Inter- and intra-cellular mechanisms of prostaglandin $F_{2\alpha}$ action during corpus luteum regression in cattle

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The bovine corpus luteum (CL) grows very fast and regresses within a few days at luteolysis. Mechanisms controlling development and secretory function of the bovine CL may involve many factors that are produced both within and outside the CL. In the cow, luteolysis is initiated by uterine prostaglandin (PG)$F_{2\alpha}$ released at the late luteal stage. It can also be induced by injection of exogenous $PGF_{2\alpha}$ given at the mid luteal stage. Luteolysis consists of a phase of rapid decrease in progesterone (P4) production by the CL, followed by a phase of structural regression. Although uterine $PGF_{2\alpha}$ is known to be the main luteolytic factor, its direct action on the CL is mediated by the products of accessory luteal cells: immune cells, endothelial cells, pericytes and fibroblasts. There are studies showing that beside endothelin-1, cytokines (tumor necrosis factor-α, interferons) and nitric oxide play critical roles in functional and structural luteolysis in cattle by stimulating leukotrienes and $PGF_{2\alpha}$, decreasing P4 secretion and apoptosis induction. Because of luteal blood flow and P4 concentrations decrease in parallel during both spontaneous and $PGF_{2\alpha}$-induced luteolysis, a decrease in luteal blood flow resulting in hypoxia has been proposed as one of the main luteolytic mechanisms in the cow. Hypoxia inhibits P4 synthesis in luteal cells by inhibiting the steroidogenic enzymes and promotes apoptosis of luteal cells by increasing pro-apoptotic proteins. Although reduction of luteal blood flow and hypoxia contribute to the late events of luteolysis, little is known about the physiological relevance and the cause of the transient increase in luteal blood flow and reactive oxygen species during the initial step of luteolysis.

Introduction

When animals do not become pregnant, regression of the corpus luteum (CL), named luteolysis, is essential for the normal cyclicity as it allows for the development of a new ovulatory follicle. Luteolysis occurs when there is no pregnancy on Day 16–18 after oestrus in cattle (McCracken *et al.* 1999). In the cow, luteolysis is initiated by prostaglandin (PG)$F_{2\alpha}$ released from the uterus at the late luteal stage (McCracken *et al.* 1999). During luteolysis, $PGF_{2\alpha}$ of uterine origin enters the ovarian artery from the utero-ovarian vein via a countercurrent exchange mechanism (McCracken *et al.* 1999). This allows uterine $PGF_{2\alpha}$ to pass directly into the ovary, without entering the pulmonary circulation, where it is enzymatically inactivated in the lungs. It has been shown

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that *in vivo* and *in vitro* $PGF_{2\alpha}$ induces a number of mechanisms leading to the regression of the CL, i.e. a decrease in progesterone (P4) secretion, down-regulation of receptors for luteotropic hormones, inhibition of cholesterol cellular uptake, inhibition of cholesterol transport through the cell and/or across the mitochondrial membranes, inhibition of steroidogenic enzymes expression and activity, and finally increase of free calcium ions [Ca^{2+}] in both types of steroidogenic luteal cells (Davis *et al.* 1987; Alila *et al.* 1990; Skarzynski & Okuda 1999). Although *in vivo* $PGF_{2\alpha}$ decreases P4 concentrations in number of species (McCracken *et al.* 1999), it is even luteotropic in the monolayer cultured steroidogenic luteal cells (Alila *et al.* 1988; Okuda *et al.* 1998; Korzekwa *et al.* 2008a). In the cultured bovine luteal cells, supplied with lipoprotein (LP)s as a source of cholesterol-enhancing P4 secretion, $PGF_{2\alpha}$ treatment decreased only LH- and LPs-stimulated P4 secretion (Pate & Condon 1989; Wiltbank *et al.* 1990). Thus, the direct influence of $PGF_{2\alpha}$ on steroidogenesis in luteal cells is still controversial and depends on the methodology of examination. Therefore, the luteolytic action of $PGF_{2\alpha}$ within the bovine CL seems to be mediated by the products of the immune and endothelial luteal cells (reviewed by Pate & Keyes 2001; Meidan *et al.* 2005; Berisha & Schams 2005).

The bovine CL is composed of a heterogeneous mixture of cell types. There are at least two types of steroidogenic cells, large and small luteal cells, which originate from the granulosa and thecal cells of the follicle ruptured at ovulation, respectively. The bovine CL consists of not only steroidogenic luteal cells but also non-steroidogenic cells (accessory cells), *i.e.* vascular endothelial cells, pericytes and fibroblasts, and immune cells such as lymphocytes and macrophages (Lei *et al.* 1991; Hojo *et al.* 2009). Pate and her colleague suggested that immune cells and their products fulfill the part of luteolytic $PGF_{2\alpha}$ action on steroidogenic luteal cells (reviewed by Pate & Keyes 2001; Pate 2003). Additionally, nitric oxide (NO) and leukotriene (LT)C4 are suggested to serve as $PGF_{2\alpha}$ mediators during luteolysis in cattle (Blair *et al.* 1997; Jaroszewski & Hansel 2000; Skarzynski *et al.* 2003a, b; Korzekwa *et al.* 2006). Luteolytic action of $PGF_{2\alpha}$ may also be mediated by endothelin-1 (EDN1), the main product of the luteal endothelial cells (Girsh *et al.* 1996; Davis *et al.* 2003; Meidan *et al.* 2005). The above data strongly indicate that the contact between different types of luteal cells seems to be necessary for its regression. This review focuses on inter-, intracellular, as well receptor mechanisms of uterine $PGF_{2\alpha}$ secretion and action on bovine CL. Furthermore, some of our and others' data on intraluteal mediators of luteolytic actions of $PGF_{2\alpha}$ on bovine CL during luteolysis have also been reviewed. Moreover, the regulation of the blood flow into the CL as an important mechanisms of regression of bovine CL is also discussed.

Uterine prostaglandin $F_{2\alpha}$ is the main luteolytic factor in ruminants

The importance of the uterus in the control of luteal regression was first reported by Loeb (1923), who demonstrated that hysterectomy abolished the oestrous cycle and caused abnormal persistence of the CL in guinea pig. It has also been demonstrated in the cow that the CL is maintained after bilateral hysterectomy (Wiltbank & Casida 1956). In 1970, Lukaszewska & Hansel (1970) showed that a low-molecular-weight substance, extracted from the uterus, has a luteolytic action. Finally, this substance was identified as an arachidonic acid metabolite, *i.e.* $PGF_{2\alpha}$ (Hansel *et al.* 1975). The importance of uterine $PGF_{2\alpha}$ in the functional and structural demise of the bovine CL has been well established (reviewed by McCracken *et al.* 1999; Okuda *et al.* 2002).

Prostaglandins are produced from arachidonic acid (AA) liberated from phospholipid stores through the action of phospholipase (PL)s. Arachidonic acid is then converted into prostaglandin endoperoxide H_2 (PGH_2) by prostaglandin G/H synthases (PTGS, previously known as cyclooxy-

genase - COX). Two isoforms of the PTGS enzyme, types 1 and 2, are coded by different genes and catalyze the double oxygenation and reduction of AA. Among several enzymes capable of converting PGH_2 into $PGF_{2\alpha}$ (PGF-synthase, PGFS), in the endometrium of cyclic cows around the time of luteolysis only AKR1B5, an aldose reductase also exhibiting 20α-hydroxysteroid dehydrogenase (20α-HSD) activity, was shown to be up-regulated and expressed in significant amounts [Madore *et al.* 2003]. According to Madore *et al.* (2003), AKR1B5 has the ability to combine two converging functions: inactivation of P4 and generation of $PGF_{2\alpha}$. This makes AKR1B5 a multifunctional enzyme with complementary action in the endometrium during luteolysis and delivery, when P4 secretion and luteal function is terminated.

