Vascular composition, apoptosis, and expression of angiogenic factors in the corpus luteum during prostaglandin $F_{2\alpha}$ -induced regression in sheep

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Abstract

Corpora lutea and blood samples were collected from superovulated ewes 0, 4, 8, 12 and 24 h after prostaglandin $F_{2\alpha}$ (PGF) analog injection on day 10 of the estrous cycle. Changes in vascular cell and fibroblast composition, apoptosis and mRNA expression for several angiogenic factors in the corpus luteum (CL) were determined. While peripheral progesterone concentration decreased at 24 h after PGF injection, CL weight did not change. The area of positive BS-1 lectin staining (endothelial cell marker), smooth muscle cell actin (SMCA; pericyte and SMC marker), collagen type 1 (fibroblast marker), and the rate of cell death changed in luteal tissues after PGF treatment. In association with these cellular changes, mRNA for several angiogenic factors including vascular endothelial growth factor (*VEGF*) and receptors (*Flt* and *KDR*), basic fibroblast growth factor (*FGF2*) and receptor, *angiopoietin* (*ANGPT*) 1 and receptor *Tie-2*, *endothelial nitric oxide synthase* (*NOS3*), and *angiotensin II receptor 1* (*AT1*) were altered. Changes in endothelial cell marker expression were positively correlated with changes in VEGF and NO systems. In addition, changes in mRNA expression for *VEGF*, *Flt* and *KDR* were positively correlated with changes in *ANGPT2*, *Tie-2*, and *NOS3*, indicating a functional relationship. This data demonstrates that after an initial increase, the endothelial component of the vascular bed decreases during PGF-induced luteal regression. However, SMCA expression remained high during luteal regression, potentially indicating a role of pericytes and vascular SMC in luteolysis, likely to regulate tissue remodeling and to maintain the integrity of larger blood vessels. Further, it appears that early regression may increase collagen type 1 production and/or expression by fibroblasts. Expression of angiogenic factors is influenced by PGF-induced luteolysis and may serve to maintain vascular structure in order to aid luteal regression.

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Introduction

The corpus luteum (CL) is one of the few adult organs exhibiting periodic growth, differentiation and regression – playing a major role in the reproductive process (Niswender & Nett 1994, Hansel & Blair 1996, Milvae *et al.* 1996, Pate 1996). During the course of the estrous cycle (16–21 days in domestic ruminants), luteal tissues exhibit dramatic structural and functional changes including dramatic tissue growth, followed by regression, changes in density of vascular bed and progesterone secretion (Jablonka-Shariff *et al.* 1993, Reynolds *et al.* 1994, 2000).

Growth, differentiation and regression of the CL depend on a balance between luteotropic and luteolytic factors (Niswender & Nett 1994, Hansel & Blair 1996, Pate 1996). In the ewe, the major tropic factor that

controls luteal function is luteinizing hormone (LH), whereas the primary luteolytic factor is prostaglandin $F_{2\alpha}$ (PGF; Niswender & Nett 1994). A number of reported PGF actions, all of which may contribute to functional and structural luteolysis, include reduced ovarian blood flow, uncoupling of the LH receptor from adenylate cyclase, reduced steroidogenic enzyme activity and progesterone production, decreased LH receptor concentration, changes in membrane fluidity, changes in luteal cell population, and increased lysosomal enzyme activity (Niswender & Nett 1994, Hansel & Blair *et al.* 1996, Milvae *et al.* 1996, Pate 1996).

The CL consists of several cell types (e.g., steroidogenic small and large luteal cells, and nonsteroidogenic endothelial cells, pericytes, fibroblasts, and other cells), which differ in morphological and physiological

