WestVirginiaUniversity
THE RESEARCH REPOSITORY @ WVU

Graduate Theses, Dissertations, and Problem Reports

2009

Role of endothelin 1 in follicular and luteal function in the sheep

Fernando P. Perea-Ganchou
West Virginia University

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Recommended Citation

Perea-Ganchou, Fernando P., "Role of endothelin 1 in follicular and luteal function in the sheep" (2009). *Graduate Theses, Dissertations, and Problem Reports*. 2860.
https://researchrepository.wvu.edu/etd/2860

This Dissertation is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Dissertation has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

Role of Endothelin 1 in Follicular and Luteal Function in the Sheep

Fernando P. Perea-Ganchou, M.S.

Dissertation submitted to the Davis College of Agriculture,
Forestry, and Consumer Sciences at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Reproductive Physiology

E. Keith Inskeep, Ph.D., co-Chair
Jorge A. Flores, Ph.D., co-Chair
Robert A. Dailey, Ph.D.
Jianbo Yao, Ph.D.
Robert L. Goodman, Ph.D.

Faculty of Reproductive Physiology

Morgantown, WV
2009

Keywords: preovulatory follicles, granulosal cells, steroidogenesis, luteolysis, endothelin 1, sheep

ABSTRACT

Roles of endothelin 1 in follicular and luteal function

Fernando P. Perea-Ganchou., M.S.

Reproductive activity in mammals is characterized by the recurrent presence of follicles and corpora lutea (CL) in the ovaries. In addition to the roles of follicles and corpora lutea in regulating the activity of the hypothalamic–hypophyseal–ovarian axis, those ovarian structures are responsible to generate a viable female gamete and to provide the appropriate uterine physiological conditions to sustain the embryo/fetus development during pregnancy. If fertilization of the oocyte does not occur or pregnancy fails, a new ovarian cycle starts a few days later, assuring further opportunity for onset of a new gestation. Numerous locally produced ovarian factors modulate function of follicles and CL. Endothelin 1, a 21-amino acid peptide produced in the ovary, modulates steroid production by granulosal cells and mediates luteolytic actions of $PGF_{2\alpha}$. Three experiments were carried out in order to examine the role of endothelin in follicular steroid secretion in vitro and in spontaneous and $PGF_{2\alpha}$-induced luteolysis in sheep.

In experiment 1, it was hypothesized that endothelin 1 (END1) acting through its receptors ENDRA and/or ENDRB, on granulosal cells of ovine preovulatory follicles would inhibit steroid production, and therefore, prevent the premature luteinization of granulosal cells of those follicles. On day 14 of the estrous cycle (standing estrus = day 0), 17 nonpregnant Suffolk ewes were injected with 2 doses of $PGF_{2\alpha}$ (5 mg) 3 h apart. Thirty six to 40 hours after the second dose of $PGF_{2\alpha}$, ovaries were removed by mid-ventral laparotomy. The 2 or 3 largest follicles in the ovaries were dissected and granulosal cells were harvested under sterile conditions. Aliquots of 15 μL of granulosal cell suspension from each presumed preovulatory follicle were cultured in 96-well plates containing a solution of 185 μL of Ham's F12 supplemented medium and treatments (single and combined effects of LH, END1, ENDRA and ENDRB). Data from estrogen-inactive 20 follicles collected from 13 ewes were analyzed. Accumulation of E_2 and P_4 by cultured granulosal cells was not affected by either LH or END1 or the END receptor blockers. Granulosal cells from follicles with the greatest concentration of E_2 in FF produced on the average significantly more E_2 and P_4 than follicles with lower concentrations of E_2 in FF. In conclusion, END1 did not affect steroid production by granulosal cells from preovulatory follicles in sheep, and therefore the working hypothesis was not supported.

In the second experiment, the ENDRA antagonist BQ-610 was delivered into the CL via an osmotic minipump to examine the expression of genes related with P4 production and structural luteolysis at 6 and 24 h after exogenous $PGF_{2\alpha}$. It was hypothesized that sustained blockade of ENDRA would prevent upregulation of genes stimulated by $PGF_{2\alpha}$ during structural luteolysis. Minipumps loaded with either vehicle (2:1 methanol/saline solution) or 2.5 mg of BQ-610, were implanted in the mesovarium, with a catheter into a CL, on day 7.5 to 9 of the estrous cycle. A dose of $PGF_{2\alpha}$ (25 mg) or saline was injected 48 h after surgery in all animals, respectively. Six or 24 h later ewes were euthanized, ovaries were collected and CL were dissected and stored for later determinations of hormones and gene expression. Data for luteal weight, and concentrations of serum and luteal P_4, mostly at 24 h after $PGF_{2\alpha}$, indicated

that in 9 of 10 treated ewes, BQ-610 did not prevent luteolysis, and the pattern of gene expression confirmed this finding. Exogenous $PGF_{2\alpha}$ downregulated expression of StAR, 3βHSD, VEGF, TIMP-1 and eNOS ($P \leq 0.02$), whereas it upregulated 20αHSD, MMP-14 Bcl-2 ($P \leq 0.03$). Expression of Bcl-2 and Bax were reduced and Fas-R was increased from 6 to 24 h after $PGF_{2\alpha}$. In BQ-610-treated ewes, mRNA expression was upregulated more for 20αHSD, and caspases 3 and 8 than in vehicle-treated and control ewes. In conclusion, $PGF_{2\alpha}$ induced luteolysis in both vehicle-treated and BQ-610-treated ewes. Given that in BQ-610-treated ewes, certain genes related with P_4 metabolism and apoptosis were differentially upregulated, the greater dosage of BQ-610 used in this study, or its combination with methanol may have activated ENDRA. If so, these findings would confirm that END1 via ENDRA in luteal cells is associated with structural luteolysis as previously reported.

In the third experiment, it was hypothesized that chronic delivery of BQ-610 into the CL would prevent luteal regression induced by endogenous $PGF_{2\alpha}$, thereby increasing length of the estrous cycle and maintaining functional and structural characteristics of the CL. Ewes were assigned receive an osmotic mini-pump containing 2 mg of BQ-610 (n = 12) or vehicle (n = 9), implanted surgically on d 9 of the estrous cycle. Corpora lutea were collected 12 h after onset of estrus, or on the afternoon of d 21 in ewes that had not returned to estrus, and from untreated ewes on d 10 to 12 of the estrous cycle (mid-phase CL). Three of 12 BQ-610-treated ewes did not show estrus before d 21 compared to 0 of 9 vehicle-treated ewes ($P = 0.33$); CL of the remaining nine ewes treated with BQ-610 were excluded from further analysis. Estrous cycles in vehicle-treated ewes averaged 15.5 ± 0.2 d. In the three BQ-610-treated ewes, luteal weights on d 21were greater than in vehicle-treated ewes on the day of estrus (0.62 ± 0.05 versus 0.39 ± 0.03 g, respectively; $P < 0.001$), as were luteal contents of P_4 (20.9 ± 1.8 versus 1.3 ± 1.0 μg/g, respectively; $P < 0.0001$). Serum concentrations of P_4 in the three BQ-610-treated ewes remained above 1.5 ng/ml through d 21 ($P < 0.01$). Their luteal tissue appeared normal with 53.3 ± 5.8% of apoptotic cells, whereas luteal tissue in vehicle-treated ewes was markedly disorganized and in an advanced stage of structural regression. Expression of mRNA was lower [3βHDS ($P < 0.01$), and TIMP-1 ($P < 0.01$)]; greater [preproEND1 ($P < 0.01$), END receptor A ($P < 0.01$)]; or similar (Bax, Bcl-2, Fas-R) in CL from vehicle-treated than in CL of BQ-610-treated ewes or in mid-phase CL. Caspase 3 expression was grater in CL of vehicle-treated an BQ-610-treated ewes than mid-phase CL. In conclusion, chronic infusion of BQ-610 blocked luteolysis and lengthened the estrous cycle in 3 of 12 ewes. Furthermore, functional features of CL of those 3 ewes were similar to mid-phase CL. Overall, these results supported a role for END1 in luteal regression in sheep.

Key words: luteolysis, progesterone, END1, endothelin receptor type A, BQ-610, gene expression, sheep

ACKNOWLEDGEMENTS

I would like to thank Dr Keith Inskeep and Dr Jorge Flores for their patient guidance, support, and criticism in this unforgettable journey plenty of scientific knowledge, challenges, and life experience. The constant interest and enthusiasm to spread their knowledge for science, and particularly for reproductive physiology issues, have been a source of inspiration, satisfaction, and encouragement in my personal and professional life.

I would like thank Drs Robert Dailey, Jianbo Yao and Robert Goodman for their assistance and participation on my graduate committee. Their guidance and scientific knowledge in the field of the reproductive physiology was an invaluable contribution in my scientific and professional training in the area.

I would like to express my gratitude to Dr Paul Lewis, chair of the Division of Animal and Nutritional Sciences, for his interest and diligence to obtaining the tuition waivers during my scholar period. Also, I would like to express my appreciation to the staff members of this division for their assistance in all administrative procedures. The help and advice of Drs. Mohamed Salem and Miro Valent was very useful, thanks.

During my stay in WVU, I have had the privilege to meet and receive the invaluable help, guidance or friendship of numerous co-students, ones already graduated and others still in training. Particularly, I would like to thank Drs Ida Holásková, Madhusudan Goravanahally, Justin Rhinehart, Aimee Wurst, Brandon Lingenfelter, Jill Koch, Fredrick Odhiambo, Raghuveer Ramachandra, M.S. Adrienne Boguzs, and the graduate students Julie Mankey, Erin Soisson, Shaun Stanley, Tammy Holler, Caleb Lemley, Tina Dow, Matthew Dean, Jyothsna Tejomurtula Lei Wang and undergraduates Gail Nesselrod and Edie Johnson.

I also wish to thank Dr Margaret Minch, Jennifer Lyndon, Heather Clemmer, and Andy Holt for their helpful assistance during the surgeries and experimental activities in the Livestock Farm and FARF.

I cannot leave to mention and thank Domingo Mata, Patricia Mascaro, Jim and Marcela Whetsell, Don and Amada McDowell and Eugenia Pena for their invaluable help, advices and friendship during my life in Morgantown.

Finally, I would like to thank Romina, my beautiful wife, and Mariana and Miguel, my loved children, for their radiant joy, love, patient, and unconditional encouragement and help in this wonderful journey. To my father, Horacio, for his support, advices, and example of integrity and perseverance, I will be ever grateful.

The presence of God in my life has been an unlimited source of faith, strength, encouragement and optimism that allowed this achievement. Thanks.

TABLE OF CONTENTS

LIST OF FIGURES

REVIEW OF LITERATURE

Role of END1 in steroid production by ovine granulosal cells collected from large preovulatory follicles

Effect of blocking endothelin receptor type A on gene expression during $PGF_{2\alpha}$-induced luteolysis in sheep

Effect of chronic administration of an endothelin receptor type A antagonist on length of estrous cycle and functional characteristics of the corpus luteum in sheep

LIST OF TABLES

REVIEW OF LITERATURE

Physiologic aspects of follicular growth

Ovarian follicles are structural and functional entities that play essential physiological roles, modulating activity of the hypothalamic–pituitary–ovarian (HPO) axis and providing an appropriate metabolic and endocrine milieu to support the development and maturation of the oocyte. Eventually they facilitate the release of the oocyte into the oviduct, where fertilization takes place. Ovarian follicles develop from primordial follicles throughout different morphological stages that are conducive to attainment of the functional competence to ovulate, and thus, provide the female gamete required to assure survival of the species. Two follicular populations of steroidogenic cells (granulosa and theca) interact with each other to produce estradiol and other hormones necessary to modulate reproductive function (Mann et al., 1992). Once ovulation occurs, the remnant collapsed follicle gives rise to another essential ovarian structure, the corpus luteum. If fertilization of the oocyte does not occur, the corpus luteum undergoes functional demise, and a new group of follicles begins to mature a few days later. Thus, ovarian follicles are an essential component of reproductive cyclicity.

Preantral follicular development

Primordial follicles are the most elemental follicular structures and consist of an oocyte surrounded by a single layer of flattened granulosal cells. In domestic animals and primates, primordial follicles are formed during the embryonic/fetal period, and most of them will undergo atresia during the first years after birth (Driancourt et al., 1993). For instance, in

newborn human females, only 400,000 follicles remain in the ovary from 6 to 7 million follicles present at mid gestation (Gougeon, 1996). The large pool of primordial follicles in the ovary constitutes a follicular store from which they are activated progressively and grow through different developmental stages. Most primordial follicles are in a resting stage as a consequence of local and systemic inhibitory stimuli. Accordingly, in superficial pieces of ovarian cortex cultured in serum-free conditions, healthy primordial follicles decreased by 88%, 90% and 94% after 2, 4 and 7 days in culture, respectively, whereas healthy primary follicles increased by 260%, 209% and 197%, respectively, compared with the numbers present on d 0 (Wandji et al., 1996a). These findings indicated that certain components of the ovarian stroma may exert inhibitory control in the onset of growth of the primordial follicles (Wandji et al., 1996a). The initiation of growth of primordial follicles and subsequent development to the secondary stages can occur in absence of gonadotropins (Wandji et al., 1997). Moreover, the transition from primordial to primary follicular stage is apparently dependent upon communication exchanged between the follicle and the adjacent cells, mostly stromal/interstitial and endothelial cells, which provide both stimulatory and inhibitory signals (Skinner, 2005). Thus, once stimulatory factors prevail over the inhibitory influences, primordial follicles start to grow toward subsequent follicular stages (McGee and Hsueh, 2000; Durlinger et al., 2002; Kezele et al., 2002; 2003; Holt et al., 2006; McNatty et al, 2007).

The transition from primordial to primary follicular stage is a process by which a primordial follicle increases in size (Wandji et al., 1996a), as granulosal cells proliferate and change in morphology from flattened to cuboidal (Lundy et al., 1999). As the follicle develops new layers of granulosa, the size of the follicle cells increase, concomitant with the increment of oocyte

diameter (Lundy et al., 1999). Numerous factors from local or systemic origin modulate growth of primary follicles and successive preantral follicular stages (Wandji et al., 1996b; Fortune; 2003; McNatty et al, 2007). Although FSH did not appear to be essential for development of preantral follicles (Halpin et al., 1996; McNatty et al., 1990), evidence indicated the presence of FSH receptors in granulosal cells from primary and secondary follicles in ruminants (Wandji et al., 1992; Tisdall et al., 1995; Xu et al., 1995a; Bao et al., 1997a). FSH and bFGF stimulated a greater increment of the diameter of bovine preantral follicles than EGF (Wandji et al., 1996b). Moreover, granulosal cells from preantral follicles treated with FSH produced more progesterone than control or bFGF- and EGF-treated cells (Wandji et al., 1996b). Thus, it was evident that FSH as well as other locally produced factors such as insulin, IGF1, GDF9, BMP15, EGF, activin, and steroids, can modulate the rate of preantral follicle development (Hulshof et al., 1995; 1997; Gutierrez et al., 2000; Juengel et al., 2002; Souza et al., 2002; Zhao et al., 2003). In addition, granulosa-oocyte communication appears to be essential for normal preantral follicular development (McGee and Hsueh, 2000). During the development of preantral stages, growing oocytes synthesize the zona pellucida, and epithelial cells adjacent to the follicle differentiate into thecal cells (Driancourt et al., 1993). Although mRNA for LH receptor has been detected after formation of the theca interna, its role in these early stages of follicular development has not been elucidated (Bao and Garverick, 1998).

Antral follicular development

At the end of the preantral stage, following formation of the theca interna around the granulosal cells, thecal cells express mRNA for LH receptor and important steroidogenic enzymes such as P450scc, P450c17 and 3βHSD as well (Xu et al., 1995a; Xu et al., 1995b; Bao

et al., 1997a; Bao et al., 1997b). Around the beginning of the antral stage, granulosal cells start to express mRNA for 450arom (Xu et al., 1995b). Moreover, P450c17 mRNA was identified exclusively in thecal cells, while 450arom mRNA was expressed solely in granulosal cells, while both cell types expressed mRNA for P450scc and 3βHSD (Xu et al., 1995b; Bao et al., 1997b). So, it was clear that thecal cells had the capacity to synthesize progestins and androstenedione, whereas granulosal cells from antral follicles synthesized progestins and estradiol. Therefore, it was suggested that thecal cells under LH stimulus synthesize androgens and granulosal cells under FSH influence actively aromatize exogenous androgens to estrogens (Fortune and Armstrong, 1978; Fortune, 1986). Furthermore, granulosal cells produced considerable amounts of pregnenolone that were used by thecal cells as a precursor of androgen production (Fortune, 1986), and androgens produced by the theca interna increased the ability of granulosal cells to synthesize pregnenolone (Fortune, 1986).

Once follicles become progressively gonadotropin-dependent and the metabolic machinery is expressed completely in steroidogenic cells, follicles start to form a fluid filled cavity or antrum. According to Webb and Campbell (2007), "antrum formation marks the transition from a proliferative to a differentiative phenotype for the follicular somatic cells", which occurs concomitant with a manifest increase in the rate of follicular atresia and significant changes in gene expression (Webb et al., 2003). At the beginning of the antral stage, follicular fluid is synthesized exclusively by thecal cells, and in later stages granulosal cells acquire the metabolic capacity to synthesize both progestins and estradiol (Bao et al., 1997a; Bao et al., 1997b).

Although during the antral developmental stage, gonadotropic stimulus is essential for normal development of the follicles; other locally produced factors originated from the oocyte and somatic cells are important autocrine/paracrine modulators of the follicular growth. Several members of BMP family (BMP 2, 4 and 6) increased FSH-stimulated production of estradiol and inhibin A by ovine granulosal cells (Souza et al., 2002; Campbell et al., 2006). Also, BMP 4, 6 and 7 stimulated estradiol, inhibin A, activin A, and follistatin production by granulosal cells without presence of FSH in the culture media (Gliser et al., 2004). Moreover, BMP 4, 6 and 7 increased the ability of IGF-I to stimulate secretion of estradiol, inhibin A, activin A, and follistatin by granulosal cells (Gliser et al., 2004) and suppressed basal and LH-stimulated androgen and progesterone production by bovine thecal cells (Gliser et al., 2005). Also, GDF9, an oocyte derived factor, stimulated proliferation of granulosal and thecal cells and decreased FSH-/IGF-I- and LH-stimulated steroid production in both cell types, respectively (Spicer et al., 2006; 2008). The inhibitory effect of GDF9 on androgen production by thecal cells was associated with a reduction of LH receptor and P450ssc mRNA (Spicer et al., 2008); whereas, the ability of GDF9 to stimulate thecal cell proliferation was more evident in small than in large follicles (Spicer et al., 2008). Apparently, the role of GDF9 was to prevent premature differentiation of both thecal and granulosal cells (Spicer et al., 2006; 2008).

In addition to the previously described factors, estradiol and inhibin are important modulators of follicular function. Estradiol decreased progesterone production induced by LH, LH and insulin or LH and IGF-I in thecal cells. In contrast, estradiol had a potent stimulatory effect on production of androstenedione by thecal cells in either absence or presence of LH, LH and insulin, or LH and IGF-I (Spicer et al., 2005). So, estrogen synthesized in granulosal cells

appeared to form part of a paracrine positive feedback mechanism that stimulated further production of follicular estradiol by increasing androgen production by thecal cells (Spicer et al., 2005). On the other hand, inhibin, which is produced primarily by granulosal cells, plays important autocrine and paracrine roles in follicular physiology. Apparently, inhibin limits follicular estradiol production, because treatment of granulosal cells with an inhibin antibody increased estradiol production by 2 to 15 fold, compared with controls (Jimenez-Krassel et al., 2003). Moreover, treatment of granulosal cells isolated from estrogen-active and estrogen-inactive dominant follicles and subordinate follicles with anti-inhibin significantly increased estradiol production, indicating that inhibin was suppressing estradiol production by estrogen-inactive and subordinate follicles (Jimenez-Krassel et al., 2003).

In addition to the stimulatory role of FSH and LH in follicular development, two circulating substances, insulin and IGF-I, play important roles in modulating follicular growth (Spicer et al., 2002). Insulin and IGF-I increased FSH-stimulated estradiol production by granulosal cells from either small or large follicles. Likewise, individual or combined treatment of insulin and IGF-I stimulated basal and FSH-induced proliferation of granulosal cells from small and large follicles in a dose dependent manner (Spicer et al., 2002). Both IGF-I and IGF-II were locally identified in granulosa and theca, respectively, from follicles of different morphologic and functional characteristics (Yuan et al., 1998). Although IGF-II mRNA was detected in bovine thecal cells from diverse follicular developmental stages, no evidence for IGF-I mRNA expression was observed at any stage of development (Armstrong et al., 2000). It seems that IGF-II plays an autocrine role because IGF-II stimulated androstenedione and progesterone production by thecal cells, and this effect was mediated by IGF type I receptors (Spicer et al.,

2004). Hence, both IGF-II and IGF-I play significant roles in steroidogenesis during follicular development.

Follicular selection and dominance

Ruminants have been used intensively in studies of ovarian follicular development. Particularly in cattle, morphological changes of the follicles during the estrous cycle have been associated with circulating hormones and functional characteristics of the follicles at different developmental stages. In addition to the feasibility to perform interesting in vivo studies, sheep represented a useful model to study aspects of follicular physiology in vitro.

In ruminants, the later stages of antral follicle development occur in waves of follicular growth. During each reproductive cycle, follicular development was characterized by recruitment of several small follicles that grew progressively until the stage in which one or more were selected and continued their development; while the remaining follicles became smaller and progressively disappeared from the ovarian cortex (Ireland et al., 2000). In ruminants and other domestic species, emergence of each follicular wave is preceded or accompanied by a small increment of FSH concentration (Evans, 2003a) followed by upregulation of P450scc and P450arom in granulosal cells and gonadotropin receptors in steroidogenic cells (Bao and Garverick, 1998). Thus, the resultant greater steroidogenic capacity is an important physiological fact that influences the subsequent stages of follicular development.

Follicular selection is the mechanism by which one or two follicles become morphologically (greater size) and functionally (greater steroidogenic capacity) dominant (Xu et al., 1995b; Bao

et al., 1997b) and continue growing toward ovulation. The remaining follicles decreased in size and underwent atresia, most likely due to down regulation of LH receptor, StAR and steroidogenic enzymes (Xu et al., 1995a; Xu et al., 1995b; Bao et al., 1998), with the consequent reduction in estradiol synthesis that preceded apoptosis of granulosal cells (Austin et al., 2001; Clark et al., 2004). Follicular dominance was associated with greater expression of mRNA for LH receptor, P450scc, P450c17 and 3βHSD in thecal cells, and P450arom in granulosal cells, as compared with previous follicular stages (Xu et al., 1995b; Bao et al., 1997a; Bao et al., 1997b). Dominant follicles (DF) had lower concentrations of IGF binding proteins (IGFBP) 2, 4, and 5 and greater concentrations of IGF-I than follicles prior to selection (Mihm et al., 1997; Austin et al., 2001). Likewise, onset of expression of LH receptor and 3βHSD in granulosal cells was a remarkable indicator of the greater steroidogenic capacity of selected follicles (Bao et al., 1997a; Bao et al., 1997b; Beg et al., 2001), which apparently switch in gonadotropin dependence from FSH to LH (Mihm et al., 2006). Moreover, follicular size was highly correlated with concentrations of mRNA for P450arom ($r = 0.77$) and LH receptors in theca ($r = 0.71$) and granulosa ($r = 0.54$; Bao et al., 1997a). Expression of StAR, that was observed only in thecal cells, progressively increased as the dominant follicle grew (Bao et al., 1998), indicating that increasing synthesis of estradiol may require an increment of cholesterol transport in thecal cells to produce greater amounts of androgen, the substrate utilized by granulosal cells to synthesize estradiol.

After the transient increase of FSH that precedes the emergence of each follicular wave, circulating concentrations of FSH decreased progressively within the following 24 h (Mihm et al., 1997; Austin et al., 2001), probably due to negative feedback exerted by increasing

amounts of estradiol and inhibin secreted by selected follicles (Kaneko et al., 1997; Ginther et al., 2000a; Bergfelt et al., 2000). It was suggested that the decrease in circulating concentrations of FSH after the transient rise is associated with selection or deviation of the DF and atresia of the remaining subordinate follicles (Adams et al., 1993; Mihm et al., 1997; Beg et al., 2001). Administration of physiological concentrations of FSH delayed selection of the DF and subordinate follicles (Mihm et al., 1997). Apparently, follicles with greater amounts of gonadotropin receptors and steroidogenic capacity (Xu et al., 1995a; Xu et al., 1995b; Bao et al., 1998a; Bao et al., 1998b; Bao et al., 1998; Beg et al., 2001) were more competent to grow in an endocrine milieu low in FSH (Mihm et al., 1997; Ginther et al., 1999; Ginther et al., 2000b). In contrast, subordinate follicles depended on FSH support and the reduced FSH concentration was a critical event that conferred them with metabolic disadvantages to grow at the same rate as the DF (Adams et al., 1993). However, subordinated follicles continued growing if adequate FSH was provided (Adams et al., 1993) or became dominant if the DF was removed (Ko et al., 1991; Ginther et al., 2001), indicating that they maintained the metabolic capacity to grow and were able to reach ovulatory status under appropriate endocrine influence.

Evidence indicates that the IGF system is involved in follicular selection and dominance. The IGF system consists of two ligands (IGF-I and IGF-II), two receptors (IGFR1 and IGFR2), several IGF binding proteins (IGFBP) and IGFBP proteases (Spicer and Echternkamp, 1995; Monget et al., 1996). As documented above, IGF ligands play relevant functions in ovarian follicular development by stimulating granulosal cell proliferation and steroidogenesis (Spicer et al., 2002). The role of the IGF system in follicular deviation and subsequent dominance has been described. Using an in situ hybridization approach, IGF-I and IGF-II were detected in

granulosa and theca, respectively, with a significantly greater abundance in DF than subordinate follicles in both early- and mid-dominance (Yuan et al., 1998). Also, amounts of IGFBP2 were lower in DF than subordinate follicles in both follicular stages (Yuan et al., 1998) and treatment of granulosal cells with FSH decreased IGFBP2 mRNA expression (Armstrong et al., 1998). In agreement with the previous findings, concentrations of IGF-I in follicular fluid were greater in the largest than in the smaller follicles (Echternkamp et al., 1994; Beg et al., 2001). The IGFBP2 concentrations were significantly greater in the largest compared with the smaller follicles (Beg et al., 2001), coincident with the finding that proteolytic activity on IGFBP was greater in follicular fluid of estrogen-active than estrogen-inactive follicles (Echternkamp et al., 1994; Rivera et al., 2001). Samples of follicular fluid from the two or three largest follicles collected on d 3 of the estrous cycle indicated that estradiol was greater and IGFBP4 was lower in the presumptive DF compared with subordinated follicles (Mihm et al., 2000). Apparently, an increment in the proteolytic activity on IGFBP and the consequent reduction of IGFBP concentrations in follicular fluid is a functional feature that distinguishes DF from subordinate follicles. Thus, the increased bioavailability of IGF-I under an appropriate gonadotropin stimulus mediates the greater capacity of DF to produce estradiol.

In the ewe, as in the cow, emergence of each follicular wave is preceded by a transient increase of FSH (Souza et al., 1997; Evans, 2003b). During the estrous cycle, 2 to 4 groups of follicles grow in a wave-like pattern (Souza et al., 1998; Bartlewski 1999; Evans et al., 2000; Duggavathi et al., 2003). In the ewe, follicular dominance appears to be less evident than in the cow (Duggavathi et al., 2004; 2005; Barrett et al., 2006). Although follicular dominance was

documented in sheep (Lopez-Sebastian et al., 1997; Gonzalez-Bulnes et al., 2001; 2004), recent authors have questioned the concept of follicular dominance as it occurs in cattle. The presence of a large estrogen-active growing follicle in the ovary did not prevent the emergence of a new follicular wave induced by exogenous FSH during the inter-wave FSH nadir (Duggavathi et al., 2004). In addition, induction of physiological peaks of circulating FSH by exogenous administration of FSH every 2 to 2.5 d, stimulated the emergence of an extra follicular wave, but modified neither the occurrence of endogenous FSH peaks nor the subsequent follicular wave emergence (Duggavathi et al., 2004). Moreover, emergence and development of the induced follicular wave was not inhibited by the presence of the largest growing follicles belonging to the preceding endogenous FSH-induced follicular wave (Duggavathi et al., 2005). Using a different experimental approach, the peak and the amplitude of endogenous FSH was altered by elevated concentrations of estradiol released from implants placed on d 4 of estrous cycle (Barrett et al., 2006); as a consequence, emergence of a new follicular wave was prevented. However, despite supra-physiological concentrations of estradiol, administration of a physiological dose of FSH restored the emergence of follicles (Barrett et al., 2006). To support further the previous data, 2 or more large follicles from ultimate and penultimate waves of the estrous cycle may reach ovulatory status and eventually ovulate simultaneously (Souza et al., 1997; Bartlewski 1999).

After luteolysis is initiated by uterine $PGF_{2\alpha}$, concentrations of progesterone drop rapidly and the negative feedback exerted by this steroid in the hypothalamus and pituitary decline. The subsequent increased frequency of LH pulses during proestrus stimulates greater secretion of estradiol by the DF that acts in a positive feedback loop to enhance LH and further estradiol

secretion (Goodman and Inskeep, 2006). Thus, elevated concentrations of estradiol trigger an LH/FSH surge (Karsch et al., 1983). Greater mRNA concentrations for P450scc and 3βHSD in both granulosa and theca and P450-17α-hydroxylase in thecal cells were responsible for the elevated steroidogenic capacity of preovulatory follicles, mostly by providing substrate for the aromatization of androgen to estradiol in granulosal cells (Tian et al., 1995). Nevertheless, after an LH surge, the pattern of steroid secretion from the preovulatory follicle changed abruptly (Murdoch and Dunn, 1982; Fortune and Hansel, 1985). In the ewe and cow, a remarkable reduction of estradiol and androgen production followed the LH surge. Likewise, an abrupt increment in progesterone concentrations and the switch in pregnenolone/progesterone ratio from 1.6 h prior to LH to 0.18 h after the LH surge was indicated (Murdoch and Dunn, 1982; Fortune and Hansel, 1985). In addition, the transition in steroid production by preovulatory follicles from estrogen to progesterone dominance was associated with downregulation of P450-17α-hydroxylase and P450arom in thecal and granulosal cells, respectively, and upregulation of progesterone receptor (PR) in the two types of steroidogenic cells (Voss and Fortune, 1993a; Komar et al., 2001; Jo et al., 2002). Thus, the switch in steroid production by steroidogenic cells after the LH surge is part of the mechanism of differentiation of granulosal and thecal cells into large and small luteal cells of the insipient corpus luteum. Moreover, the amount of PR mRNA increased markedly and transiently in follicular cells of bovine periovulatory follicles within a few hours after the LH surge, followed by a second increase near the time of ovulation (Jo et al., 2002). Therefore, the first rise in PR mRNA may be associated with the sudden and simultaneous increases in follicular production of oxytocin, prostaglandins, and other important substances for ovulation during the periovulatory period;

whereas the second rise in progesterone and PR mRNA may play an important role in the subsequent formation of the CL (Murdoch et al., 1986; Voss and Fortune, 1993a; Voss and Fortune, 1993b).

The events that characterize follicular growth from the pool of primordial follicles throughout different developmental stages are summarized in Figure 1. Under appropriate stimuli by local and systemic hormones and growth factors, one or more large follicles become morphologically and functionally dominant while others undergo atresia. Ovulation occurs at the end of the follicular phase, once progesterone declines and increasing amounts of estradiol produced by the preovulatory follicle trigger the LH/FSH surge.

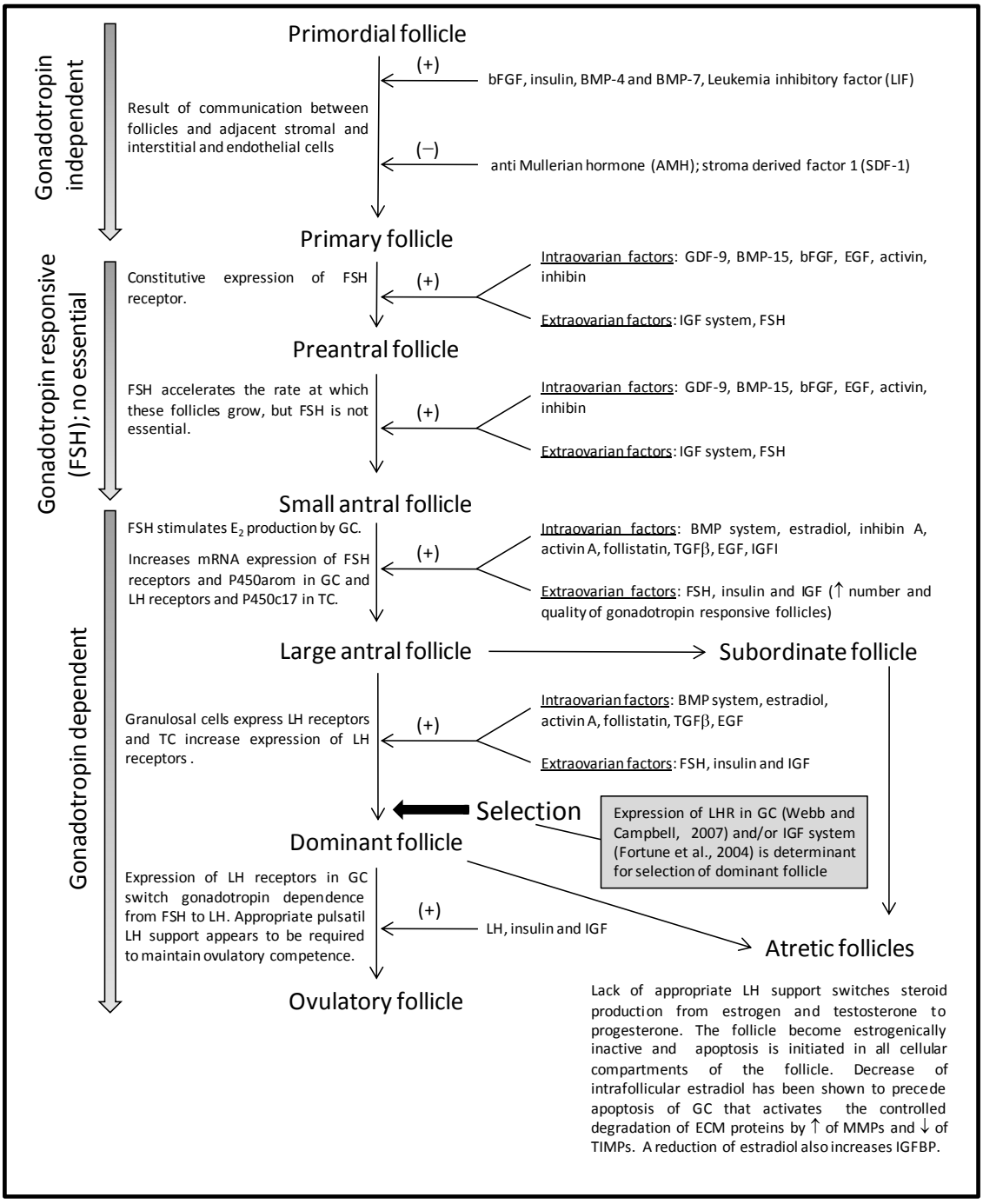

Figure 1. Summary of the events that characterize follicular development throughout different developmental stages in the cow.

Physiologic aspects of luteal function

After ovulation, the collapsed ovulatory follicle undergoes dramatic cellular and structural changes that confer to the new ovarian structure, the corpus luteum (CL), endocrine characteristics aimed to create physiological conditions that favor early embryonic development, implantation, placentation, and in general, assure success of gestation and survival of the specie. Thus, the CL constitutes the primary source of circulating progesterone. However, if fertilization of the oocyte does not occur, CL function decreases and a new reproductive cycle is initiatea a few days later. This physiological process is known as "corpus luteum regression" or "luteolysis". Therefore, CL function is essential in all mammals for implantation and early maintenance of pregnancy and in the event of an infertile cycle its disappearance assures further opportunity for onset of a new gestation (Hoyer, 1998).

Morphological characteristics of the corpus luteum

The corpus luteum is a transient, highly vascularized, ovarian tissue that develops in a few days from a "corpus hemorrhagicus" to a mature CL, producing progressively greater amounts of progesterone (P4). From morphological and functional points of view, the CL has two different populations of cells: endocrine and non-endocrine cells that vary in proportion and number throughout the luteal phase (Rodgers and O'Shea, 1982; O'Shea et al., 1984; Rogers et al., 1984; Farin et al., 1986). Steroidogenic cells (SC) represent 55% of the cellular volume of the CL and consist of large (LLC) and small (SLC) luteal cells originated from granulosal and thecal cells of the ovulatory follicle (Hansel and Dowd, 1986), respectively. Nonsteroidogenic cells have been identified as fibroblastic, endothelial and immune cells (O'Shea et al., 1984;

Rogers et al., 1984) and represent around 16% of the volume of the CL, considering that cellular matrix occupies 27% (Farin et al., 1986). Non-steroidogenic cells provide the structural architecture of the CL and synthesize several products that interact with steroidogenic cells and regulate steroid production (Girsh et al., 1996b; Juengel et al., 1998a; Korzekwa et al., 2008).

Irregular shaped SLC, with a diameter < 20 μm, produce low basal concentrations of progesterone (P4) under the influence of LH (Rodgers and O'Shea, 1982; Hoyer et al., 1984; Mamluk et al., 1998; Wiltbank, 1994), whereas round or polyhedral shaped LLC, whose diameter varies between 20 and 40 μm in different mammalian species, secrete considerably greater amounts of P4 without pituitary luteotropic stimulus (Rodgers and O'Shea, 1982; Fitz et al., 1982; Bourdage et al., 1984; Wiltbank, 1994). Although number of LLC did not appear to change through different stages of luteal phase, including early periods of regressed CL, SLC significantly increased from Day 4 to 8 of the estrous cycle and maintained stable numbers until time of luteal regression when they began to decline (Farin et al., 1986). Apparently, hypertrophy of LLC and hyperplasia of SLC, fibroblasts and endothelial cells account for the dramatic increase in size and weight of the CL, often compared with tumor tissues (Juengel et al., 1998a). During CL development, a vast network of capillaries is established assuring sufficient blood flow to sustain the high metabolic rate of a completely mature CL (Bruce and Moor, 1976).

Functional characteristics of the corpus luteum

In most mammalian species, a luteotropic stimulus is partially or totally required to maintain luteal secretion of P4, particularly during the estrous cycle and in some species during early

stages of gestation. However, the influence of a specific pulsatile pattern of LH secretion appears to differ among species. In primates, for instance, luteolysis (Dubourdieu et al., 1991; Webley et al., 1991) or inhibition of luteal angiogenesis and progesterone production (Dickson and Fraser, 2000) were consequences of interruption of pulsatile LH secretion by using GnRH antagonists. In ruminants, lack of an appropriate pattern of LH secretion disrupted function of developing CL in heifers (Peters et al., 1994) but did not affect substantially any stage of luteal development in sheep (McNeilly et al., 1992). In other mammalian species, like the rabbit, estrogen is the main luteotropic hormone, while in the rat prolactin is primary and LH and estrogen play secondary roles (Holt, 1989; Freeman, 2006; Stouffer, 2006).

Lifespan of the cyclic CL varies in different mammalian species. In primates and farm animals, cyclic CL persist between 12 and 16 days, whereas in rodents luteal phase is shorter (4 or 5 day cycle) and is extended for about 1 to 2 or 3 weeks only in the event of pseudopregnancy or a fertile cycle, respectively (Leymarie and Martal, 1993; Freeman, 2006). In other animals like carnivores (dogs, wolves, foxes, cats, etc), the length of the luteal phase in pregnant or nonpregnant females does not differ appreciably (Stouffer, 2006). On the other hand, while in many mammals the CL is not absolutely indispensable throughout gestation (sheep, horse, rat, human, monkey) because luteal P_4 is replaced by placental secretion of P4, some animals such as cow, pig, goat and rabbit require luteal function during the entire gestation period (Tanabe, 1966; McDonald et al., 1952; Allen, 2001).

At the end of the ovarian cycle, the absence of viable embryos in the uterus allows luteolysis to take place. In many species, including domestic farm animals (such as the ewe, cow, goat, mare, sow) and laboratory animals (the rat, mouse, hamster, guinea, pig and and rabbit), an

endogenous luteolytic substance, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), released from the uterine endometrium, restrains P4 production and induces CL regression (Wiltbank and Casida, 1956; Malven and Hansel, 1964; McCracken et al., 1970; Hilliard, 1973; Currie and Thorburn, 1974; Gleeson et al., 1974; Douglas and Ginther, 1976; Douglas et al., 1976). In most of these species, transfer of PGF2α from utero-ovarian vein toward ovarian artery appears to be an important component of the mechanism by which this hormone reaches the luteal tissue (Ginther, 1974); given that close anatomical relationship between uterine vein and ovarian artery (Del Campo and Ginther, 1972; 1973a; 1973b; Ginther and Del Campo, 1974), and functional connection among uterus and CL (Wiltbank and Casida, 1956; Malven and Hansel, 1964; Inskeep and Butcher, 1966; Barley et al., 1966; Currie and Thorburn, 1974; Gleeson et al., 1974; Gleeson, 1974; Pexton et al., 1975a) were demonstrated. In contrast, in the mare, uterine and ovarian vasculature seems to be anatomically more independent (Ginther et al., 1972; Del Campo and Ginther, 1973a) and plays a less significant role in luteolysis (Douglas and Ginther, 1975). Although in primates an extensive area of contact between utero-ovarian vein and ovarian artery exists (Ginther et al 1974), raising the possibility that a local diffusion mechanism takes place, no functional association among uterus and CL has been indicated (Neill et al., 1969; Castracane et al., 1979), and even, the role of $PGF_{2\alpha}$ as a luteolytic agent in these species has been controversial and several substances have proposed as luteotropic factors (Zeleznik and Clifford, 2006). However, a cumulative bulk of evidence indicates that luteal $PGF_{2\alpha}$ mediates luteolysis in primates (Auletta et al, 1984; Johnson et al., 1988; Houmards et al., 1989; Zelinski-Wooten, et al., 1990; Bennegard et al., 1991; Bogan et al., 2008; Priyancka et al., 2009).

Factors affecting lifespan of the corpus luteum

The events conducive to secretion of $PGF_{2\alpha}$ from the uterus have been described in detail in ruminants. Luteolysis during the estrous cycle was believed to occur as consequence of a reciprocal positive feedback mechanism between luteal oxytocin and uterine $PGF_{2\alpha}$ (McCracken et al., 1984; Lafrance and Goff, 1988; Lamsa et al., 1989). This mechanism was proposed to be responsible for the episodic secretion of $PGF_{2\alpha}$ necessary to complete luteal regression (Flint et al., 1990); furthermore, this process occurs at a specific time of the estrous cycle (Lafrance and Goff, 1988; Silvia et al., 1991; Kieborz-Loos et al., 2003). Progesterone suppressed secretion of PGF2α when more P_4 receptors were present, presumably by inhibition of synthesis of oxytocin receptors early in the estrous cycle when the uterus was more responsive to P_4 (McCracken et al., 1984; Hazzard et al., 1998; Kieborz-Loss et al., 2003). However, as the estrous cycle progressed, concentrations of P_4 receptors decreased, which in turn allowed the synthesis of estrogen and oxytocin receptors (McCracken et al., 1984). As a result, the uterus became more responsive to luteal oxytocin and a positive feedback mechanism was apparently established between oxytocin and endometrial $PGF_{2\alpha}$.

