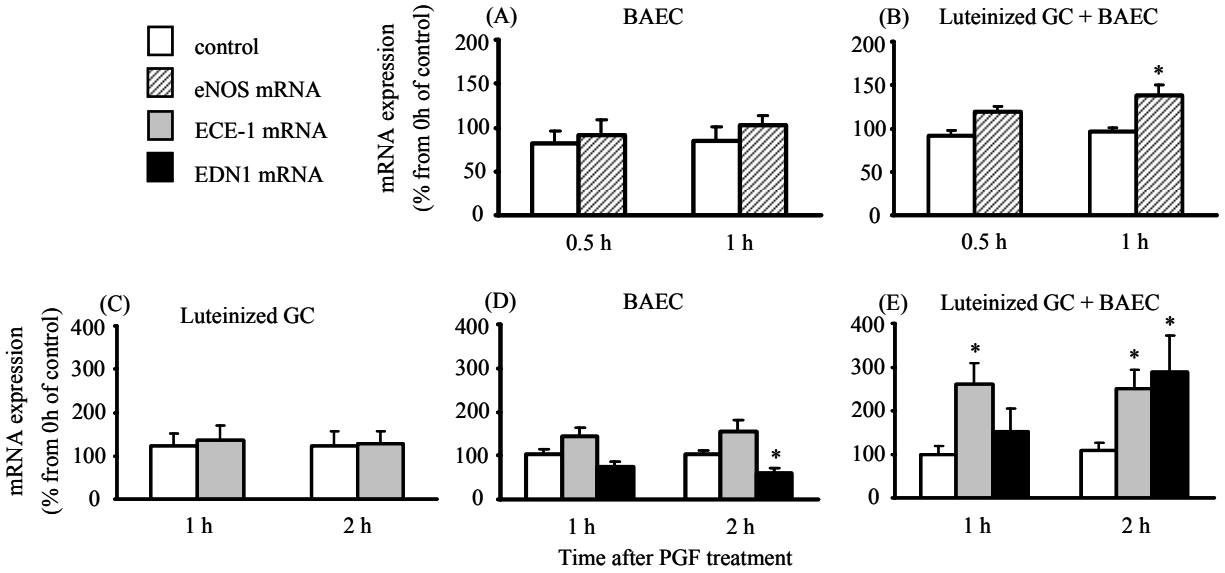


Figure 5. Effects of $PGF_{2\alpha}$ treatment on mRNA expression of eNOS (Fig. 5A-B), ECE-1 and EDN1 in co-cultures of endothelial cells with "fully-luteinized" granulosa cells (Fig. 5C-E). Fig. 5A-B: A) bovine aorta endothelial cell (BAEC) alone; B) co-cultures of BAEC with fully-luteinized GCs (8 days-cultured). Fig. 5C-E: C) Fully-luteinized GCs alone (8 days-cultured); D) bovine aorta endothelial cell (BAEC) alone; E) co-cultures of BAEC with fully-luteinized GCs (8 days-cultured). Data are shown as the mean ± SEM for each time point. The data are expressed as the percentage from individual pretreatment levels. The white bars indicate control groups and colored bars indicate $PGF_{2\alpha}$ treated groups. Asterisk indicates significantly different values compared to control of each time point ($P < 0.05$). These figures were modified from Shirasuna *et al.* (2007b, 2008c).

Proposed concept and Conclusion

On the basis of the current information described in the present review, we propose a possible sequence of events leading to luteolysis in the cow, with a main focus on vasoactive molecules (Fig. 6). Endogenous (from the uterus) or exogenous $PGF_{2\alpha}$ enters the ovarian artery and is transported to the luteal microvessels. $PGF_{2\alpha}$ directly activates eNOS and apelin in the peripheral area of the mature CL, so that the strong vasorelaxant NO is drastically induced in the periphery of the CL, especially in large luteal vascular vessels. Therefore the luteal blood flow in the peripheral area is acutely increased as results of the vasodilation induced by NO. This suggests that $PGF_{2\alpha}$-induced increase in luteal blood flow is one of the earliest physiological signals of the luteolytic cascade in the cow. After the onset of the luteolytic cascade, endometrial $PGF_{2\alpha}$ and luteal OXT comprise a positive feedback mechanism acting between the uterus and the CL to enhance luteal regression. The OXT may have a role in regulating the amplitude of pulsatile secretion of uterine-derived $PGF_{2\alpha}$ after the onset of luteolysis in the cow. In the CL, following an increase in luteal blood flow, $PGF_{2\alpha}$ directly stimulates the production of EDN1 and Ang II from luteal endothelial cells and luteal $PGF_{2\alpha}$ from luteal cells. In addition to causing strong vasoconstriction, these vasoactive substances directly induce the decrease of P4 secretion from the CL. During the course of functional luteolysis, the gap and adherence junctions composed of cell adhesion molecules (CAMs) are maintained such that active cell-to-cell communication occurs to transport luteolytic signals between the luteal cells and luteal endothelial cells. After the onset of functional luteolysis, vasoactive molecules are maintained at high concentrations within the CL to regulate luteal blood vessels and potentiate the severe and chronic vasoconstriction that cuts off the blood supply. Therefore, the decrease of P4 secretion and apoptosis in both luteal cells and endothelial cells are stimulated by direct and/or indirect action of vasoactive substances. The subsequent decrease of CAMs in this phase promotes cell apoptosis.