characteristics. These cell types interact to maintain normal luteal function during growth, differentiation and regression (Grazul-Bilska *et al.* 1997, Niswender *et al.* 2000, Reynolds *et al.* 2000). Perivascular cells comprise of more than 50% of the total cells in the CL (Reynolds *et al.* 1994). During luteal regression, endothelial cells degenerate, but the number of smooth muscle cells and pericytes remains high (Redmer *et al.* 2001, Davis *et al.* 2003). These vascular changes occur simultaneously with a reduction of the functional status of the CL (Davis *et al.* 2003). Determining the profile of angiogenic factors responsible for vascular changes may prove to be important in determining how the composition of the CL is altered during regression. While there are several factors which have been shown to influence vascularity of different tissues, some of the primary regulators of ovarian angiogenesis and vascular function include: the vascular endothelial growth factor (VEGF) system (including the VEGF receptors and the neuropilins; Ferrara *et al.* 1992, Shalaby *et al.* 1995, Neufeld *et al.* 1999, Stacker & Achen 1999), fibroblastic growth factors (FGF) and receptors (Klagsbrun & D'Amore 1991, Neuvians *et al.* 2004a,b), the angiopoietins (ANGPT) and their receptor Tie-2 (Sato *et al.* 1993, Davis & Yancopoulos 1999, Hazzard *et al.* 2000, Wulff *et al.* 2000, Tanaka *et al.* 2004), the nitric oxide (NO) system (Motta *et al.* 2001, Jaroszewski *et al.* 2003, Klipper *et al.* 2004, Shi *et al.* 2004, Al-Gubory *et al.* 2005), and the renin-angiotensin system (Hansel & Blair 1996, Hayashi & Miyamoto 1999, Kobayashi *et al.* 2001). While it is most certain that there are several other angiogenic factors playing a role in the regulation of vascular function, selected members from these families were investigated in the current experiment.

The objective of this study was to evaluate changes in the composition of vascular cells (endothelial cells, pericytes, smooth muscle cells and fibroblasts), the rate of apoptosis, and alterations in angiogenic factor mRNA expression – VEGF, VEGF receptors *Flt* and *KDR*, basic FGF (FGF2), FGF receptor (*FGF-R*), *ANGPT-1*, *ANGPT-2*, the *ANGPT* receptor (*Tie-2*), endothelial NO synthase (*NOS3*), guanylate cyclase 1 soluble $\beta 3$ (*GUCY1B3*), and angiotensin II receptors (*AT1* and *AT2*) – during PGF-induced luteolysis in sheep.

Materials and Methods

Animal treatments and tissue processing

The Institutional Animal Care and Use Committee at NDSU approved all animal procedures utilized in this study.

Mature non-pregnant ewes of mixed breeds ($n=25$) were injected twice daily (morning and evening) with FSH-P (FSH with 10% LH; Sioux Biochemical, Sioux Center, IA) on days 13 (5 units/injection, day 0=estrus), 14 (4 units/injection) and 15 (3 units/injection) of the estrous cycle (total dose=24 units) to induce superovulation (Grazul-Bilska *et al.* 2001). Superovulation was

necessary to collect multiple CL from each ewe for all analyses. McClellan *et al.* (1975) and Hild-Petito *et al.* (1987) demonstrated that CL from superovulated and non-superovulated ewes is comparable in hormonal production and cellular composition. Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized rams. Ovariectomy was performed at 0, 4, 8, 12, and 24 h ($n=5$ ewes/group) after injection of an analogue of PGF (Estrumate; cloprostenol sodium; 2 ml, 250 μ g/ml) on day 10 of the estrous cycle. At the time of ovariectomy, blood samples were collected to determine progesterone concentration with radioimmunoassay. Several CL ($n=2-4$ from each ewe) were dissected from the ovaries, and fixed in Carnoy's or formalin solution for determination of vascular/accessory cells composition by histochemistry or immunohistochemistry, and cell death by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. To determine the vascular composition, tissue sections were stained for the presence of lectin BS-1, which binds to endothelial cells (Augustin *et al.* 1995), and for the presence of smooth muscle cell actin (SMCA), which is expressed by pericytes and smooth muscle cells (Redmer *et al.* 2001). To determine fibroblast expression, tissue sections were stained for the presence of collagen type 1, a specific product of fibroblasts (Freshney 2005). The remaining CL was snap frozen using isopentane and liquid nitrogen and RNA was extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA).

Progesterone RIA

Progesterone concentrations in extracted plasma were measured as previously reported (Jablonka-Shariff *et al.* 1993). The sensitivity of the assay was 12.5 pg/tube and intra-assay coefficient of variation was 4.5%. All samples were analyzed in a single assay.

Histochemistry/immunohistochemistry in luteal tissues

Histochemical localization of lectin BS-1 binding

Detection of lectin BS-1 (from *Bandeiraea simplicifolia*) binding was performed as previously described (Redmer *et al.* 2001). Briefly, to localize endothelial cells, luteal tissue sections (4 μ m) fixed in Carnoy's solution were incubated with fluorescein-labeled lectin BS-1 (a specific marker of endothelial cells; 20 μ g/ml; Sigma) overnight at 4 °C. After rinsing in PBS, sections were covered with coverslips and mounted with aqueous mounting medium containing anti-fading agent (Gel-mount, Biomed, Foster City, CA, USA).