For a long time, the factor(s) that can induce the luteolytic $PGF_{2\alpha}$ output for the bovine uteri have been defined and discussed (reviewed by McCracken et al. 1999; Okuda et al. 2002). It was established in the 80´s in the previous century that $PGF_{2\alpha}$ stimulates oxytocin (OT) released from the ovine CL, and that OT stimulates endometrial PG production (Flint & Sheldrick 1982). Therefore, at the time of luteolysis, CL-derived OT and uterus-derived $PGF_{2\alpha}$ were believed to create a positive feedback loop in ruminants. In fact, the importance of ovarian and / or hypohysial OT in the control of luteolysis in cows was first reported by Armstrong & Hansel (1956). Later, Newcomb *et al.* (1977) reported that injection of OT increased $PGF_{2\alpha}$ secretion in cows. Finally, it has been demonstrated that OT-stimulated $PGF_{2\alpha}$ secretion is associated with the activity of protein kinase (PK)C (Burs *et al.* 1997, Skarzynski *et al.* 2000a) and the gene expression of several enzymes involving in PGs synthesis, *e.g.*, PLA2, PTGS-2, and PGFS (Madore *et al.* 2003). However, there is increasing evidence that OT is not essential for the initiation of $PGF_{2\alpha}$ output during luteolysis in the cow (Parkinson *et al.* 1992; Kotwica *et al.* 1997, 1998; Douglas & Britt 2000). Concentrations of OT in blood (Parkinson et al. 1992; Kotwica et al. 1998), and in intact (Parkinson et al. 1992) and microdialyzed CL (Douglas & Britt 2000) are extremely low at the time of spontaneous luteolysis. Moreover, the blockade of uterine OT receptors with a specific OT antagonist from Day 15 to Day 22 of the cycle affected neither luteolysis nor the duration of the oestrous cycle in heifers (Kotwica *et al.* 1997). Therefore, $PGF_{2\alpha}$ secretion by the endometrium may be regulated not only by OT but also by one or more other factors (i.e. cytokines, steroids and peptide hormones) in cattle (reviewed by Okuda et al. 2002).

Tumor necrosis factor-α (TNF) and its receptors have been demonstrated to be present in the bovine cyclic endometrium (Miyamoto *et al.* 2000; Okuda *et al.* 2010). TNF stimulates $PGF_{2\alpha}$ output by the bovine uterus *in vitro* (Miyamoto *et al.* 2000; Skarzynski et al. 2000a; Murakami *et al.* 2001) as well as in the conscious cows (Skarzynski *et al.* 2003b; 2007; 2009). Moreover, we have shown that the infusion of the lower dose of TNF (1 mg) increased plasma concentrations of PGFM (metabolite of luteolytic $PGF_{2\alpha}$) and inhibited P4 production finally resulting in shortening of the oestrous cycle (Skarzynski *et al.* 2003b). Furthermore, the inhibition of PG production in the bovine uterus and/or CL by a non-selective PTGS inhibitor (indomethacin) preinfusion has completely withdrawn the luteolytic action of TNF showing that action on TNF on the bovine oestrous cycle is mediated by PG production and action (Skarzynski et al. 2007). This cytokine augmented $PGF_{2\alpha}$ production only in the bovine endometrial stromal cells via the activation of PLA_2 and NO synthase *in vitro* (Skarzynski *et al.* 2000a) and *in vivo* (Skarzynski *et al.* 2007), as well as via increasing *PTGS-2* mRNA expression (Okuda et al. 2004a). Because the expression of TNF protein and mRNA have been found only in epithelial cells of the bovine endometrium, TNF seems to play a role as a paracrine factor for regulating endometrial function in the cow (Okuda *et al.* 2010). Therefore, we have proposed a hypothesis that endometrial (Okuda *et al.* 2010) and / or luteal TNF (Shaw & Britt 1995; Sakumoto *et al.* 2000) is a trigger for the output of $PGF_{2\alpha}$ from the uterus in the initiation of luteolysis (Fig. 1).

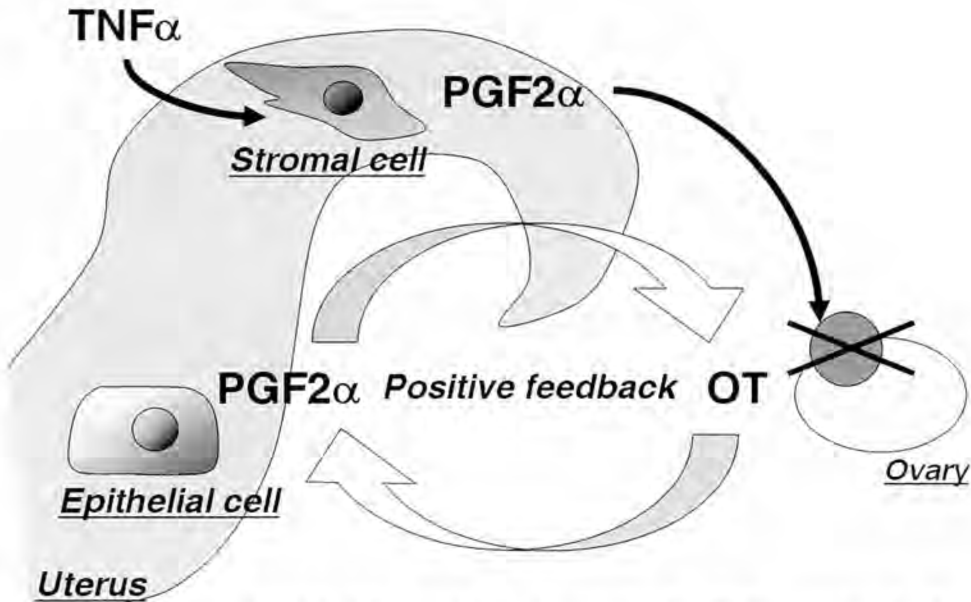

Fig. 1. Hypothetical model of the regulation of prostaglandin (PG)$F_{2\alpha}$ secretion by the bovine endometrium during luteolysis (see text for details).

For the initiation of bovine luteal regression, the pulsatile character of $PGF_{2\alpha}$ is much more important and plays a mandatory role rather than its absolute levels (Schramm et al. 1983; Lamsa *et al* 1989; Ginther *et al*. 2009, 2010). $PGF_{2\alpha}$ released from the endometrium, especially the inter-caruncular region of the surface epithelium of the uterus (Asselin et al. 1998), in a pulsatile manner causes regression of the bovine CL (Ginther *et al*. 2009, 2010). It has recently been shown, that the excessive, bolus $PGF_{2\alpha}$ doses may stimulate nonphysiologic P4 responses and only sequential $PGF_{2\alpha}$ pulses are required and able to stimulate the natural luteolysis in cattle (Ginther *et al*. 2009, 2010). In fact, such a long-lasting and permanent exposition of CL to $PGF_{2\alpha}$ action desensitized the ovine (Lamsa *et al*. 1989) and bovine CL (Skarzynski & Okuda 1999; Bah *et al*. 2006) on the luteolytic effect of supplementary-extraordinary $PGF_{2\alpha}$. A rest period of 6 h is required to restore the normal response to $PGF_{2\alpha}$ (Lamsa *et al*. 1989). However, TNF in concert with other inflammatory mediators including NO may sensitize the bovine CL to luteolytic $PGF_{2\alpha}$ action (Skarzynski *et al*. 2000b; Ohtani *et al*. 2004).