A period of progestational influence appears to be important in the timing of luteal regression. Treatment with exogenous P_4 on day 0 or 1 after estrus advanced the occurrence of the first peak of $PGF_{2\alpha}$ and shortened the length of the estrous cycle in sheep (days 8 and 11 for P4-treated ewes and days 12.3 and 17 for control ewes, respectively; Ottobre et al., 1980). In addition, the blockade of P4 receptors by mifepristone (RU 486) during the early to mid-luteal phase inhibited luteolysis and preserved a functional corpus luteum beyond day 20, presumably

due to delayed $PGF_{2\alpha}$ secretion from the endometrium (Morgan et al., 1993). Abolition of P_4 source on days 12 or 14 by removal of either ovaries or CL in sheep, resulted in a temporal increase of $PGF_{2\alpha}$ followed by a gradual decline (Ottobre et al., 1984; Vincent et al., 1986); it was suggested that continued secretion of P_4 is necessary for secretion of $PGF_{2\alpha}$ to continue beyond day 14 of the estrous cycle. Apparently, P_4 regulates secretion of $PGF_{2\alpha}$ early in the estrous cycle by timing the initial peaks of secretion, and thereafter, by modulating secretion of $PGF_{2\alpha}$ until luteal regression is completed. However, in postpartum cows and anestrous ewes the absence of a previous exposure of the uterus to P_4 followed by estrogen prior to first ovulation alters the physiological conditions of the uterus and as a consequence, the timing of luteolysis is premature (Inskeep, 2004).

At the time when $PGF_{2\alpha}$ was identified as the luteolytic agent (McCracken et al., 1970), and the anatomical and functional relationship between uterus and CL were pointed out (Ginther, 1974), suppression of $PGF_{2\alpha}$ secretion was the most likely mechanism to prevent luteolysis in pregnant animals. However, secretion patterns of $PGF_{2\alpha}$ in pregnant and nonpregnant sheep in the late luteal phase were similar (Pexton et al., 1975b), indicating that in pregnant females luteolysis is prevented by mechanisms other than suppression of $PGF_{2\alpha}$ secretion, for example amounts of $PGF_{2\alpha}$ transported or metabolized, or amount of $PGF_{2\alpha}$ assimilated by luteal tissue (Pexton et al., 1975c; Lewis et al., 1977). Beside the luteoprotective effect of the embryos in the uterus (Inskeep et al., 1975; Pratt et al., 1977; Thatcher et al., 1986), uterine PGE_2 appears to be important to prevent luteolysis in pregnant sheep (Lewis et al., 1978; Pratt et al., 1979; Silvia et al., 1984).

Physiological and molecular aspects of luteal regression

Luteal regression involves two consecutive and complementary mechanisms whereby the CL loses steroidogenic capacity (functional luteolysis) without apparent changes in cellular structure and undergoes subsequent degradation of all cellular and structural components (structural luteolysis). The latter mechanism implies a concomitant reduction of luteal weight and size; and finally, the CL becomes a fibrotic nonfunctional tissue named "corpus albicans". These mechanisms are triggered at the end of the luteal phase after uterine $PGF_{2\alpha}$ activates its receptors in different luteal cell types. Hence, luteolysis is a complex and orchestrated physiologic processes involving not only steroid secreting cells but also endothelial and immune cells that through autocrine and paracrine mechanisms drive a sequence of cellular and vascular events conducive to demise of the CL (reviewed by Hoyer, 1998; McCracken et al., 1999; Niswender et al., 2000; Stocco et al., 2007).

$PGF_{2\alpha}$ reduces essential enzymes for progesterone synthesis during functional luteolysis

An abrupt increase in the amplitude, duration and number of pulses of $PGF_{2\alpha}$ at the end of the luteal phase (Pexton et al., 1976; Nett et al., 1976a; Zarco et al., 1988a) marks the onset of luteal regression. A few hours later, concentrations of peripheral P_4 are minimum and size and weight of the CL are smaller (Zarco et al., 1988b; Tsai et al., 2001). Furthermore, reduction in P_4 secretion after exogenous PGF2α administration was accompanied by a simultaneous decrease in blood flow (McCracken et al., 1970; Nett et al., 1976b; Niswender et al., 1975), which implies less nutrient and luteotropic stimuli to sustain steroid production by large and small luteal cells (Niswender et al., 1976).

After PGF2α binds to its luteal receptor, it triggers changes in expression of genes and activity of proteins associated with P_4 synthesis. Apparently, P450ssc mRNA and protein activity are not down regulated by $PGF_{2\alpha}$ before plasma P_4 declines below functional concentrations (Tian et al., 1994; Rogers et al., 1995; Diaz and Wiltbank, 2005). Although 3β-HDS mRNA decreased concurrently with P_4 concentration ($r = 0.76$, $p < 0.005$) during the first 12 h after a luteolytic dose of $PGF_{2\alpha}$ (Tian et al., 1994; Tsai et al., 2001), neither the amount of protein nor enzyme activity decreased within 24 h following $PGF_{2\alpha}$ (Rogers et al., 1995; Juengel et al., 1998b). Tubulin, a component of the cytoskeleton associated with transport of cholesterol to the outer mitochondrial membrane, decreased markedly before decline of P_4 in $PGF_{2\alpha}$-induced luteolysis in sheep (Murdoch, 1996). Moreover, mRNA encoding steroidogenic acute regulatory protein (StAR), a protein involved in transport of cholesterol from the outer to the inner mitochondrial membrane, was down regulated a few hours after exogenous $PGF_{2\alpha}$ in sheep, sows and cows (Juengel et al., 2000; Tsai et al., 2001; Diaz and Wiltbank, 2005). In addition, transcript concentration for LH receptor in luteal tissue decreased significantly in a lapse of 4 to 9 h after $PGF_{2\alpha}$-induced luteolysis in cows and sheep (Juengel et al., 2000; Tsai et al., 2001) but not in sows (Diaz and Wiltbank, 2005). Thus, abolition of luteotropic stimulus and transport of cholesterol through cytoplasm and the mitochondrial membrane appears to be the target of $PGF_{2\alpha}$ to reduce steroidogenic capacity of CL during functional luteolysis. However, reduction of P_4 production and oligonucleosome formation after a subluteolytic dose of $PGF_{2\alpha}$ was reversed a few hours later (Juengel et al., 2000). Apparently a continuous pattern of episodic $PGF_{2\alpha}$ secretion is necessary to induce irreversible changes in the functional and structural characteristics of CL.

Role of blood flow in luteolysis

The effect of endogenous and exogenous $PGF_{2\alpha}$ on reduction of luteal blood flow concurrently with diminution of P_4 concentrations during the estrous cycle has been described as a normal physiological event in the ewe (McCracken et al., 1970; Niswender et al., 1975; Niswender et al., 1976; Nett et al., 1976). Whether $PGF_{2\alpha}$ has a direct effect on ovarian blood flow or blood flow declines as a consequence of the reduction of P_4 production by luteal cells (or vice versa) has been uncertain. However, in recent years evidence has accumulated indicating a role of $PGF_{2\alpha}$ in the hemodynamic changes associated with luteolysis. The presence of $PGF_{2\alpha}$ receptors (FP) in endothelial cells was reported in the cow and sow (Mamluk et al., 1998; Zanoni et al., 2007). Also, acute increases in blood flow followed by a progressive reduction at the end of the luteal phase and after administration of $PGF_{2\alpha}$ in mid-phase bovine CL were associated as a component of the luteolytic cascade (Acosta et al., 2002; Shirasuna et al., 2008). Moreover, this acute increase in luteal blood flow during spontaneous luteolysis was accompanied by an increase in plasma PGFM (15-keto, 13,14dihydro-$PGF_{2\alpha}$, inactive metabolite of $PGF_{2\alpha}$) (Shirasuna et al., 2008).

Endothelin 1 (END1), a vasoactive peptide produced by luteal endothelial cells, increased at the end of the luteal phase or after an exogenous dose of $PGF_{2\alpha}$ (Girsh et al., 1996b; Ohtani et al., 1998; Wright et al., 2001; Choundhary et al., 2004; Shirasuna et al., 2008). END1, whose potent vasoconstrictor effect has been demonstrated in many tissues, activates receptors in luteal endothelial cells (Mamluk et al., 1999; Klipper et al., 2004) and may be responsible for reducing blood supply to the CL during structural luteolysis. In addition to the previous

physiological effects, END1 decreased P_4 concentrations in vitro and in vivo and is involved in $PGF_{2\alpha}$-induced luteolysis in sheep and cows (Hinckley and Milvae, 2001; Doerr et al., 2008; Keator et al., 2008), as will be described in detail later in this review.

Nitric oxide (NO), another vasoactive substance synthesized by luteal endothelial cells) was reported to modulate luteal production of P_4 in vivo and in vitro (Jaroszewski and Hansel, 2000; Jaroszewski et al., 2002; Jaroszewski et al., 2003; Skarzynski et al., 2003; Korzekwa et al., 2004) and may play a role acting as a component of autocrine and paracrine actions during luteolysis (Jaroszewski et al., 2003). Nitric oxide, a gas synthesized by deamination of the amino acid L-arginine through catalytic action of NO synthase (NOS), is secreted from endothelial cells causing relaxation of vascular smooth muscular cells by binding and activating guanylyl cyclase to produce cyclic GMP (Alberts et al., 2002); and is an important modulator of local blood flow in the tissues (Moncada and Higgs, 2006). Luteal endothelial NOS (eNOS) mRNA and protein increased at the end of the luteal phase (coincident with the time of onset of luteolysis) or after exogenous $PGF_{2\alpha}$ in cows and sheep, respectively (Skarzynski et al., 2003; Vonnahme et al., 2006; Shirasuna et al., 2008), whereas only eNOS protein increased after PGF2α in the rabbit (Boiti et al., 2003).

Recently, it was reported that administration of NO donor into the CL induced an acute increase in luteal blow flow within 30 min after injection and reduced the length of the estrous cycle in cows (Shirasuna et al., 2008). In contrast, intraluteal injections of a NO inhibitor suppressed the increase of luteal blood flow and delayed the onset of luteolysis in response to exogenous $PGF_{2\alpha}$ (Skarzynski et al., 2003; Shirasuna et al., 2008). In addition, partial pressure of oxygen and concentrations of nitrite/nitrate (metabolites of NO) were augmented

significantly in cows after a luteolytic dose of $PGF_{2\alpha}$ during the mid-luteal phase (Skarzynski et al., 2003; Acosta et al., 2008); and both events were temporally associated with the increase of luteal blood flow and preceded the decline of P_4 concentrations (Acosta et al., 2008). Moreover, infusion of a NO inhibitor increased concentration of P_4, decreased plasma concentration of nitrite/nitrate and extended the lifespan of the CL (Skarzynski et al., 2003). Accordingly, the increase in luteal blood flow caused by NO and the consequent enhancement of vascular permeability may promote infiltration of immune cells and other substances into the CL, contributing to the successive events involved in the mechanism of structural luteolysis (Acosta et al., 2008). Whether the increase in blood supply to the CL within few minutes after PGF2α triggers the luteolytic cascade as was suggested (Acosta and Miyamoto, 2004) or is only a component of the intricate mechanism of luteolysis have to be further confirmed.

Active participation of immune system in luteolysis

According to Pate and Keyes (2001), the role of immune cells in luteal regression may be explained by their participation in the loss of steroidogenic capacity and destruction of the luteal tissue, and by modulation of the potential inflammatory conditions originated by degenerating luteal cells with the aim of preserving basic environmental conditions for those cells that have not died. Population of immune cells in CL is represented by T-lymphocytes, macrophages-monocytes and eosinophils (Bagavandoss et al., 1988; Pate, 1994; Bukovsky et al., 1995; Best et al., 1996; Penny et al., 1999; Bauer et al., 2001 Townson et al., 2002), whose number increased as the mid-luteal phase progressed (Bauer et al., 2001; Townson et al., 2002); reaching a considerably greater number of cells during spontaneous luteolysis and after

PGF2α-induced luteolysis (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002). Leucocytes increased around 20% at the end of the luteal phase and 70% at late luteal regression; this increment was due to infiltration of monocytes and local proliferation of macrophages when capillaries were no longer present (Bauer et al., 2001). During structural luteolysis macrophages play an important role by phagocytizing dead cells and cell debris (Paavola and Boyd, 1979) as well as releasing cytokines, oxygen radicals and substances that inhibit steroidogenesis (Hoyer, 1998; Pate and Keyes, 2001),

Infiltration of macrophages appears to be regulated by chemokines (Pate and Keyes, 2001), particularly monocyte chemoattractant protein 1 (MCP-1), a cytokine detected in greater quantity during luteal regression in coincidence with the influx of macrophages (Bowen et al., 1996; Penny et al., 1998) or 4 to 8 h after $PGF_{2\alpha}$-induced luteolysis (Haworth et al., 1998; Tsai et al., 1997; Penny et al., 1999). Analysis of MCP-1 mRNA expression in bovine CL revealed a significant increment as early as 0.5 h after $PGF_{2\alpha}$-induced luteolysis and continued for at least 64 h (Kliem et al., 2009); whereas the expression of its receptor was greater from 12 until 64 h after $PGF_{2\alpha}$. MCP-1 was detected in endothelial cells (Cavicchio et al., 2002). Once expressed in vascular tissue, MCP-1 stimulated migration of immune cells, particularly monocytes, macrophages and T-lymphocytes, from blood vessels into tissues undergoing inflammation (Mukaida et al., 1998). Townson et al. (2002) documented that MPC-1 mRNA and protein expression as well as immune cell accumulation in bovine CL preceded the onset of spontaneous luteolysis by several days. If luteolysis was prevented between days 16 to 18 of the cow estrous cycle, no increase in MCP-1 or macrophage infiltration was observed (Penny, 2000). In addition, in vitro studies have failed to demonstrate an effect of $PGF_{2\alpha}$ on MCP-1

secretion by endothelial cells (Cavicchio et al., 2002), indicating that this chemoattractant may be released as an indirect action of $PGF_{2\alpha}$. According to the proposed model by Penny (2000), at the end of the luteal phase $PGF_{2\alpha}$ stimulated, directly or indirectly, secretion of MCP-1 and other cytokines by several luteal cell types (lymphocytes, macrophages, fibroblasts, and steroidogenic and endothelial cells) causing an influx of macrophages that play an essential role during structural regression of the CL .

Once immune cells become activated within an inflamed tissue, they secrete cytokines, such as tumor necrosis α (TNF-α), interferon γ (IFN-γ) and interleukin 1β (IL-1β). In addition to modulating the immune response during luteal regression, these cytokines directly affect luteal steroidogenic cells, suppressing progesterone production and inducing apoptosis (Friedman et al., 2000; Pate and Keyes, 2001; Petroff et al., 2001). A significant increase of mRNA expression of TNF-α, IFN-γ, and IL-1β was detected as early as 2 h after PGF2α-induced luteolysis (Neuvians et al., 2004a). Moreover, this increment was simultaneous with a decline of LHR and P450scc mRNA (Neuvians et al., 2004a); which supports the suppressive effect of these immune mediators on P_4 production by luteal tissue (Fairchild and Pate, 1991; Benyo and Pate, 1992; Young et al., 1997). In another study, increases of TNF-α and IFN-γ mRNA 0.5 and 2 h after $PGF_{2\alpha}$, respectively, were reported (Kliem et al., 2009), followed by increases in TNF-α receptor mRNA and protein at 2 and 12 h, respectively. TNF-α receptor was identified in endothelial cells from microvascular tissue of bovine CL (Okuda et al., 1999) as well as in SLC and LLC (Friedman et al., 2000). In addition, expression of TNF-α receptor mRNA increased significantly after a luteolytic dose of $PGF_{2\alpha}$ (Friedman et al., 2000).

Evidence indicates that both TNF-α and IFN-γ are highly cytotoxic (Benyo and Pate, 1992; Friedman et al., 2000; Petroff et al., 2001). Cultured luteal cells treated with TNF-α and IFN-γ had less cell viability than untreated control cells and these cytokines induced DNA fragmentation by 48 h after cytokine treatment (Petroff et al., 2001). Immune cytokines stimulated greater secretion of luteal $PGF_{2\alpha}$ in vitro than control untreated cells (Fairchild and Pate, 1991; Benyo and Pate, 1992; Petroff et al., 2001). However, IFN-γ-induced elevation of luteal $PGF_{2\alpha}$ was reversed by P4 (Fairchild and Pate, 1991), indicating a protective effect of P_4 on luteal function that was also demonstrated by reducing TNF-α-induced cytotoxicity in endothelial cells treated with P_4 (Friedman et al., 2000). In this regard, Juengel et al. (2000) observed that a subluteolytic dose of $PGF_{2\alpha}$ (3 mg/6 kg BW) in the ewe transiently suppressed serum concentrations of P_4 and caused formation of oligonucleosomes 9 h after treatment and thereafter disappeared without any effect on CL weight.

In conclusion, after luteolysis is initiated by uterine $PGF_{2\alpha}$, secretion of MCP-1 by luteal cells (Penny, 2000) induces infiltration of macrophages and T-lymphocytes into the CL (Penny et al., 1999; Townson et al., 2002). These leukocytes secrete immune cytokines such as TNF-α, IFN-γ and IL-1β that stimulate luteal $PGF_{2\alpha}$ secretion and suppress P_4 production by steroidogenic cells, reinforcing the cascade of luteolytic events (Pate and Keyes, 2001). In addition, immune cytokines have a direct effect on viability of endothelial and steroidogenic cells, inducing apoptosis (Petroff et al., 2001) and degradation of cellular matrix (Hagedorn et al., 2001).

Apoptosis during structural luteolysis

After uterine $PGF_{2\alpha}$ disrupts synthesis and secretion of P_4 from steroidogenic cells, luteal $PGF_{2\alpha}$ directly and/or through several luteolytic mediators (like NO, END1, TNF-α, IFN-γ, IL-1β) triggers structural luteolysis that involves cellular apoptosis, phagocytosis of endothelial and steroidogenic cells, and degradation of cellular matrix (Hoyer, 1998; Pate and Keyes, 2001; Smith et al., 2002; Stocco et al., 2007).

Apoptosis or programmed cell death is a highly regulated physiological mechanism for reducing non-necessary populations of cells for the normal function of the tissue, or during specific developmental stages. Albert et al. (2002) described structural changes of the apoptotic cell as follow: "the cell shrinks and condenses, the cytoskeleton collapses, the nuclear membrane disassembles and the nuclear DNA breaks up into fragments". As a final cellular event of apoptosis, fragmentation of DNA occurs due to activation of Mg^{++}/Ca^{++}-dependent endonucleases that cleave DNA into 185-bp DNA fragments (Tilly, 1996). So, degradation of DNA and the consequent formation of oligonucleosomes is considered a hallmark of apoptosis (Compton, 1992) and has been identified during luteal regression in human beings (Shikone et al., 1995; Sugino et al., 2000a), sheep (Sawyer et al., 1990; Rueda et al., 1995a), cows (Friedman et al., 2000; Petroff et al., 2001), sows (Bacci et al., 1996) and rats (Matsuyama et al., 1996).

During functional luteolysis, the anti-apoptotic effect of P_4 is suppressed and luteal $PGF_{2\alpha}$, directly or via autocrine and paracrine mediators, initiates progressive destruction of all cellular populations of the CL. Juengel et al. (1993) reported that apoptotic signals appeared after P_4

concentrations declined and structural regression started; evidence that also was observed by Murdoch (1995). Content of DNA in bovine CL decreased from about 9.5 mg/CL at the mid-luteal phase to 2.2 mg/CL at the time of luteolysis (days 18 to 21 of the estrous cycle) (Zheng et al. 1994); whereas weight of CL and luteal content of P_4 decreased 73% and 98% respectively, and the number of cells dying during luteolysis was approximately 77.8×10^6 per day. Fragmentation of DNA was first observed 12 h after exogenous $PGF_{2\alpha}$ in the ewe (Sawyer et al., 1990; Rueda et al., 1995) and by 6 h in the sow (Bacci et al., 1996). Endothelial cells appeared to be the first population of cells targeted by $PGF_{2\alpha}$-induced apoptosis (Sawyer et al., 1990; Rueda et al., 1995; Bacci et al., 1996; Gaytan et al., 1998). In the sow, loss of capillaries increased progressively from 15% at 6 h after $PGF_{2\alpha}$-induced luteolysis to 49% 36 h later (Bacci et al., 1996). However, new morphological signs of apoptosis in parenchymal cells were not observed until 48 h after $PGF_{2\alpha}$ (Bacci et al., 1996). In regressing ovine CL, endothelial cells with normal nuclei declined significantly at 12 h after $PGF_{2\alpha}$, followed by steroidogenic cells and fibroblasts at 24 and 36 h, respectively (Sawyer et al., 1990). Also, apoptotic cells in buffalo cows increased 18 h after $PGF_{2\alpha}$ (Yadav et al., 2005).

Apoptosis is mediated by a variety of proteins activated by stimuli originated inside (intrinsic pathway) or outside (extrinsic pathway) the cell. In the first case, several factors (such as hypoxia, hyperoxia, hormone deprivation, radiation, etc) can induce changes in the permeability of the mitochondrial membrane that trigger a cascade of intracellular events that involve several proteases named caspases. In the second case, an apoptotic cascade is originated by activation of a membrane receptor (Fas receptor; FasR) by Fas ligands (FasL) present in the surface of killer lymphocytes (Alberts et al., 2002). Also, intrinsic apoptosis

during cellular stress is regulated by changes in the Bcl-2 family of proteins that have anti-apoptotic (Bcl-2 and others) and pro-apoptotic (Bax, Bad, etc) activity (Tilly, 1996). The ratio of Bax to Bcl-2 appears to be important to determine cell survival. Concentrations of Bax but not Bcl-2 mRNA and protein increased within 4 h after $PGF_{2\alpha}$-induced luteolysis in buffalo cows (Yadav et al., 2005); the Bax/Bcl-2 ratio was 4 fold greater compared with time 0 (PGF2α injection). Both caspases 3 and 9 proteins and activity increased after $PGF_{2\alpha}$, but caspase 9 increased earlier (4-12 h) than caspase 3 (18 h) (Yadav et al., 2005). During induced luteolysis in cows, mRNA expression of p53 (another apoptotic protein) and Bax increased significantly from 24 to 64 h after $PGF_{2\alpha}$, whereas Bcl-XL showed a significant increase only from 48 to 64 h (Kliem et al., 2009). In addition, caspases 3, 6 and 7 were greater at 12, 2 and 2 h after $PGF_{2\alpha}$, respectively, than at time 0.

In human CL, the expression of Bcl-2 and Bax mRNA was examined during the menstrual cycle. Results revealed that Bcl-2 protein was lower in the regressing CL than in mid-luteal phase and early pregnancy (Sugino et al., 2000a), whereas Bax protein was greater in the regressing CL than during mid-luteal phase and early pregnancy. In addition, mRNA expression during luteolysis followed the same pattern with greater expression level for Bax and lower for BCL-2 (Sugino et al., 2000a). Interestingly, hCG incubated with luteal tissue significantly decreased mRNA and protein of Bax and significantly increased those of Bcl-2 (Sugino et al., 2000a). In this regard, blockade of P_4 receptor in cultured bovine luteal cells increased FasR and caspase 3 mRNA expression and caspase 3 activity (Okuda et al., 2004), supporting the concept that apoptosis is a hormone-regulated process in which luteotropic stimuli inhibit and luteolytic stimuli promote apoptosis.

Intrinsic apoptosis during luteal regression has been associated with an increase of reactive oxygen species (ROS), and with the consequent oxidative stress (Hoyer, 1998). During luteolysis, production of ROS was increased in the CL by several mechanisms involving PGF2α, macrophages and hemodynamic changes (Sugino, 2006). As a consequence, accumulation of ROS causes DNA and protein damage and cell membrane alterations that trigger apoptosis (Sugino, 2006). In addition, the CL and other tissues have specific enzymes that counteract superoxide radical accumulation (Sugino et al., 2000b). Copper-zinc and manganese superoxide dismutase (SOD) mRNA and protein, and lipid peroxide concentrations during the human menstrual cycle and early pregnancy were evaluated (Sugino et al., 2000b). Copper-zinc SOD mRNA and activity reached the lowest amount during luteal regression and the greatest amount during pregnancy. In contrast, manganese SOD mRNA and activity were greater in regressing CL than at any other luteal stage (Sugino et al., 2000b), concurrently with higher amounts of lipid peroxide concentration.

On other hand, FasL was significantly up-regulated 0.5 h after $PGF_{2\alpha}$ and attained the highest increment at 12 h; while FasR increased from 2 h until 64 h after $PGF_{2\alpha}$ (Kliem et al., 2009). Both proteins, FasL and FasR, have been implicated in the regulation of cellular apoptosis during structural luteolysis (Taniguchi et al., 2002). FasR transcript was detected in bovine CL with a significantly greater expression during luteal regression than in any other stage (early, mid and late CL) (Taniguchi et al., 2002). Also, FasR mRNA expression increased 1.4 and 1.8 fold in cultured luteal cells treated with INFγ alone and INFγ combined with TNFα, respectively (Taniguchi et al., 2002). Moreover, treatment of luteal cells with FasL combined with INFγ or with INFγ and TNFα induced death of 60 and 85% of cultured luteal cells;

whereas FasL alone was not cytotoxic. Although FasR was expressed in luteal cells in the absence of cytokines, the presence of FasL in the culture did not induce apoptosis, supporting the role of the immune cytokines in the sequence of events leading to luteal regression.

Cellular matrix degradation and structural luteolysis

In the ovary, as in other tissues, the space between cells is occupied by a network of macromolecules, proteins and polysaccharides that constitute the structural support of the tissue and interact with the cells, influencing their survival, development, migration, proliferation, shape and function (Albert et al., 2002). This "substance around the cells" is named extracellular matrix (ECM). In the ovary, ECM is object of continuous remodeling to provide basic cellular conditions to each developmental stage and functional structure. Stability of ECM is a consequence of differential expression of two important groups of proteins: matrix metalloproteinases (MMP) that degrade certain components of ECM (Pitzel et al., 2000) and tissue inhibitors of metalloproteinases (TIMP) that inhibit MMP activity (Ricke et al., 2002a).

MMP proteins are zinc- and calcium-dependent enzymes that degrade proteinaceous components of the ECM and cleave signaling proteins. This group consists of more than 26 members synthesized as inactivated enzymes (pro-MMP) and classified in collagenases, gelatinases, stromelysins and membrane type MMP (Smith et al., 2002). TIMP include a group of 4 proteins (TIMP-1, -2, -3 and -4) that specifically inhibit MMP and can regulate cellular functions by mechanisms different to MMP inhibitor activity (Smith et al., 2002). TIMP are able to modulate proliferation of endothelial cells and fibroblasts (Rho et al., 2007; Seo et al.,

2008), inhibit angiogenesis (Seo et al., 2003) and apoptosis (Guedez et al., 1998) and promote steroidogenesis (Shores and Hunter, 2000) and other functions.

During its lifespan, a CL undergoes an intensive remodeling of the ECM that is hormonally regulated by luteotropic, luteolytic and steroidogenic stimuli at the level of transcription, secretion and activation of proenzymes (Smith et al., 2002; Vassilev et al., 2002). Modifications of MMP and TIMP mRNA expression and protein activity were associated with structural regression of the CL in rats (Liu et al., 1999), pigs (Pitzet el al., 2000) and sheep (Ricke et al., 2002 a, b). Members of MMP and TIMP families have been identified and monitored in luteal tissue of several species during the estrous cycle and luteolysis (Liu et al., 1999; Pitzel el al., 2000; Ricke et al., 2002 a, b, c; Towle et al., 2002; Ribeiro et al., 2006 , 2007; Kliem et al., 2007). Expression of TIMP-2 mRNA was demonstrated in ovine preovulatory follicles and CL throughout the luteal phase (Smith et al., 1995). Concentrations of TIMP-2 in luteal tissue were maximal during the early luteal phase (d 3 and 7 post estrus) and minimal on d 16, when probably luteolysis was taking place. Also, TIMP-2 mRNA concentration was 10 times greater in LLC than in SLL, indicating that probably LLC are the primary source of TIMP-2 as reported elsewhere (Smith et al., 1994; Ricke et al., 2002b). Amounts of MMP-2 and MMP-14 mRNA followed a different expression pattern during the estrous cycle in sheep (Ricke et al., 2002c). At day 5, MMP-14 attained greater expression and decreased in mid- and late-luteal phase; whereas MMP-2 was greater on day 10 compared with earlier stages.

In ovine tissue of mid-luteal phase, TIMP-1 and TIMP-2 decreased abruptly (60% and 90% from controls, respectively) as early as 1 h after $PGF_{2\alpha}$-induced luteolysis, remaining low until

h 8. In contrast, MMP-2 and MMP-9 activities increased 165% at 8 h after $PGF_{2\alpha}$, coinciding with the lowest concentration of P_4 (Towle et al., 2002). In another report in sheep, TIMP-1 concentration significantly decreased 15 min after administration of $PGF_{2\alpha}$ (time 0) and remained at basal values through 48 h (Ricke et al., 2002a). In addition, TIMP-1 mRNA decreased 1 h after $PGF_{2\alpha}$ and increased at 4 and 6 h, whereas TIMP-2 and TIMP-3 mRNA remained lower throughout the first 6-h period and then significantly increased (Ricke et al., 2002a). The study of mRNA expression and activity of various members of the MMP family (MMP-1, -2, -3 , -7 , -13 and -14) in the ewe CL after $PGF_{2\alpha}$-induced luteolysis revealed an increase in mRNA of all MMP members (except MMP-2) 15 to 30 min post $PGF_{2\alpha}$ (Ricke et al., 2002b). All MMP mRNAs were greater than at time 0 (previous to PGF2α) by 6 h after $PGF_{2\alpha}$, and MMP activity increased significantly by 15 min and remained elevated through 48 h post- $PGF_{2\alpha}$ (Ricke et al., 2002b). Thus, modification in the expression pattern and activity of MMP and TIMP mRNA and protein may increase the MMP/TIMP ratio, facilitating ECM degradation and consequently favoring structural luteolysis (Smith et al., 2002).

In mid-phase bovine CL, MMP-1 mRNA expression increased abruptly 2 h after $PGF_{2\alpha}$-induced luteolysis, followed by MMP-9 (12 h), and MMP-2 and MMP-14 (24 h). TIMP-2 mRNA initially remained at same amounts of h 0 luteal tissue (previous to $PGF_{2\alpha}$) and decreased precipitously at h 48; whereas TIMP-1 increased at h 12 and declined 36 h later (Kliem et al., 2007). In addition, mRNA expression of plasminogen activator system (PAS) revealed an abrupt increase as early as 2 to 4 h after $PGF_{2\alpha}$. PAS is involved in ECM degradation in the CL (Liu, 2004).

Roles of uterine and luteal PGF2α in luteal regression

As mentioned above, absence of viable embryos in the uterus at the end of each luteal cycle allows increased secretion of $PGF_{2\alpha}$ (Zarco et al., 1988) that through diffusion from venous to arterial blood vessels reaches the ovary and binds to specific $PGF_{2\alpha}$ receptors (FP) localized in the surface of LLC (Fitz et al., 1984; Balapure et al., 1989; Gadsby et al., 1990). Activation of FP triggers a cascade of molecular events conducive to the demise of the CL and the onset of a new reproductive cycle.

Biochemical characteristics, synthesis, and transport of $PGF_{2\alpha}$

Prostaglandins (PG) are members of a group of biomolecules containing 20-carbon backbone structure known as eicosanoids, that play essential functions in the reproductive physiology of mammals (Weems et al., 2006; Flores and Barlund, 2009). In addition to PG, the eicosanoid family includes thromboxanes and leukotrienes. Biologically active prostaglandins include 3 members: PGI_2, PGE_2 and $PGF_{2\alpha}$. Prostaglandins are synthesized from arachidonic acid (an important polyunsaturated fatty acid of the cellular membrane) and hence have a lipid nature (Marnett et al., 1999). Phospholipase A_2 catalyzes release of arachidonic acid from cellular membrane, followed by oxygenation and peroxidation of arachidonic acid in the reticulum endoplasmic to yield PGG_2 and PGH_2 (Marnett et al., 1999). This step is comprised of two successive reactions catalyzed by a single bifunctional enzyme: prostaglandin endoperoxide synthase or prostaglandin G/H synthase (Cox). At least 2 forms of Cox have been identified; Cox-1 is expressed constitutively and Cox-2 is an inducible isoform (Smith et al., 2000). Formation of PGG_2 and PGH_2 is catalyzed by cyclooxygenase and peroxidase

components of the enzyme, respectively (Smith et al., 2000); and is considered a rate-limiting step in the biosynthesis of PG (Sirois et al., 2004). Conversion of PGH_2 into the different biological forms of PG is catalyzed by cell-specific terminal synthases (Flores and Barlund, 2009). For instance, PGES and PGFS synthesize PGE_2 and $PGF_{2\alpha}$, respectively, whereas prostaglandin dehydrogenase catabolizes $PGF_{2\alpha}$ into 13, 14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) (Tai et al., 2002).

Although PG have a lipid nature, at physiological pH they behave as organic anions and do not diffuse easily across cellular plasma membranes (Shuster, 1998). Instead, PG utilize carrier-mediated transport that includes three aspects: transport and metabolic clearance by the lung, transport by non-lung tissues that metabolize PGs, and transport by tissues that do not metabolize PGs (Shuster, 1998). Recently, PG transporter (PGT) in ovine endometrium was cloned and characterized, and its participation in $PGF_{2\alpha}$-induced luteolysis was demonstrated (Banu et al., 2008). Inhibition of PGT-mediated transport of PGF2α from the uterus prevented luteolysis and extended lifespan of the CL and length of the estrous cycle. Moreover, inhibition of PGT reduced the ability of the endometrium to secrete $PGF_{2\alpha}$ in response to oxytocin (Banu et al., 2008). Apparently, ERK1/2 pathways modulate efflux of $PGF_{2\alpha}$, whereas JNK/SAPK pathways regulate both efflux and influx of $PGF_{2\alpha}$ in ovine endometrial cells (Banu et al., 2008). In ovine CL, expression of both PGT mRNA and protein was greater on d 7 to 21 than 1 to 6 of the estrous cycle with maximal expression values on d 13 to 15 (Arosh et al., 2003). Also, PGT protein was expressed highly in LLC.

Characteristics, variants, and localization of $PGF_{2\alpha}$ receptor

$PGF_{2\alpha}$ induces changes in intracellular functions by binding to a specific cellular membrane receptor that was cloned and characterized in several mammalian species (Abramovitz et al., 1994; Graves et al., 1994; Kitanaka et al., 1994; Lake et al., 1994; Sakamoto et al., 1994). FP belongs to the family of G-protein-coupled receptors that, once activated, couples to Gq to trigger the inositol trisphosphate (IP_3) second-messenger pathway. In cattle and sheep, FP protein has 362 amino acids, while human being and rat have 359 and 366, respectively. Molecular weight of FP is similar among species, varying between 40060 and 40983 in human beings and ruminants (Abramovitz et al., 1994; Graves et al., 1994; Kitanaka et al., 1994; Sakamoto et al., 1994).

Two FP isoforms have been identified in the sheep CL (Graves et al., 1994; Pierce et al., 1997). Both FPA and FPB have similar amino acid (AA) sequences through the seven transmembrane domains, but differ in the carboxyl terminus site, in which FPA has 45 more AA than FPB (Pierce et al., 1997). Thus, FPB isoform is a truncated version of the FPA isoform. Activation of FP receptor variants leads to an increase in inositol phosphate accumulation, protein kinase C (PKC) activation, and intracellular calcium release consistent with activation of G-proteins of the Gq family (Abramovitz et al., 1994; Graves et al., 1994; Sakamoto et al., 1994). However, FPA and FPB activation led to Rho-dependent changes in cell morphology, formation of actin stress fibers, and tyrosine phosphorylation of p125 FAK (Pierce et al., 1999).

In cells transfected with either FPA or FPB, $PGF_{2\alpha}$ elicited a similar maximum IP accumulation; nevertheless, basal accumulation of IP was about 130% greater in cells expressing FPA than in those expressing FPB isoforms (Pierce et al., 1997). Moreover, FP isoforms are regulated differentially by PKC. In human embryonic kidney cells that steadily expressed FPA and FPB, PKC inhibited $PGF_{2\alpha}$-stimulated IP formation by FPA but not by FPB (Fujino et al., 2000a). Also, a stronger PKC-mediated agonist dependent phosphorylation was observed in FPA, but not in FPB (Fujino et al., 2000a), indicating that under conditions of acute activation or down-regulation of PKC, the FPA isoform might be more responsive to submaximally stimulating concentrations of agonist than the FPB variant. In addition, agonist-induced stimulation of inositol phosphate formation, and mobilization of intracellular Ca^{2+} indicate that the FPB variant resensitizes more slowly than the FPA (Fujino et al., 2000b). Apparently, the carboxyl terminus of the FPA isoform is critical for resensitization because the absence of 45 of 46 AA in the carboxyl terminus of FPB slowed resensitization and prolonged signaling (Fujino et al., 2000b). Overall, these findings demonstrated molecular and signaling differences between variants of FP that may contribute to elucidation of physiological actions of $PGF_{2\alpha}$ in the CL. Possibly, differential expression of the two isoforms in response to functional stages of the CL may alter intracellular responsiveness to $PGF_{2\alpha}$ (Hoyer, 1998).

In ovine CL, northern blot analysis revealed similar expression of FP mRNA at mid-luteal phase (d 10, 2.73 ± 0.17; d 12 2.47 ± 0.91) but greater on d 16 of pregnancy (Rueda et al., 1995b). A subsequent study corroborated that concentrations of FP mRNA did not vary throughout the estrous cycle (Juengel et al., 1996), although a significant reduction in FP mRNA expression was observed as early as 4 h (Juengel et al., 1996; Tsai et al., 2001) or 12 h

(Tsai et al., 1998a) during $PGF_{2\alpha}$-induced luteolysis in sheep, cow and pig (Rueda et al., 1995b; Sakamoto et al., 1995; Estill et al., 1995; Mamluk et al., 1998). These findings are consistent with the reduction of number of FP by the time of luteolysis (Wiepz et al., 1992), and the decline of FP mRNA concentration in cultured LLC treated with a PKC activator or calcium ionophore (Tsai et al., 2001). Thus, reduction of FP protein and mRNA concentrations were associated with significantly lower concentrations of P_4 and luteal weight (Juengel et al., 1996; Tsai et al., 1998a). On the basis of the previous evidence, FP seems to be downregulated by a mechanism of homologous ligand that could imply reduction of mRNA FP expression for $PGF_{2\alpha}$ at the same time that FP are downregulated by mechanisms of receptor internalization and degradation (Tsai et al., 1998a).

Both FP mRNA and protein are considerably more abundant in luteal tissue than in any other ovine tissue (Tsai et al., 1998a). Amounts of FP mRNA and proteins were approximately 26 and 148 times greater, respectively, in luteal tissue than in ovarian stroma, and this figure was even greater when compared with other tissues. FP were localized primarily in LLC from several mammalian species (Balapure et al., 1989; Gadsy et al., 1990; Juengel et al., 1996; Mamluk et al., 1998; Boonyaprakob et al., 2001; Bogan et al., 2008). Using separated populations of LLC and SLC purified by elutriation, high affinity binding sites (Kd = 19 to 64 nM in pigs and 17.4 ± 2.3 nM in ewes) were identified exclusively in LLC, whereas low affinity binding sites (Kd = 262-3103 nM in pigs and 409 ± 166 nM in ewes) were present in LLC and SLC (Balapure et al., 1989; Gadsby et al., 1990). Number of high affinity $PGF_{2\alpha}$-binding sites was low during the early luteal phase of the pig (before d 12) but increased thereafter (day 13, 14 and 16-17) (Gadsby et al., 1990). In bovine CL, in situ hybridization

allowed detection of FP mainly in LLC (Sakamoto et al., 1995). However, in a subsequent study using RT-PCR with elutriated SLC and LLC, FP mRNA was detected in both steroidogenic cells, although FP mRNA expression in LLC was around 2.5 times greater than in SLC (Mamluk et al., 1998). Immunohistochemical analysis (IHC) in primate CL revealed expression of FP protein in LLC and vascular endothelial cells (Bogan et al., 2008). Amounts of FP were lower during early and mid-luteal phase and increased in the following days of the menstrual cycle.

Albeit evidence of FP in the vascular tissue of the CL has been controversial over the years, presence of FP in endothelial cells was documented in pigs, cows and primates (Mamluk et al., 1998; Zannoni et al., 2007; Bogan et al., 2008). Using in situ hybridization, Boonyaprakob et al. (2003) did not identify FP in endothelial cells in pig CL. However, in a recent report, microvascular endothelial cells were isolated from porcine CL by an immunomagnetic separation protocol and FP protein and mRNA were identified and quantified by immunofluorescence and RT-PCR (Zanoni et al., 2007); FP mRNA and protein expression significantly increased from early- to mid-luteal phase, attaining maximum amounts during pregnancy. FP mRNA was identified in luteal endothelial cells from bovine CL (Mamluk et al., 1998), whereas in primates FP in vascular tissue was identified by IHC (Bogan et al., 2008).

Luteolysis is an intricate process in which substances locally produced by different cellular components modulate luteal regression by paracrine and autocrine mechanisms (Korzekwa et al., 2008). In luteal steroidogenic cells cultured in monolayer, $PGF_{2\alpha}$ increased P4 accumulation after 24 h incubation. Nevertheless, $PGF_{2\alpha}$ reduced production of P4 in culture of luteal steroidogenic, endothelial and immune cells (Korzekwa et al., 2008). So, interactions

among different cellular components appear to be necessary so that synthesis and secretion of P_4 is disrupted, and hence, subsequent steps of luteolysis take place. In addition, two important vasoactive factors, NO and END1, produced by endothelial cells (Girsh et al., 1996a; Skarzynski et al., 2003) appear to be involved in luteolysis. Moreover, they are regulated by $PGF_{2\alpha}$ (Manluk et al., 1999; Skarzynski et al., 2003; Shirasuna et al., 2008), and $PGF_{2\alpha}$ itself is involved in regulating blood flow in extraovarian tissues (Ogletree et al., 1982). Hence, presence of FP in endothelial cells is consistent with experimental evidence about the role of luteal vascular tissue in luteolysis.

Signal transduction pathways involved in $PGF_{2\alpha}$-mediated luteolysis

Activation of luteal FP by $PGF_{2\alpha}$ during luteolysis modifies the molecular conformation of the receptor allowing activation of G protein (Alberts et al., 2002). G protein (GTP-binding protein) is a trimeric molecule composed of three subunits, α, β, and γ, which remain inactive while receptor is not bound to its ligand. Once G-protein is stimulated, GDP in the α subunit is exchanged by GTP, and as a consequence, the trimeric G-protein dissociates into two activated components, α subunit and $\beta\gamma$ complex (Levitzki, 1987). In Gq-protein-coupled receptors, as is the case of FP, activated α subunit triggers the inositol phosphate (IP_3) second-messenger pathway. Briefly, α subunit activates the enzyme phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] into two molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge et al., 1983; Davis et al., 1987; Davis et al., 1988). Both molecules act as intracellular messengers with different functions (Berridge et al., 1987). The IP_3 leaves the plasma membrane and diffuses through the cytosol to the

endoplasmic reticulum (ER). In the ER membrane, IP_3 binds and opens IP_3-gated Ca^{2+}-release channels increasing concentrations of intracellular Ca^{2+} (Streb et al., 1984; Spat et al., 1986; Davis et al., 1987a; Williamson and Monk, 1989). In the cellular membrane, DAG together with the increased cytosolic Ca^{2+} activates the enzyme protein kinase C (PKC) (Berridge et al., 1987). The PKC, via mitogen-activated protein kinase (MAP kinase) activates PLA, which in turn stimulates release of AA from the membrane (Qiu and Leslie, 1994; Chen et al., 1998) and promotes synthesis of $PGF_{2\alpha}$. Thus, uterine $PGF_{2\alpha}$ induces synthesis of luteal $PGF_{2\alpha}$.