Immunohistochemical localization of smooth cell muscle actin and collagen type 1

Detection of SMCA was performed as previously described (Redmer *et al.* 2001). Briefly, sections of luteal

tissues fixed in Carnoy's solution were rinsed several times in PBS containing Triton-X100 (0.3%, v/v) and were then treated for 20 min with blocking buffer – PBS containing normal goat serum (1–2%, v/v) – followed by treatment with mouse monoclonal antibody against SMCA (Oncogene Research Products, San Diego, CA, USA) or rabbit polyclonal antibody against mouse collagen type 1 (Calbiochem, San Diego, CA, USA) overnight at 4°C. Primary antibody was detected by using a biotinylated secondary antibody (for SCMA, horse anti-mouse IgG; for collagen type 1, goat anti-rabbit IgG) and a streptavidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Collagen type 1 primary antibody was also detected by Alexa fluor 488 labeled anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). After immunofluorescent detection of collagen type 1, counterstaining with propidium iodide (PI; 500 ng/ml; Molecular Probes) was performed to visualize cell nuclei in selected slides. Control sections were incubated with normal mouse or rabbit serum in the place of primary antibody.

Evaluation of apoptosis

To localize apoptotic cells in luteal tissue sections fixed in formalin, TdT-FragEL, DNA fragmentation detection kit was used according to the manufacturer's protocol (Oncogene Research Products).

Image analysis

All images were taken from the parenchymal area where distribution of steroidogenic and accessory cells is relatively uniform; areas with connective tissue tracts were avoided. To determine changes in the cellular composition of the CL, the percentage of the total area that exhibited positive fluorescent staining for BS-1 (occupied by endothelial cells), positive colorimetric staining for SMCA (pericytes and smooth muscle cells) or collagen type 1 (expressed and secreted by fibroblasts)

was evaluated quantitatively with an image analysis system (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA) as described previously (Grazul-Bilska *et al.* 2001). For each CL, 8–10 randomly chosen fields (0.025 mm² per field) were evaluated in each tissue section ($n=8-10$ measurements/CL). Background fluorescence for BS-1 or collagen type 1 staining was minimal and was adjusted to the same level for each section by the image analysis system. The data for BS-1, SMCA, and collagen type 1 are reported as the percentage change from 0 h. Data were expressed as mean percentage $\pm$ S.E.M. of the total area that exhibited positive staining within each field. To determine the rate of apoptosis, images were taken from 10 randomly chosen fields of each CL tissue section. Then, the number of apoptotic cells per area was determined by using image analysis. The data are reported as the mean number of cells $\pm$ S.E.M. per area.

RT-PCR

Messenger RNA levels for a cadre of angiogenic factors and their receptors (Table 1) were determined using quantitative RT-PCR as described by Redmer *et al.* (2005). TaqMan probe-primer sets for ovine *VEGF*, *Flt*, *KDR*, *ANGPT-1*, *ANGPT-2*, *Tie-2*, *FGF2*, *NOS3*, and *GUCY1B3* were used as described previously (Redmer *et al.* 2005). Further, sheep-specific TaqMan probe-primer sets for *FGF-R*, *NP-1*, *AT1* and *AT2* were designed (Table 2). The quality and quantity of total cellular RNA were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA; Redmer *et al.* 2005). All reagents were purchased from Applied Biosystems (Foster City, CA) as described previously (Redmer *et al.* 2005).

Statistical analysis

Data for peripheral progesterone concentration, CL weights, positive staining, the number of apoptotic

Table 1 The genes of interest and the proposed function of each angiogenic gene.

Gene of interest	Proposed function
<i>Vascular endothelial growth factor (VEGF)</i>	Potent angiogenic factor; causes endothelial proliferation and migration, acts via KDR and Flt receptors
<i>VEGF receptor-1 (VEGFR-1 or Flt)</i>	Receptor for VEGF
<i>VEGF receptor-2 (VEGFR-2 or KDR)</i>	Receptor for VEGF
<i>Neuropilin-1 (NP-1)</i>	Aids in VEGF attachment to their receptors
<i>Angiopoeitin-1 (ANGPT-1)</i>	Stabilization of blood vessels
<i>Angiopoeitin-2 (ANGPT-2)</i>	Vascular regression in the absence of VEGF; vascular sprouting in the presence of VEGF
<i>Tie-2</i>	Receptor for ANGPT-1 and ANGPT-2
<i>Basic fibroblast growth factor (FGF2)</i>	Promotes cell growth, differentiation, transformation and angiogenesis
<i>Fibroblast growth factor receptor FGFR</i>	Receptor for FGF
<i>Endothelial nitric oxide synthase (NOS3)</i>	Endothelial-derived synthase which aids in NO production
<i>Soluble guanyl cyclase (GUCY1B3)</i>	Receptor for NO
<i>Angiotensin II receptor 1 (AT1)</i>	Receptor for angiotensin II; functions include generalized vasoconstriction
<i>Angiotensin II receptor 2 (AT2)</i>	Receptor for angiotensin II; functions include potential vasodilation via increasing NO and/or cGMP; may also function in increasing apoptosis via increasing caspase activity