Intra- and intercellular mechanisms of $PGF_{2\alpha}$ action

The receptor for $PGF_{2\alpha}$ (FPr) is coupled to phospholipase C, generating two second messengers, inositol triphosphate (IP3), which is involved in the release of intracellular [Ca^{2+}] and diacylglycerol (DAG), an activator of PKC (Sakamoto *et al*. 1995). FPr was cloned in the bovine species by Sakamoto *et al*. (1994). This receptor was considered as a contractile receptor with two well recognized isoforms (FPr-α and FPr-β), and generated by alternative splicing of C terminal of a single gene identified in ovine and bovine CL (Ezashi *et al*. 1997). Bovine FPr-β and other, new discovered FPr isoforms may act as a negative regulator to attenuate the normal FPr-α-mediated

protein kinase C function. However, the data on FPr-β and other FPr isoforms (FPr-δ, -γ, -ε and -ζ) in the bovine CL are at a preliminary stage (Akabane et al. 2008).

As mentioned, the effects of $PGF_{2\alpha}$ in bovine CL appear to be mediated through the PKC second messenger system (Davis *et al.* 1987; Wiltbank *et al.* 1990; Sen *et al.* 2005; Choundhary *et al.* 2005). $PGF_{2\alpha}$ activates PLC, which causes hydrolysis of membrane phosphatidylinositol (PIP2) to IP3 and DAG. IP3 stimulates the release of $[Ca^{2+}]$ from intracellular stores, while DAG enhances the affinity of PKC for calcium, resulting in an increase of free intracellular $[Ca^{2+}]$ concentration and activation of PKC (Davis *et al.* 1987). It has been demonstrated that $PGF_{2\alpha}$ activates the Raf/MEK1/mitogen-activated protein kinase signaling cascade in bovine luteal cells (Chen *et al.* 1998; Arvisais *et al.* 2010). Treatment of midluteal-phase cows *in vivo* with a luteolytic dose of $PGF_{2\alpha}$ resulted in a rapid increase in ERK and mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70S6K1) signalling and a rapid suppression of Akt phosphorylation in luteal tissue (Arvisais *et al.* 2010). Also *in vitro* treatment of primary cultures of luteal cells with $PGF_{2\alpha}$ as well, resulted in an increase in ERK and mTOR/p70S6K1 signalling and a diminished capacity of IGF-I to stimulate PI3K, Akt, and PKC (Arvisais *et al.* 2010). In fact, the actions of $PGF_{2\alpha}$ were mimicked by a PKC activator (PMA). Therefore, the activation of Raf/MEK1/mitogen-activated protein kinase by $PGF_{2\alpha}$ may provide a mechanism to transduce signals initiated by FPr_B on the cell surface into the nucleus and may be associated with transcriptional activation of luteal genes (Chen *et al.* 1998; Arvisais *et al.* 2010).

The phosphorylation events associated with the activation of PKC and the IP3-mediated sustained elevations of $[Ca^{2+}]$ are believed to regulate OT secretion, to inhibit P4 and cause a cytotoxic effect (Wiltbank *et al.* 1990). Therefore, the luteolytic action of $PGF_{2\alpha}$ is attended by PKC and $[Ca^{2+}]$ as secondary messengers inside the steroidogenic luteal cells (Sen *et al.* 2005; Choundhary *et al.* 2005). Nevertheless, it has been shown that PKC also mediates the luteotropic action of $PGF_{2\alpha}$ in the steroidogenic cell culture from the mid stage of oestrous cycle (Alila *et al.* 1990; Okuda *et al.* 1998). Thus, it should be emphasized that the various actions of $PGF_{2\alpha}$ on the bovine CL upon binding to its G protein-coupled receptor are initiated by the same PLC / DAG-IP3 / Ca^{2+} - PKC pathway.

The possible reason for such different effects are differences in the cell culture system and consequently differences in the cell-to-cell contact, gap junctions and connection-communication between large and small luteal cells and immune or/and endothelial cells (Okuda et al. 1998; Korzekwa *et al.* 2004, 2008a; Miyamoto *et al.* 1993; Sen *et al.* 2006; Shibaya *et al.* 2005; Bah *et al.* 2006). In contrast from the monolayer long-term system of the culture (at least 48-h of culture) used in the study by Okuda *et al.* (1998), Tsai & Wiltbank (1997) and Sen *et al.* (2006) conducted their experiments in luteal cell suspension, incubated and stimulated the cells for a shorten time. Luteal cells cultured in such a system of suspension are in contact with each other and keep their original shape during culture, thus the cytoskeleton works correctly (Shibaya *et al.* 2005). The cytoskeleton keeps a variety of cellular components in place and it has been implicated in a variety of cellular processes, such as cell motility, cell migration, spatial distribution of cell organelles, intracellular communication and cellular responses to membrane events (Shibaya *et al.* 2005). We have shown that the aggregate culture system that ensures three-dimensional, cell-to-cell contact created more physiological conditions for cell function than the *in vitro* monolayer system (Shibaya *et al.* 2005; Korzekwa *et al.* 2008a). Thus, the cell culture suspension system is better than the monolayer one to show the inhibitory effect of $PGF_{2\alpha}$ during luteolysis. The disorders in the cell shape and organization and in the gap junctions may disturb the action of PKC in the cells. Therefore, the model ensuring cell-to-cell contact and shape seems to be necessary for studying the influence of some biologically active substances (i.e. cytokines, $PGF_{2\alpha}$, END1, LT, NO) on bovine luteal function during luteolysis

(Skarzynski & Okuda 1999; Shibaya *et al.* 2005; Sen *et al.* 2006; Korzekwa *et al.* 2008a). Korzekwa *et al.* (2008a) have shown that the cell coculture model, including the main types of CL cells (i.e. steroidogenic, immune and endothelial luteal cells), is the most approximate to study $PGF_{2\alpha}$ role *in vitro* (Fig. 2). The cell-to-cell contact and interactions between endothelial and immune cells with steroidogenic CL cells are needed for luteolytic $PGF_{2\alpha}$ action within the bovine CL *in vitro*. Moreover, concerning *in vivo* studies, Ginther *et al.* (2009) pointed out that such unexpected observed dose-sensitive effects apparently represented a non-physiologic response to unnatural doses and delivery of $PGF_{2\alpha}$. It seems that many of the reported studies during the past few decades on the nature of the luteolytic process in cattle may have resulted in dubious interpretations, owing to potential artifactual or pharmacologic responses to unnatural doses or unnatural delivery of $PGF_{2\alpha}$ to the CL. Therefore, a dose and method of delivery that approximated the endogenous system is needed to study $PGF_{2\alpha}$ action on the bovine CL (Ginther *et al.* 2009). Nevertheless, as suggested by the authors, some reservations are required, owing to the unnatural time of treatment during mid luteal phase.

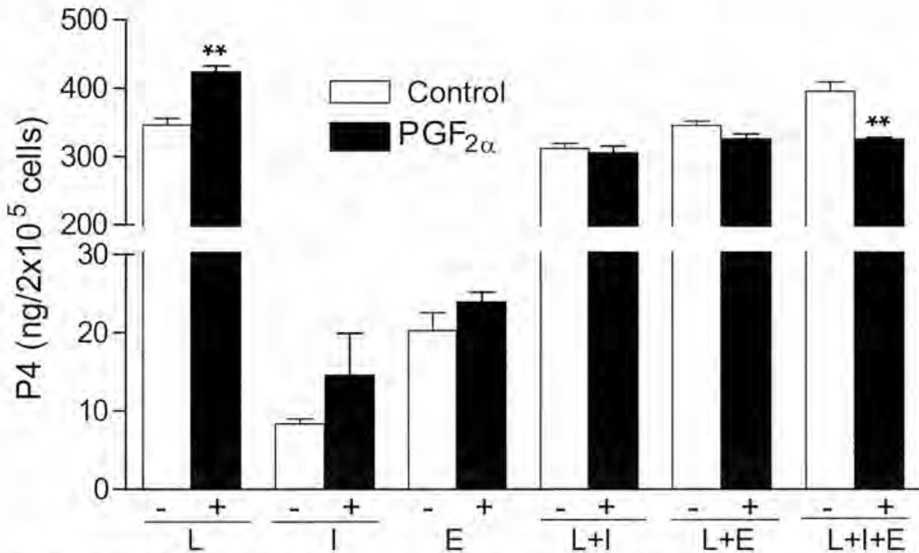

Fig. 2. Effect of prostaglandin $(PG)F_{2\alpha}$ on progesterone (P4) secretion by co-cultured bovine luteal cells (steroidogenic cells-L, endothelial cells-E and/or immune cells-I) obtained on Day 15/16 of the oestrous cycle. The cells were enzymatically isolated, pre-incubated in glass tubes in a shaking water bath for 2 h and then exposed to $PGF_{2\alpha}$ (10-6M) for 22 h. Asterisks indicate significant differences ($P < 0.05$) between the $PGF_{2\alpha}$-treated and Control. Modified from Korzekwa *et al.* 2008a.