Recently, it was demonstrated that the interaction of $PGF_{2\alpha}$ with FP receptor activates an additional intracellular signaling pathway, the mitogen activated protein kinases (MAPKs), that is involved in the transduction of extracellular signals into the nucleus to regulate gene expression (Chen et al., 1998). This pathway has been widely described in the signaling of receptor-linked tyrosine kinases activated by several growth factors, and involves phosphorylation of numerous serine/threonine-selective protein kinases, such as RAF, MEK and MAPK, that regulate the activities of several transcription factors (Alberts et al., 2002). Apparently, the RAF/MEK/MAPK signaling cascade also mediates the cellular response to various ligands that bind G protein-coupled receptors, including $PGF_{2\alpha}$ (Chen et al., 1998; Tai et al., 2001; Hou et al., 2008). Three isoforms of Raf family were detected in the bovine CL, but only Raf-1 and B-Raf were activated by $PGF_{2\alpha}$ and phorbol 12-mysristate 13-acetate (PMA; a pharmacological PKC activator); and subsequently MEK1, $p42^{mapk}$ and $p44^{mapk}$ kinases were activated. Activation of this protein kinase pathway by $PGF_{2\alpha}$ may represent a mechanism by which the intracellular signal initiated by FP is transmitted to the nucleus to regulate transcriptional activity related with luteolysis (Chen et al., 1998). Moreover, $PGF_{2\alpha}$,

via MEK/ERK signaling pathway, stimulated mRNA and protein expression of early growth response 1 (EGR1) both in vivo and in vitro (Hou et al., 2008). EGR1 has been associated to various reproductive functions (Espey et al., 2000; Russell et al., 2003). During $PGF_{2\alpha}$-stimulated luteal regression in cow, EGR1 induced expression of TGFβ1, an important tissue remodeling protein also involved in apoptosis (Hou et al., 2008).

The first attempts to understand the intracellular mechanisms by which $PGF_{2\alpha}$ disrupts synthesis and secretion of P_4 were carried out in rat luteal cells. Treatment of rats with $PGF_{2\alpha}$ down-regulated LH luteal receptors within 12-24 h in parallel with the decrease of P_4; nevertheless, reduction of P_4 concentrations preceded a decrease in LH receptors by several hours (Grinwich et al., 1976). Also, it was demonstrated that LH or $PGF_{2\alpha}$ in absence of LH, stimulated P_4 production in a dose dependent manner in dissociated luteal cells (Thomas et al., 1978). In addition, LH induced a significant increase of adenylate cyclase activity and accumulation of cAMP; whereas adding $PGF_{2\alpha}$ to LH-supplemented cultured luteal cells reduced both adenylate cyclase activity and cAMP accumulation by about 50 to 70% (Thomas et al., 1978; Dorflinger et al., 1983; Kenny and Robinson, 1986). Moreover, addition of dibutyryl cyclic AMP (dbcAMP; analogue of cAMP) reversed PGF2α-induced inhibition of P_4 (Thomas et al., 1978; Dorflinger et al., 1983 Kenny and Robinson, 1986).

From these early studies, it was evident that the PGF2α-induced block of LH-stimulated P_4 secretion was not due to inhibition of specific binding activity of LH receptor, cAMP degradation, direct action on adenylate cyclase or direct damage of luteal cells by $PGF_{2\alpha}$ (Grinwich et al., 1976; Thomas et al., 1978; Dorflinger et al., 1983). Instead, $PGF_{2\alpha}$-induced

inhibition of LH-stimulated P_4 may be due to inhibition of activation of cAMP accumulation by LH and inhibition of luteal cell response to cAMP (Thomas et al., 1978; Dorflinger et al., 1983). However, when dbcAMP or LH was added to dispersed luteal cells from rats treated with $PGF_{2\alpha}$ in vivo, P4 production was reduced significantly, indicating that $PGF_{2\alpha}$-induced inhibition of P_4 production may occur at a site beyond cAMP formation in the steroidogenic pathway (Jordan, 1981). Because $PGF_{2\alpha}$ was more effective to inhibit dbcAMP-stimulated steroidogenesis in vivo (Jordan, 1981) than in vitro (Thomas et al., 1978; Jordan, 1981) it was suggested that $PGF_{2\alpha}$-induced inhibition of P_4 may be the result of both direct and indirect effects (Jordan, 1981). In addition, $PGF_{2\alpha}$ was able to inhibit activity of 3β-HDS and cholesterol esterase (Dwyer and Church, 1979), as well luteal adenylate cyclase activity concomitant with the decrease of P_4 concentrations in a 24-h period (Agudo and Smith, 1982). Thus, on the basis of the experimental evidence it was likely that $PGF_{2\alpha}$ may have multiple sites of action and that its luteolytic actions involve other substances or mediators, indicating the complex mechanism of luteolysis.

Luteal regression was associated to structural changes in cellular membranes (Carlson et al., 1984) and hydrolysis of phosphoinositides with the concomitant increment of radiolabeled inositol phosphates (Raymond et al., 1983; Leung et al., 1986). At that time, increasing evidence from other tissues strengthened the idea that inositol polyphosphates may function as a second messenger for mobilizing Ca^{2+} from intracellular stores (Burguess et al., 1984); and this mechanism may be responsible for inhibition of gonadotropin-induced cAMP and progesterone production (Leung et al., 1986; Davis et al., 1987). In this regard, incubation of luteal cells in Ca^{2+} free medium increased cAMP accumulation in response to LH. Also, the

Ca^{2+} ionophore A23187 inhibited LH-stimulated cAMP accumulation in a dose-dependent manner, although the inhibition was dependent on presence of extracellular Ca^{2+} (Dorflinger et al., 1984). In addition, Ca^{2+} directly inhibited LH-sensitive adenylate cyclase activity at concentrations consistent with known (IC_{50}, ~ 10μM) intracellular values (Dorflinger et al., 1984). Moreover, the inhibitory effect of Ca^{2+} was not due to stimulation of an increase in cAMP degradation because inhibition occurred in cells incubated in presence or absence of a cAMP phosphodiesterase inhibitor (Dorflinger et al., 1984). On the other hand, the effect of PGF2α on LH-stimulated cAMP accumulation was independent of the presence of Ca^{2+} in the medium, and the blockade of Ca^{2+} channels by verapimil did not affect the magnitude of inhibition induced by PGF2α as well. Thus, it appeared that the luteolytic effect of $PGF_{2\alpha}$ was not dependent on an influx of extracellular Ca^{2+}, instead, it seemed to be dependent on an increase in intracellular Ca^{2+} by other means (Dorflinger et al., 1984; Davis et al., 1987).

In bovine luteal cells, $PGF_{2\alpha}$ increased turnover of inositol phospholipid inducing a rapid (10 sec) and sustained (up to 60 min) increment in the concentrations of inositol phosphates (IP), that was greater in magnitude in IP_3 than IP_2 and IP_1 and independent of extracellular Ca^{2+} (Davis et al., 1987a; Davis et al., 1988). Also, $PGF_{2\alpha}$ augmented concentrations of Ca^{2+} by 2 to 3 fold, again independent of extracellular Ca^{2+}. In cultured SLC, removal of Ca^{2+} from the medium reduced hormone-stimulated but not basal P4 production, whereas in LLC both basal and stimulated P_4 production were dependent on Ca^{2+} (Alila et al., 1990). Interestingly, the finding that LH was able to enhance accumulation of cAMP as well concentrations of IP_3 and Ca^{2+} in bovine luteal cells indicated that LH actions are mediated by at least two second messenger systems (Davis et al., 1987b). Hence, this fact implies that both LH and $PGF_{2\alpha}$ use

the same second messenger systems to produce opposite effects on P_4 production by luteal steroidogenic cells (Wiltbank et al., 1991).

Measurements of free intracellular Ca^{2+} in ovine individual SLC and LLC demonstrated differential response of both cell types to PGF2α and calcium ionophore (A23187). Although A23187 increased free intracellular Ca^{2+} in the two cell types, SLC underwent an abrupt and marked increase in free Ca^{2+} that was highest by 30 s and gradually returned to baseline in the time course of 3 min. Instead, LLC reached the greatest free Ca^{2+} by 90 s and required more than 30 min to return to baseline values (Wiltbank et al., 1989a). In addition, $PGF_{2\alpha}$ induced a remarkable increase of intracellular Ca^{2+} in LLC but not in SLC; whereas LH did not affect free Ca^{2+} concentration in either luteal cell type (Wiltbank et al., 1989a). The increment of intracellular Ca^{2+} in $PGF_{2\alpha}$-stimulated LLC was dependent upon influx of extracellular Ca^{2+}, because inclusion of a Ca^{2+} channel blocker decreased and removal of extracellular Ca^{2+} eliminated the response to $PGF_{2\alpha}$ (Wiltbank et al., 1989a).

At this point, it is clear that the IP_3 increase after FP activation by $PGF_{2\alpha}$ is associated to an abrupt and transient increment of intracellular Ca^{2+} released from endoplasmic reticulum, in whose membrane IP_3 binds specific receptors (Vermassen et al., 2004). Nevertheless, other intracellular organelles, such as the Golgi apparatus and secretory vesicles, may act as IP_3-sensitive Ca^{2+} stores (Vermassen et al., 2004). The IP_3 receptor has been characterized as a tetrameric intracellular ligand-gated Ca^{2+} channel (250-300 kDa), bearing a large cytosolic domain in which several regulatory molecules can bind, including IP_3 and Ca^{2+}, and cooperatively modulate channel gating (Dawson, 1997). Three of the 4 IP3-receptor subunits

are encoded by different genes and determine three IP_3 receptor isoforms (types 1, 2 and 3) (Südhof et al., 1991; Blondel et al., 1993; Yamada et al., 1994; Yamamoto et al., 1994). The structure of the type 1 IP_3 receptor has been better characterized and consists of a ligand-binding domain in the NH_2 terminal portion, a coupling or modulatory domain in the middle portion and a Ca^{2+} channel domain in the COOH-terminal portion (Mignery and Südhof, 1990). The three IP_3 receptor isoforms were identified in mouse granulosal cells (Díaz-Muñoz et al., 2008); but only IP_3 receptor type 2 was observed in granulosal and thecal cells of growing follicles and luteal cells of developing CL in pigs (Steffl et al., 2004).

As indicated above, protein kinase C (PKC) is associated to $PGF_{2\alpha}$-induced inhibition of P_4 production by luteal steroidogenic cells (Wiltbank et al., 1991). In ovine luteal cells, activity of PKC was 4 times greater in LLC than SLC. Activation of PKC by phorbol 12-myristate 13-acetate (PMA) caused an abrupt and dose-dependent inhibition of P_4 production in LLC and LH-, forskolin- and dbcAMP-stimulated SLC (Wiltbank et al., 1989b). Moreover, PMA reduced P4 production in LLC treated with 25-hydroxycholesterol but that effect was not demonstrated in LH-stimulated SLC. Apparently, the inhibitory action of PKC in SLC occurs after LH binding and cAMP production because in forskolin-, dbaAMP- and LH-stimulated cells, SLC P_4 production was similarly inhibited. It is likely that PKC inhibition occurs prior to enzymatic cleavage of cholesterol because inclusion of 25-hydroxycholesterol in the culture medium did not affect P_4 production by SLC in response to PMA (Wiltbank et al., 1989b). The presence of lipoproteins in the culture medium increased P_4 production in both LLC and LH-stimulated SLC with a greater effect of high density lipoproteins (HDL) than low density lipoproteins (LDL). In LLC, $PGF_{2\alpha}$ significantly reduced P_4 production in the presence but not

in the absence of HDL or LDL; whereas in SLC stimulated or not with LH and/or HDL or LDL, $PGF_{2\alpha}$ did not reduce P_4 production (Wiltbank et al., 1990). To further demonstrate if PKC was mediating the inhibition of P_4 by PGF2α, PKC-deficient or normal LLC stimulated with LDL or HDL were cultured in presence or absence of $PGF_{2\alpha}$. As was expected, $PGF_{2\alpha}$ reduced P_4 production in PKC-normal LLC with LDL or HDL, but had no effect in PKC-deficient LLC (Wiltbank et al., 1990).

It seems to be evident that PKC mediates the antisteroidogenic actions of $PGF_{2\alpha}$ in luteal cells; nevertheless, the locus at which PKC blocks P_4 production in LLC and SLC is apparently different. Consistent with the differences in the IP_3−Ca^{2+}−PKC second messenger system indicated above, SLC and LLC have other morphological and functional differences. For instance, Fitz et al. (1982) reported that SLC in the ewe had 11 times greater LH receptors than LLC (33260 and 3074 for SLC and LLC, respectively), whereas LLC contained 32 times greater $PGF_{2\alpha}$ receptors than SLC (62143 and 2115 for LLC and SLC, respectively). In contrast, a later study in sheep from the same group indicated no differences in the number of LH receptors between both types of steroidogenic cells during the mid-luteal phase (Harrison et al., 1987). Although SLC were considerably more responsive to LH stimulus, LLC produced 6 to 8 times more P_4 than SLC (Ursely and Leymarie, 1979; Harrison et al., 1987). Moreover, pharmacological or physiological activation of the cAMP second messenger pathway did not increase P_4 production by LLC (Hoyer and Niswender, 1984). It was suggested that this signaling pathway is constitutively activated in these steroidogenic cells and for that reason stimulation of LLC with LH did not cause a further increment in P_4 output (Hoyer and Niswender, 1984; Diaz et al., 2002; Niswender et al., 2007).

From the numerous PKC isoenzymes described in the literature, five forms, α, βI, βII, ε, and μ were detected in bovine luteal tissue (Sen et al., 2004). Whereas α, βI, βII variants were detected in both luteal steroidogenic and endothelial cells, PKCε was located exclusively in steroidogenic cells of the CL (Sen et al., 2006). PKCβII and PKCε were expressed more in day-10 than day-4 CL (Sen et al., 2004). In addition, $PGF_{2\alpha}$ induced a significantly lower increase in intracellular Ca^{2+} concentration in 4-day than 10-day CL in both LLC and SLC (Sen et al., 2005; Choudhary et al., 2005). A PKCε inhibitor reduced the $PGF_{2\alpha}$-induced calcium increase in both LLC and SLC on day 10 (Sen et al., 2005). The magnitude of P_4 reduction induced by a pharmacological increase of Ca^{2+} concentrations was lower in day-10 than day-4 CL (Goravanahally et al., 2007). Moreover, down-regulation of PKCε expression by siRNA technology or blocking PKCε activation reduced the effectiveness of $PGF_{2\alpha}$ in reducing LH-stimulated production of P_4 (Sen et al., 2005; Goravanahally et al., 2007). Hence, these findings strongly indicate that differential expression of certain components associated to signal transduction triggered by $PGF_{2\alpha}$ may account for lack of responsiveness of CL during early development. Previously it was demonstrated that the insensibility of early CL to $PGF_{2\alpha}$ was not due to the lack of FP (Wiltbank et al., 1995; Mamluk et al., 1999), or limited capacity of $PGF_{2\alpha}$ to stimulate END1 (Choudhary et al., 2004; Choudhary et al., 2005) as had been suggested (Levi et al., 2000). Instead, a lower expression of PGT and a greater PGE_2 production in the early CL may contribute to explain its resistance to luteolytic actions of $PGF_{2\alpha}$ (Arosh et al., 2004). However, new findings about differential expression of signaling mediators of $PGF_{2\alpha}$ strengthen the thesis espoused above (Goravanahally et al., 2009).

Luteal $PGF_{2\alpha}$ and luteolysis: a proposed model to explain the intraluteal mechanism that regulates luteal regression in ruminants

As indicated elsewhere (Diaz et al., 2002; Wiltbank and Ottobre, 2003; Niswender et al., 2007), the corpus luteum of several mammalian species has the capacity to synthesize $PGF_{2\alpha}$. Luteal $PGF_{2\alpha}$ is synthesized and secreted by LLC, and by autocrine and paracrine mechanisms this hormone coordinates and participates in the successive intracellular and intercellular events that are conducive to the functional and structural disappearance of the CL. In the bovine CL, LLC have the molecular and metabolic machinery related with the synthesis, transport, and signaling of both $PGF_{2\alpha}$ and PGE_2 (Arosh et al., 2004). For instance, Cox-2, PGES, PGT, EP_2, EP_3 and FP were greatly regulated, whereas Cox-1, PGFS and PGDH were expressed uniformly throughout the luteal lifespan. Accordingly, synthesis of PG during the bovine estrous cycle is directed selectively toward PGE_2 in early luteal phase and toward $PGF_{2\alpha}$ during luteal regression, but it is nonselective during mid-luteal phase (Arosh et al., 2004). Likewise, in sheep, exogenous $PGF_{2\alpha}$ induced expression of Cox-2 but not Cox-1; Cox-2 mRNA expression increased about 10 fold in less than 1 h after $PGF_{2\alpha}$ was added to cultured LLC (Tsai and Wiltbank, 1997). Also, Cox-2 mRNA was greater expressed in LLC than SLC (4507 vs 323 copies/cell). These findings indicate that LLC respond to uterine $PGF_{2\alpha}$ by increasing synthesis of luteal $PGF_{2\alpha}$ within 1 h or less, and in that way intraluteal $PGF_{2\alpha}$ signal is amplified several times by a positive feedback loop (Tsai and Wiltbank, 1997), in which luteal oxytocin and other mediators may be involved (Townson and Pate, 1994; 1996; Mann, 1999; Kotwica, 1999; Shirasuna et al., 2007).

The importance of intraluteal production of $PGF_{2\alpha}$ during luteolysis was demonstrated by applying a different experimental approach (Niswender et al., 2007). On day 10 of the ovine estrous cycle, an implant with 0, 1 or 10 mg of indomethacin (inhibitor of PG synthesis) was delivered into the CL. Progesterone production was significantly greater on days 13 through 16 of the cycle and CL were heavier in indomethacin(10 mg)-treated than in control ewes (Niswender et al., 2007). Thus, it seems that luteal $PGF_{2\alpha}$ is required in both functional (decreasing steroidogenic capacity) and structural (inducing apoptosis and tissue degradation) phases of luteolysis.

Recently, Niswender et al. (2007) proposed a model to explain the intraluteal mechanism that regulates luteal regression in ruminants. In this model, the authors suggested that although luteolysis is initiated from extra-luteal influence (endometrial $PGF_{2\alpha}$), the mechanisms that ultimately lead to the demise of the CL are orchestrated by the corpus luteum itself through autocrine and paracrine actions of luteal $PGF_{2\alpha}$ and oxytocin. Thus, once the uterine signal initiates luteal regression, increased luteal secretion of $PGF_{2\alpha}$ triggers mobilization of intracellular Ca^{2+} in large luteal cells and secretion of oxytocin by these cells. Oxytocin in turn, increases concentrations of Ca^{2+} in small luteal cells. Apoptosis follows Ca^{2+} mobilization in both cell types. Likewise, as was indicated elsewhere in this review, several other factors secreted from nonsteroidogenic cells of luteal origin (vascular smooth muscular cells, endothelial cells, and immune cells) interact with steroidogenic cells to support luteolytic actions of luteal $PGF_{2\alpha}$ (Figure 2).

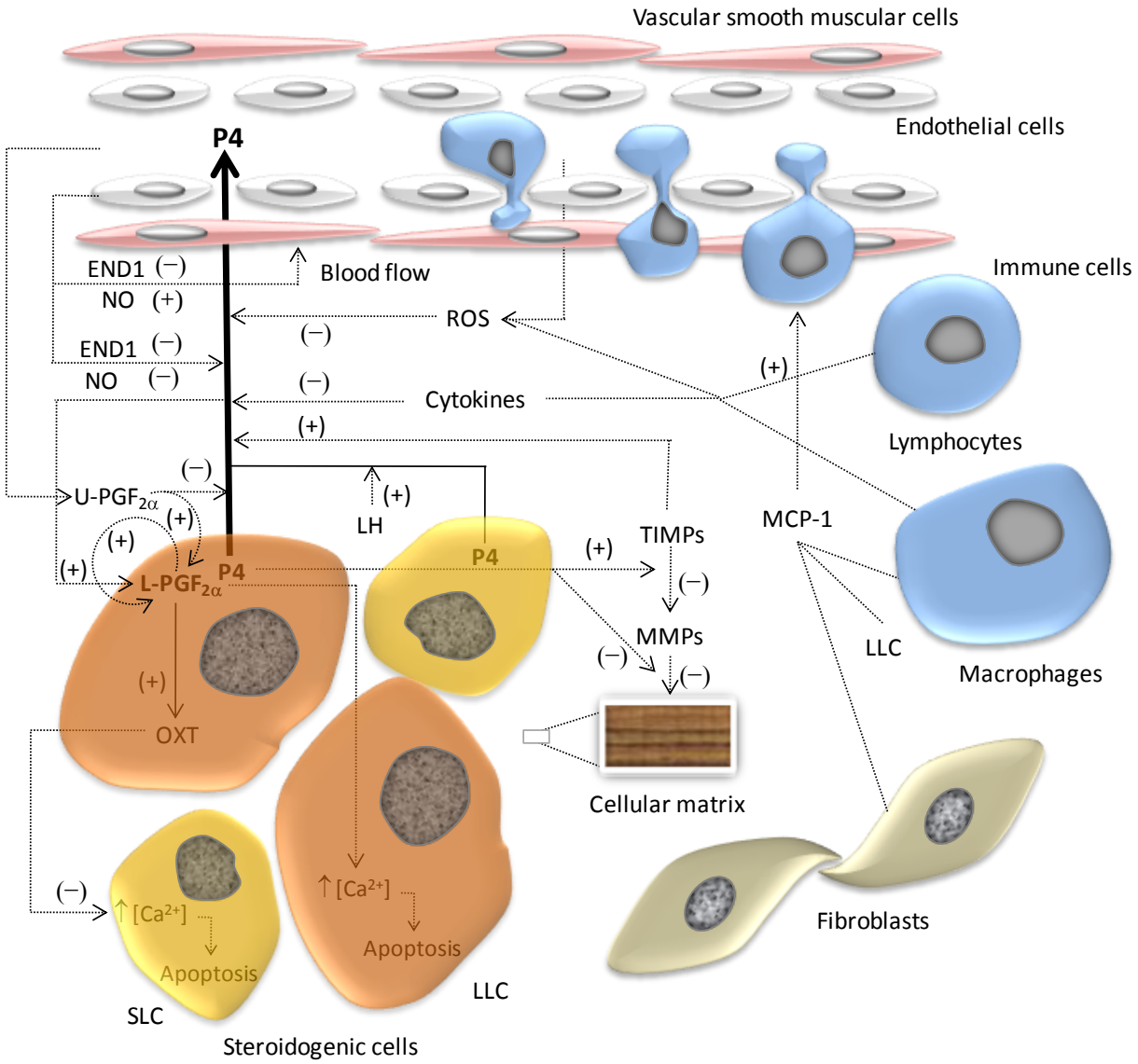

Figure 2. Cellular interactions during luteal regression.

Endothelin system: a modulator of ovarian function

Ovarian function is regulated mainly by the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), through their receptors. However, numerous factors such as steroid hormones, peptides and growth factors, produced locally or not, play essential modulatory roles in follicular and luteal function (Tisdall et al., 1995; Schams and Berisha, 2002; Webb et al., 2002; Lopez et al., 2005).

Endothelins (END), a family of 21-amino acid peptides constituted by three different isoforms (END1, END2 and END3), are involved in a wide variety of biological functions in diverse tissues (Kedezierski and Yanagisawa, 2001). The most relevant END family member associated with female reproductive function, END1, has been identified in the ovary of several mammalian species (Usuki et al., 1991; Iwai et al., 1991; Flores et al., 1992; Kamada et al., 1993; Tedeschi et al., 1994; Mancina et al., 1997; Flores et al., 2000; Hinckley and Milvae, 2001; Schams et al., 2003; Boiti et al., 2005).

In the corpus luteum, END1 plays an important role in mediating PGF2α-induced luteal regression. END1 reduced both basal and LH-stimulated progesterone secretion in a dose-dependent manner, and its inhibitory effect was prevented by the addition of a selective ENDRA receptor antagonist (Girsh et al., 1996a; Hinckley and Milvae, 2001; Doerr et al., 2008). In antral follicles, END1 acts as a modulator of the steroidogenic function of granulosal cells. Both LH- and FSH-stimulated progesterone and cAMP production in granulosal cells were suppressed by END1 in a dose-dependent manner (Iwai et al., 1991; Flores et al., 1992; Kamada et al., 1993; Flores et al., 1999). Thus it appears that END1, acting through autocrine

and paracrine mechanisms, inhibits the luteinization of granulosal cells until near time to ovulate, when its activity is abolished and all events conducive to ovulation take place.

Brief description of endothelin system

The endothelin system consists of a group of peptides (ligands) and proteins (enzymes and receptors) involved in a wide variety of physiologic and pathologic roles in numerous mammalian tissues (Nussdorfer et al., 1999; Kedezierski and Yanagisawa, 2001). These molecules have been described as two G-protein-coupled receptors, three peptide ligands, and two activating peptidases (Pinet et al., 2004).

Endothelin-1 (END1), a peptide constituted of 21 amino acids, originally was isolated from porcine aortic endothelial cells as a potent vasoconstrictor (Yanagisawa et al., 1988). Endothelin-2 (END2) and endothelin-3 (END3) were discovered later and constitute two structurally-related peptides that differ from END1 by two and six amino acids, respectively (Fig. 3; Inoue et al., 1989). Endothelins are synthesized as large molecules (~200 amino acids) named preproendothelins (ppEND), which are cleaved into three physiologically active endothelin peptides (Fig. 4) through two consecutive enzymatic steps (Rubanyi and Polokoff, 1994). The first step involves a furin-like protease that converts pp-ENDs into an inactive END precursor (p-ENDs; ~37 to 41 amino acids). The second step is performed by a specific endopeptidase (zinc metalloendoeptidase) known as endothelin-converting enzyme (ECE), which consists of two isoforms: ECE1 and ECE2 (Xu et al., 1994; Emoto and Yanagisawa, 1995).

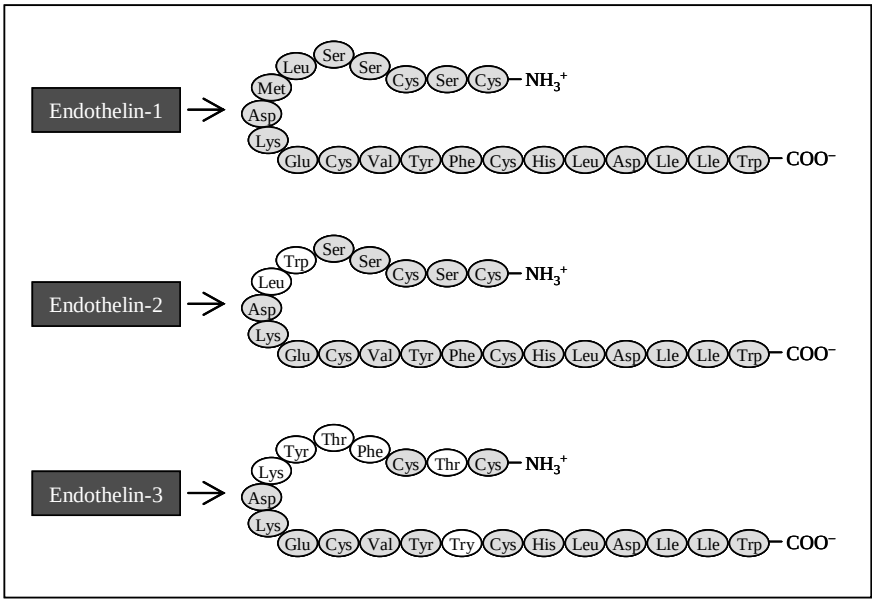

Figure 3. Molecular structure of endothelin peptides. The unshaded circles illustrate the variable amino acid sequence of human endothelins showing the amino acid substitution of END2 and END3 with respect to END1 (Modified from Nussdorfer et al., 1999). Each END peptide is encoded by a different gene. The three genes have different chromosomal localization and are differentially regulated. END isoforms are molecularly similar to cardiotoxic peptides isolated from the venom of *Atractacpis engaddensis*.

Two membrane receptors (ENDRA and ENDRB) have been identified as responsible for the intracellular signals that cause the different biological effects of ENDs described in the scientific literature (reviewed by Nussdorfer et al., 1999 and Kedezierski and Yanagisawa, 2001). ENDRA has a very high affinity for END1 and END2 and around 70 to 100% lower affinity for END3, while ENDRB binds with high and similar affinity to all three END peptides. Each END receptor is encoded by a different gene.

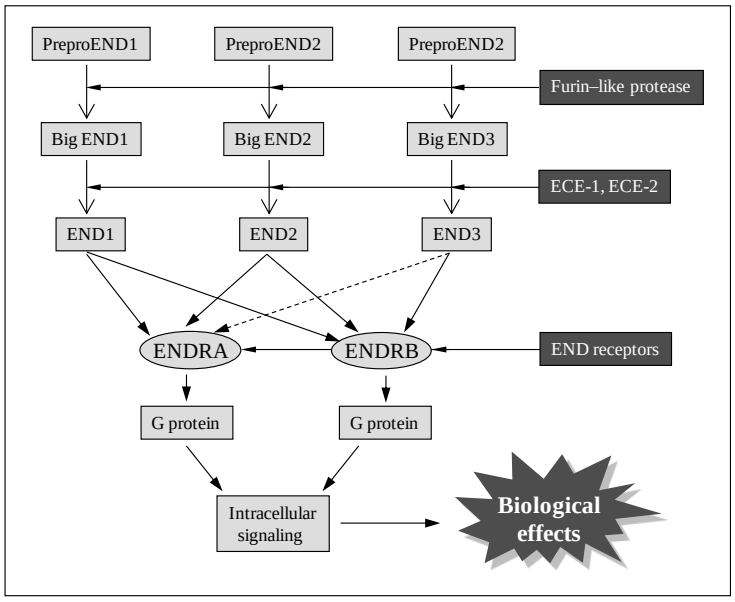

Figure 4. Molecular components of endothelin pathway (Modified from Pinet et al., 2004). Although END1 was described originally as a potent vasoconstrictor, currently innumerable biological effects of this peptide family are known in a wide variety of tissue, taking part not only in physiological but also in pathological functions in human beings and animals. ENDRA receptor has around 100 fold more affinity for END1 and END2 than END3, while ENDRB receptor interacts on a non-selective manner with three END peptides.

Molecular characteristics of endothelin receptor type A

Endothelin receptors have been isolated and cloned from mammalian tissues and the structures of the mature receptors have been deduced from the nucleotide sequences of the cDNA (Arai et al., 1990; Sakurai et al., 1990). ENDRA structure is consistent with a seven-transmembrane domain (7TM), G protein-coupled receptor belonging to the rhodopsin-type receptor family. Binding of END to ENDRA and ENDRB activate at least three classes of $G\alpha$ proteins ($G\alpha_s$, $G\alpha_1$ and $G\alpha_q$) (Miyauchi and Masaki, 1999). ENDRA has an extracellular NH_2-terminal domain, a seven transmembrane-spanning domain, and intracellular COOH-terminal

domain. The transmembrane domains consist of seven amphipathic membrane-spanning helices joined together by three intracellular and three extracellular loops. The ENDRA receptor has an N-terminal signal sequence with a relatively long extracellular N-terminal portion preceding the first transmembrane domain (Figure 5). There are two separate ligand-interaction sub-domains, and the extracellular loops, particularly between transmembrane domains 4 to 6, determine selectivity (Devenport and Maguire, 2006).

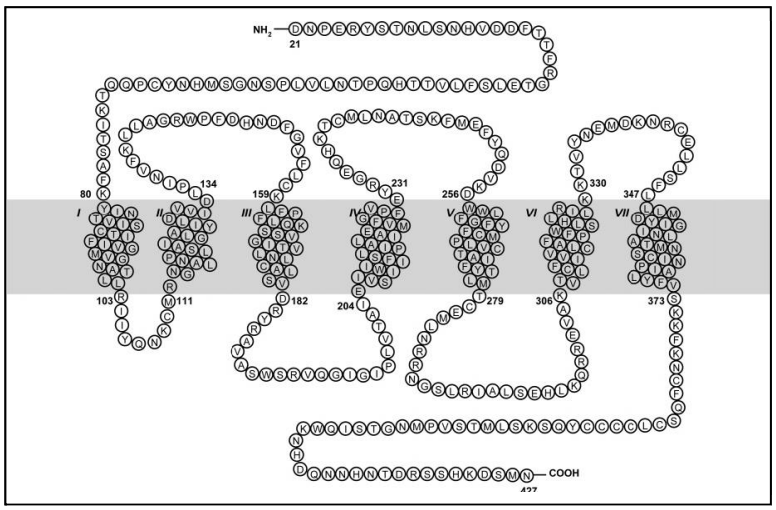

Figure 5. Structure of endothelin receptor type A

Activation of ENDRA receptors produces a range of biological effects in different tissues. END1 is known as one of the most potent vasoconstrictors. In the ovary of Chinese hamster, for example, END1 through ENDRA receptor induces the release of arachidonic acid (AA) from the cell membrane (Trevesi et al., 2002). END1-induced vascular contraction is accompanied by an increase in calcium (Ca^{2+}) through its mobilization from intracellular stores and as result of transmembrane Ca^{2+} influx. Also, extracellular Ca^{2+} influx plays a critical role

in END1-induced arachidonic acid (AA) release from membrane lipids by activating ENDRA receptor. In the ovary of hamsters, AA was released by activating two types of Ca^{2+} permeable nonselective cation channels and a store-operated Ca^{2+} channel, which were activated by G_q and G_{12} proteins coupled to ENDRA receptor (Kawanabe et al., 2003).

Binding of END1 to ENDRA changes its conformation and alters the conformation of the G protein bound to the receptor. The alteration of α subunit of the G protein allows it to exchange its GDP for GTP and causes the G protein to break up into two active components, α subunit and $\beta\gamma$ complex. Immediately α subunit or $\beta\gamma$ complex activates phospholipase C (PLC) and the IP_3 and DAG pathway (Trevisi et al., 2002) described above for $PGF_{2\alpha}$. Alternatively, in human vascular endothelium, PKC and non- receptor protein tyrosine kinase (PTK) activated by END1 through its receptor ENDRA, regulate the expression of endothelial nitric oxide synthase (eNOS) at the genomic level and stimulate NO production (Marsen et al., 1999).

In addition, in various cell types, ENDRA receptors activate PLC to produce IP3 as well as DAG. The IP3 stimulates release of Ca^{2+} from IP3-sensitive stores (endoplasmic reticulum) and subsequent activation of store-operated Ca^{2+} channels. After or concomitant with the release of Ca^{2+} from intracellular stores, a second phase is initiated in which Ca^{2+} moves into the cell from the extracellular compartment. The resulting Ca^{2+} influx is responsible for an additional component of the END1-induced contraction of rat main pulmonary artery (Hyvelin et al., 1998).

Role of END1 in follicular function

END family members have been identified in ovarian antral follicles from pig (Iwai et al., 1991; Kamada et al., 1993; Flores et al., 1995; Flores, 2000), rat (Iwai et al., 1993; Tedeschi et al., 1994), cow (Acosta et al., 1998; Levy et al., 2003) and human being (Magini et al., 1996; Mancina et al., 1997). Their general role appears to be to regulate steroid production by granulosal and thecal cells of antral follicles (Iwai et al., 1991; Tedeschi et al., 1992; Flores et al., 1992; Kamada et al., 1993) by acting as a local modulator of ovarian function.

Measurable amounts of END1 (Iwai et al., 1991) were lower in porcine follicular fluid of small (1 to 2 mm) than medium (3 to 5 mm) or large (6 to 11 mm) follicles (9.4 ± 1.1 vs 12.2 ± 1.1 b and 14.2 ± 1.8 pg/ml, respectively; $P<0.05$); and greater in porcine granulosal cells cultured with LH than FSH (56 ± 9.3 and 4.9 ± 1.8 pg/106 cells × h, respectively) (Iwai et al., 1991). Also, cultured porcine granulosal cells collected from large antral follicles (9 to 10 mm) had three fold greater ($P<0.05$) binding capacity for END1 than granulosal cells from small follicles (2 to 5 mm; Flores et al., 1995). Studies in porcine ovaries have demonstrated that greater binding capacity for END1 (Flores et al., 1995) and higher immunoreactivity of END1 (Flores, 2000) were restricted to vascular and granulosal cell compartments of antral follicles. END1 gene expression in ovarian follicles started around the antral stage; it increased as follicles developed toward the periovulatory stage, and it decreased immediately after ovulation (Flores, 2000). Likewise, large antral follicles expressed higher immunoreactivity to END1 (66.7%) and underwent less atresia (20%) than small antral follicles (16.8 and 40%, respectively).

Multiple evidence has indicated that END can modulate steroidogenic function of GC in several mammalian species (Iwai et al., 1991; Kamada et al., 1992; Flores et al., 1992; Tedeschi et al., 1992; Kamada et al., 1993; Tedeschi et al., 1994; Magini et al., 1996; Calogero et al., 1998; Plonowski et al., 1999; Denkova et al., 2000; Denkova et al., 2002; Yaneva et al., 2003). Significantly less LH- (Iwai et al., 1991), hCG- (Kamada et al., 1993) and FSH-stimulated (Flores et al., 1992; Kamada et al., 1993) accumulation of progesterone was quantified in GC cultured in presence of END1. Both FSH- and LH-stimulated progesterone accumulation decreased in a dose-dependent manner (Iwai et al., 1991; Kamada et al., 1993). Similarly, LH- and FSH-stimulated cAMP production in granulosal cells was suppressed by END1 in a dose-dependent manner (Iwai et al., 1991; Flores et al., 1992; Flores et al., 1999). The cellular mechanism by which END1 reduced the FSH-stimulated progesterone production in the rat, could be by inhibiting some progesterone-forming enzymes (Flores et al., 1999) and/or by stimulating progesterone-degrading enzymes (Tedeschi et al., 1992). This mechanism would imply the activation of phosphoinositide-calcium-PKC pathway and the obstruction of FSH-stimulated steroidogenesis by inhibiting cAMP production (Flores et al., 1999).

Three END isoforms had similar inhibitory effects on progesterone production by porcine (Iwai et al., 1991; Kamada et al., 1993) and murine (Tedeschi et al., 1994) granulosal cells. Therefore, it has been suggested that the nonselective receptor, ENDRB, could be involved in that action (Iwai et al., 1991; Tedeschi et al., 1994). Iwai et al. (1993) documented the presence of abundant ENDRB mRNA on granulosal cells of developing follicles in rat. However, competitive binding assays and receptor agonist competition experiments, performed in cultured granulosal cells, indicated that type A is the most important END receptor in the

porcine ovary (Flores et al., 1995). The latter authors suggested that such divergences could be attributed to different methodologies and species used in these studies.

Role of END1 in luteolysis

Prostaglandin $F_{2\alpha}$ has been documented as the main luteolytic factor in many mammalian species (Niswender et al., 2000). However, it is currently known that numerous other factors participate in luteolysis (McCracken et al., 1999), some of which act in a paracrine or/and autocrine manner (Girsh et al., 1996a; Shirasuna et al., 2006). $PGF_{2\alpha}$ suppressed the P_4 secretion from large luteal cells only in the presence of endothelial cells, so Girsh et al. (1995) suggested that one or more factors produced in the microvascular tissue of the CL could be involved in that process. At that time, END1 was proposed as a possible mediator in $PGF_{2\alpha}$-induced luteal regression, and subsequent evidence corroborated that hypothesis (Girsh et al., 1996a; Miyamoto et al., 1997; Doerr et al., 2008).

Studies in ruminants confirmed that END1 reduces both basal and LH-stimulated P4 secretion in a dose-dependent manner (Girsh et al., 1996a; Hinckley and Milvae, 2001), and that the inhibitory effect could be prevented by the addition of a selective ENDRA receptor antagonist (Girsh et al., 1996a; Doerr et al., 2008). Apparently, END1 is synthesized and secreted only by vascular endothelial cells, because END1 could not be detected in either luteal-like cells or luteal cell extract (Girsh et al., 1996a). On the other hand, a significantly greater content of ENDRA and expression of END1 mRNA were detected in CL collected on day 18 of the bovine estrous cycle compared with those collected on day 5 and 10 of the cycle (Girsh et al., 1996b). In concordance with the latter finding, greater amounts of ECE-1 mRNA

expression were observed at middle (days 7 to 12) and late (days 13 to 16) stages of luteal phase than at earlier stages (Levy et al., 2001, 2003). The greater END content and mRNA expression coincided with high amounts of $PGF_{2\alpha}$ present during luteolysis and concur with the fact that END1 secretion increases in response to $PGF_{2\alpha}$ in vitro (Girsh et al., 1996a; Miyamoto et al., 1997) and in vivo in microdialysed CL (Ohtani et al., 1998). However, END1 is not regulated by $PGF_{2\alpha}$ early in the cycle and it is present in the natural CL (Wright et al., 2001; Choudhary et al., 2004).

Peripheral plasma concentrations of END1 during the bovine estrous cycle remained low from days 2 to 12, increased on days 13 to 19 and reached the greatest values on days 20 to 22 (Ohtani et al., 1998). Low expression of END1 peptide and mRNAs for END1, ECE1 and ENDRA (Ohtani et al., 1998; Mamluk et al., 2001; Levy et al., 2001; Wright et al., 2001; Levy et al., 2003; Choudhary et al., 2004) at early stages of the luteal phase are coincident with the time when the CL is refractory to luteolytic doses of $PGF_{2\alpha}$ (Braun et al., 1988; Levy et al., 2000). However, CL has enough PGF2α receptors from day 2 of estrous cycle for initiating luteolysis (Wiltbank et al., 1995). So, the lack of response of CL to the luteolytic action of $PGF_{2\alpha}$ may be because incomplete neovascularization of CL in that developmental stage limits END1 synthesis (Levy et al., 2000). However, subsequent evidence indicated that endothelin system in the bovine CL is regulated by $PGF_{2\alpha}$ in a dependent or independent manner according to the phase of the estrous cycle (Choudhary et al., 2004). Before day 10 of estrous cycle, the amounts of mRNA encoding END1, ECE1, ENDRA and ENDRB increased independently of $PGF_{2\alpha}$; whereas $PGF_{2\alpha}$ differentially affected expression of ppEND1 and ENDRA after day 10 of estrous cycle (Choudhary et al., 2004). So, these findings do not

support the involvement of END1 in the differential sensitivity of CL to $PGF_{2\alpha}$ in early versus later stages of luteal phase.

In recent years, data from various in vivo studies have confirmed and strengthened the concept that END1 is an important mediator of $PGF_{2\alpha}$-induced luteal regression. Infusions of BQ-610 (500μg), a highly specific ENDRA antagonist, every 12 h on d 16 through 18 of the estrous cycle in heifers delayed luteolysis for about 2 days (Keator et al., 2008). Chronic administration of BQ-610 into the CL through a minipump implanted in the ovary during the mid-luteal phase in sheep, decreased serum P_4 concentration throughout first 12 h after a single injection of $PGF_{2\alpha}$. However, P_4 progressively increased during the next 36 h, attaining a concentration similar to the control group 48 h after $PGF_{2\alpha}$ (Doerr et al., 2008). Apparently, a direct action of PGF2α decreased serum P_4 during early luteolysis, whereas END1 was required in later stages as indicated by the rise of P_4 in BQ-610-treated ewes beginning 12 h after $PGF_{2\alpha}$; this would imply that END1 may participate in structural rather than functional luteolysis. In contrast, administration of an ENDRB antagonist (BQ-788) in the same experiment did not reverse the reduction of P_4 after $PGF_{2\alpha}$ (Doerr et al., 2008). This finding agreed with the fact that ENDRB mRNA expression was almost absent in mid-phase CL (Doerr et al., 2008), indicating that END1 participates in the luteolytic cascade by activating ENDRA.

Intraluteal injections of ENDRA antagonists (BQ-610, BQ-123 and LU-13522) were less effective to address the role of END1 $PGF_{2\alpha}$-induced luteolysis. A single injection of BQ-123 (100 μg) on days 8 to 9 of the estrous cycle in sheep diminished, but did not completely block peripheral P_4 concentration during 24 h in response to a luteolytic dose of $PGF_{2\alpha}$ (Hinckley and

Milvae, 2001). The authors suggested that activation of ENDRB may have contributed to the incomplete inhibition of END system or BQ-123 was less effective in blocking ENDRA than other antagonists. Also, single or multiple intraluteal injections of ENDRA antagonists during bovine mid-luteal phase were ineffective to prevent P_4 decline as was reported in sheep. In both heifers (Keator et al., 2008) and cows (Watanabe et al., 2006) P4 concentration after a luteolytic dose of $PGF_{2\alpha}$ decreased parallel to control females.