In conclusion, the acute increase of luteal blood

flow is one of the earliest physiological signals for the luteolytic cascade and luteal NO may be a crucial factor in the initiation of luteolysis 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 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. Furthermore, it is suggested that vasoactive molecules produced from the CL have many critical roles as both regulators of luteal blood flow and accelerators/mediators of the luteolytic cascade in the cow.

Proposed concept for luteolytic cascade with a main focus on vasoactive molecules in the cow

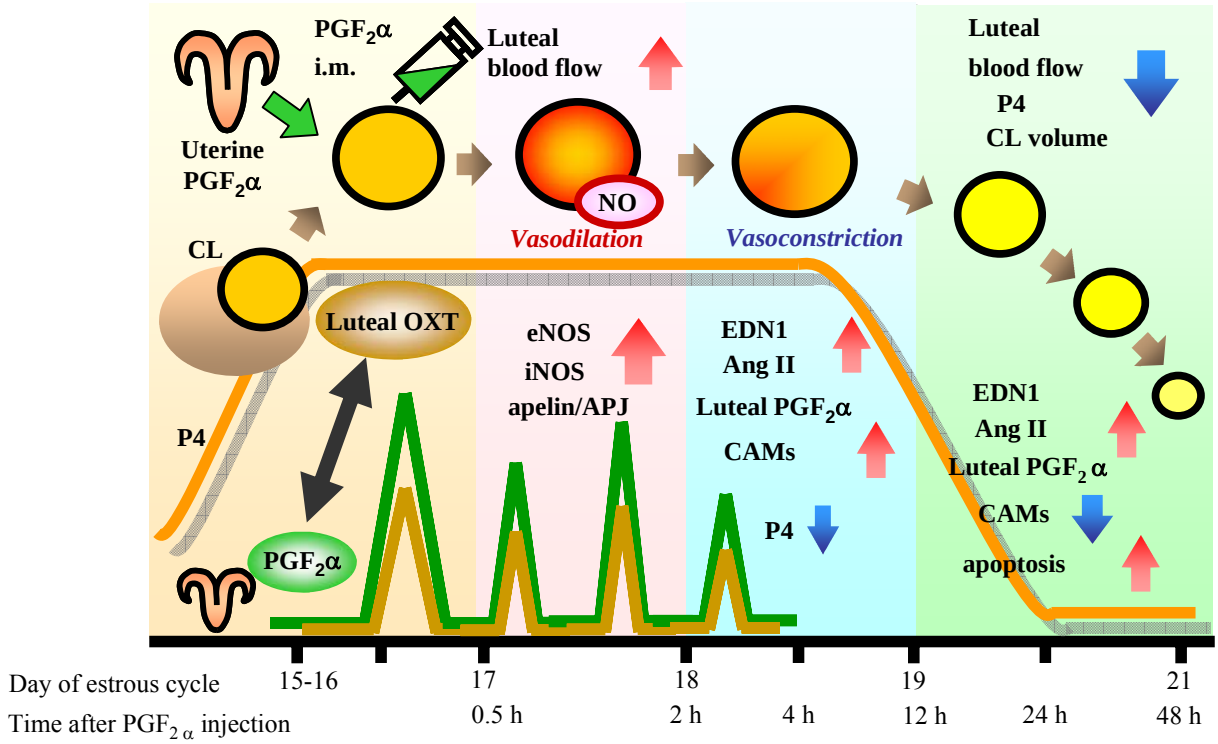


Figure 6. Proposed model for luteolytic cascade with a main focus on vasoactive molecules in the cow. Endogenous (from the uterus) or exogenous $PGF_{2\alpha}$ enters the ovarian artery and is transported to the luteal microvessels. $PGF_{2\alpha}$ directly activates eNOS and apelin in the peripheral area of the mature CL. Therefore the luteal blood flow in the peripheral area is acutely increased as results of the vasodilation induced by NO. After the onset of the luteolytic cascade, endometrial $PGF_{2\alpha}$ and luteal OXT comprise a positive feedback mechanism acting between the uterus and the CL to enhance luteal regression. In the CL, following an increase in luteal blood flow, $PGF_{2\alpha}$ directly stimulates the production of EDN1 and Ang II from luteal endothelial cells and luteal $PGF_{2\alpha}$ from luteal cells. During the course of functional luteolysis, the gap and adherence junctions composed of cell adhesion molecules (CAMs) are maintained such that active cell-to-cell communication occurs to transport luteolytic signals between the luteal cells and luteal endothelial cells. After the onset of functional luteolysis, vasoactive molecules are maintained at high concentrations within the CL to regulate luteal blood vessels and potentiate the severe and chronic vasoconstriction that cuts off the blood supply. The subsequent decrease of CAMs in this phase promotes cell apoptosis.

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