Intraluteal mediators of luteolytic $PGF_{2\alpha}$ action

It was previously observed that $PGF_{2\alpha}$ stimulated (Alila *et al.* 1988; Okuda *et al.* 1998; Korzekwa *et al.* 2004), inhibited (Pate & Condon 1989) or had no direct effect on P4 secretion in cultured steroidogenic luteal cells. Therefore, it is suggested that the luteolytic action of $PGF_{2\alpha}$ on bovine CL is mediated by local factors, i.e. EDN1, cytokines and NO. However, the secretory functions of bovine luteal cells are regulated not only by PGs but also by others AA metabolites - LTs (Milvae *et al.* 1986, Korzekwa *et al.* 2010a, b). We have shown that LTs are produced and released in the bovine CL and modulate the production of P4 and PGs in the bovine reproductive tract

during the oestrous cycle, influence on the lifespan of CL and may serve as luetolitic mediators (Jaroszewski *et al.* 2003b; Skarzynski et al. 2003b, Korzekwa et al. 2008b, 2010a, c). In addition, prokineticin 1 (PROK1, also termed endocrine gland-derived vascular endothelial growth factor - endocrine gland-derived VEGF) is involved in the recruitment of monocytes to regressing CL and their consequent activation therein (Kisliouk *et al.* 2007). Apelin (APJ, putative receptor protein related to angiotensin type 1 receptor - a G-protein-coupled receptor) and its receptors have been also identified as a novel regulators of blood flow and as an angiogenic factor during luteolysis in cattle (Shirasuna *et al.* 2008a). Moreover, Berisha *et al.* (2010) have recently shown that *in vivo* during $PGF_{2\alpha}$-induced luteolysis, insulin growth factor-1 (IGF1) and vascular endothelial growth factor (VEGFA) protein already decreased after 0.5 h after an analogue of $PGF_{2\alpha}$ treatment. By contrast, angiopoietin-2 (ANGPT2) protein and mRNA, as well as the *IGFBP1 mRNA* significantly increased during the first 2 h of $PGF_{2\alpha}$-induced luteolysis, followed by a steep decrease after 4 h. Moreover, tissue OT peptide and *OTR* mRNA decreased significantly after 2 h, followed by a continuous decrease of *OT* mRNA. Taking together Berisha *et al.* (2010) data, the acute decrease in luteotropic (P4, OT, IGF1) as well pro-angiogenic factors (i.e. VEGF, IGF1) with acute increase in capillary destabilization factors (i.e. ANGPT2) and modulation of vascular stability may be a key components in the cascade of the early events leading to functional luteolysis

Endothelin-1 and vasoactive peptides

Endothelin-converting enzyme 1 (ECE-1) is a key enzyme in the biosynthesis of EDN1, a potent regulator/modulator of $PGF_{2\alpha}$ action during luteolysis in cattle (Levy *et al.* 2000). The rapid increase in EDN1 during luteal regression, the ability of EDN1 to inhibit steroidogenesis *in vitro* and *in vivo*, and the inhibition of the luteolytic effects of $PGF_{2\alpha}$ by pretreatment with type A EDN1 (ETA) receptor antagonists suggest that this peptide functions as an important component of the luteolytic cascade (Girsh *et al.* 1996; Miyamoto *et al* 1997; Ohtani *et al.* 1998; Levy *et al.* 2001). In fact, a pivotal role for the main endothelial cells product - EDN1 in $PGF_{2\alpha}$-induced luteal regression in cows has been well documented by Meidan's group (Girsh *et al.* 1996; Mamluk *et al.* 1999; Meidan *et al.* 2005; Levy *et al.* 2000, 2003; Rosiansky-Sultan *et al.* 2006). mRNA expression of *EDN1* as well as its concentration in bovine CL increased at 2-10 h after $PGF_{2\alpha}$ treatment (Ohtani *et al.* 1998; Wright *et al.* 2001; Shirasuna *et al.* 2004). EDN1 inhibited P4 secretion, in a dose dependent manner via selective EDN1 bindings sites (EBN_A). The expression of members of the EDN system (EDN1, EDN converting enzymes, and EDN_A and EDN_B receptors) increases during luteal regression (Levy et al. 2001; Rosiansky-Sultan *et al.* 2006). Moreover, $PGF_{2\alpha}$ up-regulates EDN1 and EDN_A expression within the CL (Mamluk *et al.* 1999). Besides EDN1, other vasoactive peptides (i.e. angiothensin 2 – ANG2, atrial natriuretic peptide – ANP) are considered important factors in mediating $PGF_{2\alpha}$ luteolytic action (Berisha and Schams 2005; Berisha et al. 2010). These vasoactive peptides decreased blood flow and triggered the luteolytic cascade and consequently inhibited P4 secretion (Shirasuna *et al.* 2004; Miyamoto *et al.* 2005).

However, it has been showed that the EDN1 system (EDN1, EDN_A and EDN converting enzymes) exists in the bovine CL through the oestrous cycle and $PGF_{2\alpha}$ increased *EDN1* mRNA expression *in vivo* only at 10 h after treatment (Wright *et al.* 2001). In fact, up-regulation of EDN1 and ANG2 occurred mainly during structural luteal regression (Berisha & Schams 1995). Therefore, EDN1 seems to be additionally involved in the process of structural egression of the bovine CL by promoting leukocyte migration and stimulating macrophages to release cytokines (i.e. TNF, IFNG; Friedman et al. 2000; Meidan et al. 2005).