To test the role of END1 in $PGF_{2\alpha}$-induced luteolysis, sheep were injected (im) with saline, 7.5 mg of PGF2α, 100 μg of END1, or 7.5 mg of $PGF_{2\alpha}$ plus 100 μg of END1 during mid-luteal phase (Hinckley and Milvae, 2001). END1 transiently increased plasma P_4 concentrations that thereafter decreased progressively attaining minimal values simultaneously with saline-treated group. Dosage of $PGF_{2\alpha}$ first decreased concentrations of P_4 for about 6 h and then P_4 increased to similar values as in saline-treated ewes. Interestingly, combination of $PGF_{2\alpha}$ and END1 reduced plasma P_4 concentration from 0.25 h to 48 h post-treatment, time at which P4 reached luteolytic concentrations (less than 0.5 ng/mL). The combination also shortened the estrous cycle (10.3 d) compared with the rest of the groups (about 16 d each). These findings support the role of END1 as a mediator of the intricate luteolytic cascade initiated by uterine PGF2α and modulated by luteal PGF2α and other local mediators. Apparently, presence of PGF2α is required so that END1 exerts its luteolytic effect as demonstrated by Miyamoto et al. (1997) in an in vitro study.

Role of END1 in steroid production by ovine granulosal cells collected from large preovulatory follicles

Introduction

In sheep, follicular growth occurs in a wave-like pattern with typically 3 to 4 follicular waves during every estrous cycle (Ginther et al., 1995; Bartlewski et al., 1999; Evans et al., 2003). Follicular dominance is not as marked in sheep as is in the cow (Lopez-Sebastian et al., 1997; Gonzalez-Bulnes et al., 2001; 2004). Neither the presence of a large follicle induced by exogenous FSH nor supraphysiological concentrations of estradiol released from implants prevented emergence of a new wave of follicles (Duggavathi et al., 2004; 2005; Barrett et al., 2006). Likewise, more than 2 antral follicles from ultimate and penultimate waves of the cycle may reach ovulatory status and eventually ovulate (Souza et al., 1997; Bartlewski et al., 1999).

Preovulatory follicles produce greater amounts of estradiol (Duggavathi et al., 2003); and elevated concentrations of circulating estradiol trigger the LH/FSH surge and the consequent ovulation process (Karsch et al., 1983). After the LH surge, the predominant pattern of steroid production by the preovulatory follicles changes rapidly from estrogen to progesterone as the main steroid secreted by granulosal and thecal cells (Murdoch and Dunn, 1982; Fortune and Hansel, 1985). The downregulation of P450-17α-hydroxylase and P450arom in thecal and granulosal cells, respectively, and upregulation of progesterone receptor in the two types of steroidogenic cells appear to support this change (Voss and Fortune, 1993a; Komar et al., 2001; Jo et al., 2002). Likewise, steroid production by granulosal and thecal cells of large follicles is regulated by numerous factors through autocrine and paracrine actions: insulin (Spicer et al.,

2002), BMPs (Gliser et al., 2004; Campbell et al., 2006), BMP15 (Juengel et al., 2002), GDF9 (Spicer et al., 2006; 2008), inhibin (Jimenez-Krassel et al., 2003), estradiol (Spicer et al., 2005), IGFs (Spicer et al., 2002; 2004), and END1 (Flores et al., 1992; Kamada et al., 1993; Acosta et al., 1998), modulate steroid production through autocrine and paracrine mechanisms, and might prevent premature differentiation of granulosal and thecal cells of preovulatory follicles prior to LH/FSH surge.

Endothelin 1, a 21-amino acid peptide, was described originally as a potent vasoconstrictor (Yanagisawa et al., 1988) and later associated with modulation of steroidogenesis in follicular and luteal tissue from rats (Tedeschi et al., 1992; 1994; Colagero et al., 1998), pigs (Iwai et al., 1991; Flores et al., 1992; Kamada et al., 1992; 1993), cows (Girsh et al., 1996a; Miyamoto et al., 1997), ewes (Hinckley and Milvae, 2001; Doerr et al., 2008) and human beings (Denkova et al., 2000; Yaneva et al., 2003). Endothelin 1 is synthesized and secreted from ovarian granulosal cells, and greater amounts of END1 were detected in larger than in smaller antral follicles (Iwai et al., 1991; Flores et al., 2000). The antisteroidogenic effect on granulosal cells appeared to be mediated via ENDRA in pigs (Flores et al., 1995) and ENDRB in rats (Iwai et al., 1993). The END1 mRNA expression in pigs was detected first around the antral stage, increased as the follicles grew, and decreased rapidly after ovulation (Flores, 2000). In the rat ovary, inhibition of P_4 production in granulosal cells by END1 was associated with downregulation of P450scc and 3β-HSD and upregulation of 20α-HSD, 5α-reductase and 3α-HSD (Tedeschi et al., 1992), enzymes implicated in synthesis and metabolism of P_4, respectively. Moreover, in bovine microdialyzed mature follicles, END1 inhibited P_4 secretion and stimulated E_2 secretion (Acosta et al., 1998; 1999). Although there is no evidence of a role

of END1 in regulating steroidogenesis in sheep follicles, on the basis of previous experimental evidence, it is possible to speculate that END1 acting through autocrine and paracrine mechanisms, might prevent premature luteinization of granulosal cells prior to the LH surge in sheep as suggested in other species (Tedeschi et al., 1992; Flores, 2000).

The objective of this study was to examine the effect of blocking ENDRA and ENDRB on follicular steroid production by granulosal cells from preovulatory follicles in sheep. The hypothesis was that END1 acting through ENDRA and/or ENDRB would inhibit steroid production, and therefore, prevent the premature luteinization of granulosal cells of ovine preovulatory follicles.

Materials and methods

General experimental procedure

Seventeen nonpregnant cycling Suffolk ewes with at least one previous estrous cycle of normal length (15 to 19 days) were used in the experiment, conducted in fall of 2006 (September-December) with ewes from the West Virginia University research flock. For observation of estrus (12 h intervals), ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket; standing estrus was confirmed by teasing with another vasectomized ram. Fourteen days after estrus (estrus = day 0) ewes were injected (i.m.) with two doses (5 mg each) of $PGF_{2\alpha}$ (Lutalyse®, Pfizer Animal Health, New York, NY) 3 h apart (Hawk, 1973) to induce luteolysis. To verify that a surge of LH had not occurred before collection of follicles, jugular blood samples (8 ml) were collected at h -3, 0, 6, 12, 18, 24, 30

and 36 in relation to the second dose of $PGF_{2\alpha}$ (Fig. 6). Blood samples were refrigerated for 24 h to allow them to clot, and then centrifuged for 20 minutes at 2500 rpm. Serum was collected and kept at -20° C until radioimmunoassayed for LH.

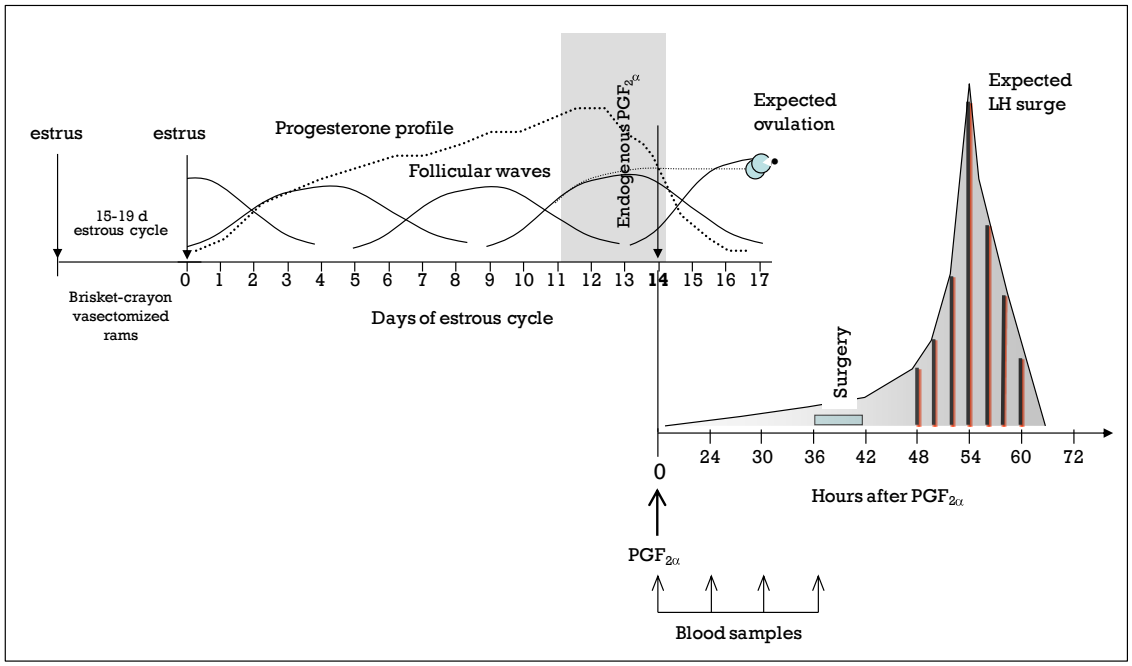

Figure 6. Protocol for collection of preovulatory follicles in sheep

Thirty six to 40 hours after the second dose of $PGF_{2\alpha}$, ewes were anesthetized with 0.3 mg/kg diazepam (Valium, 5 mg/ml; Roche Pharmaceutivals, Nutley, NJ) and 7 mg/kg ketamine (Vetamine, 100 mg/ml; Mallinckrodt Veterinary, Mundelein, IL), and then placed on a mixture of halothane (Halocarbon Laboratries, Riveredge, NJ), oxygen (2.0 L/min) and nitrous oxide gas (1.0 L/min) until bilateral ovariectomy was carried out through a mid-ventral incision. Immediately after ovariectomy, ovaries of each ewe were placed in cold phosphate buffered saline (PBS) and stored on ice until completion of the surgery. Follicles were dissected and

granulosal cells were harvested. All animal procedures were approved by WVU Institutional Animal Care and Use Committee (ACUC # 05-1205).

Culture of granulosal cells and treatments

Ovarian stroma was removed from 28 presumed preovulatory follicles (1.7 ± 0.8 follicles/ewe) and the diameter of each dissected follicle was recorded (9.2 ± 1.5 mm). Using a 22-gauge needle attached to 3 mL syringe, follicular fluid (FF) of each follicle was aspirated and centrifuged at 400 × g at 4°C for 5 min and the supernatant stored at -20°C for subsequent steroid determination by RIA; the remaining granulosal cells were added to those granulosal cells obtained directly from the follicle (see below).

Each follicle was bisected under sterile conditions and granulosal cells were scraped gently from the basement membrane with a spatula into F12 Nutrient Mixture (Ham's F12) media supplemented with sodium bicarbonate (0.01 M), antibiotics (1.5 mg of penicillin/mL and 2.5 mg of streptomycin/mL) and 19-OH androstenedione (1 μM) (Jimenez-Krassel and Ireland, 2002). Granulosal cells were rinsed in cold Ham's F12 supplemented medium, centrifuged at 400 × g for 5 min at 4°C and resuspended in 200 μL of cold Ham's F12 supplemented medium. This last procedure was repeated twice. The number of cells and cell viability were estimated using a hemocytometer and trypan blue exclusion dye, respectively. According to the number of granulosal cells harvested from each follicle, additional volume of Ham's F12 supplemented medium was added to cell suspension. Approximately 5.9 ± 2.6 × 10^5 cells were cultured in each well. Nevertheless, because differentiating viable and non-viable cells by the trypan blue method was sometime difficult, steroid concentrations per well were normalized against total

protein content per well and expressed as pg of steroid per μg of protein per 24 h. Total protein at end point of the incubation period was quantified by colorimetric method using the Coomassie Protein Assay (Pierce Biotechnology, Inc., Rockford, IL).

Aliquots of 15 μL of granulosal cell suspension obtained from each presumed preovulatory follicle were cultured in 96-well plates containing a solution, previously equilibrated at 37°C, of 185 μL of Ham's F12 supplemented medium and treatments (described in Table 1). Cultures were incubated at 37 °C in a humidified atmosphere (5% CO2 and 95% air) for 24 h. Once finished the incubation period, the medium was removed carefully from each well, centrifuged (3000 × g for 1 min at room temperature) and stored at -20°C for subsequent steroid assays by RIA. Cultured granulosal cells and the remaining volume of granulosal cells in suspension were stored at -20°C.

Radioimmunoassays for LH, progesterone, and estradiol

Concentrations of P_4 and E_2 in FF and culture media, and LH concentrations in serum were measured in duplicate by RIA as previously described (Sheffel et al., 1982; Tortonese et al., 1990; Goodman et al., 2004). LH assay sensitivity averaged 0.54 ng/ml, and the intraassay coefficient of variation was 15%. Intraassays coefficient of variation and sensitivities for P_4 and E_2 in unextracted FF were 8.6%, and 34 pg/tube for P4 and 10.6%, and 32 pg/tube for E_2, respectively. For P_4 and E_2 in culture media, the inter- and intra-assay coefficient of variation and sensitivities were P_4, 12.3%, 14.4%, and 0.20 ng/ml; and E_2, 13.0%, 14.9%, and 0.25 pg/ml.

Table 1. Treatments used to evaluate the role of END1 in steroid production by granulosal cells from preovulatory follicles in sheep.

	Treatments	Description	Doses
1	Control	Ham's F12 (Sigma-Aldrich, Milwaukee, WI) supplemented media	–
2	LH	Luteinizing hormone (LH, NIADDK-oLH-25 AFP 5551B;)	150 ng/mL
3	END1	Endothelin-1 peptide (END1; Bachem Bioscience Inc, King of Prussia, PA)	100 nM
4	LH+ END1		–
5	ENDRAa	selective END receptor type A antagonist (ENDRA): BQ-610 (Azepane-1-carbonyl-leu-D-Trp(For)-D-Trp-OH; Bachem Bioscience Inc, King of Prussia, PA)	1500 nM
6	ENDRAa+LH+ END1		–
7	ENDRBa	selective END receptor type B antagonist (ENDRB): BQ-788 (N-cis-2,6-Dimethylpiperidinocarbonyl)-D-Nle-OH; Bachem Bioscience Inc, King of Prussia, PA)	1000 nM
8	ENDRBa +LH+END1		–
9	ENDRAa+ ENDRBa	END receptor types A and B antagonist (ENDRA+B)	–
10	ENDRAa+ENDRBa+ LH+END1		–

Statistical Analysis

Concentrations of P_4 and E_2 in culture media were evaluated by one-way analysis of variance using the General Linear Model (GLM) procedure of SAS (SAS Institute; Cary, NC). Differences among means were compared by the LSM procedure of SAS. Data were expressed as mean ± SEM. Antral follicles with greater concentrations of P_4 and androgens than E_2 were considered atretic (Morr et al., 1978; Ireland and Roche, 1982), and estrogen-inactive (P_4>E_2) (Ireland and Roche, 1983) follicles were removed from the analysis. Estrogen-active follicles (E_2>P_4) were categorized according to E_2 concentration in FF into three groups (1: ≤ 250

ng/ml; 2: > 250 ≤ 350 ng/ml, and 3: > 350 ng/ml) and included as a discrete variable in the statistical model. Two ewes had concentrations of LH greater than 180 ng/ml in samples collected at 24 or 30 h after the second dose of $PGF_{2\alpha}$, and their follicles were excluded from the analysis. The final number of follicles examined was 20, which were collected from 13 ewes. For 9 of these follicles, cells were cultured in duplicate and the average value was utilized.

Results

Accumulation of E_2 and P_4 was not affected by either LH or END1 or the END receptor blockers (Fig 7). Correlations between concentration of E_2 in FF and E_2 and P4 accumulation in the culture media were 0.30 ($P < 0.0001$) and 0.26 ($P < 0.0004$), respectively. Granulosal cells from follicles with the greatest concentration of E_2 in FF produced on the average significantly more E_2 and P_4 than follicles with lower concentrations of E_2 in FF (Table 2). Treatments did not affect accumulation of E_2 and P4 in any of the categories of follicles considered (Fig 8). However, for all treatments, granulosal cells from preovulatory follicles with the greatest concentration of E_2 in FF produced greater amounts of E_2 and P_4 than preovulatory follicles with lower concentration of E_2 in FF (Fig 8).

Table 2. Steroid accumulation in media from cultured granulosal cells according to estradiol concentration in follicular fluid from preovulatory follicles

Steroid	Concentration of E_2 in follicular fluid (ng/ml)			Significance level
	≤ 250	> 250 ≤ 350	> 350	
Estradiol	0.75 ± 0.06 [a]	0.80 ± 0.08 [a]	1.97 ± 0.07 [b]	[a,b] P<0.0001
Progesterone	145.0 ± 3.9 [a]	170.8 ± 5.9 [b]	203.4 ± 7.6 [c]	[a,b,c] P<0.0001

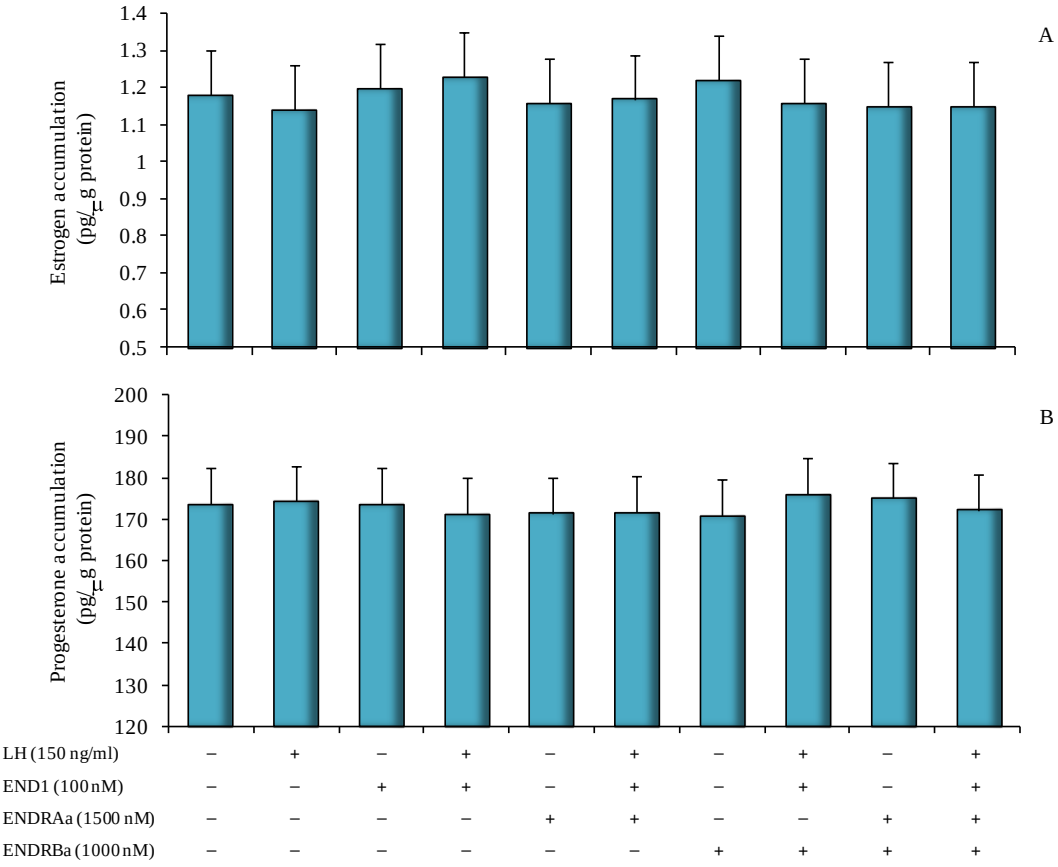

Figure 7. Effects of LH, END1, ENDRAa (BQ-610) and ENDRBa (BQ-788) on production of estradiol and progesterone by ovine granulosal cells isolated from preovulatory follicles. Bars represent mean ± SEM of estradiol (panel A) or progesterone (panel B) accumulation expressed in pg/μg of protein in 24 h.

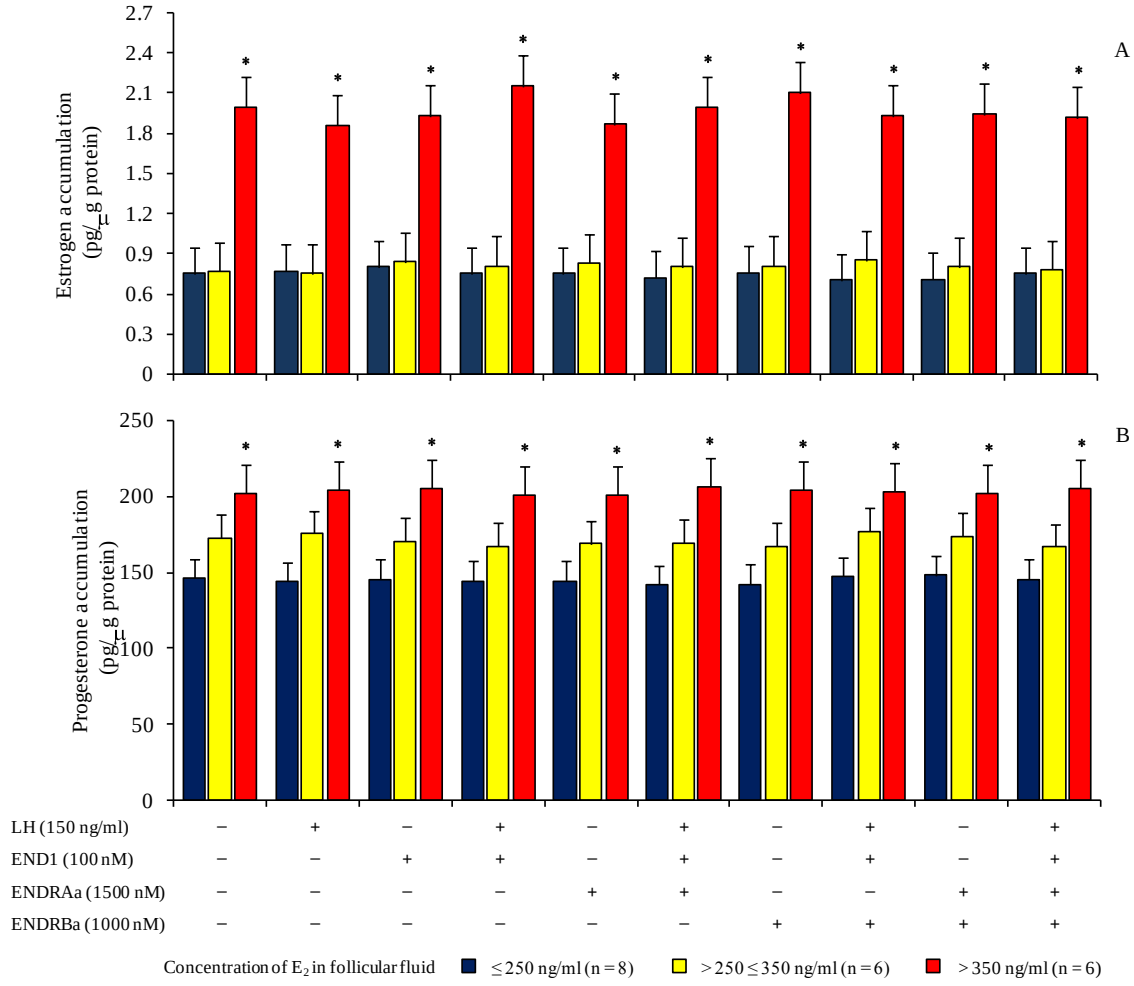

Figure 8. Effects of LH, END1, ENDRAa (BQ-610) and ENDRBa (BQ-788) on production of estradiol and progesterone by ovine granulosal cells isolated from preovulatory follicles. According to concentration of estradiol in follicular fluid, preovulatory follicles were categorized into three groups (1: ≤ 250 ng/ml; 2: > 250 ≤ 350 ng/ml, and 3: ≥ 350 ng/ml). Bars represent mean ± SEM of estradiol (panel A) and progesterone (panel B) accumulation expressed in pg/μg of protein in 24 h. Values were statistically different between groups 1 and 2 versus group 3 ($^{*}P < 0.001$) for estradiol (panel A) and between groups 1 and 3 ($^{*}P < 0.02$) for progesterone.

Discussion

Granulosal cells from preovulatory follicles with greater concentrations of estradiol produced more E_2 and P_4, most likely due to the greater steroidogenic capacity of these follicles (Tian et al., 1995) compared to follicles with lower estrogenic production. Dominant follicles expressed greater amounts of mRNA for LH receptor, P450scc, P450c17 and 3βHSD in thecal cells and P450arom in granulosal cells than subordinate follicles (Xu et al., 1995b; Bao et al., 1997a; Bao et al., 1997b). Moreover, follicular dominance was associated with onset of expression of LH receptor and 3βHSD in granulosal cells (Bao et al., 1997a; Bao et al., 1997b; Beg et al., 2001). Hence, in the preovulatory follicles collected in this study, greater steroid production may be supported by a greater expression of LH receptor and steroidogenic enzymes in the two populations of endocrine cells, theca and granulosa. It is clear that some preovulatory follicles collected from either the same or different animals may have had functional advantages over others with lower rank. Thus, granulosal cells from those follicles reflected more steroidogenic capacity with greater accumulation of E_2 and P_4 after 24 h of culture. In addition, given that the substrate for synthesis of estradiol was provided in culture media, aromatization of androstenedione to estradiol by granulosal cells was carried out rapidly.

Unexpectedly, LH did not induce an increase of either E2 or P4. Evidence indicated that LH and FSH stimulated steroid production by granulosal cells in vitro and END1 prevented that increment in other species (Iwai et al., 1991; Tedeschi et al., 1992; Kamada et al., 1993; Flores et al., 1999). However, responsiveness of LH receptors in follicles exposed to sustained activation by LH decreased progressively (Zor et al., 1976; Rao et al., 1977; Hunzicker-Dunn,

1981), most likely because components of the downstream signaling pathway were disrupted (Ekstrom and Hunzicker-Dunn, 1989; Gudermann et al., 1995). Aggregation of receptors and receptor internalization are other mechanisms of agonist-induced desensitization of LH receptors (Amsterdam et al., 1980; Ghinea et al., 1992; Gudermann et al., 1995). In this study preovulatory follicles were collected at a specific follicular stage prior to LH surge, but were exposed to increasing concentrations of LH over time (Karsch et al., 1983; Fortune and Hansel, 1985). In that circumstance, presence of elevated and sustained concentrations of LH in culture media may have induced deactivation of LH receptors in granulosal cells, and prevented stimulation of E_2 and P_4 production.

It was hypothesized that END1, via either ENDRA or ENDRB, would regulate follicular steroid production and prevent premature luteinization of granulosal cells before the LH surge in sheep, as suggested for other species (Tedeschi et al., 1992; Flores, 2000). In this experiment, neither individual nor combined presence of LH and END1 in the culture medium affected the patterns of E_2 and P_4 secretion by granulosal cells isolated from preovulatory follicles. Hence, the hypothesis that END1 may modulate steroid production by granulosal cells from preovulatory follicles was not supported. Although antisteroidogenic actions of END1 were demonstrated in follicles of rats (Tedeschi et al., 1992; 1994), pigs (Iwai et al., 1991; Flores et al., 1992; Kamada et al., 1993) and human beings (Denkova et al., 2000; Yaneva et al., 2003), such a role of END1 has not been reported in sheep. Attempts to identify END1 by immunohistochemistry in follicles from days 2 to 6 of the estrous cycle in sheep were not productive (Perea et al., unpublished data). However, in microdialyzed large bovine follicles, END1 stimulated E_2 secretion and inhibited P_4 and androgen production (Acosta et al., 1998;

1999). Moreover, LH increased END1 release from mature follicles (Acosta et al., 1998), and the presence of ENDRA and ENDRB was detected in the theca of bovine follicles (Acosta et al., 1999). Because the presence of END1 in preovulatory follicles was associated with other vasoactive peptides such as angiotensin II and atrial natriuretic peptide in microdialyzed mature follicles (Acosta et al., 1999), a role of END1 in modulating blood flow into the follicle may be one of its functions.

In conclusion, these results did not demonstrate effects of END1 on LH-stimulated steroid production by granulosal cells from preovulatory follicles. Future research is needed to clarify whether END1 has a role of in follicular function in the sheep. Given that earlier in vitro studies in pigs, FSH-simulated P_4 production by granulosal cells collected from small- and medium-sized follicles was inhibited by END1 (Kamada et al., 1993; Flores et al., 1992; 1999), such future research should include preovulatory follicles collected earlier of and incorporation of FSH and serum in culture media.

Effects of blocking endothelin receptor type A during spontaneous and PGF2α-induced luteolysis on morphological and functional characteristics of the corpus luteum and length of the estrous cycle in the sheep

Introduction

After ovulation, the collapsed ovulatory follicle undergoes dramatic cellular and structural changes that result in formation of the corpus luteum. The corpus luteum is a transient endocrine gland that produces increasing amounts of P_4 until the mid-luteal phase, at which time P_4 reaches steady state concentrations. Progesterone plays important roles modulating the hypothalamic–hypophyseal axis and promoting appropriate uterine physiological conditions to sustain development of the embryo/fetus during pregnancy. In the absence of an embryo in the uterus, CL demise in function and a new estrous cycle is initiated a few days later.

Luteal regression involves two consecutive and complementary processes through which the CL loses steroidogenic capacity without apparent changes in the structure of the tissue (functional luteolysis), followed by degradation of all cellular and structural components of the CL, with the concomitant reduction of luteal weight and size (structural luteolysis). Functional luteolysis was associated with decline of circulating concentrations of P_4 and a transient increase followed by progressive reduction in blood flow to the CL (McCracken et al., 1970; Niswender et al., 1975; 1976; Nett et al., 1976; Acosta et al., 2003). Once P_4 decreases, structural changes imply degradation of the cellular matrix and apoptosis of all cellular components of the CL (Hoyer, 1998; Pate and Keyes, 2001; Smith et al., 2002; Stocco et al., 2007). Luteolysis is a complex and orchestrated process initiated by uterine $PGF_{2\alpha}$ at the end of

the luteal phase, or after an injection of exogenous $PGF_{2\alpha}$ during mid-luteal phase of the estrous cycle (Zarco et al., 1988a; Niswender et al., 2000; Tsai et al., 2001). Luteolysis initiated by uterine $PGF_{2\alpha}$ is mediated by numerous local (intraluteal) factors such as NO (Jaroszewski et al., 2003), angiotensin II (Shirasuna et al., 2004), TNF-α, INF-γ, IL-1β (Neuvians et al., 2004), and END1 (Girsh et al., 1996a; Hinckley and Milvae, 2001) secreted by different luteal cells.

Substantial evidence demonstrated that END1 is a relevant mediator of luteolytic actions of $PGF_{2\alpha}$ (Girsh et al., 1996a; 1996b; Miyamoto et al., 1997; Ohtani et al., 1998; Hinckley and Milvae, 2001; Keator et al., 2008). Endothelin 1, a 21-amino acid peptide, originally isolated from porcine aortic endothelial cells as a potent vasoconstrictor (Yanagisawa et al., 1988), is involved in a wide variety of physiologic and pathologic roles in numerous mammalian tissues (Nussdorfer et al., 1999; Kedezierski and Yanagisawa, 2001). Two G protein-coupled receptors have been described for END1 (Nussdorfer et al., 1999). Type A (ENDRA) mediated luteolytic actions of $PGF_{2\alpha}$ (Girsh et al., 1996b; Doerr et al., 2008; Keator et al., 2008). In the ruminant CL, END1 reduced both basal and LH-stimulated P_4 secretion in a dose-dependent manner (Girsh et al., 1996a; Hinckley and Milvae, 2001), and the inhibitory effect was prevented by the addition of a selective ENDRA receptor antagonist (Girsh et al., 1996a).

In recent years, data from various in vivo studies have confirmed and strengthened the concept that END1 is an important mediator of $PGF_{2\alpha}$-induced luteal regression. Although intraluteal injection of BQ-123 (an ENDRA antagonist) did not reverse completely the luteolytic effect of $PGF_{2\alpha}$, intramuscular injection of both END1 (100 μg) and $PGF_{2\alpha}$ (7.5 mg)

effectively reduced P_4 concentrations by 48 h post-treatment, and shortened the length of the cycle (Hinckley and Milvae, 2001). However, separate administration of either END1 or $PGF_{2\alpha}$ at the same dosage, did not induce luteolysis in sheep (Hinckley and Milvae, 2001). Intrauterine infusions of BQ-610, a highly specific ENDRA antagonist, every 12 h on d 16 through 18 of the estrous cycle in heifers delayed luteolysis for about 2 days (Keator et al., 2008). Chronic administration of BQ-610 into the sheep CL through a minipump implanted in the ovary during the mid-luteal phase allowed decreased serum P_4 concentration throughout the first 12 h after a single injection of $PGF_{2\alpha}$. However, P_4 progressively increased during the next 36 h, attaining a concentration similar to the control group by 48 h after $PGF_{2\alpha}$ (Doerr et al., 2008). Apparently, a direct action of PGF2α decreased serum P_4 during early luteolysis, whereas END1 was required in later stages as indicated by the rise of P_4 in BQ-610-treated ewes beginning 12 h after $PGF_{2\alpha}$. In addition, blockade of ENDRA during $PGF_{2\alpha}$-induced luteolysis in cows, prevented the increase of caspase 3 mRNA by 24 h compared with controls (Watanabe et al., 2006). These findings indicate that END1 may participate in structural rather than functional luteolysis, stimulating expression of pro-apoptotic factors.

Two experiments were carried out to study the effects of END1 on functional and structural changes of the CL during either spontaneous or $PGF_{2\alpha}$-induced luteolysis in the sheep. In the first experiment, the ENDRA antagonist BQ-610 was delivered into the CL through an osmotic minipump to examine the expression of genes related with P_4 production and structural luteolysis at 6 and 24 h after exogenous $PGF_{2\alpha}$. It was hypothesized that sustained blockade of ENDRA would prevent upregulation of genes stimulated by $PGF_{2\alpha}$ during structural luteolysis.

In the second experiment, the same approach was used to examine the effects of the sustained blockade of ENDRA during spontaneous luteolysis on the length of the estrous cycle and functional and structural characteristics of the CL. It was hypothesized that chronic delivery of BQ-610 into the CL would prevent or delay the luteal regression induced by endogenous $PGF_{2\alpha,}$ increasing the length of estrous cycle.

Experiment 2: Effect of blocking endothelin receptor type A on gene expression during PGF2α-induced luteolysis in sheep

Materials and methods

General experimental procedure

Forty five nonpregnant mixed-breed ewes with at least one previous estrous cycle of normal length (15 to 19 days) were used in the experiment, conducted in fall of 2007 and 2008 (September-December) with ewes from the West Virginia University research flock. For observation of estrus (12 h intervals), ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket; standing estrus was confirmed by teasing with another vasectomized ram. Treatment was begun at day 7.5 to 9 of the estrous cycle (standing estrus = day 0). Ewes were assigned randomly to receive one of the following treatments: Alzet® mini-osmotic pump (model 2002; Durect Corporation, Cupertino, CA) loaded with either vehicle (200 μL of 2:1 methanol/saline solution) or 2.5 mg of END receptor A antagonist, BQ-610 (Azepane-1-carbonyl-leu-D-Trp(For)-D-Trp-OH), diluted in 200 μL of vehicle. A third experimental group did not receive mini-osmotic pumps (control). The Alzet®

mini-osmotic pumps (Bachem Bioscience Inc, King of Prussia, PA), designed to deliver 0.52 ± 0.02 μL/h for approximately 14 days, were kept overnight in sterile 0.9% saline solution at 37°C until implantation in the ovarian pedicle. Thus, the expected delivered dosage was 6.25 μg/h of BQ-610.

Ewes were initially anesthetized with 0.3 mg/kg diazepam (Valium, i.v., 5 mg/ml; Roche Pharmaceuticals, Nutley, NJ) and 7 mg/kg ketavet (Ketamine HCL, i.v.,) and then placed on a mixture of halothane, oxygen (2.0 L/min), and nitrous oxide gas (1.0 L/min) until mid-ventral laparotomy was finished. Ovaries were examined carefully, and in the presence of multiple CL, one was selected for implantation of the catheter connected to the mini pump. Additional CL in the same ovary were enucleated and an absorbable gelatin sponge was placed inside the remaining cavity (Gelfoam®, Pfizer Inc, New York, NY) as a hemostatic component. The contralateral ovary was removed if it contained additional CL. Each minipump was positioned in the mesoovarium and the attached catheter stabilized within the CL following the surgical procedure previously described (Doerr et al., 2008). All ewes received a dose (i.m.) 3×10^6 IU of penicillin G immediately after surgery.

A luteolytic dose of $PGF_{2\alpha}$ (25 mg; Lutalyse®, Pfizer Animal Health, New York, NY) was injected (i.m.) 48 h after surgery, and 6 or 24 h later ewes were euthanized, the correct position of the pumps and catheters was verified, and ovaries were collected. Ewes in the control group (no minipump implantation) received a saline injection (5 ml; i.m.) on day 9, 10 or 11 of the estrous cycle, and 6 or 24 h later ovaries were removed by mid-ventral laparatomy as described above. Jugular blood samples (8 ml) were collected at 3 h intervals from hour 0 to 6 (6-h group) or 0 to 24 (24-h group) after $PGF_{2\alpha}$ or saline injection (h 0) to monitor P_4

concentrations. Blood samples were refrigerated for 24 hours to allow them to clot, and then centrifuged for 20 minutes at 2500 rpm. Serum was collected and stored at -20° C until radioimmunoassay for P4. All animal procedures were approved by WVU Institutional Animal Care and Use Committee (ACUC # 05-1205).

Once removed, ovaries were refrigerated in ice-cold PBS, transported to the laboratory, and each CL was dissected, weighed, and sectioned into pieces that were weighed individually, then frozen in liquid nitrogen and stored at -80°C. One piece of each CL was homogenized in PBS (1 ml/100 mg of tissue), centrifuged, and supernatant stored at -20 °C until assayed for P4. Number of ewes per treatment and times of tissue collection are shown in Table 3.

Table 3. Numbers of ewes per treatment and time of tissue collection

Time of tissue collection	Treatment		
	BQ-610	Vehicle	Control
6-h	9	6	6
24-h	10	7	7
Total	19	13	13

Real-Time RT-PCR

The relative expression of a group of genes associated with luteal function or involved in functional and structural luteolysis, as reported elsewhere (Juengel et al., 2000; Tsai et al., 2001; Diaz et al., 2002; Smith et al., 2002; Taniguchi et al., 2002; Kliem et al., 2007; 2009), was quantified by real-time PCR. Total RNA was isolated from frozen luteal tissue using Tri-reagent (MRC, Cincinnati, OH) following the recommendations of the manufacturer. Once

solubilized in RNAse-free water, the quality and quantity of each RNA sample were assessed by 1% agarose gel electrophoresis and by spectrophotometry (Nano Drop Technologies, Wilmington, DE), respectively. Purified RNA samples were treated with DNAse (Invitrogen, Carlsbad, CA) to remove residual DNA contamination. To assure that low expression of a gene was not negatively affecting the Ct values and confirming variability within replicates, the RT-PCR was tested at total RNA concentrations of 0.5, 1.0 and 2.0 μg/μL. The gene expression data reported in this study were from the 2.0 μg of total mRNA concentration.

To generate first-strand cDNA, total RNA was reverse transcribed in a reaction volume of 20 μL containing sample RNA (2.0 μg), dNTP mix (10 nM), oligo dT primer (450 ng) and Superscript II reverse transcriptase (200 U) (Invitrogen, Carlsbad, CA). Real-time PCR was carried out using the approach reported by Pfaffl (2001) and following the procedure described by Goravanahally et al. (2009). Briefly, representative RNA samples from each treatment were pooled and used to produce first-strand cDNA as described above. Five consecutive dilutions (1:10) of cDNA were used to generate a standard curve to estimate the efficiency of the specific primers for each gene. The optimum efficiency of the PCR reactions was standardized between 90 to 110%. Melting curve from optimization reactions of each set of primers assured the absence of secondary products.

Oligonucleotide primers were designed with primer3 software (Rozen and Skaletsky, 2000) based on the mRNA sequences of *Ovis aries*. In the event of mRNA sequences not previously described in sheep, *Bos taurus* mRNA sequences were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the reference gene to normalize the expression values of the genes of interest. Table 4 shows the species, accession number, sequences and additional

characteristics of each gene primer included in the study. Quantitative PCR reactions were carried out in duplicate for each cDNA sample in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using a total reaction volume of 25 μL that included SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 μM of each primer and non-diluted target cDNA (2 μL). Real-time PCR conditions were: 30 sec at 95°C followed for 40 cycles at 95°C for 30 sec to denature, 30 sec at 60°C to anneal, and 1 min at 72°C for extension.

Radioimmunoassays for progesterone

Concentrations of P4 in serum and luteal tissue were measured in duplicate by RIA as previously described (Sheffel et al., 1982). Assay sensitivity averaged 0.15 ng/ml, and inter- and intraassay coefficients of variation were 13.8% and 13.0%, respectively.

Statistical Analysis

The effect of treatments on concentrations of serum and luteal P4 and luteal weight for each time of tissue collection (6-h and 24-h groups) was evaluated by one-way analysis of variance using the General Linear Model (GLM) procedure of SAS (SAS Institute; Cary, NC). Differences among means were compared by the LSM procedure of SAS. To examine differences in gene expression according to treatment, time, and treatment × time, a complete block design was applied and analyzed by the GLM procedure of SAS. Differences among means were compared by orthogonal contrasts. Contrasts tested were (a) PGF2α versus control and (b) BQ-610 versus vehicle. Data were expressed as mean ± SEM.

Table 4. Characteristics of primers used in quantitative real-time RT-PCR

Gene	Accession number	Species	Primer sequence	Product size (bp)	Annel. Temp. (°C)	Slope Standard curve
StAR	NM_001009243	*O. aries*	F: 5'-TGCTGAGTAAAGTGATCCCTGA-3' R: 5'-AGGACCTTGATCTCCTTGACAC-3'	148	60	-3.631
3βHSD	NM_174343	*B. taurus*	F: 5'-CCACACCAAAGCTACGATGA-3' R: 3'-TGTAAATTGGACTGAGCAGGAA-3'	148	60	-3.419
20αHSD	XM_868164	*B. taurus*	F: 5'-CTGAACGCATTGCTGAGAAC-3' R: 3'-AACTCCTCGTGAAAGGGGTAA-3'	148	60	-3.601
VEGF	NM_001025110	*O. aries*	F: 5'-TTGCTGCTCTACCTTCACCAT-3' R: 5'-TCTGGGTACTCCTGGAAGATGT-3'	158	60	-3.370
eNOS	DQ015701	*O. aries*	F: 5'-TGTTGGATTCCCTGTACTATCTCA-3' R: 5'-AACTTCTGGTCCAGTCTCTTATGG-3'	240	60	-3.262
TIMP-1	NM_001009319	*O. aries*	F: 5'-CCAGACATCCGATTCATCTACA-3' R: 3'-GCAGAACTCATGCTGTTCCA-3'	167	60	-3.558
TIMP-2	NM_174472	*B. taurus*	F: 5'-AGTTCTTCGCCTGCATCAA-3' R: 3'-AGCTGGACCAGTCTGAACTCTT-3'	159	60	-3.376
Bcl-2	DQ152929	*O. aries*	F: 5'-TGGATGACCGAGTACCTGAA-3' R: 3'-CAGCCAGGAGAAATCAAACAG-3'	120	60	-3.314
Bax	AF163774	*O. aries*	F: 5'-GAAGCGCATTGGAGATGAA-3' R: 3'-AAGTAGAAAAGGGCGACAACC-3'	159	60	-3.286
MMP-2	AF267159	*O. aries*	F: 5'-TTCATCTGGCGAACAGTGAC-3' R: 3'-CAGTATTCGTTCCCTGCAAAG-3'	149	60	-3.264
MMP-14	AF267160	*O. aries*	F: 5'-TGAGAGGAAGGATGGCAAGT-3' R: 3'-TCCAGAAGAGAGCAGCATCA-3'	149	60	-3.376
Fas-R	NM_001123003	*O. aries*	F: 5'-CGGAAGAATGGTATGGAGGA-3' R: 3'-TGCAAGAGCTTTTGGGAGAT-3'	162	60	-3.375
Caspase 3	AF068837	*O. aries*	F: 5'-GGATTATCCTGAAATGGGTTTATG-3' R: 3'-GATCGTTTTTAATCCTGACTTCGT-3'	155	60	-3.266
Caspase 8	NM_001045970	*B. taurus*	F: 5'-TGTCACAATCGCTTCCAGAG-3' R: 3'-AGTATCCCCGAGGTTTGCTT-3'	126	60	-3.311
GAPDH	AF030943	*O. aries*	F: 5'-TCTCAAGGGCATTCTAGGCTAC-3' R: 3'-TGTAGCCGAATTCATTGTCG-3'	155	60	-3.254

Results

Corpus luteum weight

The weights of the CL collected 6 h after injection of saline or $PGF_{2\alpha}$ were not affected by treatments. Apparently, treatment with BQ-610 did not prevent the luteolytic effect of exogenous $PGF_{2\alpha}$, because CL from BQ-610-treated ewes were lighter ($P < 0.02$) than those from control ewes 24-h after $PGF_{2\alpha}$ or saline (Fig 9).

Serum and luteal progesterone

Given that control ewes had one or more CL in the ovaries, while treated ewes had only one CL, concentrations of P_4 for each collection time were compared as the change from hour 0 (time of $PGF_{2\alpha}$ or saline injection). In ewes from which CL were removed at 6 h, change in concentration of P_4 3 h after $PGF_{2\alpha}$ was less negative in control than in BQ-610- and vehicle-treated ewes ($P < 0.01$). By 6 h, the P_4 decrease was similar in all treatments (Fig 10). In contrast, P_4 concentrations in BQ-610-treated and vehicle-treated ewes from which CL were removed at 24 h, declined progressively by greater than 0.5 ng/ml by 24 h after $PGF_{2\alpha}$, while in control ewes P_4 remained more than 0.5 ng/ml above the initial value throughout the sampling period (Fig. 11). Six hours after onset of induced luteolysis, content of luteal P_4 in BQ-610-treated and vehicle-treated ewes that received $PGF_{2\alpha}$ had decreased by ~ 65% and ~ 61%, respectively, with respect to luteal content of P_4 in control ewes. By 24 h after $PGF_{2\alpha}$ injection, luteal content of P_4 in BQ-610-treated and vehicle-treated ewes was around 1/9 and 1/3, respectively, of the luteal content of P_4 in control ewes ($P < 0.0005$; Fig. 12). In the 24-h group,

one ewe had a secretion pattern of serum P_4 similar to that reported by Doerr et al. (2008) in sheep treated with BQ-610. In this responsive BQ-610-treated ewe, P_4 concentration declined progressively from 2.5 ng/ml at time 0 to 0.9 ng/ml 9 h after $PGF_{2\alpha}$ injection, remained below 1 ng/ml until h 15 and then increased to 1.5 ng/ml by h 24 after $PGF_{2\alpha}$. Concentration of luteal P_4 in this animal was 12.8 μg/g and the luteal pattern of gene expression was similar to mid-cycle CL from the control ewes.

Gene expression

Expression of StAR and 3β-HSD mRNA were affected by treatment ($P = 0.05$ and $P < 0.0001$, respectively), and the expression of each gene was lower in $PGF_{2\alpha}$-treated than in control ewes ($P < 0.03$; $P < 0.0001$, respectively); but did not differ between BQ-610- and vehicle-treated CL at any time (Fig 13). For 20α-HSD mRNA, there was an effect of treatment ($P < 0.0001$), time ($P < 0.01$) and treatment × time ($P < 0.0001$), reflected in the upregulation of this gene in $PGF_{2\alpha}$-treated compared with control animals ($P < 0.02$) and in BQ-610-treated ewes compared with vehicle-treated animals ($P < 0.02$; Fig 13). Expression of VEGF mRNA was affected by treatment ($P < 0.0001$), time ($P < 0.01$) and treatment × time ($P < 0.04$), specifically being lower in $PGF_{2\alpha}$-treated than in control ewes ($P < 0.0001$; Fig 14). Treatment ($P < 0.0001$) and treatment × time ($P < 0.0001$) affected mRNA expression of TIMP-1 (Fig 14), which was lower in $PGF_{2\alpha}$-treated than in control ewes ($P < 0.0001$). In contrast, TIMP-2 mRNA at 6 and 24 h averaged 1.35 ± 0.28 and 1.03 ± 0.27, respectively, and did not differ with treatment, time or the interaction.

Expression of MMP-1 mRNA was affected by time, while the contrast BQ-610 versus $PGF_{2\alpha}$ only tended to be different (0.90 ± 0.20 versus 0.57 ± 0.20, respectively; P<0.10). For MMP-14, treatment (P = 0.0002), time (P < 0.0001) and treatment × time (P = 0.0005) differed, and its expression was lower in $PGF_{2\alpha}$-treated versus control ewes (P < 0.0001; Fig 15); and as judged by least squares means, this transcript was greater in vehicle-treated than in BQ-610-treated ewes followed by control animals at 24 h (P < 0.01). Time and treatment affected expression of eNOS (Fig 15), which was lower in $PGF_{2\alpha}$-treated than in control ewes (1.03 ± 0.14 and 1.47 ± 0.13, respectively; P = 0.001).

In regard to the genes involved in structural luteolysis, Bcl-2 expression was affected by treatment (P = 0.06) and time (P < 0.002; Fig 16), whereas its expression in $PGF_{2\alpha}$-treated ewes was greater than in control ewes (1.22 ± 0.30 versus 0.63 ± 0.28, respectively; P < 0.03). Time (P < 0.0001) and treatment × time (P < 0.005) affected Bax mRNA expression (Fig 16), with a trend for a greater expression in BQ-610-treated than in vehicle-treated CL (0.86 ± 0.1 versus 0.68 ± 0.1; P < 0.09). The Bcl-2/Bax ratio at 6 h was greater in BQ-610-treated than in control ewes (P < 0.01) (2.29 ± 0.38 versus 0.84 ± 0.38, respectively). In contrast, at 24 h, Bcl-2/Bax ratio tended to be lower in BQ-610-treated ewes than in control ewes (P < 0.09) (0.79 ± 0.38 versus 1.70 ± 0.33, respectively). For Fas-R transcript, time (P < 0.0001) and treatment × time (P = 0.001) differed, and treatment (P = 0.09) and $PGF_{2\alpha}$ versus control tended to differ (P < 0.09; Fig 16). For caspases 3 and 8, mRNA expression was affected by treatment, time and treatment × time (all P ≤ 0.01), and both contrasts, $PGF_{2\alpha}$ versus control and BQ-610 versus vehicle were significant. As judged by least squares means, mRNA expression in BQ-610-

treated ewes for both caspases 3 and 8 was upregulated at 24 h compared to either vehicle-treated or control animals with the effect already apparent at 6 h for caspase 3 (Fig 17).

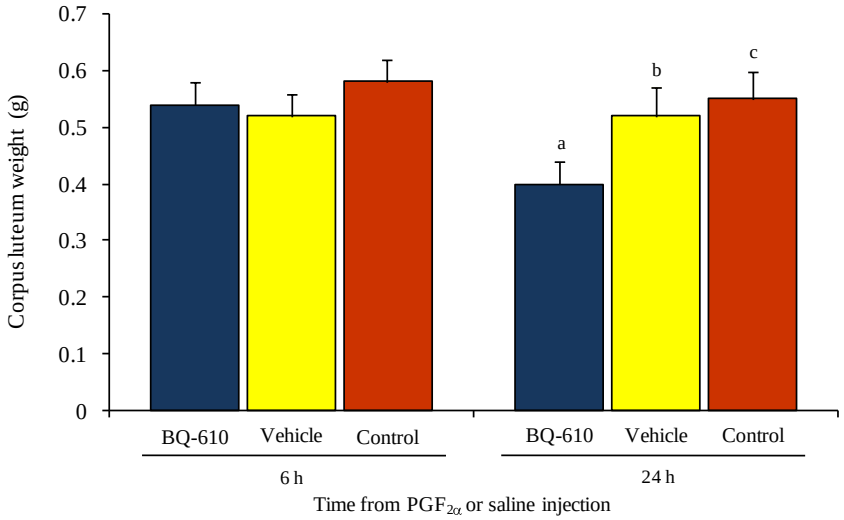

Figure 9. Effect of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on corpus luteum weight in sheep. Corpora lutea were collected 6 h or 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of corpus luteum weight (g). Values differed among treatments ($^{a,b}P < 0.07$; $^{a,c}P < 0.02$; ^{b,c}NS).

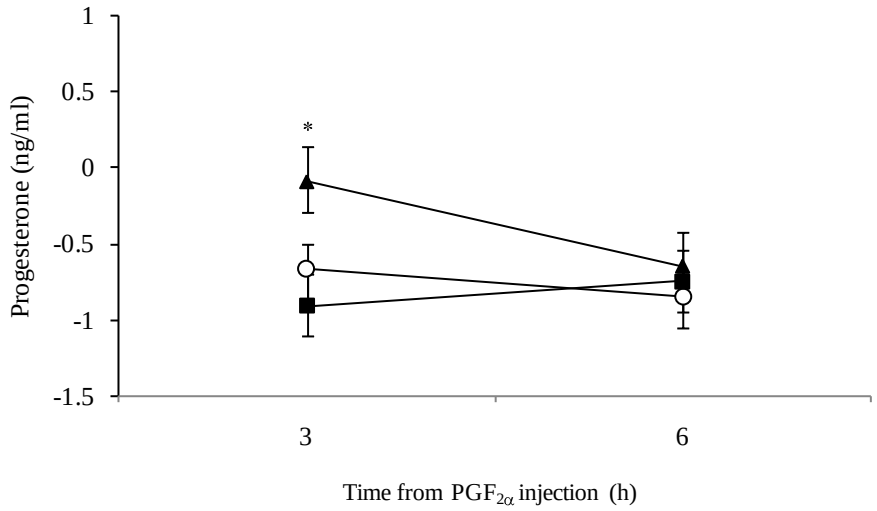

Figure 10. Effect of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on changes in serum concentrations of progesterone in ewes from which CL were removed at 6 h. Jugular blood samples were collected at 3-h intervals from hour 0 to 6 after $PGF_{2\alpha}$ injection (time 0) in BQ-610- (○) and vehicle-treated (■) ewes or after saline injection in control ewes (▲). Data points represent differences in progesterone (ng/ml) from hour 0 (mean ± SEM). Values in control and $PGF_{2\alpha}$-treated ewes were different ($^{*}P < 0.01$) at hour 3.

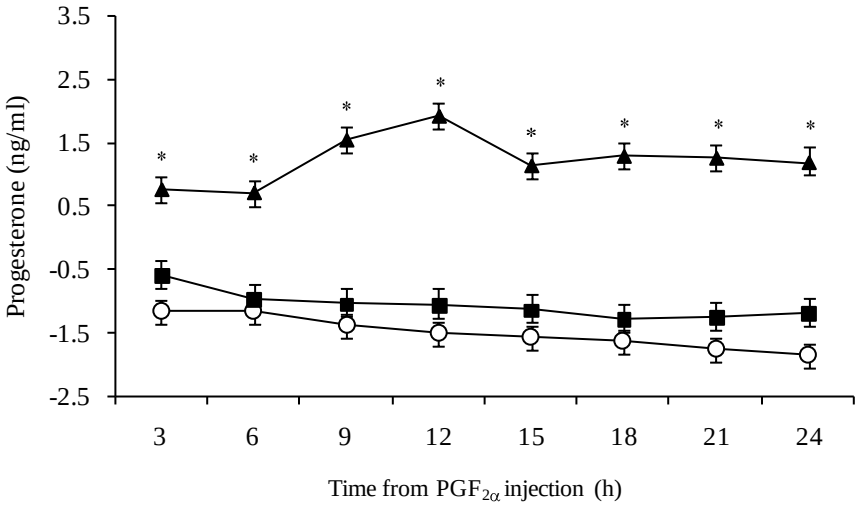

Figure 11. Effect of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on changes in serum concentration of progesterone in ewes from which CL were removed at 24 h. Jugular blood samples were collected at 3-h intervals from hour 0 to 24 (24-h group) after $PGF_{2\alpha}$ injection (time 0) in BQ-610- (○) and vehicle-treated (■) ewes or after saline injection in control ewes (▲). Data points represent differences in progesterone (ng/ml) from hour 0 (mean ± SEM). Values in control and $PGF_{2\alpha}$-treated ewes were statistically different ($^{*}P < 0.001$).

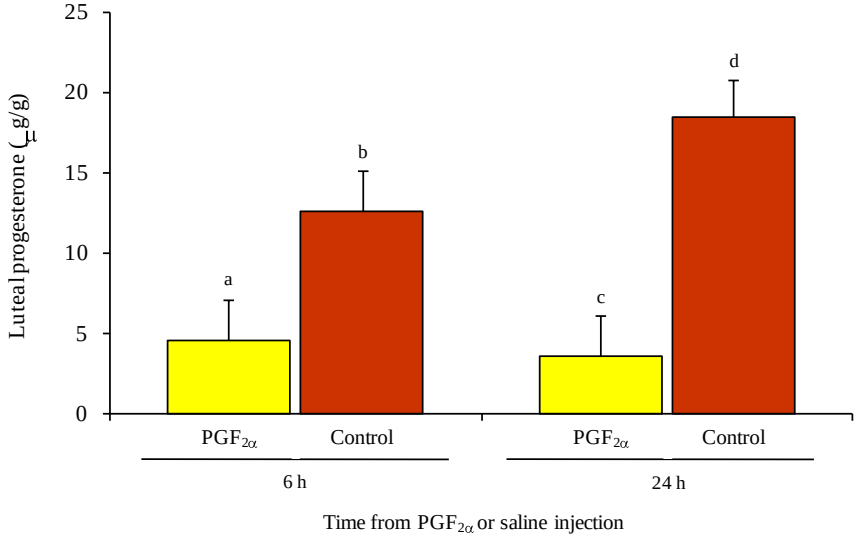

Figure 12. Effect of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on luteal content of progesterone in sheep. Corpora lutea were collected 6 h or 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of homogenized corpus luteum extracts (μg/g). Values differed among treatments ($^{a,b}P < 0.03$; $^{c,d}P < 0.0005$).

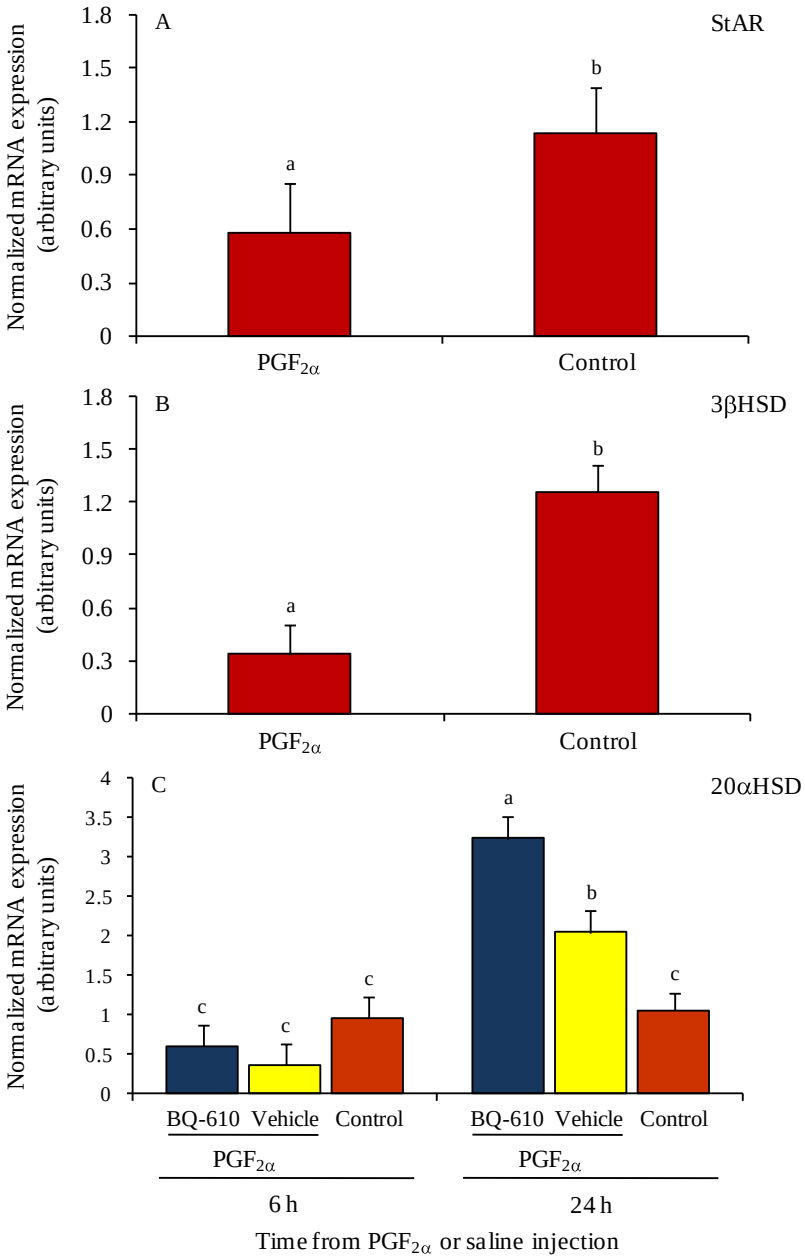

Figure 13. Effects of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on gene expression. Corpora lutea were collected 6 h and 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of normalized mRNA expression for StAR (panel A), which was affected by treatment ($P = 0.05$) and the contrast $PGF_{2\alpha}$ versus control ($^{a,b}P < 0.03$); 3βHSD (panel B), which varied with treatment ($P < 0.0001$), and the contrast $PGF_{2\alpha}$versus control ($^{a,b}P < 0.0001$); and 20αHSD (panel C), which varied with time ($P < 0.0001$), treatment ($P < 0.01$), time × treatment ($P < 0.0001$), the contrasts $PGF_{2\alpha}$ versus control and and BQ-10 versus vehicle ($P < 0.02$); least squares means for treatments at 24 h differed ($^{a,b}P = 0.004$; $^{a,c}P < 0.0001$; $^{b,c}P < 0.01$).

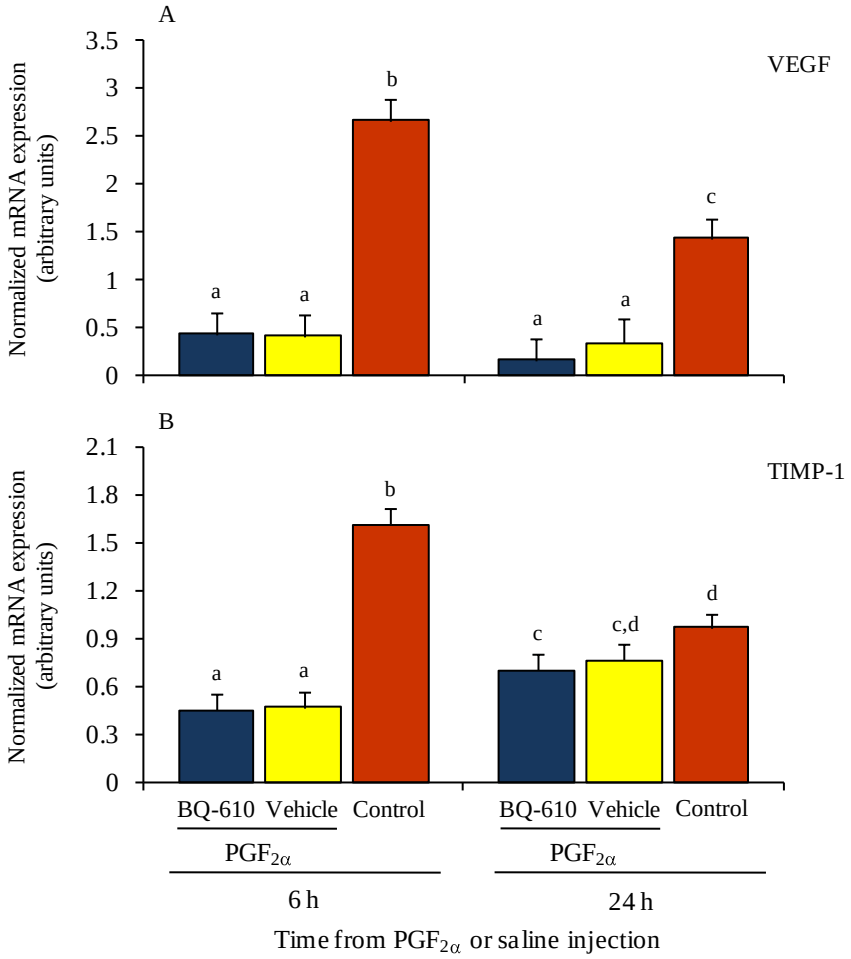

Figure 14. Effects of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on gene expression. Corpora lutea were collected 6 h and 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of normalized mRNA expression for VEGF (panel A), which varied with time ($P < 0.01$), treatment ($P < 0.0001$), time × treatment ($P < 0.04$), and the contrast $PGF_{2\alpha}$ versus control ($^{a,b}P < 0.0001$), least squares means for treatments differed ($^{a,b}P < 0.0001$; $^{a,c}P < 0.003$; $^{b,c}P < 0.0001$); and TIMP-1 (panel B), which was affected by treatment ($P < 0.0001$), time × treatment ($P < 0.0001$), and the contrast $PGF_{2\alpha}$ versus control ($^{a,b}P < 0.0001$), least squares means for treatments differed ($^{a,b}P < 0.0001$; $^{b,c}P < 0.0001$; $^{c,d}P = 0.05$).

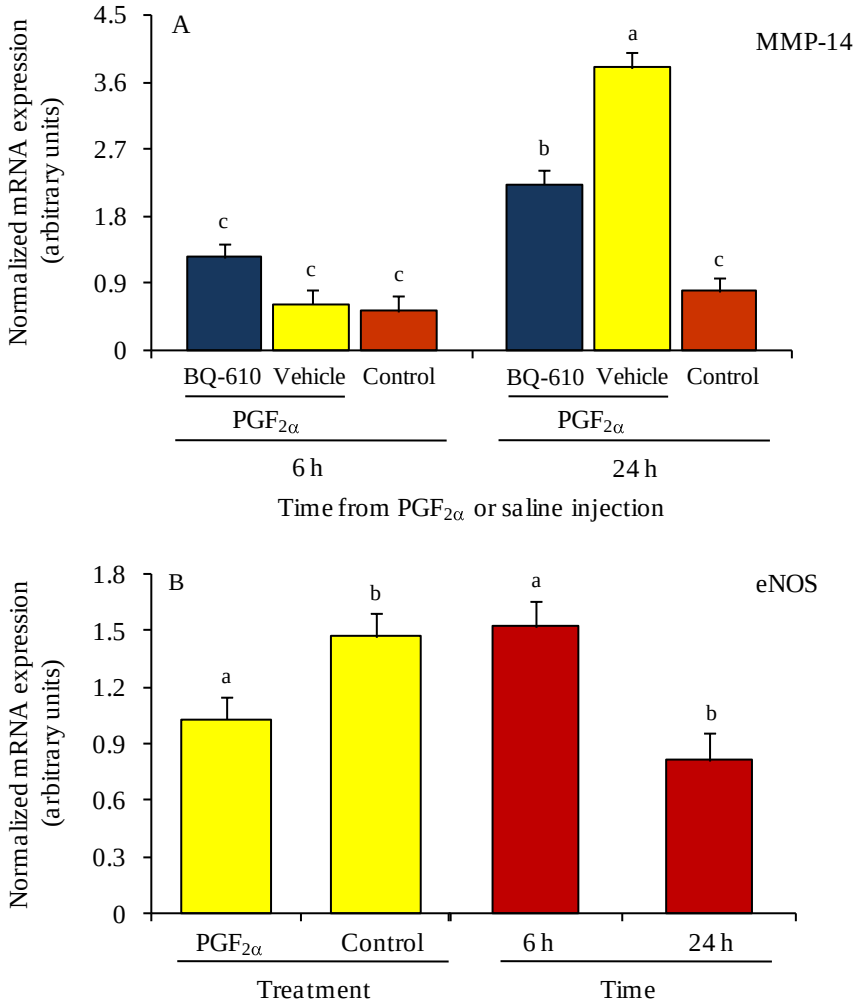

Figure 15. Effects of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on gene expression. Corpora lutea were collected 6 h and 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of normalized mRNA expression for MMP-14 (panel A), which was affected by time ($P < 0.0001$), treatment ($P = 0.0002$), time × treatment ($P = 0.0005$), and the contrast $PGF_{2\alpha}$ versus control ($P < 0.0001$), least squares means for treatments at 24 h differed ($^{a,b}P < 0.01$; $^{a,c}P < 0.0001$); and eNOS (panel B), which varied with time ($P < 0.0001$), treatment ($P < 0.005$), and the contrast $PGF_{2\alpha}$ versus control ($P = 0.001$).

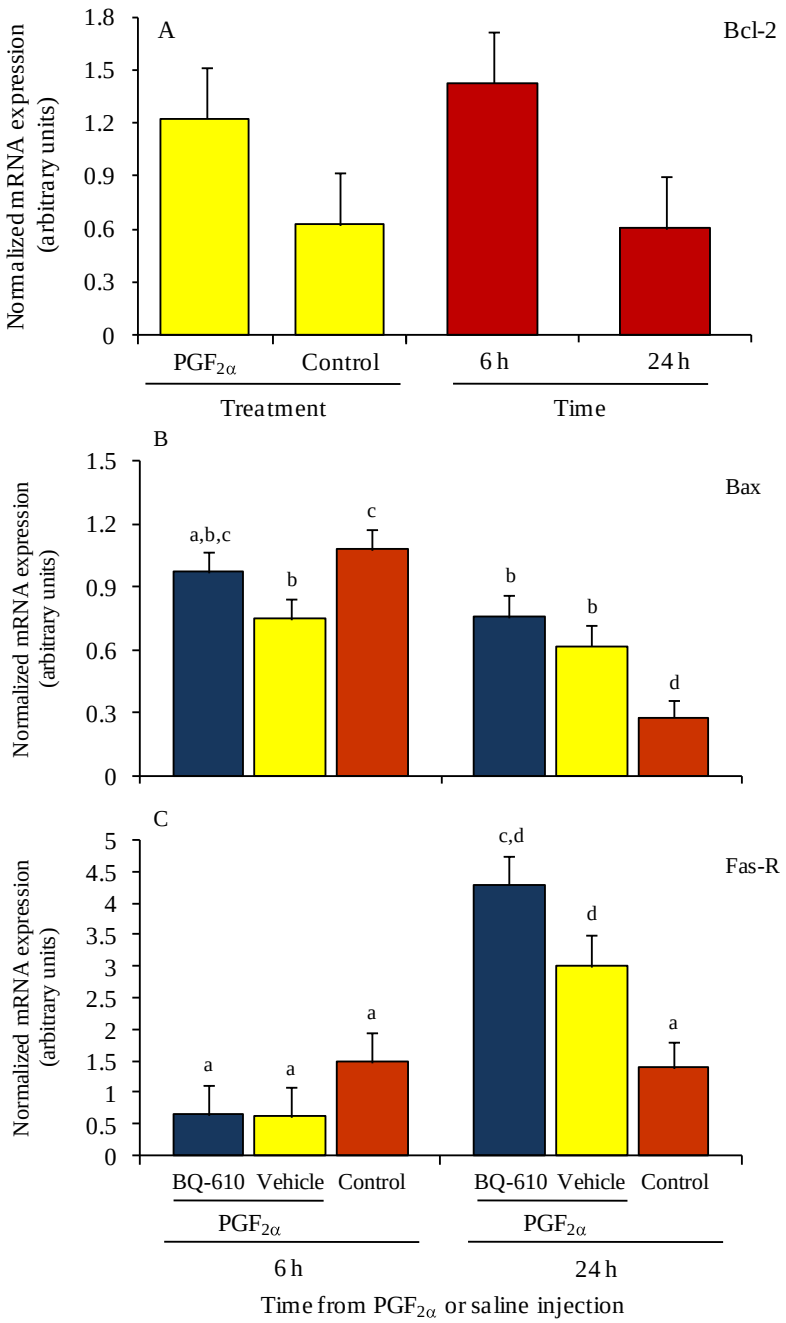

Figure 16. Effects of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on gene expression. Corpora lutea were collected 6 h and 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of normalized mRNA expression for Bcl-2 (panel A), which was affected by time ($P = 0.002$), and treatment ($P = 0.06$); Bax (panel B), time ($P < 0.0001$), which was affected by time × treatment ($P = 0.003$), and the contrast BQ-610 versus vehicle ($P < 0.09$), least squares means for treatments differed ($^{a,e}P < 0.0001$; $^{b,c}P < 0.04$; $^{b,e}P < 0.002$; $^{c,b}P < 0.04$; $^{c,d}P < 0.0001$); and Fas-R (panel C), which varied with time ($P < 0.0001$), and time × treatment ($P = 0.001$), least squares means for treatments differed ($^{a,c}P \leq 0.0002$; $^{a,d}P \leq 0.04$).

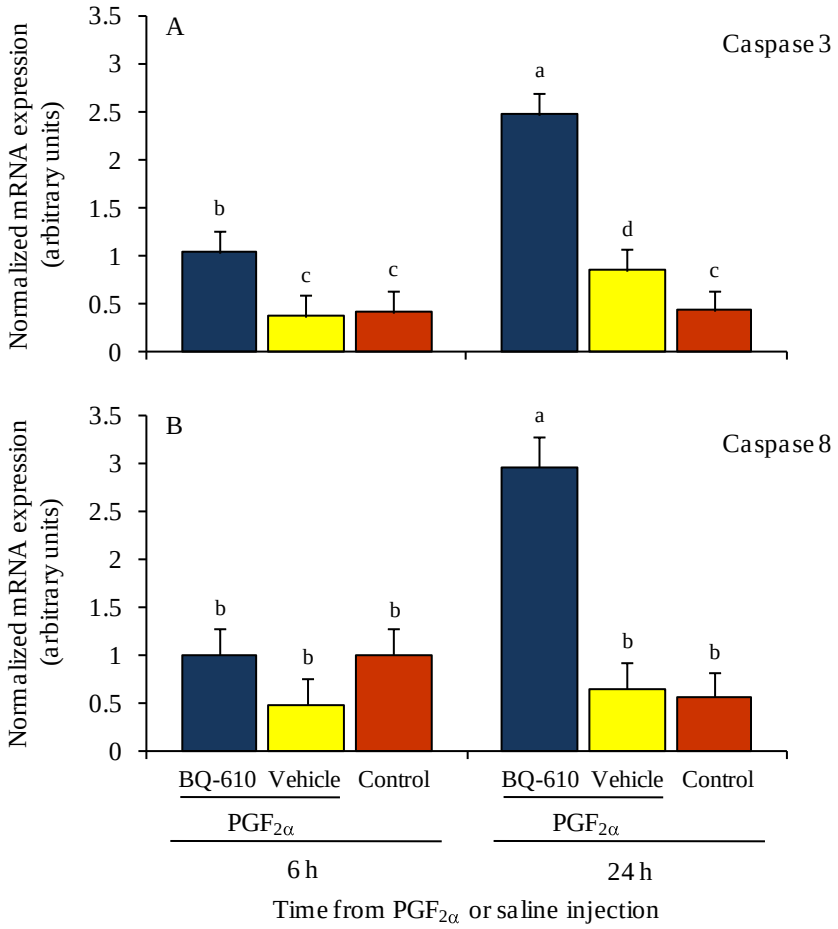

Figure 17. Effect of chronic administration of either BQ-610 or vehicle during $PGF_{2\alpha}$-induced luteolysis on gene expression. Corpora lutea were collected 6 h and 24 h after $PGF_{2\alpha}$ injection in BQ-610- and vehicle-treated ewes or after saline injection in control ewes. Bars represent mean ± SEM of normalized mRNA expression for caspase 3 (panel A), time ($P = 0.001$), which varied with treatment ($P < 0.0001$), time × treatment ($P = 0.01$), the contrasts $PGF_{2\alpha}$ versus control ($P < 0.0005$) and BQ-10 versus vehicle ($P < 0.0001$), least squares means for treatments at 6 and 24 h differed ($^{a,b,c,d}P<0.0005$; $^{b,c}P<0.06$; ^{c,d}NS); and caspase 8 (panel B), which was affected by time ($P < 0.01$), treatment ($P = 0.0001$), time × treatment ($P = 0.0005$), the contrasts $PGF_{2\alpha}$ versus control ($P < 0.02$) and BQ-10 versus vehicle ($P = 0.0001$), least squares means for treatments at 24 h differed ($^{a,b}P < 0.0001$).

Discussion

Data from serum and luteal concentrations of P4 (Fig. 10 and 11) as well as CL weight (Fig. 9) at 24 h indicated that BQ-610 did not prevent luteolysis in 90% (9/10) of the treated ewes. Because control ewes had an average of 1.7 ± 0.5 and 1.8 ± 0.4 CL in 6-h and 24-h groups, respectively, compared to only one CL in the treated ewes, data were presented as changes in concentrations of P_4 with respect to h 0. Accordingly, at 6 h, serum P_4 had declined equally in all groups of ewes with respect to h 0. In contrast, P_4 concentrations decreased to sub-luteal values by 24 h in both groups of $PGF_{2\alpha}$-treated ewes, while in control animals P_4 concentrations rose more than 0.5 ng/ml with respect to h 0. Similarly, luteal P_4 in the 24-h group was significantly lower in both groups of $PGF_{2\alpha}$-treated ewes than in control ewes. In contrast to the present results, Doerr et al. (2008) reported that blocking ENDRA with BQ-610, using a similar experimental approach, effectively reversed the decrease in concentrations of P_4 induced by exogenous $PGF_{2\alpha}$ in all of 6 treated animals. In that study, P_4 concentration in BQ-610-treated ewes decreased rapidly after a luteolytic dose of $PGF_{2\alpha}$, and remained below 1 ng/ml until hour 12 after $PGF_{2\alpha}$, but increased thereafter, reaching similar concentrations to the saline-injected ewes, around 48 h after $PGF_{2\alpha}$. Thus, it was suggested that during the first 12 h, $PGF_{2\alpha}$ exerted its antisteroidogenic actions independent of mediation by END1, whereas in later stages END1 was required to suppress P_4 production (Doerr et al., 2008). Nevertheless, only one ewe had a similar pattern of P_4 secretion at 24 h in this study. In that animal, luteal concentration of P_4 was 12.8 μg/g, an amount considerably greater than mean luteal P_4 in vehicle-treated ewes 24 h after $PGF_{2\alpha}$ (2.0 ± 2.1 μg/g). Moreover, the overall pattern of gene

expression in this responsive BQ-610-treated animal was similar to the control ewes. Hence, the physiological response to the antagonist in the responsive BQ-610-treated animal indicated that luteolysis was prevented and the functional status of the CL was like a saline-injected ewe.

Although CL weight did not differ among treatments at 6 h after $PGF_{2\alpha}$, luteal content of P_4 was significantly lower in both groups of $PGF_{2\alpha}$-treated ewes with respect to control animals. Consistent with the present findings, neither luteal volume nor CL weight decreased after 4 h of $PGF_{2\alpha}$-induced luteolysis in mid-cycle bovine CL, whereas serum and luteal P_4 concentrations were significantly lower than in saline-injected cows (Tsai et al., 1998b). Judged by CL weight, it seems that at least 24 h are required for $PGF_{2\alpha}$ to induce structural changes in the luteal tissue (Juengel et al., 1996; Tsai et al., 1998a). The first hours after $PGF_{2\alpha}$ initiates luteal regression are associated with rapid decreases of circulating concentrations of P_4, concomitant with the reduction of the luteal blood flow (Niswender et al., 1996; Wanatabe et al., 2006). Nevertheless, a transient increase in blood flow was associated with $PGF_{2\alpha}$-stimulated release of NO from vascular tissue (Acosta et al., 2002, 2008).

Loss of steroidogenic capacity occurs mostly by down regulation of enzymes and proteins involved in P_4 synthesis (Juengel et al., 1995; Tsai and Wiltbank, 1998; Tsai et al., 2001). It is likely that $PGF_{2\alpha}$ reduced synthesis of P_4 and transport of cholesterol, because both StAR and 3βHSD were reduced equally by 6 h after $PGF_{2\alpha}$. However, as judged by the increment in the expression of 20αHSD at 24 h, metabolism of P_4 may have contributed to decrease circulating and luteal concentrations of P_4. Interestingly, treatment with BQ-610 upregulated even more 20αHSD transcripts than vehicle, supporting the fact that BQ-610 did not prevent luteolysis, as

shown by concentrations of serum P_4. Moreover, as indicated by luteal P_4 and weight and some other genes, the degree of luteolysis in BQ-610-treated ewes appeared to be greater than in vehicle-treated animals. In bovine luteal tissue, mRNA expression for StAR and 3βHSD decreased as early as 4 h after exogenous $PGF_{2\alpha}$ (Tsai et al., 2001). Six hours after $PGF_{2\alpha}$ is likely too early to determine if BQ-610 was able or not to effectively prevent luteolysis. During this early period, antisteroidogenic actions of $PGF_{2\alpha}$ were independent of END1, as indicated by the profile of P_4 concentration in BQ-610-treated ewes reported by Doerr et al. (2008). Even if structural luteolytic changes were induced by lower doses of $PGF_{2\alpha}$, (3 mg PGF2a/60 kg body weight) removal of luteolytic stimulus allowed increased serum P_4 concentrations and reversed the effects of $PGF_{2\alpha}$ in luteal tissue (Juengel et al., 2000).

Apart from 3βHSD and StAR, other genes that promote luteal function, VEGF and TIMP-1, were downregulated by $PGF_{2\alpha}$ in both 6 and 24-h groups. VEGF, an angiogenic and potent mitogenic growth factor, was highly expressed during early luteal development and downregulated at the end of the luteal phase (Schams and Berisha, 2004; Ribeiro et al., 2006; 2007). In agreement with these data, studies in ruminants indicated a reduction of VEGF mRNA within 8 to 12 after exogenous $PGF_{2\alpha.}$ (Neuvians et al., 2004b; Vonnahme et al., 2006). TIMP family members are associated with the maintenance of the ECM stability by reducing activity of MMP (Smith et al., 2002). TIMP-1 mRNA expression in ovine CL decreased 1 h after $PGF_{2\alpha}$, increased by 4 and 6 h, and decreased again from 12 through 48 h of $PGF_{2\alpha}$-induced luteolysis (Ricke et al., 2002); and as occurred in this study, TIMP-2 remained unchanged from 6 through 48 h after $PGF_{2\alpha}$ (Ricke et al., 2002a). In the light of this finding, it seems that downregulation of the genes associated with stability of luteal function by $PGF_{2\alpha}$ is

required in order for structural changes to take place. In this experiment, only MMP-14 was upregulated by $PGF_{2\alpha}$, and its expression increased from 6 to 24 h in all groups. In contrast, MMP-2 was similar among treatments and declined over time. In ovine luteal tissue, MMP-2 transcript varied throughout the examined time after $PGF_{2\alpha}$ with peaks of expression at 6 and 24 h (Ricke et al., 2002b); whereas MMP-14 was upregulated by $PGF_{2\alpha}$ as early as 6 h and remained unchanged over time. The lower expression of MMP-2 at 24 h than at 6 h in this study might be associated with fluctuation of this gene as reported by Ricke et al. (2002b). Once the antiluteolytic effect of P_4 is withdrawn, $PGF_{2\alpha}$ stimulates upregulation of factors involving in degradation of cellular matrix (Smith et al., 2002). In the current study, upregulation of MMP-14 is consistent with that concept, and with reports in ewes and cows that monitored expression of this transcript during a period of time after $PGF_{2\alpha}$-induced luteolysis (Ricke et al., 2002b; Kliem et al., 2007).

In general, the expression pattern of genes that stimulate synthesis of P_4 or inhibit its metabolism tended to favor luteal function in control ewes, but not in the BQ-610-treated group in which the ENDRA antagonist was expected to prevent luteolysis and maintain the functional status of the CL, as reported elsewhere for $PGF_{2\alpha}$-treated ewes (Doerr et al., 2008) and seen in spontaneous luteolysis (experiment 3). Moreover, BQ-610 appeared to promote the expression of genes related with structural luteolysis, because at 24 h after $PGF_{2\alpha,}$ expression of caspases 3 and caspases 8 were significantly greater in BQ-610-treated ewes than in vehicle-treated or control ewes (Fig 16). Moreover, Bcl-2/Bax ratio decreased over time only in the BQ-610-treated group. These factors are involved in apoptosis and are upregulated within a few hours after $PGF_{2\alpha}$ (Yadav et al., 2005; Kliem et al., 2009). Structural luteolysis follows the functional

demise of the CL, and occurs as a consequence of degradation of luteal cellular and structural components (Hoyer, 1998; Pate and Keyes, 2001; Smith et al., 2002; Stocco et al., 2007). Upregulation of factors related with apoptosis, as shown for caspase 3 and 8 in this study, is an integral part of luteolysis and follows functional demise of the CL.

A reasonable explanation why in this experiment BQ-610 treatment was ineffective to inhibit the luteolytic actions of END1 in $PGF_{2\alpha}$-induced luteolysis in the majority of the animals is not readily available. The experimental design of the current study was based on the findings reported by Doerr et al. (2008). Because concentrations of luteal P_4 were ~ 42% lower in BQ-610/BQ-788-treated ewes (1 mg each) and approximately 26% lower in BQ-610-treated ewes (2 mg) than in saline-injected animals (Doerr et al., 2008), it was evident that BQ-610 treatment in that concentration was not able to completely reverse the antisteroidogenic effects of $PGF_{2\alpha}$ mediated by END1. Therefore, on the basis of that finding, the amount of BQ-610 used in this study was increased from 2 to 2.5 mg/pump and the vehicle in which the antagonist was totally solubilized was 1 to 2 saline/methanol. The proportion of methanol in the BQ-610 solution was considerably greater than (2.5 times) that reported by Doerr et al. (2008; 3 to 1 saline/methanol). As described in material and methods, in a few cases, technical problems derived from catheter deattachment, presence of partial clots into the catheter or in the tissue around the end of the catheter might have interfered with the delivery of the BQ-610 or vehicle solution into the luteal tissue. At the moment of CL collection, 4 catheters (2 from 6-h vehicle, 1 from 6-h BQ-610 and 1 from 24-h BQ-610) were not attached to the treated CL. It was considered that the manipulation of the reproductive tract inside of abdominal cavity during

necropsy was probably the cause. In addition, in 6 ewes (2 in 6-h vehicle, 2 in 6-h BQ-610 and 2 in 24-h BQ-610) the end of the catheter attached to the treated CL was partially clotted.