Cytokines

The increase in the immune cell numbers in the bovine CL has been observed during luteolysis (Lobel & Levy 1968; Penny *et al.* 1999; Towson *et al.* 2002). Moreover, production of monocyte chemoattractant protein 1 (MCP1), which stimulated proliferation and activation of the immune cells, was observed in the bovine CL from days 12-18 of the oestrous cycle (Townson et al. 2002). The number of leukocytes (i.e. T lymphocytes, macrophages) increased at the time of structural luteolysis and play a central role in structural luteolysis of both steroidogenic and endothelial luteal cells (Benyo & Pate 1992; Friedman et al. 2000; Pate & Keyes 2001; Pate 2003). The most important immune factors involved in the process of regression of bovine CL are pro-inflamatory cytokines: TNF and interferon-γ (IFNG) (Pate & Keyes 2001; Skarzynski *et al.* 2008). Shaw & Britt (1995) using a microdialysis of bovine CL system showed that TNF is released during spontaneous and $PGF_{2\alpha}$-induced luteolysis in cows. mRNAs for *TNF* and its specific receptors (*TNFR type-I*) has clearly been shown to be present in the bovine CL during luteolysis (Sakumoto *et al.* 2000; Neuvians *et al.* 2004; Korzekwa *et al.* 2008b). TNF acting via TNFR type-I in combination IFNG, reduced P4 production, induced apoptosis and $PGF_{2\alpha}$ production by luteal cells *in vitro* (Sakumoto *et al.* 2000; Petroff *et al* 2001; Korzekwa *et al.* 2006). Specific binding sites for TNF are also present in endothelial cells derived from bovine CL (Okuda *et al.* 1999). Furthermore, TNF induces EDN1 production by endothelial cells that may lead to the structural regression of the CL (Okuda *et al.* 1999; Friedman *et al.* 2000). However, mostly all *in vitro* studies indicated that TNF induces luteolysis only in combination with INFG or other intraluteal factors i.e. EDN1 (reviewed by Pate & Keyes 2001; Meidan *et al.* 2005; Skarzynski *et al.* 2008). Therefore, we tested *in vivo* whether TNF acts as a luteolytic factor *in vivo*, and whether it changes the lifespan of bovine CL (Skarzynski *et al.* 2003a). Lower doses of TNF increased $PGF_{2\alpha}$ and nitrite/nitrate (stable metabolites of NO), decreased P4 level and consequently resulted in shortening of the oestrous cycle. Surprisingly, higher doses of TNF stimulated the synthesis of P4 and PGE_2 and consequently resulted in prolongation of the lifespan of bovine CL (Skarzynski *et al.* 2003a; Skarzynski *et al.* 2007; Korzekwa *et al.* 2008b). However, it is difficult to state how these observed concentrations/doses relate to the local concentrations achieved at the CL and the endometrium, because very high TNF concentrations may be achieved locally to exert paracrine and autocrine effects. Thus TNF, depending on its concentrations, influences the secretory function of the CL and uterus in cattle (Skarzynski *et al.* 2009).

TNF belongs to the TNF super family (TNF-SF), which consists of eighteen members and as a classical cytokine is pleiotropic in nature. Regulating reproductive processes, TNF also has a diverse spectrum of biological activities, including stimulation of cell proliferation and differentiation, induction of cell apoptotic death (reviewed by Terranova *et al.* 1995). The pleiotropic TNF properties may depend on the differences in receptors and second messenger actions. Two immunologically distinct TNFRs have now been identified, TNFRI and TNFRII have different intracellular signalling pathways (Beutler & Van Huffel 1994). TNFRI contains an intracellular death domain, which is required for signalling pathways associated with apoptosis. In contrast, TNFRII can induce gene transcription for cell survival, growth, and differentiation (Beutler & Van Huffel 1994). Therefore, TNF may play multiple physiological roles in reproductive tract (Terranova *et al.* 1995; Pate & Keyes 2001; Skarzynski *et al.* 2008).

In our *in vivo* study, we have shown that the inhibition of PG synthesis by indomethacin, a non-specific PTGS inhibitor, injected into the *aorta abdominalis*, blocked the actions of TNF indicating that TNF acts mainly through mediation of AA metabolites (Skarzynski *et al.* 2007). In addition to TNFR, other cytokine membrane receptors, second messengers, including $[Ca^{2+}]$ and regulatory proteins are involved in apoptosis of steroidogenic and endothelial luteal cells (Friedman *et al.* 2000; Petroff *et al.* 2001; Taniguchi *et al.* 2002). Fas ligand (FASL), a member

of the TNF super family, primarily engages its receptors (FAS) to induce apoptosis (Taniguchi *et al.* 2002; Okuda *et al.* 2004b). The expression of *FAS* mRNA was increased by IFNG, and TNF augmented the stimulatory action of IFNG on *FAS* expression (Taniguchi *et al.* 2002). Moreover, apoptotic bodies were observed in the luteal cells treated with FASL in the presence of IFNG and/ or TNF, showing that leukocyte-derived TNF and IFNG play important roles in FASL-FAS-mediated luteal cell death in the bovine CL.

Leukotrienes

Leukotrienes, especially LTB_4 and LTC_4 could be engaged in the process of luteolysis (Milvae *et al.* 1986). Blair *et al.* (1997) showed using a microdialysis system *in vivo* that at Day 12 of the oestrous cycle after $PGF_{2\alpha}$ infusion increased both LTC_4 and LTB_4 levels in luteal perfusate. Other *in vivo* and *in vitro* studies also indicated that the levels of LTC_4 in the perfusate of bovine CL (Jaroszewski & Hansel 2000; Jaroszewski *et al.* 2003a,b), cultured luteal cells (Jaroszewski *et al.* 2003a; Korzekwa *et al.* 2010a), as well as in the blood of conscious cattle (Jaroszewski & Hansel 2000; Jaroszewski *et al.* 2003a,b; Skarzynski *et al.* 2003b) are elevated after $PGF_{2\alpha}$, NO and TNF treatment at the late luteal phase of oestrous cycle, suggesting that LTs play some role in luteolysis.

Leukotrienes are synthesized by 5-lipoxygenase (5-LO) and commonly known as potential inflammatory factors that cause oedema in respiratory tract diseases, but they also have got the roles in reproduction and may enhance the action of PGs. Receptors for LTs are classified into two separate groups in respect of structure and cell location: LTRI for LTB_4 and LTRII for cysteinyl LTs (LTC_4, LTD_4 and LTE_4). There are two isoforms of receptors for LTB_4 and at least two receptors for cysteinyl LTs (Izumi *et al.* 2002). We have recently shown that mRNA for *5-LO* and *LT receptors* are expressed in the bovine ovarian cell types, i.e. steroidogenic and endothelial luteal, and granulosa cells (Korzekwa *et al.* 2010b). Leukotriene B_4 seems to play a luteotropic role in the bovine CL, stimulating P4 and PGE_2 secretions, whereas LTC_4 stimulates the secretion of luteolytic $PGF_{2\alpha}$ and may enhance the luteolytic cascade (Korzekwa *et al.* 2010a). These *in vitro* results has been recently supported by *in vivo* studies (Korzekwa *et al.* 2010c). Thus, LTs were found to be auto / paracrine factors modulating the secretory functions of ovarian cells depending on the stage of the cycle and type of LTs (Blair *et al.* 1997; Korzekwa *et al.* 2010a, c).

The highest level of mRNA expressions for *LTRs* and *5-LO* in luteal tissue were observed on Day 16-18 of the cycle (Korzekwa *et al.* 2010a). More specifically, a similar pattern of mRNA expression of *LTRs* and *5-LO* and production/secretion of LTs during the cycle was described recently by us in two main cell population of bovine luteal steroidogenic and endothelial cells (Korzekwa *et al.* 2010a, b). The greatest mRNA expression for *LTR-II* and *5-LO* was found at the late luteal phase of the cycle in endothelial luteal cells, whereas *LTR-I* mRNA expression did not differ among cell types. These findings indicate that endothelial cells possess the greatest potential for LTs production among ovarian cells, being these cells the main source of LTs in the bovine ovary (Korzekwa *et al.* 2010a, b). Our most recent data indicate that endogenous LTC_4 inhibits P4 secretion in the oestrous cycle, since Azelastine a specific LTC_4 antagonist, elevated P4 output and prolonged the lifespan of bovine CL *in vivo* (Korzekwa *et al.* 2010c). Consequently, the data obtained in the recent studies strengthen the concept that LTs are factors regulating reproductive processes as auto- and/or paracrine factors in CL during the oestrous cycle (Korzekwa *et al.* 2010a,b,c). We have shown: (i) LTs production and secretion in luteal tissue and its localization in the ovary; and (ii) the influence of LTs on PGs secretion and action during the estrous cycle. Based on the above findings, LTs action on the bovine reproductive tract has been suggested to be dependent on the LT type, and LTC_4 may serve as a mediator of luteolytic $PGF_{2\alpha}$ action (Milvae *et al.* 1986; Blair *et al.* 1997; Jaroszewski *et al.* 2003 Skarzynski *et al.* 2003b; Korzekwa *et

al. 2008b, 2010a,c). Although we have shown that LTC_4 was strongly elevated in the bovine CL between 2- and 4-h after the pharmacological dose of an analogue of $PGF_{2\alpha}$ infusion (dinoprost, 5 mg; Jaroszewski *et al.* 2003b), no stimulatory effect of the luteolytic dose of TNF on LTC_4 output was observed during 4-h (Skarzynski *et al.* 2003b). Moreover, the peaks of luteolytic LTC_4 have been demonstrated in bovine CL on Day 18 after ovulation in heifers undergoing spontaneous luteolysis (Milvae *et al.* 1986), and their frequency increased within the 12-h period during which the onset of P4 decline occurred (Blair *et al.* 1997). Therefore, the release of LTC_4 from the CL may be one of the late results of the activation of the luteolytic cascade induced by uterine $PGF_{2\alpha}$.

Nitric oxide

Nitric oxide which plays roles in a variety of physiological mechanisms, including blood flow regulation appears to be a good candidate to mediate the $PGF_{2\alpha}$ action during the first steps of the luteolytic cascade in cattle (Skarzynski *et al.* 2003a; Shirasuna *et al* 1998; Acosta *et al.* 2009). NADPH-d localization (a marker for nitric oxide synthase - NOS) and immunostaining of both isoforms of NOS (inducible - iNOS and endothelial - eNOS) were detected in steroidogenic cells and in blood vessels of the bovine CL during the entire estrous cycle with increasing activity from the early to the late luteal phases (Skarzynski *et al.* 2003b). However, other studies presented the highest level of eNOS mRNA and protein expression in the early bovine CL (Rosiansky-Sultan *et al.* 2006; Shirasuna *et al.* 2010). Luteolytic or luteotropic actions of NO on the bovine CL during the oestrous cycle is strictly dependent on the stage of CL, and cell interactions (cell-to-cell contact) and composition (Jaroszewski *et al.* 2003a; Weems *et al.* 2004; Klipper *et al.* 2004; Rosiansky-Sultan *et al.* 2006). NO donor (S-NAP) stimulated PGE_2 secretion by steroidogenic luteal cells in the early and mid-luteal phases (Skarzynski & Okuda 2000; Skarzynski *et al.* 2000b). These data show that there is an inverse relationship between NOS and EDN1 throughout the life span of bovine CL, and imply that this pattern may be the result of their interaction between luteal endothelial and immune cells. Thus, NOS are expressed in a physiologically relevant manner: elevated NO at an early luteal stage is likely to play an important role in angiogenesis, whereas reduced levels of eNOS during luteal regression may facilitate the sustained upregulation of EDN1 levels during luteolysis (Rosiansky-Sultan *et al.* 2006; Shirasuna *et al.* 2010). During the development and maintenance of the CL, PGE_2 which is both luteotropic and antiluteolytic, may stimulate NO production. Moreover, NO donors and EDN1 increased PGE_2 secretion by bovine luteal slices *in vitro* on Days 13-14 of the cycle without any direct effect on P4 secretion (Weems *et al.* 2004). Therefore, the increased NO production during early stages of the cycle and pregnancy is likely to play a role in the development and angiogenesis of bovine CL (Skarzynski & Okuda 2000; Weems *et al.* 2004; Rosiansky-Sultan *et al.* 2006) and suggests multiple roles of NO in the regulation of the bovine luteal function (Skarzynski *et al.* 2008). However, Shirasuna *et al.* (2010) have observed many of eNOS positive areas in the periphery of the mid, late and regressing bovine CL, where many arteriolovenous vessels are located. As suggested by the authors, the distribution of capillaries, arteriolovenous and eNOS expression differs between early, mid, late and regressing bovine CL. Thus, this structural change from the early (homogeneous, regarding cell types distribution and vasculature composition) to late (heterogeneous) luteal phase is related to the differences in the CL response to $PGF_{2\alpha}$ due to the blood regulating factors, including NO. Moreover, it has been recently shown that not only eNOS is involved in NO production during luteolysis in cattle (Lee et al. 2009). Bovine endothelial luteal cells are a target for $PGF_{2\alpha}$ and that $PGF_{2\alpha}$ stimulates iNOS expression and iNOS activity. Stimulation of the NO generating system and iNOS activity by $PGF_{2\alpha}$ may result in increasing local NO production followed by luteolysis (Lee *et al.* 2009). This *in vitro* observation has been supported by the *in vivo* data (Acosta *et al.* 2009). We have shown *in vivo* that after $PGF_{2\alpha}$ application, concentrations of NO metabolites

in the ovarian artery blood plasma preceded the rise in the systemic circulation by about 15 to 30 min (Fig. 3). Thus, it appears that an ovary bearing a CL responds to $PGF_{2\alpha}$ injection within a shorter period of time compared with other organs. This supports the idea that the CL produces a considerable amount of NO in response to $PGF_{2\alpha}$ and that increased NO is responsible for the acute increase in luteal blood flow observed in cattle. Moreover, in the late luteal phase, $PGF_{2\alpha}$ might simulate a shear stress-like reaction of endothelial luteal cells resulting in compensative NO release during the first steps of luteolysis - up to 2-4 h after $PGF_{2\alpha}$ treatment (Skarzynski *et al.* 2003a; Acosta *et al.* 2009). These data suggest that NO produced by two NOS isoforms is involved in structural and functional changes that occur in the bovine CL during lutolysis.

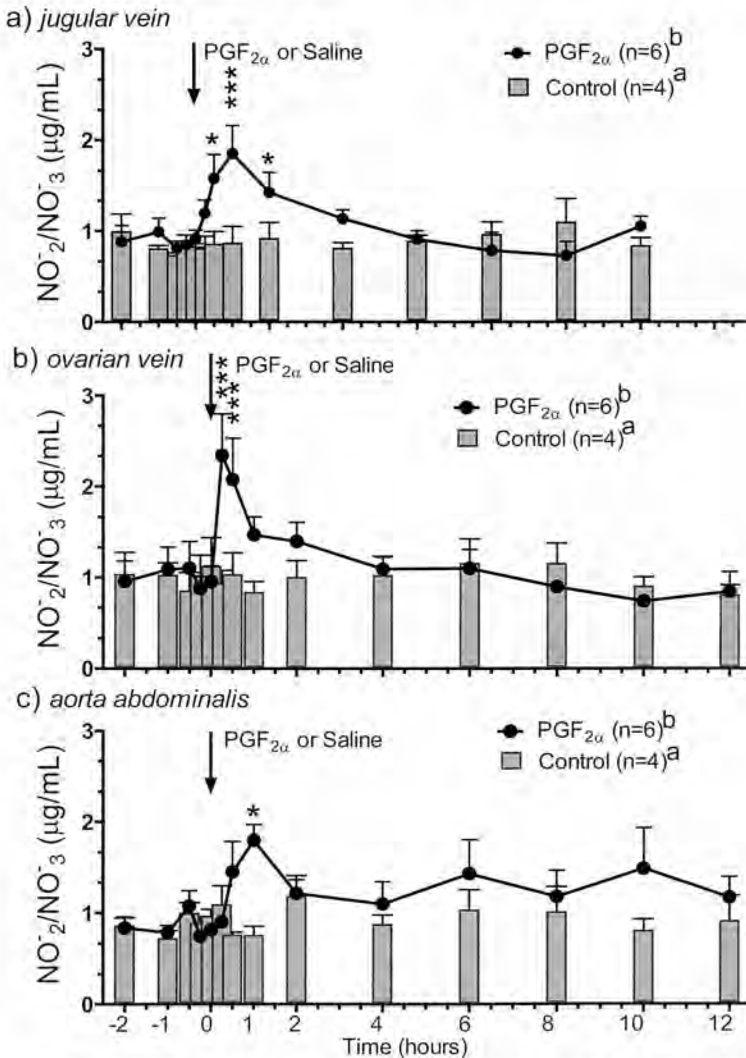

Fig. 3. Effects of prostaglandin $(PG)F_{2\alpha}$ on the concentrations of nitrite/nitrate (NO^-_2/NO^-_3), the stable metabolites of nitric oxide, in blood plasma collected from the (a) jugular vein, (b) ovarian vein and (c) *aorta abdominalis*. Cows were treated with a $PGF_{2\alpha}$ analogue (cloprostenol, 500 μg) or Saline (Controls) intramuscularly on Day 10 of the oestrous cycle. Asterisks indicate significant differences ($P < 0.05$) compared with the pretreatment period (baseline). Different superscript letters indicate significant differences ($P < 0.05$) between the $PGF_{2\alpha}$-treated and Control. Reprinted from Acosta *et al.* (2009).