Was the increased volume of methanol in the BQ-610 solution the cause of the ineffectiveness of BQ-610 treatment to reverse the luteolytic effect of $PGF_{2\alpha}$ mediated by END1? Perhaps so, if only the data from serum and luteal P_4 and CL weight are considered. One can speculate that methanol, in that elevated concentration, was cytotoxic and supported induction of luteal regression. Another possible mechanism is that methanol interfered with or prevented binding of BQ-610 to ENDRA, and thus, luteal regression was a consequence of exogenous $PGF_{2\alpha}$. Nevertheless, if either of the previous mechanisms caused or allowed luteal regression in BQ-610-treated ewes, the effects on gene expression in BQ-610-treated ewes should have been similar (as were the serum and luteal P_4 and CL weight) to those in vehicle-treated animals. However, that was not the case, particularly in the 24-h group in which the genes related with apoptosis were upregulated more in BQ-610-treated than in vehicle-treated ewes after $PGF_{2\alpha}$. So, it seems that instead of preventing luteolysis by acting as an antagonist, the greater dosage of BQ-610 synergized with or enhanced the effect of $PGF_{2\alpha}$; or alternatively, elevated concentrations of methanol may have modified the molecular structure of BQ-610 making it able to activate ENDRA, because the expression of 20αHSD, caspase 3 and caspase 8 in BQ-610-treated ewes at 24 h, was considerably greater than in vehicle treated ewes. The ratio Bcl-2/Bax in BQ-610-treated ewes changed from 2.29 in 6-h group to 0.79 in 24-h group; and tended to be lower only in BQ-610-treated with respect to control ewes. Moreover, at 24 h after PGF2α, CL weight was reduced significantly only in BQ-610-treated ewes compared to

control animals; and reduction of CL mass is a reliable evidence of the cellular and structural changes during luteolysis (Stocco et al., 2007).

From the previous argument a new question arises. Why did this hypothetical luteolytic effect attributed to BQ-610 not occur in all animals of this experiment and experiment 3 or in any animal of the Doerr et al. (2008) study? As mentioned earlier, in one ewe from this experiment and in 3 from experiment 3, the evidence indicated that BQ-610 effectively inhibited luteolysis. So, a combination of factors, including the presumptive ability of BQ-610 to act as an agonist, the greater amount of methanol in the BQ-610 solution and some other factor derived from particular characteristics of the animals may explain this physiological incongruence? One or more of these possibilities may be a reasonable physiological explanation.

Binding of END1 to type A G-protein coupled receptor activates at least three classes of $G\alpha$ proteins ($G\alpha_q$, $G\alpha_s$, $G\alpha_i$), each of them associated to a different signaling pathway (Aramori and Nakanishi, 1992; Wang et al., 1992; Ono et al., 1994; Takigawa et al., 1995). Different domains of the ENDRA structure are required for coupling to distinct $G\alpha$ proteins (Nussdorfer et al., 1999). In addition, there are at least two separable ligand interaction subdomains within the ENDRA (Sakamoto et al., 1993); and ENDRA gene can give rise to at least three transcripts by alternative RNA splicing (Miyamoto et al., 1996). Moreover, a single amino acid substitution altered the three-dimensional structure of the ligand-binding domain of ENDRA and significantly reduced the END1 binding activity (Juan, 2008). This complex molecular scenario in combination with the experimental conditions mentioned above, might make it plausible that BQ-610 activated the ENDRA, and consequently triggered downstream events

conducive to luteal regression. To support this interpretation, it was documented that abosentan, a selective non-peptide ENDRA antagonist, stimulated p38 mitogen-activated protein kinase (MAPK/ERK) phosphorylation mediated by ENDRA in mouse cardiac tissue (Marchant et al., 2009). The ERK signaling pathway stimulated mRNA and protein expression of early growth response 1 (EGR1), and during PGF2α-induced luteal regression in the cow ERG1 induced expression of TGFβ1, an important tissue remodeling protein also involved in apoptosis (Hou et al., 2008).

Intraluteal injections of ENDRA antagonists in cows did not prevent antisteroidogenic effects of PGF2α mediated by END1 (Watanabe et al., 2006; Keator et al., 2008) or did not reverse completely the luteolytic effect of $PGF_{2\alpha}$ in sheep (Hinckley and Milvae, 2001). As reported by Watanabe et al. (2006), administration of 5 intraluteal injections of LU-13522 (an ENDRA antagonist) at 2 h intervals in cows did not prevent the decrease in plasma P_4 concentration and the downregulation of mRNA for StAR and 3βHDS in response to a luteolytic dosage of $PGF_{2\alpha}$, as occurred in the vehicle-treated group. In addition, blockade of ENDRA upregulated expression of ppEND1 and ENDRB but did not affect ENDRA transcript expression (Watanabe et al., 2006).

Administration of ENDRA antagonist by multiple intraluteal injections, delayed for about 2 d the decrease of CL volume and blood flow in the area adjacent to the CL compared with vehicle-treated cows (Watanabe et al., 2006). The vasocontractive properties of END1 have been previously reported (Clarke et al., 1989; Ivi et al., 1994; Skovgaard et al., 2008), and the decrease of blood flow to the ovary at the end of the luteal phase was associated with luteal

demise (Wise et al., 1982). Likewise, reduced blood flow implies lower concentrations of oxygen in the tissues, and hypoxic conditions in the bovine CL decreased P_4 production (Nishimura et al., 2006) and induced apoptosis by upregulating pro-apoptotic factors such as caspase 3 and BNIP3 (Nishimura et al., 2008).

Interestingly, caspase 3 mRNA expression was significantly greater in vehicle-treated than antagonist-treated cows at 24 h after $PGF_{2\alpha}$ (Watanabe et al., 2006), indicating that the role of END1 during luteolysis may be associated with structural rather than functional luteolysis. This hypothesis is supported by the findings reported by Doerr et al., (2008) that the early antisteroidogenic actions of $PGF_{2\alpha}$ were independent of mediation by END1, whereas in later stages of luteolysis END1 was required to effectively induce demise of the CL. In experiment 3, the delay of endogenous luteolysis for more than 5 days in 3 of 12 ewes by blocking ENDRA did not prevent the upregulation of genes related with apoptosis and structural luteolysis. Apparently, the elevated concentrations of P_4 in the responsive BQ-610-treated animals exerted a protective effect, most likely by reducing the activity of apoptotic proteins. The protective and antiapoptotic effect of P_4 on luteal function has been widely documented (Rueda et al., 2000; Goyeneche et al., 2003; Okuda et al., 2004; Nishimura et al., 2008). The assumption that in this experiment BQ-610 activated ENDRA and upregulated apoptosis-related genes fits with the concept that actions of END1 during luteolysis are directly (inhibiting P_4 production and stimulating apoptosis) and indirectly (via reduction of luteal blood flow) associated to structural luteolysis (Watanabe et al., 2006; Doerr et al., 2008; experiment 3).

In conclusion, data from serum and luteal P_4 and CL weight indicated that $PGF_{2\alpha}$ induced luteolysis in both vehicle-treated and BQ-610-treated ewes. The pattern of gene expression further confirmed the luteolytic effect of $PGF_{2\alpha}$ in both treated groups. But, interestingly, upregulation of genes related to apoptosis and structural luteolysis was greater in BQ-610-treated than vehicle-treated ewes, indicating that instead of blocking actions of endogenous END1, BQ-610 might have stimulated actions ascribed to stimulation of ENDRA by END1 (Watanabe et al., 2006; Doerr et al., 2008). This unexpected finding indicates that BQ-610 or its combination with a greater concentration of methanol may have activated ENDRA and therefore, induced over expression of these apoptotic factors. If so, these findings would confirm that END1 via ENDRA in luteal cells is associated with structural luteolysis as previously reported (Watanabe et al., 2006; Doerr et al., 2008).

Experiment 3: Effect of chronic administration of an endothelin receptor type A antagonist on length of estrous cycle and functional characteristics of the corpus luteum during spontaneous luteolysis in sheep

Materials and methods

General experimental procedure

Twenty one nonpregnant Suffolk ewes with at least one previous estrous cycle of normal length (15 to 19 days) were used in the experiment, conducted in winter (January-February) and fall (September-November) of 2008 with ewes from the West Virginia University research flock. For observation of estrus (12 h intervals) ewes were penned with a vasectomized ram

bearing a harness with a crayon in the area of the brisket; standing estrus was confirmed by teasing with another vasectomized ram. On day 9 of the estrous cycle (standing estrus = day 0) ewes were assigned randomly to receive one of the following treatments: Alzet® mini-osmotic pump (model 2002; Durect Corporation, Cupertino, CA) loaded with either vehicle (200 μL 2:1 methanol:saline solution; n = 9), or 2.5 mg of END receptor A antagonist (n = 12), BQ-610 (Azepane-1-carbonyl-leu-D-Trp(For)-D-Trp-OH), diluted in 200 μL of vehicle. The Alzet® mini-osmotic pumps (Bachem Bioscience Inc, King of Prussia, PA) designed to deliver 0.52 ± 0.02 μL/h for approximately 14 days, were kept overnight in sterile 0.9% saline solution at 37°C previous to implantation in the ovary. Thus, the expected delivered dosage was 6.25 μg/h of BQ-610.

Minipumps were implanted surgically as described in experiment 2. Twenty four h after surgery, ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket, and estrous detection was performed as described above. Blood samples were collected (8 ml) by jugular venipuncture at 6 h intervals from day 11 until estrus or until day 21 of the estrous cycle in ewes that did not return to estrus. Blood samples were refrigerated for 24 h to allow them to clot, and then centrifuged for 20 minutes at 2500 rpm. Serum was collected and kept at -20° C until radioimmunoassay for P4. Approximately 12 h after estrous detection or in the afternoon of day 21, ewes were euthanized, the correct position of the pumps and catheters were verified, and ovaries were collected. All animal procedures were approved by WVU Institutional Animal Care and Use Committee (ACUC # 05-1205).

Luteal tissue processing

Ovaries were refrigerated in ice-cold PBS, transported to the lab within 2 to 3 h after surgery, and each CL was dissected, weighed, sectioned into 5 pieces that were weighed individually and 3 of them were frozen in liquid nitrogen and stored at -80°C. One piece of each CL was homogenized in PBS (1 ml/100 mg of tissue), centrifuged, and supernatant stored at -20 °C, for determination of luteal P4. A second fragment was placed in Bouin's fixative for at least 24 h, and processed later to assess cellular morphology and apoptosis.

Bouin's fixative was removed by rinsing luteal tissue successively in 70% alcohol. Pieces of tissue were embedded in paraffin and cut into 10 μm sections, then mounted about 8 to 14 sections on each microscope slide. After deparaffinization with xylene and rehydration through descending grades of alcohol, tissue specimens were stained with the terminal deoxynucleotide transferase-mediated deoxy-UTP nick labeling method (TUNEL) for detection of apoptotic cells by immunohistochemistry (APO-BRDU-IHC; Phoenix Flow Systems, San Diego, CA). Briefly, the procedure was performed as follows: (1) permeabilization of specimen with proteinase K (20 min at room temperature); (2) inactivation of endogenous peroxidases with 30% H_2O_2 diluted 1:10 in methanol (5 min at room temperature); (3) equilibration reaction with 1X reaction buffer followed by a labeling reaction in humid chamber (at 37°C for 90 min) using bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) and the enzyme terminal deoxynucleotidyl transferase (TdT); in the next reaction (4) an anti-BrdU antibody directly conjugated to biotin was attached to the Br-dUTP's (in darkness, for 1-1.5 hours at room temperature) followed by (5) the attachment of peroxidase-conjugated avidin to the biotin labeled Br-dUTP's; finally, (6) a DAB-H_2O_2-Urea stain reaction was performed to label the

incorporated Br-dUTP's. Slides were observed under an Olympus PROVIS AX70 microscope to identify specific brown color in the cellular nuclei indicative of apoptosis. Three fields-of-view from two different sections, at least 100 μm apart, were evaluated to quantify the number of apoptotic cells, and to estimate the rate of apoptosis for each treatment.

Assessing gene expression

To corroborate the functional status of the CL, expression of genes involved in progesterone production or structural luteolysis was determined by quantitative RT-PCR, applying a transverse-transcription real-time PCR procedure identical to that described in experiment 2. In addition to tissue from BQ-610 and vehicle ewes, luteal tissue from control ewes (no minipump; saline injection; day 10-11 of estrous cycle) obtained in experiment 2 was used. Accession number, sequence, animal source and additional characteristics of each gene primer are indicated in Table 5.

Radioimmunoassays for progesterone

Concentrations of P4 in serum and luteal tissue were measured in duplicate by RIA as previously described (Sheffel et al., 1982). Assay sensitivity averaged 0.18 ng/ml, and inter- and intraassay coefficients of variation were 9.5% and 9.8%, respectively.

Statistical Analysis

Differences in concentrations of serum and luteal P4, luteal weight, length of estrous cycle, cellular apoptosis and gene expression among treatments were evaluated by one-way analysis of variance using the General Linear Model (GLM) procedure of SAS (SAS Institute; Cary,

NC). Differences among means were compared by the LSM procedure of SAS. Data were expressed as mean ± SEM. Proportions of ewes in which the length of the estrous cycle was prolonged beyond 20 d were analyzed by Chi-square method of SAS.

Table 5. Characteristics of primers used in quantitative real-time RT-PCR

Gene	Accession number	Specie	Primer sequence	Product size (bp)	Annel. Temp. (°C)	Slope Standard curve
StAR	NM_001009243	*O. aries*	F: 5'-TGCTGAGTAAAGTGATCCCTGA-3' R: 5'-AGGACCTTGATCTCCTTGACAC-3'	148	60	-3.631
3βHSD	NM_174343	*B. taurus*	F: 5'-CCACACCAAAGCTACGATGA-3' R: 3'-TGTAAATTGGACTGAGCAGGAA-3'	148	60	-3.419
PPEND1	NM_001009810	*O. aries*	F: 5'-TCTGCAAGTTGTTCCCCTTT-3' R: 3'-ATCTCAATGGCTGTGACCAAC-3'	148	60	-3.331
ENDAR	NM_001009433	*O. aries*	F: 5'-TCTGCAAGTTGTTCCCCTTT-3' R: 3'-ATCTCAATGGCTGTGACCAAC-3'	148	60	-3.208
Bcl-2	DQ152929	*O. aries*	F: 5'-TGGATGACCGAGTACCTGAA-3' R: 3'-CAGCCAGGAGAAATCAAACAG-3'	120	60	-3.314
Bax	AF163774	*O. aries*	F: 5'-GAAGCGCATTGGAGATGAA-3' R: 3'-AAGTAGAAAAGGGCGACAACC-3'	159	60	-3.286
Fas-R	NM_001123003	*O. aries*	F: 5'-CGGAAGAATGGTATGGAGGA-3' R: 3'-TGCAAGAGCTTTTGGGAGAT-3'	162	60	-3.375
Caspase 3	AF068837	*O. aries*	F: 5'-GGATTATCCTGAAATGGGTTTATG-3' R: 3'-GATCGTTTTTAATCCTGACTTCGT-3'	155	60	-3.266
TIMP-1	NM_001009319	*O. aries*	F: 5'-CCAGACATCCGATTCATCTACA-3' R: 3'-GCAGAACTCATGCTGTTCCA-3'	167	60	-3.558
GAPDH	AF030943	*O. aries*	F: 5'-TCTCAAGGGCATTCTAGGCTAC-3' R: 3'-TGTAGCCGAATTCATTGTCG-3'	155	60	-3.254

Results

Length of the estrous cycle and corpus luteum morphology

Three of 12 (25%) ewes treated with BQ-610 (in the successive named responsive BQ-610-treated ewes) did not show estrus before d 21 compared to 0 of 9 (0%) vehicle-treated ewes ($P = 0.33$); the remaining nine BQ-610 ewes had cycles averaging 15.3 ± 0.2 d. Estrous cycles in the responsive BQ-610-treated ewes were at least 5.5 d longer than in vehicle-treated ewes (> 21 d vs. 15.5 ± 0.2 d, respectively; $P<0.0001$). Differences in size, color and weight of the CL were important aspects to assess the effect of blocking ENDRA (Fig. 18 and 19) during spontaneous luteolysis in sheep. Corpora lutea from the three responsive BQ-treated ewes were approximately 1.6 times heavier and had 2.4 times more volume than CL from vehicle-treated ewes. In addition, CL from responsive BQ-610-treated ewes were pink while CL from control ewes had pallid color.

Serum and luteal progesterone

Serum concentrations of P_4 in the three responsive BQ-610-treated ewes remained above 1.5 ng/ml through d 21 of the cycle ($P < 0.01$). In this group, P_4 concentration averaged 2.97 ± 0.08 ng/ml from d 11 through d 16 and decreased to 1.91 ± 0.10 ng/ml from d 17 to d 21 of the cycle ($P<0.0001$). In non-responsive BQ-610-treated ewes, initial concentration of P_4 was 1.75 ng/ml and decreased gradually to minimal values by d 15 of the estrous cycle. In vehicle-treated ewes, P_4 concentration remained above 2 ng/ml from d 11 to d 13 and then decreased progressively until d 15 of the estrous cycle (Fig 20). Luteal content of P_4 was 16 fold greater in responsive BQ-610-treated than in vehicle-treated ewes (Fig 21).

Cellular morphology

Luteal tissue in responsive BQ-610-treated ewes appeared normal with 53.3 ± 5.8% of apoptotic cells, whereas luteal tissue in vehicle-treated ewes was markedly disorganized and in an advanced stage of structural regression (Fig 22). In this tissue it was not possible to detect apoptotic cells.

Gene expression

To examine functional status of the CL, expression of genes involved in progesterone production or structural luteolysis was determined in ewes treated with BQ-610 or vehicle and in mid-phase CL (n = 3 per group; Table 6). Although StAR mRNA expression was about 3 to 5 times greater in mid-phase CL and responsive BQ-610-treated ewes than in vehicle-treated ewes, differences were not significant. Also, luteal tissue from responsive BQ-610-treated ewes and mid-phase CL had greater expression of transcript for 3βHSD and TIMP-1 than vehicle-treated group. In regressed CL, both ppEND1 and ENDRA mRNA expression were greater ($P \leq 0.05$) than mid-phase CL or responsive BQ-610-treated CL. The expression of Bcl-2 was not affected by treatments; nevertheless, it was 3.9 and 1.7 times greater in mid-phase CL than in CL from responsive BQ-610-treated or vehicle-treated ewes, respectively. Bax mRNA expression was significantly lower in responsive BQ-610-treated and mid-phase CL than in the vehicle-treated group. The Bcl-2:Bax ratios were 2.25, 0.69 and 0.64 for mid-phase CL, responsive BQ-610-treated and vehicle-treated ewes, respectively ($P > 0.05$). There was no significant effect of treatment on the expression of Fas-R. In contrast, mid-phase CL had lower expression of caspase 3 than either BQ-610-treated or vehicle-treated CL.

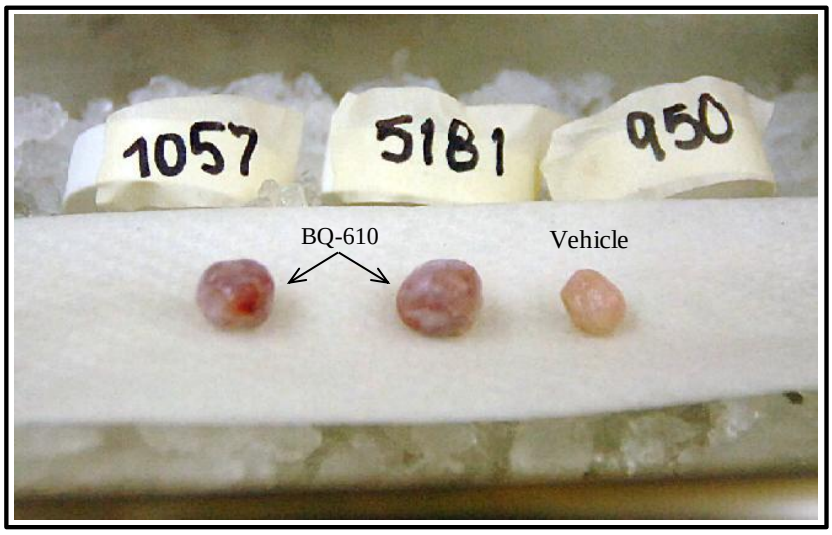

Figure 18. Representative picture of macroscopic characteristics of corpora lutea collected 12 h after onset of estrus in vehicle-treated ewes, or on the afternoon of d 21 in BQ-610-treated ewes. Corpus luteum from vehicle-treated ewe was smaller and pallid with respect to BQ-610 treated ewes.

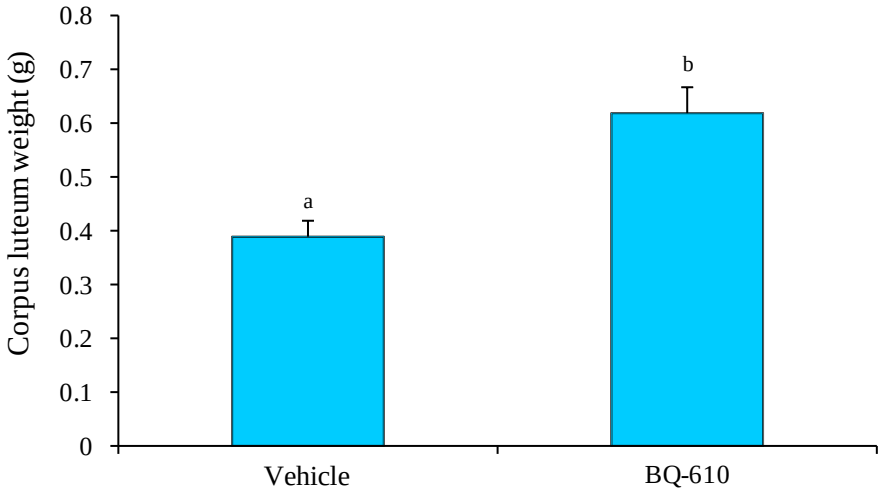

Figure 19. Effect of chronic administration of either BQ-610 (n = 3) or vehicle (n = 9) during spontaneous luteolysis on corpus luteum weight in sheep. Bars represent mean weight (g) ± SEM of corpora lutea collected 12 h after onset of estrus in vehicle-treated ewes, or on the afternoon of d 21 in BQ-610-treated ewes. Values are statistically different ($^{a,b}P<0.002$).

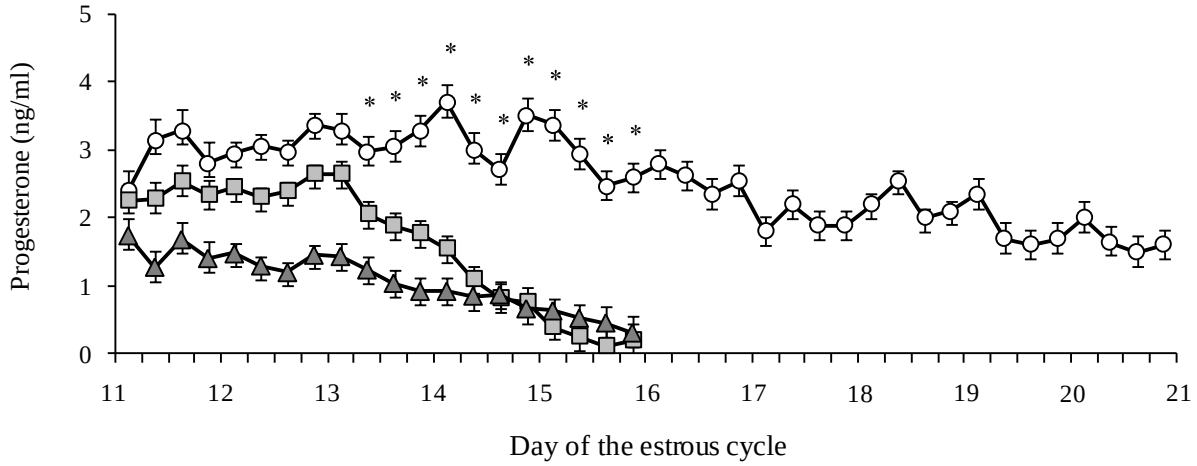

Figure 20. Effect of chronic administration of either BQ-610 or vehicle during spontaneous luteolysis on serum concentrations of progesterone in sheep. Blood samples were collected by jugular venipuncture at 6 h intervals from d 11 until estrus or until d 21 of the estrous cycle in ewes that did not return to estrus. Data points represent mean (± SEM) serum progesterone (ng/ml) per time of sampling for responsive BQ-610-treated (n = 3; ○), non-responsive BQ-610-treated (n = 9; ▲) and vehicle-treatted (n = 9; ■) ewes. Values are statistically different between treatments at designated time point (∗P<0.01).

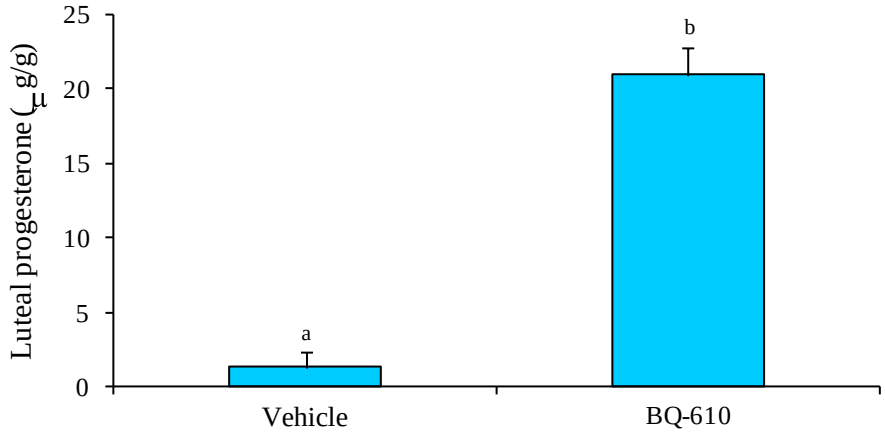

Figure 21. Effect of chronic administration of either BQ-610 (n = 3) or vehicle (n = 9) during expected spontaneous luteolysis on luteal content of progesterone in sheep. Bars represent mean (± SEM) concentrations (μg/g) of progesterone in homogenized corpus luteum extracts. Values are statistically different (^{a,b}P<0.0001).

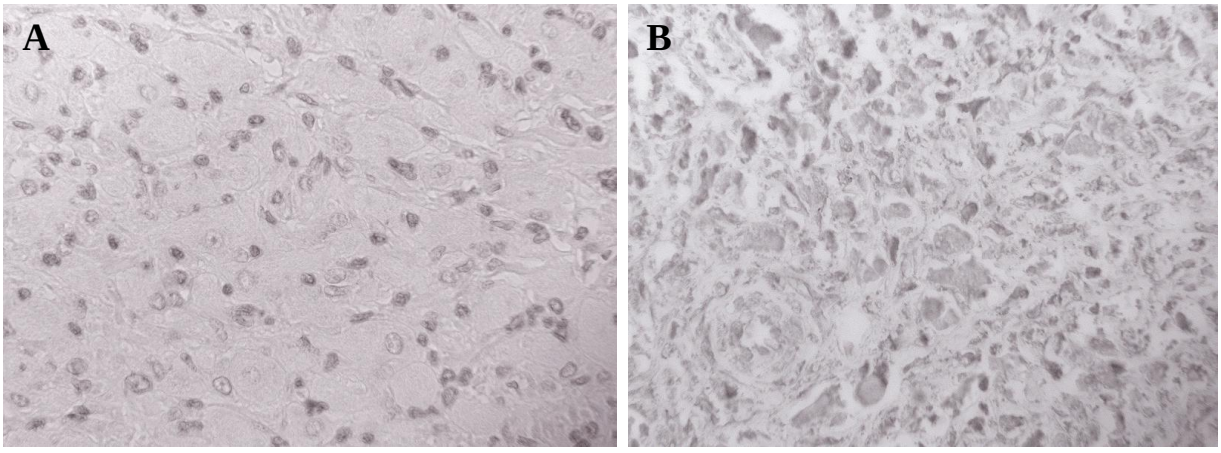

Figure 22. Representative examples of cellular morphology and apoptosis in luteal tissue after chronic administration of either (A) BQ-610 (n = 3) or vehicle (n = 9) during spontaneous luteolysis in sheep. In responsive BQ-610-treated ewes luteal tissue appeared normal with 53.3 ± 5.8% of apoptotic cells, whereas it was markedly disorganized and in an advanced stage of structural regression in vehicle-treated ewes.

Table 6. Effect of chronic administration of either BQ-610 or vehicle during expected spontaneous luteolysis on expression of genes involved in progesterone production or structural luteolysis in sheep.

Gene	Treatments *			Level of significance
	Mid-phase CL	BQ-610	Vehicle	
StAR	1.80 ± 1.15 [a]	2.49 ± 1.15 [a]	0.47 ± 1.15 [a]	P > 0.05
3βHSD	0.55 ± 0.08 [a]	1.22 ± 0.08 [b]	0.01 ± 0.08 [c]	[a,b,c] P < 0.01
TIMP-1	1.06 ± 0.11 [a]	0.81 ± 0.11 [a]	0.28 ± 0.11 [b]	[a,b] P < 0.01
ppEND1	0.69 ± 0.27 [a,b]	0.97 ± 0.27 [b]	1.96 ± 0.27 [c]	[a,c] P < 0.04; [b,c] P < 0.01
ENDRA	0.40 ± 0.39 [a]	0.56 ± 0.39 [a]	3.24 ± 0.48 [b]	[a,b] P < 0.01
Bcl-2	2.90 ± 0.86 [a]	0.76 ± 0.86 [a]	1.67 ± 1.48 [a]	P > 0.05
Bax	1.29 ± 0.23 [a]	1.26 ± 0.23 [a]	2.61 ± 0.30 [b]	[a,b] P < 0.02
Fas-R	0.77 ± 0.18 [a]	1.16 ± 0.18 [a]	0.95 ± 0.32 [a]	P > 0.05
Caspase 3	1.47 ± 0.53 [a]	3.16 ± 0.53 [b,c]	3.91 ± 0.65 [c]	[a,b] P < 0.08; [a,c] P < 0.04

* Three samples per treatment. Data are expressed as mean ± SEM.

Discussion

In this study, blocking of ENDRA prevented endogenous luteolysis for more than 5 days in only 25% of 12 BQ-treated ewes, compared with none of the 9 ewes treated with vehicle. Data on macroscopic characteristics of the CL, serum and luteal concentration of P_4 and gene expression supported this observation, and confirmed previously published in vivo studies in ruminants about the role of END1 in mediating luteolytic actions of $PGF_{2\alpha}$ (Hinckley and Milvae, 2001; Doerr et al., 2008; Keator et al., 2008). Although intraluteal injection of BQ-123 (an ENDRA antagonist) did not reverse completely the luteolytic effect of $PGF_{2\alpha}$, intramuscular injection of both END1 (100 μg) and $PGF_{2\alpha}$ (7.5 mg) effectively reduced P_4 concentrations by 48 h post-treatment, and shortened the length of the cycle (Hinckley and Milvae, 2001).

A large body of evidence indicates that END1, a 21-amino acid peptide that regulates vascular function in different tissues, participates in the luteolytic cascade initiated by uterine $PGF_{2\alpha}$ at the end of the luteal phase. In ruminants, a greater ppEND1 and ENDRA mRNA expression was observed at the end of estrous cycle near the time of luteolysis (Mamluk et al., 1999; Girsh et al., 1996b) and after exogenous $PGF_{2\alpha}$ during mid-luteal phase (Levy et al., 2000; Hinckley and Milvae, 2001; Choudhary et al., 2004). In addition, END1 reduced both basal and LH-stimulated production of P_4 and this anti-steroidogenic effect was reversed by the addition of a selective ENDRA antagonist (Girsh et al., 1996a; Hinckley and Milvae, 2001; Doerr et al., 2008). In concordance with the previous reports, expression of ppEND1 and ENDRA mRNA in vehicle-treated ewes was greater by the time of estrus than in mid-cycle CL

and responsive BQ-treated CL, which further supports the relevance of the END system in mediating luteal regression in ruminants.

Different methods for administration or delivery of ENDRA antagonists in vivo have been applied to test the role of END1 in luteal regression. Apparently, intraluteal injection of a selective ENDRA antagonist (BQ-610 or LU-13522) was ineffective to reverse the decline of P_4 production induced by $PGF_{2\alpha}$ in cows (Watanabe et al., 2006; Keator et al., 2008). In contrast, intrauterine infusions of 500 μg of BQ-610 every 12 h from d 16 through 18 of the estrous cycle delayed spontaneous luteolysis in heifers for about 2 days. Moreover, BQ-610 chronically delivered into the CL by means of an osmotic mini-pump effectively reversed the luteolytic effect of $PGF_{2\alpha}$ 12 h after its administration, allowing concentrations of serum P_4 attain similar values to the control group by 48 h after $PGF_{2\alpha}$ (Doerr et al., 2008). Apparently, a direct action of $PGF_{2\alpha}$ decreased serum P_4 during early luteolysis, whereas END1 was required in later stages, indicating that END1 might participate in structural more than functional luteolysis.

This study followed the same experimental approach utilized by Doerr et al. (2008), but the greater dosage of BQ-610 prevented spontaneous luteolysis and lengthened the estrous cycle in only 3 of 12 (25%) ewes. Endogenous $PGF_{2\alpha}$ starts to be secreted from the uterus in increased amounts around day 11 to 13 of the sheep estrous cycle (Silvia et al., 1984; Zarco et al. 1988b). The sustained delivery of BQ-610 for more than 8 days after the beginning of endogenous $PGF_{2\alpha}$ secretion effectively blocked endogenous END1 in those 3 ewes.

Different methods to assess the functional features in luteal tissue treated with BQ-610 or vehicle were used in this experiment: macroscopic and histological characteristic, steroidogenic capacity and expression of genes related to P_4 synthesis and structural luteolysis. Thus, data from this experiment evaluated both functional and structural aspects of luteolysis.

Functional regression of the CL is characterized by a rapid decrease of P_4 production by luteal steroidogenic cells coincident with a transient increased followed by a diminution of blood flow to the CL (Niswender et al., 1975; Nett et al., 1976). During this short period synthesis and secretion of P_4 is inhibited, mostly by interrupting mobilization of cholesterol throughout the cytoplasm and outer mitochondrial membrane (Murdoch, 1996; Juengel et al., 2000; Tsai et al., 2001). Thus, lacking the substrate to synthesize P_4, steroidogenic enzyme activity declines and less P_4 is secreted by large and small luteal cells. Although differences in mRNA expression for StAR were not significant, probably due to the large variation among samples (and few samples per treatment), values from responsive BQ-610 and mid-cycle CL were 5.3 and 3.8 times greater than in CL of vehicle-treated ewes. Transcript concentration for 3βHSD was greater in responsive BQ-treated and mid-cycle CL than in vehicle-treated CL. Hence, there were greater serum and luteal concentrations of P_4 in responsive BQ-610-treated ewes. These data, together with similar expression values for StAR and 3βHDS transcripts in mid-cycle CL and responsive BQ-610-treated CL, strongly indicate the cellular metabolic machinery to synthesize P_4 by luteal steroidogenic cells was completely operative in responsive BQ-610-treated ewes at day 21 of the estrous cycle.

In vehicle-treated ewes, luteal weight 12 h after estrus was 0.39 ± 0.03 g, about 37% lighter than CL from responsive BQ-610-treated ewes (0.62 ± 0.05 g) on day 21 of the cycle.

Moreover, judged by color, CL from the three responsive BQ-610-treated ewes were larger and appeared healthier than those collected from vehicle-treated ewes. As seen by Braden et al. (1988), Juengel et al. (1996) and Tsai et al. (1998a), CL weight significantly decreased 30 to 50% by 24 to 36 h after exogenous $PGF_{2\alpha}$ in sheep. The decrease in CL weight represents clear evidence that luteal regression has been already induced and implies death and phagocytosis of cellular components, mostly endothelial and steroidogenic luteal cells, and degradation of cellular matrix as well (Braden et al., 1988; Sawyer et al., 1990; Pate and Keyes, 2001; Smith et al., 2002). However, morphological alterations were not evident until 24 to 36 h after initial exposure to $PGF_{2\alpha}$ (Sawyer et al., 1990).

The expression pattern of genes related with structural luteolysis appeared to be less clear. Although the expression of Bax mRNA was significantly lower in BQ-610 and mid-cycle CL compared with vehicle CL, Bcl-2 and Fas-R expression did not differ among treatments. Moreover, the ratio between Bcl-2 and Bax mRNA was around 3.5 times greater in mid-cycle CL than in CL of either responsive BQ-610-treated or vehicle-treated ewes. Bcl-2 and Bax are proteins that prevent and promote apoptosis, respectively, and their relative abundance at a specific luteal stage could favor or oppose apoptosis (Tilly et al., 1996). While Bcl-2 prevents apoptosis by regulating mechanisms related to Ca^{2+} homeostasis and oxidative stress (Kane et al., 1993; Pinton et al., 2001), Bax promotes apoptosis by antagonizing anti-apoptotic actions of Bcl-2 (Nutt et al., 2002; Chami et al., 2004). Concentrations of Bax but not Bcl-2 mRNA and protein increased after $PGF_{2\alpha}$-induced luteolysis in ruminants (Rueda et al., 1997; Yadav et al., 2005); this change was reflected in Bax/Bcl-2 ratio for both mRNA and protein that was significantly greater at 4 h than immediately before $PGF_{2\alpha}$ injection (Yadav et al., 2005). In

addition, both protein and activity of caspases 3 and 9 increased after $PGF_{2\alpha}$ (Yadav et al., 2005). Thus, it is evident that $PGF_{2\alpha}$ upregulates proteins that induce apoptosis in luteal cells. Interestingly, $PGF_{2\alpha}$ significantly increased the expression of caspase 3 in vehicle-treated animals as well as in BQ-610-treated ewes compared with mid-cycle CL. This finding agrees with the fact that luteal tissue from the three responsive BQ-610-treated ewes had 53.3 ± 5.8% of apoptotic cells and that serum concentrations of P_4 in this group were about 36% lower from day 16 through 21 of the cycle compared with the previous days. Nevertheless, luteal concentrations of P_4 were elevated, indicating that CL from responsive BQ-610-treated ewes were completely active. To provide a physiological explanation for this discrepancy it is important to consider two aspects. First, BQ-610 blocks ENDRA but does not prevent $PGF_{2\alpha}$ from binding to its receptor. In pregnant ewes (Nett et al., 1976; Lewis et al., 1977; Vincent and Inskeep, 1986), secretion of uterine $PGF_{2\alpha}$ around d 21 post estrus is still elevated to mid range, and consequently $PGF_{2\alpha}$ may be inducing intracellular changes at mRNA or even protein levels by different pathways than END1 utilizes to mediate $PGF_{2\alpha}$-induced luteolysis. Second, anti-apoptotic and protective effects of P_4 on luteal function have been documented (Rueda et al., 2000; Goyeneche et al., 2003; Okuda et al., 2004; Nishimura et al., 2008). Thus, elevated concentrations of P_4 in BQ-610-treted ewes may counteract the luteolytic actions of $PGF_{2\alpha}$ by inhibiting or reducing activity of pro-apoptotic factors.

In summary, chronic delivery of BQ-610 into the CL during mid- to late-luteal phase prevented natural luteolysis and prolonged the estrous cycle for more than 5 days in only 3 of 12 ewes. Macroscopic and functional characteristics of those responsive BQ-610-treated CL

were similar to mid-phase CL. Overall this study indicates that END1 might plays mediatory role during spontaneous luteolysis in the ewe.

General discussion

Immediately after the LH surge, the pattern of steroid secretion from the preovulatory follicle in known to change abruptly (Murdoch and Dunn, 1982; Fortune and Hansel, 1985) from estrogen to P_4 dominance. It was postulated that END1, a 21-amino acid peptide with recognized anti-steroidogenic properties, might modulate steroid production preventing premature luteinization of granulosal and thecal cells (Tedeschi et al., 1992; Flores, 2000). In experiment 1, the protocol designed for collection of large preovulatory follicles was successful and granulosal cells from 20 of 28 follicles were cultured and challenged to 10 different treatments, resultant from combinations of LH, END1, ENDRA and ENDRB. However, neither E_2 nor P_4 accumulation in the culture media was affected by treatments after 24 h of incubation. To interpret these findings, it is important consider three aspects. First, granulosal cells from large preovulatory follicles were exposed to greater concentrations of endogenous LH for several hours and thereafter to supplemented LH during in vitro incubation. Second, removing granulosal cells in this particular follicular developmental stage and culturing them in a serum free culture medium may have triggered their luteinization; as judged by greater concentrations of P_4 than E_2 in the culture media. Third, as occurs during luteolysis, END1 may act in conjunction with other locally produced ovarian mediators regulating follicular steroid production, and therefore, the absence of these factors in the culture media may have allowed luteinization of granulosal cells. Apparently, a more appropriate culture system would be necessary to test the role of END1 in steroid production by granulosal cells in large

preovulatory follicles in sheep. Alternatively, collection of preovulatory follicles earlier, and incorporation of FSH and serum in culture media, may allow elucidation of the role of END1 in modulating steroid production by granulosal cells prior to ovulation in sheep. In earlier in vitro studies, FSH-simulated P_4 production by granulosal cells collected from small- and medium-sized swine follicles was inhibited by END1 (Kamada et al., 1993; Flores et al., 1992; 1999). However, END1 also reduced production of LH-stimulated P_4 by granulosal cells isolated from medium-sized follicles in pigs (Iwai et al., 1991).

A large body of evidence indicates that END1 plays an important role in luteal regression (Girsh et al., 1996a; 1996b; Miyamoto et al., 1997; Ohtani et al., 1998; Hinckley and Milvae, 2001; Doerr et al., 2008; Keator et al., 2008). In experiment 2, the role of END1 in structural luteolysis was not elucidated, as judged by concentrations of serum and luteal P_4 and CL weight, as well as the over expression of the genes 3β-HSD and caspases 3 and 8 in BQ-610-treated compared to vehicle-treated luteal tissue. Apparently, the increased rate of delivery of BQ-610 into the CL in combination with greater concentrations of methanol and individual animal features might have interfered with the binding ability or modified the molecular structure of BQ-610. Also, blockade of ENDRA by BQ-610 may have allowed END1 to bind ENDRB and trigger downstream signaling through this receptor contributing to an increase in the luteolytic signal. In the cow, ENDRB mRNA expression was greater at the end of luteal phase, during luteal regression (Berisha et al., 2002) and after an exogenous dose of $PGF_{2\alpha}$ (Schams et al., 2003; Watanabe et al., 2006) indicating a putative role of this receptor during luteolysis. However, concentrations of ENDRB mRNA in luteal cells were minimal during $PGF_{2\alpha}$-induced luteolysis in sheep (Doerr et al., 2008). In addition, the luteolytic effect of

$PGF_{2\alpha}$ was reversed by blocking ENDRA but not ENDRB (Doerr et al., 2008) in the ovine CL. The ENDRB is more abundant in vascular endothelial cells and its activation by END1 stimulated releases of NO (Ishiguro et al., 2001; Hirata et al., 1993), which is an important inhibitor of luteal P_4 production. It was proposed that NO acts as a local mediator of $PGF_{2\alpha}$-induced regression in bovine CL (Jaroszewski and Hansel, 2000; Jaroszewski et al., 2003). Thus, the greater availability of endogenous END1 during induced and spontaneous luteolysis in experiments 2 and 3, respectively, as well as the hypothetical increased concentrations of ENDRB at that time, may have induced direct and/or indirect luteolytic signals that caused over expression of pro-apoptotic factors in those animals in which luteolysis was not prevented.

Under the experimental conditions described above, blockage of ENDRA in experiments 1 and 2 prevented luteolysis in only 10 and 25% of the treated animals, compared to 100% in the earlier study by Doerr et al. (2008). Pharmacological dosage of $PGF_{2\alpha}$ in experiment 1 versus physiological secretion of uterine $PGF_{2\alpha}$ in experiment 2 may account for the 15 percentage point difference. In addition to the increased concentrations of methanol in the vehicle, placement of the catheter into the CL may have disrupted the functional capacity of the CL treated with BQ-610. Therefore, secretion of lower amounts of progesterone was unable to counteract the direct and indirect luteolytic actions of $PGF_{2\alpha}$., and as a consequence, luteolysis was not prevented. Perhaps lower luteal damage and greater steroidogenic capacity of the CL in those BQ-610 responsive animals during natural luteolysis might explain why BQ-610 effectively prevented luteolysis in 25% of the treated animals compared with only 10% of the animals in experiment 2, in which luteolysis was induced by a pharmacological dose of $PGF_{2\alpha}$.