Intra-luteal administration of a NOS inhibitor (L-NAME) during the late luteal phase increased P4 secretion and prolonged the functional lifespan of bovine CL (Jaroszewski & Hansel 2000; Shirasuna *et al.* 2008b). When an analogue of $PGF_{2\alpha}$ (cloprostenol) was injected on Day 15 of the cycle in combination with L-NAME, the luteolytic effect of $PGF_{2\alpha}$ was counteracted by the NOS inhibitor (Fig. 4; Skarzynski *et al.* 2003a; Jaroszewski *et al.* 2003b). Nitric oxide has been found as the most potent inhibitor of P4 secretion *in vitro* (Skarzynski *et al.* 2000; Korzekwa *et al.* 2004, 2006) and *in vivo* (Shirasuna *et al.* 2008b). Moreover, a NO donor (Spermine NONOate) strongly stimulated production of $PGF_{2\alpha}$ and LTC_4 by bovine CL both *in vitro* and *in vivo,* showing that NO is involved in the process of luteal regression by bovine CL (Jaroszewski *et al.* 2003a; Korzekwa *et al.* 2004, 2006; Shirasuna *et al.* 2008b) suggesting the role of NO in luteolysis initiation in cows. TNF and $PGF_{2\alpha}$ induce NO synthesis during $PGF_{2\alpha}$-induced luteolysis (Skarzynski *et al.* 2003a,b, 2007, 2009; Acosta *et al.* 2009) Nitric oxide is also involved in structural luteolysis stimulating *BAX* mRNA expression in the steroidogenic luteal cells (Korzekwa *et al.* 2006). Consequently, the ratio of *BCL2* to *BAX* decreased. Moreover, NO stimulates expression and activity of caspase-3. Thus, in concert with END1 and cytokines action, NO seems to play a crucial role in both functional luteolysis (inhibition of the basal and LH-stimulated P4 synthesis; blood flow) and structural luteolysis (induction of apoptosis of the luteal cells). However, Acosta *et al.* (2009) showed that *in vivo* $PGF_{2\alpha}$ induced P4 decline followed with the significant (maintaining only 0.5-2h) NO output by CL-containing ovary. Therefore, luteolytic action of NO might be limited only to the first steps of $PGF_{2\alpha}$-induced luteolytic cascade. Expression of *NOS* mRNA in bovine CL gradually reduced from two until 60-h after $PGF_{2\alpha}$ analogue injection (Neuvians *et al.* 2004; Rosiansky-Sultan *et al.* 2006). Thus, in the late stages of luteal regression, a classical - inverse relationship between $PGF_{2\alpha}$, EDN1 and NO system may exist and reduced levels of NO may facilitate the sustained up-regulation of EDN1 system in the CL (Klipper *et al.* 2004; Rosiansky-Sultan *et al.* 2006).

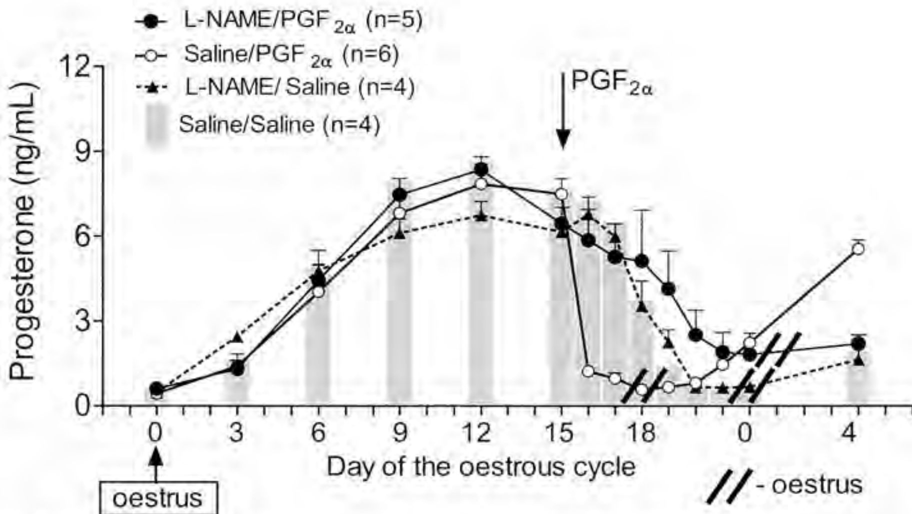

Fig. 4. The effect of 2 h infusion of saline or a nitric oxide synthase inhibitor (L-NAME; 400 mg/1h) and injection of Saline or a prostaglandin $F_{2\alpha}$ analogue ($PGF_{2\alpha}$; cloprostenol, 100 µg) at 30 min of infusion on progesterone concentrations in peripheral blood plasma of heifers on Day 15 of the oestrous cycle. Different subscript letters indicate significant differences ($P < 0.05$). Modified from Skarzynski et al. (2003a).

Role of blood flow and hypoxia in the regression of bovine CL

The dynamic changes in the ovarian blood flow have been well demonstrated in ruminants until today. Classical studies found that ovarian blood flow was low just after ovulation, gradually increased toward the luteal stage, then decreased during luteal regression in cattle (Ford & Chenault 1981; Wise *et al.* 1982). These findings have been found by using electromagnetic probes. Recently, intraovarian blood flow was monitored by color-doppler ultrasound sonography (Acosta *et al.* 2002; Ginther *et al.* 2007). These studies found that blood flow increased rapidly and temporally (up to 2h), and then decreased (after 4-8h), when a luteolytic $PGF_{2\alpha}$ was injected to cows. The rapid increase of blood flow has been suggested to be induced by vasodilators, such as PGE_2 (Miyamoto *et al.* 2005) and NO (Skarzynski *et al.* 2003a). In support, it has been shown that intraluteal application of a NO donor drastically increase luteal blood flow (Shirasuna *et al.* 2008b).

As discussed previously, NO, one of the reactive oxygen species (ROS), has been suggested to play critical roles in luteolysis in cows, because it decreases P4 secretion (Korzekwa *et al.* 2006; Shirasuna *et al.* 2008b) and promotes apoptosis in bovine luteal cells (Korzekwa *et al.* 2006). In cattle, we recently observed that an injection of $PGF_{2\alpha}$ induces a transient (1-2 h) increase in the concentrations of NO and partial pressure of O_2 (pO_2) in ovarian venous blood, and that the pO_2 of venous blood is higher in the ovarian vein than in the jugular vein (Acosta *et al.* 2009). Therefore, the luteal micro-environment seems to be high oxygen condition, especially during a short period of time (1-2 h) following $PGF_{2\alpha}$ injection. Interestingly, $PGF_{2\alpha}$ stimulates the NO generating system by increasing inducible iNOS mRNA and protein expression, and NOS activity in cultured luteal endothelial cells (Lee *et al.* 2009) as well as eNOS expression in the periphery of the bovine CL (Shirasuna *et al.* 2008b). In addition to NO, the other ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and oxygen (O_2) have been implicated in the luteolytic process (Agarwal *et al.* 2005). H_2O_2 has been shown to inhibit P4 secretion and to induce apoptotic changes in cultured bovine luteal cells (Nakamura *et al.* 2001). Therefore, generation of ROS following the changes in the blood flow may be deeply related to the local mechanisms of functional and structural luteolysis in the cow.

Following the rapid increase after $PGF_{2\alpha}$ injection to cows, blood flow decreases in a short time, *i.e.* a significant decrease was observed 8 h after $PGF_{2\alpha}$ injection (Acosta *et al.* 2002; Fig. 5). In both spontaneous and $PGF_{2\alpha}$-induced luteolysis, a decrease in luteal blood flow begins in parallel with systemic P4 concentrations (Ford & Chenault 1981; Wise *et al.* 1982). Furthermore, the pO_2 in the blood collected from the ovarian vein began to decrease at the late luteal stage (Wise *et al.* 1982). Therefore, we hypothesized that O_2 shortage (hypoxia) caused by a decrease of blood supply is one of the factors promoting luteolysis in the cow (Fig. 5). We found that hypoxia inhibits P4 synthesis in cultured bovine luteal cells by inhibiting the steroidogenic enzyme P450scc (Nishimura *et al.* 2006). Hypoxia also promotes apoptosis of luteal cells by increasing a pro-apoptotic protein BNIP3 expression, and by activating caspase-3 (Nishimura *et al.* 2008). These findings suggest that hypoxia promotes both functional luteolysis (P4 decrease) and structural luteolysis (apoptosis), and revealed that O_2 deficiency caused by a decreasing blood supply in bovine CL is one of the factors contributing to luteolysis in the cow (Hojo *et al.* 2009). However, $PGF_{2\alpha}$ is able to inhibit P4 secretion even though pO_2 remains still high and pCO_2 is not up-regulated in the bovine reproductive tract (Acosta *et al.* 2009). Moreover, Watanabe *et al.* (2006) showed that P4 decreased even when blood flow remained high in the bovine CL. Therefore, O_2 deficiency due to decreased blood flow is not mandatory for the induction of both functional and structural luteolysis in cows, but may play such a supporting role in the final steps of bovine luteal regression.

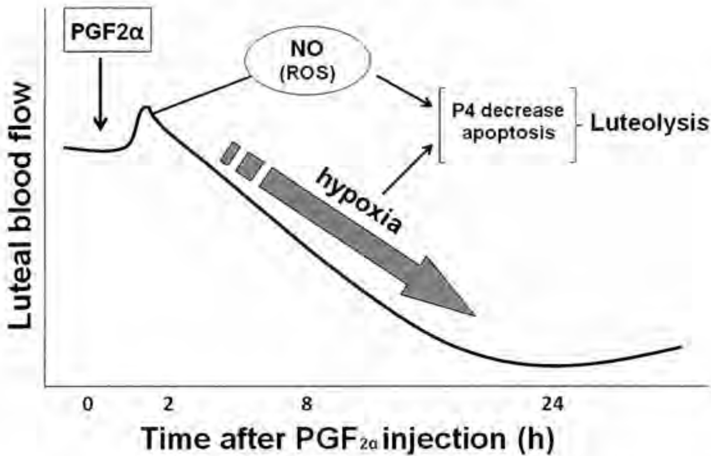

Fig. 5. Scheme of the relationship between luteal blood flow and luteolysis related factors, reactive oxygen species (ROS) and hypoxia. We propose that the temporal increase of blood flow induces ROS including nitric oxide (NO), and that the following decrease of blood flow makes hypoxic conditions in corpus luteum. Since both NO and hypoxia have been shown to decrease P4 synthesis and to induce apoptosis of bovine luteal cells, the changes of blood flow are strongly related to luteolysis by affecting these factors.

Concluding remarks

The pulsatile $PGF_{2\alpha}$ secretion in cattle was previously proposed to be generated by a positive feedback loop between ovarian and/or hypophyseal OT and endometrial $PGF_{2\alpha}$. However, since some data suggest that OT is not essential for the initiation of luteolysis, the importance of a $PGF_{2\alpha}$-OT feedback loop remains less certain in cattle. The bovine endometrium seems to possess endogenous mechanisms for initiation of $PGF_{2\alpha}$ secretion. The mechanism controlling the development, maintenance and secretory function of the CL may involve factors that are produced both within the CL and outside the ovary. Some of these regulators seem to be prostaglandins and other arachidonic acid metabolites (PGE_2, $PGF_{2\alpha}$, LT), neuropeptides, peptide hormones (OT, EDN1), growth factors and hormones (VEGF, FGF, GH, PRL) and steroids (P4 and 17β-oestradiol) that act as autocrine and/or paracrine factors. Although $PGF_{2\alpha}$ is known to be a principal luteolytic factor, its action on the CL is mediated by other intra-ovarian factors cytokines, leukotrienes, EDN1, NO (Fig 6). Nitric oxide, and TNF in combination with IFNG reduced P4 secretion, increased luteal $PGF_{2\alpha}$ production, and induced apoptosis of the luteal cells (Fig. 6). As recently suggested by Berisha et al. (2010) the cascades connected with $PGF_{2\alpha}$-induced luteolysis are: 1) luteal NO release and blood flow up-regulation, 2) up-regulation of inflammatory cytokines and luteal cell apoptosis factors, 3) strong up-regulation of vasoactive peptides in luteal cells, and 4) extracellular matrix proteases associated with luteal tissue remodelling. In addition, the decrease in luteal blood flow and hypoxia has been proposed as an important luteolytic mechanisms in the cow. Hypoxia inhibits P4 synthesis in the bovine luteal cells by inhibiting the steroidogenic enzymes and promotes apoptosis of luteal cells by increasing pro-apoptotic proteins. Although reduction of luteal blood flow and hypoxia contribute to the late events of luteolysis, little is known about the physiological relevance and the cause of the transient increase in luteal blood flow and reactive oxygen species during the initial step of luteolysis.

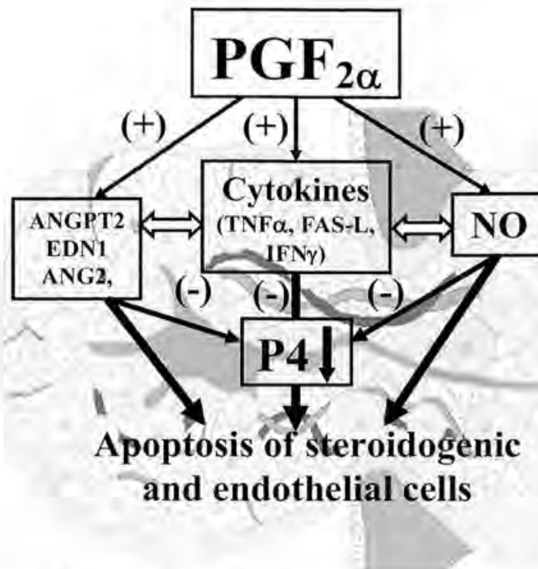

Fig. 6. Hypothetical model of the structural and functional regression of the CL (see text for the details; adapted from Skarzynski *et al.* 2008)

Acknowledgments

The work was supported by the grants-in-aid from the Polish Ministry of Sciences and Higher Education (N308 3279 33) and Japan Society for Promotion of Sciences (JSPS B 22380148). The authors are grateful to Prof. Graça Ferreira-Dias (Technical University of Lisbon, Portugal) for critical reading and editing language of the manuscript. We thank Ilona Kowalczyk-Zieba (Institute of Animal Reproduction & Food Research in Olsztyn, Poland) for technical assistance in preparation the Figures. The authors declare that they have no conflict of interest that would prejudice the impartiality of the scientific word.

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