LITERATURE CITED

Abramovitz M, Boie Y, Nguyen T, Rushmore TH, Bayne MA, Metters KM, Slipetz DM, Grygorczyk R. 1994. Cloning and expression of a cDNA for the human prostanoid FP receptor. J Biol Chem 269:2632-2636.

Acosta TJ, Miyamoto A, Ozawa T, Wijayagunawardane MPB, Sato K. 1998. Local release of steroids hormones, prostaglandin E2, and endothelin-1 from bovine mature follicle in vitro: effects of luteinizing hormone, endothelin-1, and cytokines. Biol Reprod 59:437-443.

Acosta TJ, Yoshizawa N, Ohtani M, Miyamoto A. 2002. Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F(2 alpha) injection in the cow. Biol Reprod 66:651-658.

Acosta TJ, Miyamoto A. 2004. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. Anim Reprod Sci 82-83:127-140.

Acosta TJ, Bah MM, Korzekwa A, Woclawek-Potocka I, Markiewicz W, Jaroczewski JJ, Okuda K, Skarzynski DJ. 2008. Acute changes in circulating concentrations of progesterone and nitric oxide and partial pressure of oxygen during prostaglandin f2alpha-induced luteolysis in cattle. J Reprod Dev, DOI: http://www.jstage.jst.go.jp/article/jrd/advpub/0/0812180122/_pdf.

Adams GP, Kot K, Smith CA, Ginther OJ. 1993. Selection of a dominant follicle and suppression of follicular growth in heifers. Anim Reprod Sci 30:259-271.

Agudo LSp, Smith MF. Effect of PGF2α on adenylate cyclase and phosphodiesterase activity of ovine corpora lutea. Biol Reprod 26(Suppl. 1):134A. (Abstract)

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. Molecular Biology of the Cell. Garland Sciences. Fourth Edition. New York, NY. p 838-839.

Alila HW, Davis JS, Dowd JP, Corradino RA, Hansel W. 1990. Differential effects of calcium on progesterone production in small and large bovine luteal cells. J Steroid Biochem 36:687-693.

Allen WR. 2001. Luteal deficiency and embryo mortality in the mare. Reprod Dom Anim 36:121-131.

Amsterdam A, Berkowitz A, Nimrod A, Kohen F. 1980. Aggregation of luteinizing hormone receptors in granulosa cells: a possible mechanism of desensitization to the hormone. Proc Natl Acad Sci 77:3440-4.

Armstrong DG, Baxter G, Gutierrez CG, Hogg CO, Glazyrin AL, Campbell BK, Bramley TA, Webb R. 1998. Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinology 139:2146-2154.

Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348:730-762.

Aramori I, Nakanishi S. Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. J Biol Chem 267:12468-12474.

Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. 2004. Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. Endocrinology 145:2551-2560.

Auletta FJ, Kamps DL, Pories S, Bisset J, Gibson M. 1984. An intra-corpus luteum site for the luteolytic action of prostaglandin F2 in the rhesus monkey. Prostaglandins 27:285–298.

Austin EJ, Mihm M, Evans AC, Knight PG, Ireland JL, Ireland JJ, Roche JF. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 64:839-848.

Bacci ML, Barazzoni AM, Forni M, Costerbosa GL. 1996. In situ detection of apoptosis in regressing corpus luteum of pregnant sow: evidence of an early presence of DNA fragmentation. Domest Anim Endocrinol 13:361-372.

Bagavandoss P, Kunkel SL, Wiggins RC, Keyes PL. 1988. Tumor necrosis factor-a (TNF-a) production and localization of macrophages and T lymphocytes in the rabbit corpus luteum. Endocrinology 122:1185-1187.

Balapure AK, Caicedo IC, Kawada K, Watt DS, Rexroad CE Jr, Fitz TA. 1989. Multiple classes of prostaglandin F2 alpha binding sites in subpopulations of ovine luteal cells. Biol Reprod 41:385-392.

Banu SK, Lee J, Satterfield MC, Spencer TE, Bazer FW, Arosh JA. 2008. Molecular cloning and characterization of prostaglandin (PG) transporter in ovine endometrium: role for multiple cell signaling pathways in transport of PGF2alpha. Endocrinology 149:219-231.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. 1997a. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. Biol Reprod 56:1158-1168.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. 1997b. Expression of messenger ribonucleic acid (mRNA) encoding 3beta-hydroxysteroid dehydrogenase delta4,delta5 isomerase (3beta-HSD) during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by expression of 3beta-HSD mRNA within the granulosa cell layer. Biol Reprod 56:1466-1473.

Bao B, Calder MD, Xie S, Smith MF, Salfen BE, Youngquist RS, Garverick HA. 1998. Expression of steroidogenic acute regulatory protein messenger ribonucleic acid is limited to theca of healthy bovine follicles collected during recruitment, selection, and dominance of follicles of the first follicular wave. Biol Reprod 59:953-959.

Bao B, Garverick HA. 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 76:1903-1921.

Barrett DM, Bartlewski PM, Duggavathi R, Davies KL, Rawlings NC. 2006. Suppression of follicle wave emergence in cyclic ewes by supraphysiologic concentrations of estradiol-17beta and induction with a physiologic dose of exogenous ovine follicle-stimulating hormone. Biol Reprod 75:633-641.

Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A, Rawlings NC. 1999. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. J Reprod Fertil 115:111-1124.

Bauer M, Reibiger I, Spanel-Borowski K. 2001. Leucocyte proliferation in the bovine corpus luteum. Reproduction 121:297-305.

Beg MA, Bergfelt DR, Kot K, Wiltbank MC, Ginther OJ.Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol Reprod 64:432-441.

Bennegard B, Hahlin M, Wennberg E, Noren H. 1991. Local luteolytic effect of prostaglandin F2 in the human corpus luteum. Fertil Steril 56:1070–1076.

Benyo DF, Pate JL. 1992. Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. Endocrinology 130:854-860.

Bergfelt DR, Kulick LJ, Kot K, Ginther OJ. 2000. Follicular and hormonal response to experimental suppression of FSH during follicle deviation in cattle. Theriogenology 54:1191-1206.

Berisha B, Schams D, Miyamoto A. 2002. The expression of angiotensin and endothelin system members in bovine corpus luteum during estrous cycle and pregnancy. Endocrine 19:305-312.

Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 212:473-482.

Berridge MJ. 1987. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 56:159-193

Best CL, Pudney J, Welch WR, Burger N, Hill JA. 1996. Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. Hum Reprod Apr 11:790-797.

Blondel O, Takeda J, Janssen H, Seino S, Bell GI. 1993. Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. J Biol Chem 268:11356-11363.

Bogan RL, Murphy MJ, Stouffer RL, Hennebold JD. 2008. Prostaglandin synthesis, metabolism, and signaling potential in the rhesus macaque corpus luteum throughout the luteal phase of the menstrual cycle. Endocrinology 149:5861-5871.

Boiti C, Guelfi G, Zampini D, Brecchia G, Gobbetti A, Zerani M. 2003. Regulation of nitric oxide synthase isoforms and role of nitric oxide during prostaglandin F2alpha-induced luteolysis in rabbits. Reproduction 125:807-816.

Boiti C, Guelfi G, Brecchia G, Dall'Aglio C, Ceccarelli P, Maranesi M, Mariottini C, Zampini D, Gobetti A, Zerani M. 2005. Role of endothelin-1 system in the luteolytic process of pseudopregnant rabbits. Endocrinology 146:1293-1300.

Boonyaprakob U, Gadsby JE, Hedgpeth V, Routh P, Almond GW. 2003. Cloning of pig prostaglandin F2alphaFP receptor cDNA and expression of its mRNA in the corpora lutea. Reproduction 125:53-64.

Bourdage RJ, Fitz TA, Niswender GD. 1984. Differential steroidogenic responses of ovine luteal cells to ovine luteinizing hormone and human chorionic gonadotropin. Proc Soc Exp Biol Med 175:483-486.

Bowen JM, Keyes PL, Warren JS, Townson DH. 1996. Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. Biol Reprod 54:1120-1127.

Bowen JM, Towns R, Warren JS, Landis Keyes P. 1999. Luteal regression in the normally cycling rat: apoptosis, monocyte chemoattractant protein-1, and inflammatory cell involvement. Biol Reprod 60:740-746.

Braden TD, Gamboni F, Niswender GD. 1988. Effects of prostaglandin F2 alpha-induced luteolysis on the populations of cells in the ovine corpus luteum. Biol Reprod 39:245-253.

Braun NS, Heath E, Chenault JR, Shanjs RD, Hixon JE. 1988. Effects of prostaglandins F2 alpha on degranulation of bovine luteal cells on days 4 and 12 of the estrous cycle. Am J Vet Res 49:516-519.

Bruce NW, Moor RM. 1976. Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep. J Reprod Fertil 46:299-304.

Bukovský A, Caudle MR, Keenan JA, Wimalasena J, Upadhyaya NB, Van Meter SE. 1995. Is corpus luteum regression an immune-mediated event? Localization of immune system components and luteinizing hormone receptor in human corpora lutea. Biol Reprod 53:1373-1384.

Burgess GM, Irvine RF, Berridge MJ, McKinney JS, Putney JW Jr. 1984. Actions of inositol phosphates on Ca2+ pools in guinea-pig hepatocytes. Biochem J 224:741-746.

Campbell BK, Souza CJ, Skinner AJ, Webb R, Baird DT. 2006. Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation in vitro to gonadotropins and bone morphogenic protein-2, -4, and -6. Endocrinology 147:1608-1620.

Carlson JC, Buhr MM, Riley JC. 1984. Alterations in the cellular membranes of regressing rat corpora lutea. Endocrinology 114:521-526.

Castracane VD, Moore GT, Shaikh AA. 1979. Ovarian function of hysterectomized Macaca fascicularis. Biol Reprod 3:462-472.

Cavicchio VA, Pru JK, Davis BS, Davis JS, Rueda BR, Townson DH. 2002. Secretion of monocyte chemoattractant protein-1 by endothelial cells of the bovine corpus luteum: regulation by cytokines but not prostaglandin F2alpha. Endocrinology. 143:3582-3589.

Chami M, Prandini A, Campanella M, Pinton P, Szabadkai G, Reed JC, Rizzuto R. 2004. Bcl-2 and Bax exert opposing effects on Ca2+ signaling, which do not depend on their putative pore-forming region. J Biol Chem 279:54581-54589.

Chen DB, Westfall SD, Fong HW, Roberson MS, Davis JS. 1998. Prostaglandin F2alpha stimulates the Raf/MEK1/mitogen-activated protein kinase signaling cascade in bovine luteal cells. Endocrinology 139:3876-3885.

Choudhary E, Costine BA, Wilson ME, Inskeep EK, Flores JA. 2004. Prostaglandin F2α ($PGF_{2\alpha}$) independent and dependent regulation of the bovine luteal endothelin system. Dom Anim Endocrinol 27:63-79.

Choudhary E, Sen A, Inskeep EK, Flores JA. Developmental sensitivity of the bovine corpus luteum to prostaglandin F2alpha (PGF2alpha) and endothelin-1 (ET-1): is ET-1 a mediator of the luteolytic actions of PGF2alpha or a tonic inhibitor of progesterone secretion? Biol Reprod 2005 72:633-642.

Clark LJ, Irving-Rodgers HF, Dharmarajan AM, Rodgers RJ. 2004. Theca interna: the other side of bovine follicular atresia. Biol Reprod 71:1071-1078.

Clarke JG, Larkin SW, Benjamin N, Keogh BE, Chester A, Davies GJ, Taylor KM, Maseri A. 1989. Endothelin-1 is a potent long-lasting vasoconstrictor in dog peripheral vasculature in vivo. J Cardiovasc Pharmacol 13(Suppl 5):S211-S212.

Colagero AE, Burrello N, Ossino AM 1998. Endothelin (ET)-1 and ET-3 inhibit estrogen and cAMP production by rat granulosa cells in vitro. J Endocrinol 157:209-215.

Compton MM. 1992. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev (2):105-119.

Currie WB, Thorburn GD. 1974. Luteal function in hysterectomized goats. J Reprod Fert 41:501-504.

Davis JS, Weakland LL, Weiland DA, Farese RV, West LA. 1987a. Prostaglandin F2 alpha stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilizes intracellular Ca2+ in bovine luteal cells. Proc Natl Acad Sci U S A 84:3728-3732.

Davis JS, Weakland LL, Farese RV, West LA. 1987b. Luteinizing hormone increases inositol trisphosphate and cytosolic free Ca2+ in isolated bovine luteal cells. J Biol Chem 262:8515-8521.

Davis JS, Alila HW, West LA, Corradino RA, Hansel W. 1988. Acute effects of prostaglandin F2 alpha on inositol phospholipid hydrolysis in the large and small cells of the bovine corpus luteum. Mol Cell Endocrinol 58:43-50.

Dawson AP. 1997. Calcium signalling: how do IP3 receptors work? Curr Biol 7:R544-547.

Del Campo CH, Ginther OJ. 1972. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: Guinea pigs, rats, hamsters, and rabbit. Am J Vet Res 33:2561-2578.

Del Campo CH, Ginther OJ. 1973a. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: Horses, sheep, and swine. Am J Vet Res 34:305-316.

Del Campo CH, Ginther OJ. 1973b. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: Angioarchitecture in sheep. Am J Vet Res 34:1377-1385.

Denkova R, Bourneva V, Yaneva E, Baleva K, Nikolov B, Ivanov I, Simenov K, Timeva T. 2002. Endocrine Regulations 36:19-22.

Davenport A, Maguire J. 2006. Endothelin. HEP 176/I:295-329.

Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ, Wiltbank MC. 2002. Regulation of progesterone and prostaglandin F2alpha production in the CL. Mol Cell Endocrinol 191:65-80.

Diaz FJ, Wiltbank MC. 2005. Acquisition of luteolytic capacity involves differential regulation by prostaglandin F2alpha of genes involved in progesterone biosynthesis in the porcine corpus luteum. Domest Anim Endocrinol 28:172-189.

Díaz-Muñoz M, de la Rosa Santander P, Juárez-Espinosa AB, Arellano RO, Morales-Tlalpan V. 2008. Granulosa cells express three inositol 1,4,5-trisphosphate receptor isoforms: cytoplasmic and nuclear Ca2+ mobilization. Reprod Biol Endocrinol 6:60.

Dickson SE, Fraser HM. 2000. Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate. J Clin Endocrinol Metab 85:2339-2344.

Doerr MD, Goravanahally MP, Rhinehart JD, Inskeep EK, Flores JA. 2008. Effects of endothelin receptor type-A and type-B antagonists on prostaglandin F2alpha-induced luteolysis of the sheep corpus luteum. Biol Reprod 78:688-696.

Dorflinger LJ, Luborsky JL, Gore SD, Behrman HR. 1983. Inhibitory characteristics of prostaglandin F2 alpha in the rat luteal cell. Mol Cell Endocrinol 33:225-241.

Dorflinger LJ, Albert PJ, Williams AT, Behrman HR. 1984. Calcium is an inhibitor of luteinizing hormone-sensitive adenylate cyclase in the luteal cell. Endocrinology 114:1208-1215.

Douglas RH, Ginther OJ. 1975. Route of prostaglandin F2alpha injection and luteolysis in mares (38519). Proc Soc Exp Biol Med 148:263-269.

Douglas RH, Ginther OJ. 1976. Concentration of prostaglandins F in uterine venous plasma of anesthetized mares during the estrous cycle and early pregnancy. Prostaglandins. 11:251-260.

Douglas RH, Del Campo MR, Ginther OJ. 1976. Luteolysis following carotid or ovarian arterial injection of prostaglandin F2alpha in mares. Biol Reprod 14:473-477.

Driancourt MC, Gougeon A, Royere D, Thilbault C. 1993. Ovarian function. In: Thibault C, Levasseur MC, Hunter RHF (Editors). Reproduction in Mammals and Man. Paris. Edition Marketing, pp 281-305.

Dubourdieu S, Charbonnel B, Massai MR, Marraoui J, Spitz I, Bouchard P. 1991. Suppression of corpus luteum function by the gonadotropin-releasing hormone antagonist Nal-Glu: effect of the dose and timing of human chorionic gonadotropin administration. Fertil Steril 56:440-445.

Duggavathi R, Bartlewski PM, Barrett DM, Rawlings NC. 2003. Use of high-resolution transrectal ultrasonography to assess changes in numbers of small ovarian antral follicles and their relationships to the emergence of follicular waves in cyclic ewes. Theriogenology 60:495-510.

Duggavathi R, Bartlewski PM, Barrett DM, Gratton C, Bagu ET, Rawlings NC. 2004. Patterns of antral follicular wave dynamics and accompanying endocrine changes in cyclic and seasonally anestrous ewes treated with exogenous ovine follicle-stimulating hormone during the inter-wave interval. Biol Reprod 70:821-827.

Duggavathi R, Bartlewski PM, Agg E, Flint S, Barrett DM, Rawlings NC. 2005. The effect of the manipulation of follicle-stimulating hormone (FSH)-peak characteristics on follicular wave dynamics in sheep: does an ovarian-independent endogenous rhythm in FSH secretion exist? Biol Reprod 72:1466-1474.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. 2002. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-1084.

Dwyer RJ, Church RB. 1979. Effect of prostaglandin F-2 alpha on ovarian enzyme activity in the hysterectomized guinea-pig. J Reprod Fertil 56:85-88.

Echternkamp SE, Howard HJ, Roberts AJ, Grizzle J, Wise T. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biol Reprod 51:971-981.

Ekstrom RC, Hunzicker-Dunn M. 1989. Guanosine triphosphate fulfills a complete and specific nucleotide requirement for luteinizing hormone-induced desensitization of pig ovarian adenylyl cyclase. Endocrinology 125:2470-2474.

Emoto N, Yanagisawa M. 1995. Endothelin-converting Enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. J Biol Chem 270:15262-15268.

Espey LL, Ujioka T, Russell DL, Skelsey M, Vladu B, Robker RL, Okamura H, Richards JS. 2000. Induction of early growth response protein-1 gene expression in the rat ovary in response to an ovulatory dose of human chorionic gonadotropin. Endocrinology 141:2385-91.

Estill CT, Britt JH, Gadsby JE. 1995. Does increased PGF2 alpha receptor concentration mediate PGF2 alpha-induced luteolysis during early diestrus in the pig? Prostaglandins 49:255-267.

Evans AC, Duffy P, Hynes N, Boland MP. 200. Waves of follicle development during the estrous cycle in sheep. Theriogenology 53:699-715.

Evans AC. 2003a. Characteristics of ovarian follicle development in domestic animals. Reprod Domest Anim 38:240-246.

Evans AC. 2003b. Ovarian follicle growth and consequences for fertility in sheep. Anim Reprod Sci 78:289-306

Fairchild DL, Pate JL. 1991. Modulation of bovine luteal cell synthetic capacity by interferon-gamma. Biol Reprod 44:357-363.

Farin CE, Moeller CL, Sawyer HR, Gamboni F, Niswender GD. 1986. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol Reprod 35:1299-1308.

Fitz TA, Mayan MH, Sawyer HR, Niswender GD. 1982. Characterization of two steroidogenic cell types in the ovine corpus luteum. Biol Reprod 27:703-711.

Fitz TA, Mock EJ, Mayan MH, Niswender GD. 1984. Interactions of prostaglandins with subpopulations of ovine luteal cells. II. Inhibitory effects of PGF2 alpha and protection by PGE2. Prostaglandins 28:127-138.

Flint APF, Sheldrick EL, McCann TJ, Jones DSC. 1990. Luteal Oxytocin: characteristics and control of synchronous episodes of oxytocin and PF2α secretion at luteolysis in ruminants. Dom Anim Endocrinol 7:111-124.

Flores JA, Quyyumi S, Leong DA, Valdheuis JD. 1992. Actions of endothelin-1 on swine ovarian (granulosa) cells. Endocrinology 131:1350-1358.

Flores, J.A., Winters T.A., Knight, J.W., Veldhuis, J.D. 1995. Nature of endothelin binding in the porcine ovary. Endocrinology 136:5014-5019.

Flores JA, Garmey JC, Lahav M, Veldhuis JD. 1999. Mechanisms underlaying endothelin's inhibition of FSH-stimulated progesterone production by ovarian granulosa cells. Mol Cel Endocrinol 156:169-178.

Flores J. 2000. Gene expression of endothelin in the porcine ovary: follicular development. Biol Rep 63:1377-1382.

Flores JA, Barlund C. 2009. Prostaglandins and their mechanism of actions in the cyclic ovary. Chedrese PJ (Ed). Reproductive Endocrinology. Springer Science, pp 181-189.

Fortune JE, Armstrong DT. 1977. Androgen production by theca and granulosa isolated from proestrous rat follicles. Endocrinology 100:1341-1347.

Fortune JE, Hansel W. 1985. Concentrations of steroids and gonadotropins in follicular fluid from normal heifers and heifers primed for superovulation. Biol Reprod 32:1069-1079.

Fortune JE. 1986. Bovine theca and granulosa cells interact to promote androgen production. Biol Reprod 35:292-299.

Fortune JE. 2003. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim Reprod Sci 78:135-163.

Freeman ME. 2006. Neuroendocrine control of the ovarian cycle of the rat. In: Neill JD (Editor). Knobil and Kneill's Physiology of Reproduction. Elsevier Academic Press. Third Edition. San Diego, California, pp 2327-2388.

Friedman A, Weiss S, Levy N, Meidan R. 2000. Role of tumor necrosis factor alpha and its type I receptor in luteal regression: induction of programmed cell death in bovine corpus luteum-derived endothelial cells. Biol Reprod 63:1905-1912.

Fujino H, Srinivasan D, Pierce KL, Regan JW. 2000a. Differential regulation of prostaglandin F(2alpha) receptor isoforms by protein kinase C. Mol Pharmacol 57:353-358.

Fujino H, Pierce KL, Srinivasan D, Protzman CE, Krauss AH, Woodward DF, Regan JW. 2000b. Delayed reversal of shape change in cells expressing FP(B) prostanoid receptors. Possible role of receptor resensitization. J Biol Chem 275:29907-29914.

Gadsby JE, Balapure AK, Britt JH, Fitz TA. 1990. Prostaglandin F2 alpha receptors on enzyme-dissociated pig luteal cells throughout the estrous cycle. Endocrinology 126:787-795.

Gaytán F, Morales C, García-Pardo L, Reymundo C, Bellido C, Sánchez-Criado JE. 1998. Macrophages, cell proliferation, and cell death in the human menstrual corpus luteum. Biol Reprod 59:417-425.

Ghinea N, Vu Hai MT, Groyer-Picard MT, Houllier A, Schoëvaërt D, Milgrom E. Pathways of internalization of the hCG/LH receptor: immunoelectron microscopic studies in Leydig cells and transfected L-cells. J Cell Biol 118:1347-1358.

Ginther OJ, Garcia MC, Squires EL, Steffenhagen WP. Anatomy of vasculature of uterus and ovaries in the mare. Am J Vet Res 1972 33:1561-1568.

Ginther OJ. 1974. Internal regulation of physiological processes through venoarterial pathways: a review. J Anim Sci 39:550-564.

Ginther OJ; Del Campo CH. 1974. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: Cattle. Am J Vet Res 35:193-203.

Ginther OJ, Kot K, Wiltbank MC. 1995. Associations between emergence of follicular waves and fluctuations in FSH concentrations during the oestrous cycle in ewes. Theriogenology 43:689-703.

Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 1999. Selection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of FSH concentrations. Theriogenology 52:1079-1093.

Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 2000a. Selection of the dominant follicle in cattle: role of estradiol. Biol Reprod 63:383-389.

Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 2000b. Selection of the dominant follicle in cattle: role of two-way functional coupling between follicle-stimulating hormone and the follicles. Biol Reprod 62:920-927.

Ginther OJ, Bergfelt DR, Beg MA, Kot K. 2001. Follicle Selection in Cattle: Relationships among Growth Rate, Diameter Ranking, and Capacity for Dominance. Biol Reprod 65:345-350.

Girsh E, Greber Y, Meidan R. 1995. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cella and endothelial cells. Biol Reprod 52:954-962.

Girsh E, Milvae RA, Wang W, Meidan R. 1996a. Effect of endothelin-1 on bovine luteal cell function: role in prostaglandin $PGF_{2\alpha}$-induced antisteroidogenic action. Endocrinology 137:1306-1312.

Girsh E, Wang W, Mamluk R, Arditi F, Friedman A, Milvae RA., Meidan, R. 1996b. Regulation of endothelin-1 expression in the bovine corpus luteum: elevation by prostaglandin $PGF_{2\alpha}$. Endocrinology 137:5191-5196.

Girsh E, Dekel, N. 2002. Involvement of endothelin-1 and its receptors in $PGF_{2\alpha}$-induced luteolysis in the rat. Mol Reprod Develop 63:71-78.

Gleeson AR. 1974. Luteal function in the cyclic sow after infusion of prostaglandin F2α through a uterine vein. J Repro Fert 36:487-488.

Gleeson AR, Thorburn GD, Cox RI. 1974. Prostaglandin F concentrations in the utero-ovarian venous plasma of the sow during the late luteal phase of the oestrous cycle. Prostaglandins 5:521-529.

Glister C, Kemp CF, Knight PG. 2004. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction 127:239-254.

Glister C, Richards SL, Knight PG. 2005. Bone morphogenetic proteins (BMP) -4, -6, and -7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? Endocrinology 146:1883-1892.

Gonzalez-Bulnes A, Santiago-Moreno J, Garcia-Garcia RM, del Campo A, Gómez-Brunet A, Lopez-Sebastian A. 2001. Origin of the preovulatory follicle in Mouflon sheep (Ovis gmelini musimon) and effect on growth of remaining follicles during the follicular phase of oestrous cycle. Anim Reprod Sci 65:265-272.

Goodman RL, Coolen LM, Anderson GM, Hardy SL, Valent M, Connors JM, Fitzgerald ME, Lehman MN. 2004. Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. Endocrinology 145:2959-2967.

Goodman RL, Inskeep EK. 2006. Neuroendocrine control of the ovarian cycle of the sheep. In: Neill JD (Editor). Knobil and Kneill's Physiology of Reproduction. San Diego, California. Elsevier Academic Press. Third Edition, pp 2389-2447.

Goravanahally MP, Sen A, Inskeep EK, Flores JA. 2007. PKC epsilon and an increase in intracellular calcium concentration are necessary for PGF2 alpha to inhibit LH-stimulated progesterone secretion in cultured bovine steroidogenic luteal cells. Reprod Biol Endocrinol 5:37.

Goravanahally MP, Salem M, Yao J, Inskeep EK, Flores JA. 2009. Differential gene expression in the bovine corpus luteum during transition from early to mid-phase and its potential role in acquisition of luteolytic sensitivity to prostaglandin F2 alpha. Biol Reprod 80:980-988.

Gougeon A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17:121-155.

Goyeneche AA, Deis RP, Gibori G, Telleria CM. 2003. Progesterone promotes survival of the rat corpus luteum in the absence of cognate receptors. Biol Reprod 68:151-158.

Graves PE, Pierce KL, Bailey TJ, Rueda BR, Gil DW, Woodward DF, Yool AJ, Hoyer PB, Regan JW. 1995. Cloning of a receptor for prostaglandin F2 alpha from the ovine corpus luteum. Endocrinology 136:3430-3436.

Grinwich DL, Hichens M, Behrman HR. 1976. Control of the LH receptor by prolactin and prostaglandin F2alpha in rat corpora lutea. Biol Reprod 14:212-218.

Gudermann T, Birnbaumer M, Birnbaumer L. 1995. Homologous desensitization of the murine luteinizing hormone receptor expressed in L cells. Mol Cell Endocrinol 110:125-135.

Guedez L, Stetler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M. 1998. In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. J Clin Invest 102:2002–2010.

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. 000. Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. Biol Reprod 62:1322-1328.

Hagedorn HG, Bachmeier BE, Nerlich AG. 2001. Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-beta in invasive carcinomas (Review). Int J Oncol 18:669-681.

Halpin DM, Jones A, Fink G, Charlton HM. 1986. Postnatal ovarian follicle development in hypogonadal (hpg) and normal mice and associated changes in the hypothalamic-pituitary ovarian axis. J Reprod Fertil 77:287-296.

Hansel W, Dowd JP. 1986. Hammond memorial lecture. New concepts of the control of corpus luteum function. J Reprod Fertil 78:755-768.

Harrison LM, Kenny N, Niswender GD. 1987. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrous cycle and day 25 of pregnancy. J Reprod Fertil 79:539-548.

Hawk HW, Bolt DJ. 1970. Luteolytic effect of estradiol-17β when administered after midcycle in the ewe. Biol Reprod 2:275-278.

Hawk HW. 1973. Uterine motility and sperm transport in the estrous ewe after prostaglandin induced regression of corpora lutea. J Anim Sci 37:1380-1385.

Haworth JD, Rollyson MK, Silva P, McInt*u*sh EW, Niswender GD. 1998. Messenger ribonucleic acid encoding monocyte chemoattractant protein-1 is expressed by the ovine corpus luteum in response to prostaglandin F2alpha. Biol Reprod 58:169-174.

Hazzard TM, Pinckard KL, Stormshak F. 1998. Impact of Chronic treatment of ewe with estradiol-17β or progesterone on oxytocin receptor gene transcription and ovarian oxytocin secretion. Biol Reprod 59:105-110.

Hilliard J. 1973. Corpus luteum function in Guinea Pigs, Hamster, Rats, Mice and Rabbits. Biol Reprod 8:203-221.

Hinckley ST, Milvae RA. 2001. Endothelin-1 mediates prostaglandin $F_{2\alpha}$-induced luteal regression in the ewe. Biol Reprod 64:1619-1623.

Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, Marumo F. 1993. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. J Clin Invest 91:1367-1373.

Holt JA. 1989. Regulation of progesterone production in the rabbit corpus luteum. Biol Reprod 40:201-208.

Holt JE, Jackson A, Roman SD, Aitken RJ, Koopman P, McLaughlin EA. 2006. CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. Dev Biol 293:449-460.

Hou X, Arvisais EW, Jiang C, Chen DB, Roy SK, Pate JL, Hansen TR, Rueda BR, Davis JS. 2008. Prostaglandin F2alpha stimulates the expression and secretion of transforming growth factor B1 via induction of the early growth response 1 gene (EGR1) in the bovine corpus luteum. Mol Endocrinol 22:403-414.

Houmard BS, Ottobre JS. 1989. Progesterone and prostaglandin production by primate luteal cells collected at various stages of the luteal phase: modulation by calcium ionophore. Biol Reprod 41:401–408.

Hoyer PB, Fitz TA, Niswender GD. 1984. Hormone-independent activation of adenylate cyclase in large steroidogenic ovine luteal cells does not result in increased progesterone secretion. Endocrinology 114:604-608.

Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA, van den Hurk R. 1995. Effects of fetal bovine serum, FSH and 17beta-estradiol on the culture of bovine preantral follicles. Theriogenology 44:217-226.

Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM, Vanderstichele H, van den Hurk R. 1997. Bovine preantral follicles and activin: immunohistochemistry for activin and activin receptor and the effect of bovine activin A in vitro. Theriogenology 48:133-142.

Hunzicker-Dunn M. 1981. Rabbit follicular adenylyl cyclase activity. II. Gonadotropin-induced desensitization in granulosa cells and follicle shells. Biol Reprod 24:279-286.

Hyvelin JM, Guibert C, Marthan R, Savineau JP. 1998. Cellular mechanisms and role of endothelin-1-induced calcium oscillations in pulmonary arterial myocytes. Am J Physiol 275:L269-282.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto Masaki T. 1989. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separates genes. Proc Natl Acad Sci 86:2863-2867.

Inskeep EK, Butcher RL. Local component of utero-ovarian relationships in the ewe. J Anim Sci 25:1164-1168.

Inskeep EK, Smutny WJ, Butcher RL, Pexton JE. 1975. Effects of intrafollicular injections of prostaglandins in non-pregnant and pregnant ewes. J Anim Sci 41:1098-1104.

Inskeep EK. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentration of progesterone on embryonic survival in the cow. J Anim Sci 82(E Suppl.):E24-E39.

Ireland JJ, Roche JF. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 111:2077-2086.

Ireland JJ, Roche JF. 1983. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology 112:150-156.

Ireland JJ, Mihm M, Austin E, Diskin MG, Roche JF. 2000. Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. J Dairy Sci 83:1648-1658.

Ishiguro S, Kawase J, Miyamoto A, Nishio A. 2001. Endothelin receptor mediating contraction of isolated bovine coronary artery. J Vet Pharmacol Ther 24:203-208.

Ivy DD, Kinsella JP, Abman SH. 1994. Physiologic characterization of endothelin A and B receptor activity in the ovine fetal pulmonary circulation. J Clin Invest 93:2141-2148.

Iwai M, Hasegawa M, Taii S, Sagawa N, Nakao K, Imura H, Nakanishi S, Mori T. 1991. Endothelins inhibit luteinization of cultured porcine granulosa cells. Endocrinology 129:1909-1914.

Iwai M, Hori S, Shigemoto R, Kamzaki H, Mori T, Nakanishi S. 1993. Localization of endothelin receptor messenger ribonucleic acid in the rat ovary and fallopian tube by in situ hybridization. Biol Reprod 49:675-680.

Jaroszewski JJ, Hansel W. 2000. Intraluteal administration of a nitric oxide synthase blocker stimulates progesterone and oxytocin secretion and prolongs the life span of the bovine corpus luteum. Proc Soc Exp Biol Med 224:50-55.

Jaroszewski JJ, Skarzynski DJ, Hansel W. 2003. Nitric oxide as a local mediator of prostaglandin F2alpha-induced regression in bovine corpus luteum: an in vivo study. Exp Biol Med 228:1057-1062.

Jimenez-Krassel F, Winn ME, Burns D, Ireland JLH, Ireland JJ. 2003. Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. Endocrinology 144:1876-1886.

Jo M, Komar CM, Fortune JE. 2002. Gonadotropin surge induces two separate increases in messenger RNA for progesterone receptor in bovine preovulatory follicles. Biol Reprod 67:1981-1988.

Johnson MS, Ottobre AC, Ottobre JS. 1988. Prostaglandin production by corpora lutea of rhesus monkeys: characterization of incubation conditions and examination of putative regulators. Biol Reprod 39:839–846

Jordan AW. 1981. Effects of prostaglandin F2 alpha treatment on LH and dibutyryl cyclic AMP-stimulated progesterone secretion by isolated rat luteal cells. Biol Reprod 25:327-331.

Juan CC, Au LC, Yang FY, Yang DM, Ho LT. 2008. An endothelin type A receptor-expressing cell to characterize endothelin-1 binding and screen antagonist. Anal Biochem 379:27-31.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS, Smith MF. 1993. Apoptosis during luteal regression in cattle. Endocrinology 132:249-254.

Juengel JL, Meberg BM, Turzillo AM, Nett TM, Niswender GD. 1995. Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. Endocrinology 136:5423-5429.

Juengel JL, Wiltbank MC, Meberg BM, Niswender GD. 1996. Regulation of steady-state concentrations of messenger ribonucleic acid encoding prostaglandin F2 alpha receptor in ovine corpus luteum. Biol Reprod 54:1096-1102.

Juengel JL, McIntush EW, Niswender GD. 1998a. Corpus luteum. In: E. Knobil and J Neill (Eds). Encyclopedia of Reproduction. Elsevier Academic Press. NY. pp 703-709.

Juengel JL, Meberg BM, McIntush EW, Smith MF, Niswender GD. 1998b. Concentration of mRNA encoding 3 beta-hydroxysteroid dehydrogenase/delta 5,delta 4 isomerase (3 beta-HSD) and 3 beta-HSD enzyme activity following treatment of ewes with prostaglandin F2 alpha. Endocrine 8:45-50.

Juengel JL, Haworth JD, Rollyson MK, Silva PJ, Sawyer HR, Niswender GD. 2000. Effect of dose of prostaglandin F(2alpha) on steroidogenic components and oligonucleosomes in ovine luteal tissue. Biol Reprod 62:1047-1051.

Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP, Ritvos O, McNatty KP. 2002. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. Biol Reprod 67:1777-1789.

Kamada S, Kubota T, Hirata Y, Taguchi M, Eguchi S, Marumo F, Aso T. 1992. Direct effect of endothelin-1 on granulosa cells of the porcine ovary. J Endocrinolog 134:59-66.

Kamada S, Kubota T, Hirata Y, Imai T, Taguchi M, Marumo F, Aso T. 1993. Endothelin-1 is an autocrine/paracrine regulator of porcine granulosa cells. J Endocrinolog Invest 16:425-431.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen DE. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 262:1274-1277.

Kaneko H, Taya K, Watanabe G, Noguchi J, Kikuchi K, Shimada A, Hasegawa Y. 1997. Inhibin is involved in the suppression of FSH secretion in the growth phase of the dominant follicle during the early luteal phase in cows. Domest Anim Endocrinol 14:263-271.

Karsch FJ, Foster DL, Bittman EL, Goodman RL. 1983. A role for estradiol in enhancing luteinizing hormone pulse frequency during the follicular phase of the estrous cycle of sheep. Endocrinology 113:1333-1339.

Kawanabe Y, Nozaki K, Hashimoto N, Masaki T. 2003. Characterization of Ca2+ channels and G proteins involved in arachidonic acid release by endothelin-1/endothelinA receptor. Mol Pharmacol 64:689-695.

Keator CS, Schreiber DT, Hoagland TA, McCracken JA, Milvae RA. 2008. Intrauterine infusion of BQ-610, an endothelin type A receptor antagonist, delays luteolysis in dairy heifers. Domest Anim Endocrinol 34:411-418.

Kedzierski RM, Yanagisawa M. 2001. Endothelin system: the double-edged sword in health and disease. Annu Rev Pharmacol Toxicol 41:851-876.

Kenny N, Robinson J. 1986. Prostaglandin F2 alpha-induced functional luteolysis: interactions of LH, prostaglandin F2 alpha and forskolin in cyclic AMP and progesterone synthesis in isolated rat luteal cells. J Endocrinol 111:415-423.

Kezele PR, Nilsson EE, Skinner MK. 2002. Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. Mol Cell Endocrinol 192:37-43.

Kezele PR, Skinner MK. 2003. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 144:3329-3337.

Kieborz-Loos KR, Garverick HA, Keisler DH, Hamilton SA, Salfen BE, Youngquist RS, Smith MF. 2003. Oxytocin-induced secretion of prostaglandin F2alpha in postpartum beef cows: effects of progesterone and estradiol-17beta treatment. J Anim Sci 81:1830-1836.

Kitanaka J, Hasimoto H, Sugimoto Y, Negishi M, Aino H, Gotoh M, Ichikawa A, Baba A. 1994. Cloning and expression of a cDNA for rat prostaglandin F2 alpha receptor. Prostaglandins 48:31-41.

Kliem H, Welter H, Kraetzl WD, Steffl M, Meyer HH, Schams D, Berisha B. 2007. Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum. Reproduction 134:535-547.

Kliem H, Berisha B, Meyer HH, Schams D. 2009. Regulatory changes of apoptotic factors in the bovine corpus luteum after induced luteolysis. Mol Reprod Dev 76:220-30.

Klipper E, Gilboa T, Levy N, Kisliouk T, Spanel-Borowski K, Meidan R. 2004. Characterization of endothelin-1 and nitric oxide generating systems in corpus luteum-derived endothelial cells. Reproduction 128:463-473.

Ko JC, Kastelic JP, Del Campo MR, Ginther OJ. 1991. Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers. J Reprod Fertil 91:511-519.

Ko C, Gieske MC, Al-Alem L, Hahn Y, Su W, Gong MC, Iglarz M, Koo Y. 2006. Endothelin-2 in ovarian follicle rupture. Endocrinology 147:1770-1779.

Komar CM, Berndtson AK, Evans AC, Fortune JE. 2001. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 64:1797-1805.

Korzekwa A, Jaroszewski JJ, Bogacki M, Deptula KM, Maslanka TS, Acosta TJ, Okuda K, Skarzynski DJ. 2004. Effects of prostaglandin F(2alpha) and nitric oxide on the secretory function of bovine luteal cells. J Reprod Dev 50:411-417.

Korzekwa AJ, Jaroszewski JJ, Woclawek-Potocka I, Bah MM, Skarzynski DJ. 2008. Luteolytic effect of prostaglandin F 2 alpha on bovine corpus luteum depends on cell composition and contact. Reprod Domest Anim 43:464-472.

Kotwica J, Skarzynski D, Miszkiel G, Melin P, Okuda K. 1999. Oxytocin modulates the pulsatile secretion of prostaglandin F2alpha in initiated luteolysis in cattle. Res Vet Sci 66:1-5

Lafrance M, Goff AK. 1988. Effect of progesterone and estradiol-17β on oxytocin-induced release of prostaglandin F2α in heifers. J Reprod Fert 82:429-436.

Lake S, Gullberg H, Wahlqvist J, Sjögren AM, Kinhult A, Lind P, Hellström-Lindahl E, Stjernschantz J. 1994. Cloning of the rat and human prostaglandin F2 alpha receptors and the expression of the rat prostaglandin F2 alpha receptor. FEBS Lett 355:317-325.

Lamsa JC, Kotm SJ, Elderingm JA, Nay MG, McCracken JA. 1989. Prostaglandin F2α-stimulated release of ovarian oxytocin in the sheep in vivo: threshold and dose dependency. Biol Reprod 40:1215-1223.

Leung PC, Minegishi T, Ma F, Zhou FZ, Ho-Yuen B. 1986. Induction of polyphosphoinositide breakdown in rat corpus luteum by prostaglandin F2 alpha. Endocrinology 119:12-18.

Levitzki A. 1987. Regulation of adenylate cyclase by hormones and G-proteins. FEBS Lett 211:113-118.

Levy N, Kobayashi S, Roth Z, Wolfenson D, Miyamoto A, Meidan R. 2000. Administration of prostaglandin $F_{2\alpha}$ during he early bovine luteal phase does not alter the expression of ET-1 and its type receptor: A possible cause for corpus luteum refractoriness. Biol Reprod 64:377-382.

Levy N, Gordon M, Mamluk R, Yanagisawa M, Smith MF, Hampton JH, Meidan R. 2001. Distinct cellular localization and regulation of endothelin-1 and endothelin-converting enzyme-1 expression in the bovine corpus luteum: implications for luteolysis. Endocrinology 142:5254-5260.

Levy N, Gordon M, Smith MF, Bolden-Tiller OU, Meidan R. 2003. Hormonal regulation and cell-specific expression of endothelin-converting enzyme 1 isoforms in bovine ovarian endothelial and steroidogenic cells. Biol Reprod 68:1361-1368.

Lewis GS, Wilson L Jr, Wilks JW, Pexton JE, Fogwell RL, Ford SP, Butcher RL, Thayne WV, Inskeep EK. 1977. PGF2 alpha and its metabolites in uterine and jugular venous plasma and endometrium of ewes during early pregnancy. J Anim Sci 45:320-327.

Lewis GS, Jenkins PE, Fogwell RL, Inskeep EK. 1978. Concentrations of prostaglandins E2 and F2 alpha and their relationship to luteal function in early pregnant ewes. J Anim Sci 47:1314-1323.

Leymarie P, Martal J. 1993. The corpus luteum from cycle to gestation. In: Thibault C, Levasseur MC, Hunter RHF (Editors). Reproduction in Mammals and Man. Paris. Edition Marketing, pp 413-433.

Liu K, Olofsson JI, Wahlberg P, Ny T. 1999. Distinct expression of gelatinase A [matrix metalloproteinase (MMP)-2], collagenase-3 (MMP-13), membrane type MMP 1 (MMP-14), and tissue inhibitor of MMPs type 1 mediated by physiological signals during formation and regression of the rat corpus luteum. Endocrinology 140:5330-5338.

Liu YX. Plasminogen activator/plasminogen activator inhibitors in ovarian physiology. Front Biosci 9:3356-3573.

Lopez-Sebastian A, Gonzalez de Bulnes A, Santiago Moreno J, Gomez-Brunet A, Townsend EC, Inskeep EK. 1997. Patterns of follicular development during the estrous cycle in monovular Merino del Pais ewes. Anim Reprod Sci 48:279-291.

Lopez H, Sartori R, Wiltbank ML. 2005. Reproductive hormones and follicular growth during development of one or multiple dominant follicles in cattle. Biol Reprod 72:788-795.

Lundy T, Smith P, O'Connell A, Hudson NL, McNatty KP. Populations of granulosa cells in small follicles of the sheep ovary. J Reprod Fertil 115:251-262.

Magini A, Granchi S, Orlando G, Vanneli B, Pellegrini S, Milani S, Grappone C, De Franco R, Susini T, Forti G, Maggi M. 1996. Expression of endothelin-1 gene and protein in human granulosa cells. J Clin Endocrinol Metab 81:1428-1433.

Malven PV, Hansel W. 1964. Ovarian function in dairy heifers following hysterectomy. J Dairy Sci 47:1388-1393.

Mamluk R, Chen D, Greber Y, Davis JS, Meidan R. 1998. Characterization of messenger ribonucleic acid expression for prostaglandin F2 alpha and luteinizing hormone receptors in various bovine luteal cell types. Biol Reprod 58:849-856.

Mamluk R, Levy N, Rueda B, Davis JS, Meidan R. 1999. Characterization and regulation of type A endothelin receptor gene expression in bovine luteal cell type. Endocrinology 140:2110-2116.

Mancina R, Barni T, Calogero AE, Filipi S, Amerini S, Peri A, Susini T, Vanneli GB, Burello N, Forti G, Maggi M. 1997. Identification, characterization, and biological activity of endothlial receptors in human ovary. J Clin Endocrinol Metab 82:4122-4129.

Mann GE, McNeilly AS, Baird DT. 1992. Hormone production in vivo and in vitro from follicles at different stages of the oestrous cycle in the sheep. J Endocrinol. 1992 Feb;132(2):225-34.

Mann GE. 1999. The role of luteal oxytocin in episodic secretion of prostaglandin F2alpha at luteolysis in the ewe. Anim Reprod Sci 57:167-175

Marchant D, Dou Y, Luo H, Garmaroudi FS, McDonough JE, Si X, Walker E, Luo Z, Arner A, Hegele RG, Laher I, McManus BM. 2009. Bosentan enhances viral load via endothelin-1 receptor type-A-mediated p38 mitogen-activated protein kinase activation while improving cardiac function during coxsackievirus-induced myocarditis. Circ Res 104:813-821.

Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS, Lanzo CA. 1999. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. J Biol Chem 274:22903-22906.

Marsen TA, Egink G, Suckau G, Baldamus CA. 1999. Tyrosine-kinase-dependent regulation of the nitric oxide synthase gene by endothelin-1 in human endothelial cells. Pflugers Arch 438:538-544.

Matsuyama S, Chang KT, Kanuka H, Ohnishi M, Ikeda A, Nishihara M, Takahashi M. 1996. Occurrence of deoxyribonucleic acid fragmentation during prolactin-induced structural luteolysis in cycling rats. Biol Reprod 54:1245-1251.

McCracken JA, Glew ME, Scaramuzzi RJ. 1970. Corpus luteum regression induced by prostaglandin F2-alpha. J Clin Endocrinol Metab 30:544-546.

McCracken JA, Schramm W, Okulicz WC. 1984. Hormone receptor control of pulsatile secretion of PF2α from the ovine uterus during luteolysis and its abrogation in early pregnancy. Anim Reprod Sci 7:31-55.

McCracken J, Custer EE, Lamsa JC. 1999. Luteolysis: a neuroendocrine-mediated event. Physiological Review 70:263-324.

McGee EA, Hsueh AJ. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-214.

McNatty KP, Heath DA, Hudson N, Clarke IJ. 1990. Effect of long-term hypophysectomy on ovarian follicle populations and gonadotrophin-induced adenosine cyclic 3',5'-monophosphate output by follicles from Booroola ewes with or without the F gene. J Reprod Fertil 90:515-522.

McNatty KP, Reader K, Smith P, Heath DA, Juengel JL. 2007. Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. Soc Reprod Fertil Suppl 64:55-68.

McNeilly AS, Crow WJ, Fraser HM. 1992. Suppression of pulsatile luteinizing hormone secretion by gonadotrophin-releasing hormone antagonist does not affect episodic progesterone secretion or corpus luteum function in ewes. J Reprod Fertil 96:865-874.

Miceli F, Minici F, Garcia Pardo M, Navarra P, Proto C, Mancuso S, Lanzone A, Apa R. 2001. Endothelins enhance prostaglandin (PGE(2) and PGF(2alpha)) biosynthesis and release by human luteal cells: evidence of a new paracrine/autocrine regulation of luteal function. J Clin Endocrinol Metab 86:811-817.

Mignery GA, Südhof TC. 1990. The ligand binding site and transduction mechanism in the inositol-1,4,5-triphosphate receptor. EMBO J 9:3893-3898.

Mihm M, Good TE, Ireland JL, Ireland JJ, Knight PG, Roche JF. 1997. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. Biol Reprod 57:1328-1337.

Mihm M, Austin EJ, Good TE, Ireland JL, Knight PG, Roche JF, Ireland JJ. 2000. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. Biol Reprod 63:811-819.

Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC, Ireland JJ. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. Biol Reprod 74:1051-1059.

Miyauchi T, Masaki T. 1999. Pathophysiology of endothelin in cardiovascular system. Annu Rev Physiol 61:391-415.

Miyamoto Y, Yoshimasa T, Arai H, Takaya K, Ogawa Y, Itoh H, Nakao K. 1996. Alternative RNA splicing of the human endothelin-A receptor generates multiple transcripts. Biochem J 313:795-801.

Miyamoto A, Kobayashi S, Arata S, Ohtani M, Fukui Y, Schams D. 1997. Prostaglandin F2α promotes the inhibititory action of endothelin-1 on the bovine luteal function in vitro. J Endocrinology 152:R7-R11.

Moncada S, Higgs EA. 2006. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol 147(Suppl 1):S193-201.

Monget P, Besnard N, Huet C, Pisselet C, Monniaux D. 1996. Insulin-like growth factor-binding proteins and ovarian folliculogenesis. Horm Res 45:211-217.

Morgan GL, Geisert RD, McCann JP, Bazer FW, Ott TL, Mirando MA, Stewart M. 1993. Failure of luteolysis and extension of the interoestrous interval in sheep treated with the progesterone antagonist mifepristone (RU 486). J Reprod Fertil 98:451-457.

Moor RM, Hay MF, Dott HM, Cran DG. 1978. Macroscopic identification and steroidogenic function of atretic follicles in sheep. J Endocrinol 77:309-318.

Mukaida N, Harada A, Matsushima K. 1998. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 9:9-23.

Murdoch WJ, Dunn TG. 1982. Alterations in follicular steroid hormones during the preovulatory period in the ewe. Biol Reprod 27:300-307.

Murdoch WJ, Peterson TA, Van Kirk EA, Vincent DL, Inskeep EK. 1986. Interactive roles of progesterone, prostaglandins, and collagenase in the ovulatory mechanism of the ewe. Biol Reprod 35:1187-1194.

Murdoch WJ. 1995. Temporal relationships between stress protein induction, progesterone withdrawal, and apoptosis in corpora lutea of ewes treated with prostaglandin F2 alpha. J Anim Sci 73:1789-1792.

Murdoch WJ. 1996. Microtubular dynamics in granulosa cells of periovulatory follicles and granulosa-derived (large) lutein cells of sheep: relationships to the steroidogenic folliculo-luteal shift and functional luteolysis. Biol Reprod 54:1135-1140.

Neuvians TP, Schams D, Berisha B, Pfaffl MW. 2004a. Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2alpha-induced luteolysis in bovine corpus luteum. Biol Reprod 70:473-480.

Neuvians TP, Berisha B, Schams D. 2004b. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) expression during induced luteolysis in the bovine corpus luteum. Mol Reprod Dev 67:389-395.

Neill JD, Johansson ED, Knobil E. 1969. Failure of hysterectomy to influence the normal pattern of cyclic progesterone secretion in the rhesus monkey. Endocrinology 84:464-465.

Nett TM, Staigmiller RB, Akbar AM, Diekman MA, Ellinwood WE, Niswender GD. 1976a. Secretion of prostaglandin F2alpha in cycling and pregnant ewes. J Anim Sci 42:876-880.

Nett TM, McClellan MC, Niswender GD. 1976. Effects of prostaglandins on the ovine corpus luteum: blood flow, secretion of progesterone and morphology. Biol Reprod 15:66-78.

Niswender GD, Moore RT, Akbar AM, Nett TM, Diekman MA. 1975. Flow of blood to the ovaries of ewes throughout the estrous cycle. Biol Reprod 13:381-388.

Niswender GD, Reimers TJ, Diekman MA, Nett TM. 1976. Blood flow: a mediator of ovarian function. Biol Reprod 14:64-81.

Niswender, G, Juengel JL., Silva PJ, Rollyson MK, McIntush EW. 2000. Mechanism controlling the function and life span of the corpus luteum. Physiological Review 80:1-29.

Niswender GD, Davis TL, Griffith RJ, Bogan RL, Monser K, Bott RC, Bruemmer JE, Nett TM. 2007. Judge, jury and executioner: the auto-regulation of luteal function. Soc Reprod Fertil Suppl 64:191-206.

Nussdorfer GG, Rossi GP, Malendowicz LK, Mazzocchi G. 1999. Autocrine-paracrine endothelin system in physiology and pathology of steroid-secreting tissues. Pharmacological reviews 51:403437.

Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, Swisher SG. 2002. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores. J Biol Chem 277:9219-9225.

Ogletree ML. 1982. Pharmacology of prostaglandins in the pulmonary circulation. Ann NY Acad Sci 384:191–206

Ohtani M, Kobayashi S, Miyamoto A, Hayashi K, Fukui Y. 1998. Real-time relationship between intraluteal and plasma concentration of endothelin, oxytocin, and progesterone during prostaglandin $F_{2\alpha}$-induced luteolysis in cow. Biol Reprod 58:103-108.

Okuda K, Sakumoto R, Uenoyama Y, Berisha B, Miyamoto A, Schams D. 1999. Tumor necrosis factor alpha receptors in microvascular endothelial cells from bovine corpus luteum. Biol Reprod 61:1017-1022.

Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-Potocka I, Skarzynski DJ. 2004. Progesterone is a suppressor of apoptosis in bovine luteal cells. Biol Reprod 71:2065-2071.

Ono K, Tsujimoto G, Sakamoto A, Eto K, Masaki T, Ozaki Y, Satake M. 1994. Endothelin-A receptor mediates cardiac inhibition by regulating calcium and potassium currents. Nature 370:301-304.

O'Shea JD, Rodgers RJ, Wright PJ. 1984. Morphometric analysis and function in vivo and in vitro of corpora lutea from ewes treated with LHRH during seasonal anoestrus. J Reprod Fertil 72(1):75-85.

Ottobre JS, Lewis GS, Thayne WV, Inskeep EK. 1980. Mechanism by which progesterone shortens the estrous cycle of the ewe. Biol Reprod 23:1046-1053.

Ottobre JS, Vincent DL, Silvia DJ, Inskeep EK. 1984. Aspects of regulation of uterine secretion of prostaglandins during the estrous cycle and early pregnancy. Anim Repro Sci 7:75-100.

Paavola LG, Boyd CO. 1979. Surface morphology of macrophages in the regressing corpus luteum, as revealed by scanning electron microscopy. Anat Rec 195:659-681.

Pate JL. 1994. Cellular components involved in luteolysis. J Anim Sci ;7:1884-1890.

Pate JL, Landis Keyes P. 2001. Immune cells in the corpus luteum: friends or foes? Reproduction 122:665-676.

Penny LA, Armstrong DG, Baxter G, Hogg C, Kindahl H, Bramley T, Watson ED, Webb R. 1998. Expression of monocyte chemoattractant protein-1 in the bovine corpus luteum around the time of natural luteolysis. Biol Reprod 59:1464-1469.

Penny LA, Armstrong D, Bramley TA, Webb R, Collins RA, Watson ED. 1999. Immune cells and cytokine production in the bovine corpus luteum throughout the oestrous cycle and after induced luteolysis. J Reprod Fertil 115:87-96.

Penny LA. 2000. Monocyte chemoattractant protein 1 in luteolysis. Rev Reprod 5:63-66.

Petroff MG, Petroff BK, Pate JL. 2201. Mechanisms of cytokine-induced death of cultured bovine luteal cells. Reproduction 121:753-760.

Peters KE, Bergfeld EG, Cupp AS, Kojima FN, Mariscal V, Sanchez T, Wehrman ME, Grotjan HE, Hamernik DL, Kittok RJ, Kinder JE. 1994. Luteinizing hormone has a role in development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers. Biol Reprod 51:1248-1254.

Pexton JE, Ford SP, Wilson L Jr, Butcher RL, Inskeep EK. 1975a. Prostaglandins F in uterine tissue and venous plasma of ewes with intrauterine devices. J Anim Sci 41:144-153.

Pexton JE, Weems CW, Inskeep EK. 1975b. Prostaglandins F in uterine and ovarian venous plasma from nonpregnant and pregnant ewes collected by cannulation. Prostaglandins 9:501-509.

Pexton JE, Weems CW, Inskeep EK. 1975c. Prostaglandins F in uterine venous plasma, ovarian arterial and venous plasma and in ovarian and luteal tissue of pregnant and nonpregnant ewes. J Anim Sci 41:154-159.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

Pierce KL, Bailey TJ, Hoyer PB, Gil DW, Woodward DF, Regan JW. 1997. Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor. J Biol Chem 272:883-887.

Pierce KL, Fujino H, Srinivasan D, Regan JW. 1999. Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. J Biol Chem 274:35944-35949.

Pinet F. 2004. Á quoi sert le système endothéline? Medicine/Science 20 :339-345.

Pinton P, Ferrari D, Magalhães P, Schulze-Osthoff K, Di Virgilio F, Pozzan T, Rizzuto R. 2000. Reduced loading of intracellular Ca(2+) stores and downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells. J Cell Biol 148:857-862.

Pitzel L, Lüdemann S, Wuttke W. 2000. Secretion and gene expression of metalloproteinases and gene expression of their inhibitors in porcine corpora lutea at different stages of the luteal phase. Biol Reprod 62:1121-1127.

Plonowski A, Kaplinski, AK, Radzikowska M, Borowiec M, Baranowska B. 1999. Hum Reprod 14 :2323-2327.

Pratt BR, Butcher RL, Inskeep EK. 1977. Antiluteolytic effect of the conceptus and of PGE2 in ewes. J Anim Sci 45:784-791.

Pratt BR, Butcher RL, Inskeep EK. 1979. Effect of continuous intrauterine administration of prostaglandin E2 on life span of corpora lutea of nonpregnant ewes. J Anim Sci 48:1441-1446.

Priyadarsana M, Wijayagunawardane B, Miyamoto A. 2004. Endothelin-1 system in the bovine oviduct: a regulator of local contraction and gamete transport. J Cardiovasc Pharmacol 44(Suppl. 1):S248-251.

Priyanka S, Jayaram P, Sridaran R, Medhamurthy R. 2009. Genome-wide gene expression analysis reveals a dynamic interplay between luteotropic and luteolytic factors in the regulation of corpus luteum function in the bonnet monkey (Macaca radiata). Endocrinology 150:1473-1484.

Qiu ZH, Leslie CC. 1994. Protein kinase C-dependent and -independent pathways of mitogen-activated protein kinase activation in macrophages by stimuli that activate phospholipase A2. J Biol Chem 269:19480-19487.

Rao MC, Richards JS, Midgley AR Jr, Reichert LE Jr. 1977. Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. Endocrinology 101:512-523.

Raymond V, Leung PC, Labrie F. 1983 Stimulation by prostaglandin F2 alpha of phosphatidic acid-phosphatidylinositol turnover in rat luteal cells. Biochem Biophys Res Commun 116:39-46.

Rho SB, Chung BM, Lee JH. 2007. TIMP-1 regulates cell proliferation by interacting with the ninth zinc finger domain of PLZF. J Cell Biochem 101:57-67.

Ribeiro LA, Turba ME, Zannoni A, Bacci ML, Forni M. 2006. Gelatinases, endonuclease and Vascular Endothelial Growth Factor during development and regression of swine luteal tissue. BMC Dev Biol 6:58.

Ribeiro LA, Turba ME, Bernardini C, Zannoni A, Bacci ML, Forni M. 2007. Matrix metalloproteinases -2 and -9 in swine luteal tissue angiogenesis and angioregression. Vet Res Commun 31(Suppl 1):193-196.

Ricke WA, Smith GW, McIntush EW, Smith MF. 2002a. Analysis of luteal tissue inhibitor of metalloproteinase-1, -2, and -3 during prostaglandin F(2alpha)-induced luteolysis. Biol Reprod 66:1387-1394.

Ricke WA, Smith GW, Smith MF. 2002b. Matrix metalloproteinase expression and activity following prostaglandin F(2 alpha)-induced luteolysis. Biol Reprod 66:685-691.

Ricke WA, Smith GW, Reynolds LP, Redmer DA, Smith MF. 2002c. Matrix metalloproteinase (2, 9, and 14) expression, localization, and activity in ovine corpora lutea throughout the estrous cycle. Biol Reprod 66:1083-1094.

Rivera GM, Chandrasekher YA, Evans AC, Giudice LC, Fortune JE. 2001. A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. Biol Reprod 65:102-111.

Rodgers RJ, O'Shea JD. 1982. Purification, morphology, and progesterone production and content of three cell types isolated from the corpus luteum of the sheep. Aust J Biol Sci 35:441-455.

Rodgers RJ, O'Shea JD, Bruce NW. 1984. Morphometric analysis of the cellular composition of the ovine corpus luteum. J Anat 138:757-770.

Rodgers RJ, Vella CA, Young FM, Tian XC, Fortune JE. 1995. Concentrations of cytochrome P450 cholesterol side-chain cleavage enzyme and 3 beta-hydroxysteroid dehydrogenase during prostaglandin F2 alpha-induced luteal regression in cattle. Reprod Fertil Dev 7:1213-1216.

Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365-386.

Rubanyi GM, Polokoff, MA. 1994. Endothelins: Molecular biology, biochemistry, pharmacology, physiology and pathology. Pharmacol Rev 46:325-415.

Rueda BR, Wegner JA, Marion SL, Wahlen DD, Hoyer PB. 1995a. Internucleosomal DNA fragmentation in ovine luteal tissue associated with luteolysis: in vivo and in vitro analyses. Biol Reprod 52:305-312.

Rueda1 BR, Botros1 I W, Pierce KL, Regan JW, Hoyer PB. 1995b. Comparison of mRNA levels for the PGF2α receptor (FP) during luteolysis and early pregnancy in the ovine corpus luteum. Endocrine 3:781-787.

Rueda BR, Tilly KI, Botros IW, Jolly PD, Hansen TR, Hoyer PB, Tilly JL.1997. Increased bax and interleukin-1beta-converting enzyme messenger ribonucleic acid levels coincide with apoptosis in the bovine corpus luteum during structural regression. Biol Reprod 56:186-193.

Rueda BR, Hendry IR, Hendry III WJ, Stormshak F, Slayden OD, Davis JS. 2000. Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Biol Reprod 2000 62:269-276.

Russell DL, Doyle KM, Gonzales-Robayna I, Pipaon C, Richards JS. 2003. Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3',5'-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. Mol Endocrinol 17:520–533.

Sakamoto A, Yanagisawa M, Sawamura T, Enoki T, Ohtani T, Sakurai T, Nakao K, Toyo-oka T, Masaki T. 1993. Distinct subdomains of human endothelin receptors determine their selectivity to endothelinA-selective antagonist and endothelinB-selective agonists. J Biol Chem 268:8547-8553.

Sakamoto K, Ezashi T, Miwa K, Okuda-Ashitaka E, Houtani T, Sugimoto T, Ito S, Hayaishi O. 1994. Molecular cloning and expression of a cDNA of the bovine prostaglandin F2 alpha receptor. J Biol Chem 269:3881-3886.

Sakamoto K, Miwa K, Ezashi T, Okuda-Ashitaka E, Okuda K, Houtani T, Sugimoto T, Ito S, Hayaishi O. 1995. Expression of mRNA encoding the prostaglandin F2 alpha receptor in bovine corpora lutea throughout the oestrous cycle and pregnancy. J Reprod Fertil 103:99-105.

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348:732-735.

Sawyer HR, Niswender KD, Braden TD, Niswender GD. 1990. Nuclear changes in ovine luteal cells in response to PGF2 alpha. Domest Anim Endocrinol 7:229-237.

Schams D, Berisha B. 2002. Steroids as local regulators of ovarian activity in domestic animals. Dom Anim Endocrinol 23:53-65.

Schams D, Berisha B, Neuvians T, Amselgruber W, Kraetzl WD. 2003. Real-time changes of local vasoactive peptide systems (angiotensin, endothelin) in the bovine corpus luteum after induced luteal regression. Mol Reprod Dev 65:57-66.

Schuster VL. 1998. Molecular mechanisms of prostaglandin transport. Annu Rev Physiol 60:221-242.

Sen A, Browning J, Inskeep EK, Lewis P, Flores JA. 2004. Expression and activation of protein kinase C isozymes by prostaglandin F(2alpha) in the early- and mid-luteal phase bovine corpus luteum. Biol Reprod 70:379-384.

Sen A, Choudhary E, Inskeep EK, Flores JA. 2005. Effects of selective protein kinase c isozymes in prostaglandin2alpha-induced Ca2+ signaling and luteinizing hormone-induced progesterone accumulation in the mid-phase bovine corpus luteum. Biol Reprod 72:976-984.

Sen A, Wright M, Inskeep EK, Flores JA. 2006. Participation of specific PKC isozymes in the inhibitory effect of ET-1 on progesterone accumulation in cells isolated from early- and mid-phase corpora lutea. Domest Anim Endocrinol 31:284-299.

Seo DW, Li H, Guedez L, Wingfield PT, Diaz T, Salloum R, Wei BY, Stetler-Stevenson WG. 2003. TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. Cell 114:171-180.

Seo DW, Kim SH, Eom SH, Yoon HJ, Cho YR, Kim PH, Kim YK, Han JW, Diaz T, Wei BY, Stetler-Stevenson WG. 2008. TIMP-2 disrupts FGF-2-induced downstream signaling pathways. Microvasc Res 76:145-151.

Sheffel CE, Pratt BR, Ferrell WL, Inskeep EK. 1982. Induced corpora lutea in the postpartum beef cow. II. Effects of treatment with progestogen and gonadotropins. J Anim Sci 54:830-836.

Shikone T, Yamoto M, Kokawa K, Yamashita K, Nishimori K, Nakano R. 1995. Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. J Clin Endocrinol Metab 81:2376-2380.

Shirasuna K, Asaoka H, Acosta TJ, Wijayagunawardane MP, Ohtani M, Hayashi M, Matsui M, Miyamoto A. 2004. Real-time relationships in intraluteal release among prostaglandin F2alpha, endothelin-1, and angiotensin II during spontaneous luteolysis in the cow. Biol Reprod 71:1706-1711.

Shirasuna K, Watanable S, Oki N, Wijayagunawardane MPB, Matsui M, Ohtani M, Miyamoto A. 2006. A cooperative action of endothelin-1 with prostaglandin $F_{2\alpha}$ on luteal function in the cow. Dom Anim Endocrinol 31:186-196. Mol Reprod Develop 65:57-66.

Shirasuna K, Shimizu T, Hayashi KG, Nagai K, Matsui M, Miyamoto A. 2007. Positive association, in local release, of luteal oxytocin with endothelin 1 and prostaglandin F2alpha during spontaneous luteolysis in the cow: a possible intermediatory role for luteolytic cascade within the corpus luteum. Biol Reprod 76:965-970.

Shirasuna K, Watanabe S, Asahi T, Wijayagunawardane MP, Sasahara K, Jiang C, Matsui M, Sasaki M, Shimizu T, Davis JS, Miyamoto A. 2008. Prostaglandin F2alpha increases endothelial nitric oxide synthase in the periphery of the bovine corpus luteum: the possible regulation of blood flow at an early stage of luteolysis. Reproduction 135:527-539.

Shores EM, Hunter MG. 2000. Production of tissue inhibitors of metalloproteinases (TIMPs) by pig ovarian cells in vivo and the effect of TIMP-1 on steroidogenesis in vitro. J Reprod Fertil 120:73-81.

Skinner MK. 2005. Regulation of primordial follicle assembly and development. Hum Reprod Update 11:461-471.

Silvia WJ, Ottobre JS, Inskeep EK. 1984. Concentrations of prostaglandins E2, F2 and 6-keto-prostaglandin F1 α in the utero-ovarian venous plasma of nonpregnant and early pregnant ewe. Biol Reprod 30:936-944.

Silvia WJ, Lewis GS, McCracken JA, Thatcher WW, Wilson L Jr. 1991. Hormonal regulation of uterine secretion of prostaglandin F2 alpha during luteolysis in ruminants. Biol Reprod 45:655-663.

Sirois J, Sayasith K, Brown KA, Stock AE, Bouchard N, Doré M. 2004. Cyclooxygenase-2 and its role in ovulation: a 2004 account. Hum Reprod Update 10:373-385.

Skarzynski DJ, Jaroszewski JJ, Bah MM, Deptula KM, Barszczewska B, Gawronska B, Hansel W. 2003. Administration of a nitric oxide synthase inhibitor counteracts prostaglandin F2-induced luteolysis in cattle. Biol Reprod 68:1674-1681.

Skovgaard N, Zibrandtsen H, Laursen BE, Simonsen U, Wang T. 2008. Hypoxia-induced vasoconstriction in alligator (Alligator mississippiensis) intrapulmonary arteries: a role for endothelin-1? J Exp Biol 211:1565-1570.

Smith GW, Goetz TL, Anthony RV, Smith MF. 1994. Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of messenger ribonucleic acid expression and in situ localization within preovulatory follicles and luteal tissue. Endocrinology 134:344-352.

Smith GW, McCrone S, Petersen SL, Smith MF. 1995. Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea. Endocrinology 136:570-576.

Smith MF, Ricke WA, Bakke LJ, Dow MP, Smith GW. 2002. Ovarian tissue remodeling: role of matrix metalloproteinases and their inhibitors. Mol Cell Endocrinol 191:45-56.

Smith WL, DeWitt DL, Garavito RM. 2000. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 69:145-182.

Souza CJ, Campbell BK, Baird DT. 1997. Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phases of the estrous cycle. Biol Reprod 56:483-488.

Souza CJ, Campbell BK, Baird DT. 1998. Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant. J Endocrinol 156:563-572.

Souza CJ, Campbell BK, McNeilly AS, Baird DT. 2002. Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123:363-369.

Spicer LJ, Alpizar E, Echternkamp SE. 1993. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. J Anim Sci 71:1232-1241.

Spicer LJ, Echternkamp SE. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest Anim Endocrinol 12:223-245.

Spicer LJ, Chamberlain CS, Maciel SM. 2002. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest Anim Endocrinol 22:237-254.

Spicer LJ, Voge JL, Allen DT. Insulin-like growth factor-II stimulates steroidogenesis in cultured bovine thecal cells. Mol Cell Endocrinol. 2004 Nov 30;227(1-2):1-7.

Spicer LJ, Aad PY, Allen D, Mazerbourg S, Hsueh AJ. 2006. Growth differentiation factor-9 has divergent effects on proliferation and steroidogenesis of bovine granulosa cells. J Endocrinol 189:329-339.

Spicer LJ, Aad PY, Allen DT, Mazerbourg S, Payne AH, Hsueh AJ. 2008. Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9. Biol Reprod 78:243-253.

Spät A, Bradford PG, McKinney JS, Rubin RP, Putney JW Jr. 1986. A saturable receptor for 32P-inositol-1,4,5-triphosphate in hepatocytes and neutrophils. Nature 319:514-516.

Steffl M, Schweiger M, Amselgruber WM. 2004. Oestrous cycle-regulated expression of inositol 1,4,5-trisphosphate receptor type 2 in the pig ovary. Acta Histochem 106:137-144.

Stocco C, Telleria C, Gibori G. 2007. The molecular control of corpus luteum formation, function, and regression. Endocr Rev 28:117-149.

Stouffer, RL. 2006. Structure, function, and regulation of the corpus luteum. In: Neill JD (Editor). Knobil and Kneill's Physiology of Reproduction. San Diego, California. Elsevier Academic Press. Third Edition, pp 475-526.

Streb H, Irvine RF, Berridge MJ, Schulz I. 1983. Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306:67-69.

Südhof TC, Newton CL, Archer BT 3rd, Ushkaryov YA, Mignery GA. 1991. Structure of a novel InsP3 receptor. EMBO J 10:3199-3206.

Sugino N, Suzuki T, Kashida S, Karube A, Takiguchi S, Kato H. 2000a. Expression of Bcl-2 and Bax in the human corpus luteum during the menstrual cycle and in early pregnancy: regulation by human chorionic gonadotropin. J Clin Endocrinol Metab. 85:4379-4786.

Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H. 2000b. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Mol Hum Reprod 6:19-25.

Sugino N. 2006. Roles of reactive oxygen species in the corpus luteum. Anim Sci J 77:556-565.

Tai CJ, Kang SK, Choi KC, Tzeng CR, Leung PC. 2001. Role of mitogen-activated protein kinase in prostaglandin f(2alpha) action in human granulosa-luteal cells. J Clin Endocrinol Metab 86:375-380.

Tai HH, Ensor CM, Tong M, Zhou H, Yan F. 2002. Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 68-69:483-493.

Takigawa M, Sakurai T, Kasuya Y, Abe Y, Masaki T, Goto K. Molecular identification of guanine-nucleotide-binding regulatory proteins which couple to endothelin receptors. Eur J Biochem 228:102-108.

Tanabe, T.Y., 1966. Essentiality of the corpus luteum for maintenance of pregnancy in dairy cows. J Dairy Sci 49:731 (abstract).

Taniguchi H, Yokomizo Y, Okuda K. 2002. Fas-Fas ligand system mediates luteal cell death in bovine corpus luteum. Biol Reprod 66:754-759.

Tedeschi C, Hazum E, Kokia E, Ricciarelli E, Adashi EY, Payne DW. 1992. Endothelin-1 as luteinization inhibitor: inhibition of rat granulosa cell progesterone accumulation via selective modulation of key steroidogenic steps affecting both progesterone formation and degradation. Endocrinology 131:2476-2478.

Tedeschi C, Lohman C, Hazum E, Ittoop O, Ben-Shlomo I, Resnick CE, Payne DW, Adashi EY. 1994. Rat ovarian granulosa cell as a site of endothelin reception and action: attenuation of gonadotropin-stimulated steroidogenesis via perturbation of the A-kinase signaling pathway. Biol Reprod 51:1058-1065.

Thatcher WW, Bazer FW, Sharp DC, Roberts RM. 1986. Interrelationships between uterus and conceptus to maintain corpus luteum function in early pregnancy: sheep, cattle, pigs and horses. J Anim Sci 62(Suppl 2):25-46.

Thomas JP, Dorflinger LJ, Behrman HR. 1978. Mechanism of the rapid antigonadotropic action of prostaglandins in cultured luteal cells. Proc Natl Acad Sci U S A 75:1344-1348.

Tian XC, Berndtson AK, Fortune JE. 1994. Changes in levels of messenger ribonucleic acid for cytochrome P450 side-chain cleavage and 3 beta-hydroxysteroid dehydrogenase during prostaglandin F2 alpha-induced luteolysis in cattle. Biol Reprod 50:349-356.

Tian XC, Berndtson AK, Fortune JE. 1995. Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and P450 17 alpha-hydroxylase, but not P450 aromatase. Endocrinology 136:5102-5110.

Tilly JL. 1996. Apoptosis and ovarian function. Rev Reprod 1:162-172.

Tisdall DJ, Watanabe K, Hudson NL, Smith P, McNatty KP. 1995. FSH receptor gene expression during ovarian follicle development in sheep. J Mol Endocrinol 15:273-281.

Tortonese, D.J., Lewis, P.E., Papkoff, H., Inskeep, E.K., 1990. Roles of the dominant follicle and the pattern of oestradiol in induction of pre-ovulatory surges of LH and FSH in prepubertal heifers by pulsatile low doses of LH. J Reprod Fertil 90:127-135.

Towle TA, Tsang PC, Milvae RA, Newbury MK, McCracken JA. 2002. Dynamic in vivo changes in tissue inhibitors of metalloproteinases 1 and 2, and matrix metalloproteinases 2 and 9, during prostaglandin F(2alpha)-induced luteolysis in sheep. Biol Reprod ;66:1515-1521.

Townson DH, Pate JL. 1994. Regulation of prostaglandin synthesis by interleukin-1 beta in cultured bovine luteal cells. Biol Reprod 51:480-485.

Townson DH, Pate JL. 1996. Mechanism of action of TNF-alpha-stimulated prostaglandin production in cultured bovine luteal cells. Prostaglandins 52:361-373.

Townson DH, O'Connor CL, Pru JK. 2002. Expression of monocyte chemoattractant protein-1 and distribution of immune cell populations in the bovine corpus luteum throughout the estrous cycle. Biol Reprod 66:361-366.

Trevisi L, Bova S, Cargnelli G, Ceolotto G, Luciani S. 2002. Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle via extracellular signal-regulated kinases pathway. Biochem Pharmacol 64:425-431.

Tripiciano A, Peluso C, Morena AR, Palombi F, Stefanini M, Ziparo E, Yanagisawa M, Filippini A. 1999. Cyclic expression of endothelin-converting enzyme-1 mediates the functional regulation of seminiferous tubule contraction. J. Cell. Biol. 145:1027-1038.

Tsai SJ, Wiltbank MC. 1997. Prostaglandin F2alpha induces expression of prostaglandin G/H synthase-2 in the ovine corpus luteum: a potential positive feedback loop during luteolysis. Biol Reprod 57:1016-22.

Tsai SJ, Juengel JL, Wiltbank MC. 1997. Hormonal regulation of monocyte chemoattractant protein-1 messenger ribonucleic acid expression in corpora lutea. Endocrinology 138:4517-4520.

Tsai SJ, Anderson LE, Juengel J, Niswender GD, Wiltbank MC. 1998a. Regulation of prostaglandin F2 alpha and E receptor mRNA by prostaglandin F 2 alpha in ovine corpora lutea. J Reprod Fertil 114:69-75.

Tsai SJ, Wiltbank MC. 1998b. Prostaglandin F2alpha regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. Biol Reprod 58:346-352.

Tsai SJ, Kot K, Ginther OJ, Wiltbank MC. 2001. Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies in vivo. Reproduction 121:905-913.

Ursely J, Leymarie P. 1979. Varying response to luteinizing hormone of two luteal cell types isolated from bovine corpus luteum. J Endocrinol 83:303-310.

Usuki S, Suzuki N, Matsumoto H, Yanagisawa M, Masaki T. 1991. Endothelin-1 in luteal tissue. Mol Cell Endocrinol 80:147-151.

Vassilev V, Pretto CM, Cornet PB, Delvaux D, Eeckhout Y, Courtoy PJ, Marbaix E, Henriet P. 2005. Response of matrix metalloproteinases and tissue inhibitors of metalloproteinases messenger ribonucleic acids to ovarian steroids in human endometrial explants mimics their gene- and phase-specific differential control in vivo. J Clin Endocrinol Metab 90:5848-5857.

Vermassen E, Parys JB, Mauger JP. 2004. Subcellular distribution of the inositol 1,4,5-trisphosphate receptors: functional relevance and molecular determinants. Biol Cell 96:3-17.

Vincent DL, Inskeep EK. 1986. Role of progesterone in regulating uteroovarian venous concentrations of PGF2 alpha and PGE2 during the estrous cycle and early pregnancy in ewes. Prostaglandins 31:715-733.

Vonnahme KA, Redmer DA, Borowczyk E, Bilski JJ, Luther JS, Johnson ML, Reynolds LP, Grazul-Bilska AT. 2006. Vascular composition, apoptosis, and expression of angiogenic factors in the corpus luteum during prostaglandin F2alpha-induced regression in sheep. Reproduction 131:1115-1126.

Voss AK, Fortune JE. 1993a. Levels of messenger ribonucleic acid for cytochrome P450 17 alpha-hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after the luteinizing hormone surge. Endocrinology 132:2239-2245.

Voss AK, Fortune JE. 1993b. Estradiol-17b has a biphasic effect on oxytocin secretion by bovine granulosa cells. Biol Reprod 48:1404–1409.

Wandji SA, Fortier MA, Sirard MA. 1992. Differential response to gonadotropins and prostaglandin E2 in ovarian tissue during prenatal and postnatal development in cattle. Biol Reprod 46:1034-1041.

Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. 1996a. Initiation in vitro of growth of bovine primordial follicles. Biol Reprod 55:942-948.

Wandji SA, Eppig JJ, Fortune JE. 1996b. FSH and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. Theriogenology 45:817-832.

Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE. 1997. Initiation of growth of baboon primordial follicles in vitro. Hum Reprod 12:1993-2001.

Wang Y, Simonson MS, Pouyss´egur J, Dunn MJ. 1992. Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. Biochem J 287:589–594.

Webb R, Woad KJ, Amstrong DG. 2002. Corpus luteum (CL) function: local control mechanisms. Dom Anim Endocrinol 23:277-285.

Webb R, Nicholas B, Gong JG, Campbell BK, Gutierrez CG, Garverick HA, Armstrong DG. 2003. Mechanisms regulating follicular development and selection of the dominant follicle. Reprod Suppl 61:71-90.

Webley GE, Hodges JK, Given A, Hearn JP. 1991. Comparison of the luteolytic action of gonadotrophin-releasing hormone antagonist and cloprostenol, and the ability of human chorionic gonadotrophin and melatonin to override their luteolytic effects in the marmoset monkey. J Endocrinol 128:121-129.

Weems CW, Weems YS, Randel RD. 2006. Prostaglandins and reproduction in female farm animals. Vet J 171:206-228.

Wiepz GJ, Wiltbank MC, Nett TM, Niswender GD, Sawyer HR. 1992. Receptors for prostaglandins F2 alpha and E2 in ovine corpora lutea during maternal recognition of pregnancy. Biol Reprod 47:984-991.

Williamson JR, Monk JR. 1989. Hormone effects on cellular Ca^{2+} fluxes. Annu Rev Physiol 51:107-124.

Wiltbank JN, Casida, LE. 1956. Alteration of ovarian function by hysterectomy. J Anim Sci 15:134-140.

Wiltbank MC, Guthrie PB, Mattson MP, Kater SB, Niswender GD. 1989a. Hormonal regulation of free intracellular calcium concentrations in small and large ovine luteal cells. Biol Reprod 41:771-778.

Wiltbank MC, Knickerbocker JJ, Niswender GD. 1989b. Regulation of the corpus luteum by protein kinase C. I. Phosphorylation activity and steroidogenic action in large and small ovine luteal cells. Biol Reprod 40:1194-1200.

Wiltbank MC, Diskin MG, Flores JA, Niswender GD. 1990. Regulation of the corpus luteum by protein kinase C. II. Inhibition of lipoprotein-stimulated steroidogenesis by prostaglandin F2 alpha. Biol Reprod 42:239-245.

Wiltbank MC, Diskin MG, Niswender GD. 1991. Differential actions of second messenger systems in the corpus luteum. J Reprod Fertil 43(Suppl):65-75.

Wiltbank MC. 1994. Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci 72:1873-1883.

Wiltbank MC, Shiao TF, Bergfelt DR, Ginther OJ. 1995. Prostaglandin $F_{2\alpha}$ receptors in the early bovine corpus luteum. Biol Reprod 52:74-78.

Wiltbank MC, Ottobre JS. 2003. Regulation of intraluteal production of prostaglandins. Reprod Biol Endocrinol 1:91.

Wise TH, Caton D, Thatcher WW, Barron DH, Fields MJ. 1982. Ovarian function during the estrous cycle of the cow: ovarian blood flow and progesterone release rate. J Anim Sci 55:627-637.

Wright MF, Sayre B, Inskeep EK, Flores JA. 2001. Prostaglandin F(2alpha) regulation of the bovine corpus luteum endothelin system during the early and midluteal phase. Biol Reprod 65:1710-1717.

Xu D, Emoto N, Giaid A, Slaugther C, Kaw S, deWit D, Yanagisawa M. 1994. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell 78:473-485.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. 1995a. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 53:951-957.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. 1995b. Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome p450 17 alpha-hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. Endocrinology 136:981-989.

Webb R, Nicholas B, Gong JG, Campbell BK, Gutierrez CG, Garverick HA, Armstrong DG. 2003. Mechanisms regulating follicular development and selection of the dominant follicle. Reprod Suppl 61:71-90.

Webb R, Campbell BK. 2007. Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. Reprod Fertil Suppl 64:141-163.

Yadav VK, Lakshmi G, Medhamurthy R. 2005. Prostaglandin F2alpha-mediated activation of apoptotic signaling cascades in the corpus luteum during apoptosis: involvement of caspase-activated DNase. J Biol Chem 280:10357-10367.

Yamada N, Makino Y, Clark RA, Pearson DW, Mattei MG, Guénet JL, Ohama E, Fujino I, Miyawaki A, Furuichi T, Mikoshiba K. 1994. Human inositol 1,4,5-trisphosphate type-1 receptor, InsP3R1: structure, function, regulation of expression and chromosomal localization. Biochem J 302 :781-790.

Yamamoto-Hino M, Sugiyama T, Hikichi K, Mattei MG, Hasegawa K, Sekine S, Sakurada K, Miyawaki A, Furuichi T, Hasegawa M, Mikoshiba K. 1994. Cloning and characterization of human type 2 and type 3 inositol 1,4,5-trisphosphate receptors. Receptors Channels 2:9-22.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415.

Young JE, Friedman CI, Danforth DR. 1997. Interleukin-1 beta modulates prostaglandin and progesterone production by primate luteal cells in vitro. Biol Reprod 56:663-667.

Yuan W, Bao B, Garverick HA, Youngquist RS, Lucy MC. 1998. Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. Domest Anim Endocrinol 15:55-63.

Zannoni A, Bernardini C, Rada T, Ribeiro LA, Forni M, Bacci ML. 2007. Prostaglandin F2-alpha receptor (FPr) expression on porcine corpus luteum microvascular endothelial cells (pCL-MVECs). Reprod Biol Endocrinol 20:31.

Zarco L, Stabenfeldt GH, Basu S, Bradford GE, Kindahl H. 1988a. Modification of prostaglandin F-2 alpha synthesis and release in the ewe during the initial establishment of pregnancy. J Reprod Fertil 83:527-536.

Zarco L, Stabenfeldt GH, Quirke JF, Kindahl H, Bradford GE. 1988b. Release of prostaglandin F-2 alpha and the timing of events associated with luteolysis in ewes with oestrous cycles of different lengths. J Reprod Fertil 83:517-526.

Zelinski-Wooten MB, Stouffer RL. 1990. Intraluteal infusions of prostaglandins of the E, D, I, and A series prevent PGF2-induced, but not spontaneous, luteal regression in rhesus monkeys. Biol Reprod 43:507–516

Zeleznik AJ, Clifford RP. 2006. Control of follicular development, corpus luteum function, the maternal recognition of pregnancy, and the neuroendocrine regulation of the menstrual cycle in higher primates. In: Neill JD (Editor). Knobil and Kneill's Physiology of Reproduction. San Diego, California. Elsevier Academic Press. Third Edition, pp 2449-2510.

Zhao J, Taverne MA, Van Der Weijden GC, Bevers MM, Van Den Hurk R. 2001. Insulin-like growth factor-I (IGF-I) stimulates the development of cultured rat pre-antral follicles. Mol Reprod Dev 58:287-296.

Zheng J, Fricke PM, Reynolds LP, Redmer DA. 1994. Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. Biol Reprod 51:623-632.

Zor U, Lamprecht SA, Misulovin Z, Koch Y, Lindner HR. 1976. Refractoriness of ovarian adenylate cyclase to continued hormonal stimulation. Biochim Biophys Acta 428:761-765.

Digitally signed by John H. Hagen
DN: cn=John H. Hagen, o=West Virginia University Libraries, ou=Acquisitions Department, email=John.Hagen@mail.wvu.edu, c=US
Reason: I am approving this document.
Date: 2009.06.22 15:43:53 -04'00'