Table 2 Sequence of TaqMan primers and probes for ovine FGF 2 receptor, neutropilin-1 and angiotensin II receptors.

Oligonucleotide	Nucleotide sequence	GenBank accession no. ^a
FGFR2 IIIc FP	5'-TGTTAACACACCGGACAAAGAAA-3'	AJ320477
FGFR2 IIIc RP	5'-CCCAGCATCCTCAAAAGTTACAT-3'	
FGFR2 IIIc Probe	5'/(6FAM)-TGA GGT TCT CTA TAT TCG-(MGBNFQ)-3'	
NP-1 FP	5'-GAT TGC GGT GGA CGA TAT TAG C-3'	AF395335
NP-1 RP	5'-GGT TTT GCG CAG TCC TCT TG-3'	
NP-1 probe	5'/(6FAM)-TCA ATA ACC ACA TCC C-(MGBNFQ)3'	
AT1 FP	5'-AAT CCA CCG CAA TGT ATT TTT CA -3'	AF254119
AT1 RP	5'- TTC GTA ATG GAA AGC GCA AAC-3'	
AT1 probe	5'/(6FAM) -CGA GAA TAC CAA TAT CAC - (MGBNFQ)3'	
AT2 FP	5'-AGT TCC CCT TGG TTG GTG TAT G -3'	S81979
AT2 RP	5'- CTG ACA TCC CGG AAA TAA AAC G-3'	
AT2 probe	5'/(6FAM) -CTT GTC TGT CCT CAT TGC - (MGBNFQ)3'	

^aNucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (2004) database.

cells, and real time RT-PCR analyses were analyzed separately using general linear model (GLM) procedures of SAS Statistical Software (1999). When an F-test was significant, differences between specific means were evaluated by using least significant difference test (Kirk 1982). Means were considered different when $P < 0.05$ unless otherwise stated. Correlations between specific variables were determined by using the PROC CORR correlation analysis of SAS.

Results

Superovulation resulted in an average of 23 ± 1 CL with a range of 5–44 CL per ewe. Circulating progesterone concentrations decreased at 8 h post-PGF injection (Table 3). At 24 h post-PGF injection, peripheral progesterone concentrations decreased to 24% of 0 h control (100%; Table 3). Based on the luteal tissue mass present in the ovaries, progesterone concentration decreased at 4 h post-PGF injection, attaining 9% of 0 h control (100%) at 24 h post-PGF injection (Table 3). Even though progesterone concentration decreased, the mean weight of the CL did not change within 24 h post-PGF injection (Table 3).

Presence of endothelial cells, smooth muscle cells and/or pericytes, and fibroblasts in the CL during luteal regression was detected by using specific markers, BS-1 lectin, SMCA, and collagen type 1 respectively (Fig. 1A–F). While BS-1 lectin (Fig. 1A, B) and SMCA (Fig. 1C, D) staining were detected solely

in the cytoplasm, collagen type 1 (Fig. 1E, F) staining was located in the extracellular matrix and in the cytoplasm of luteal cells (Fig. 2). Positive BS-1 lectin staining increased ($P < 0.01$) at 4 h post-PGF injection, thereafter decreasing ($P < 0.01$) at 8, 12, and 24 h post-PGF injection (Fig. 3A). Smooth muscle cell actin immunostaining increased ($P < 0.01$) at 4 h post-PGF injection, returned to 0 h levels at 8 and 12 h, increasing again at 24 h post-PGF injection (Fig. 3B). Collagen type 1 immunostaining increased ($P < 0.01$) above 0 h levels at 8 and 12 h, and further increased ($P < 0.01$) at 24 h post-PGF injection (Fig. 3C). The number of apoptotic cells, determined by TUNEL staining (Fig. 1G, H), began to increase at 12 h post-PGF injection, further increasing to 18-fold over 0 h controls at 24 h post-PGF injection (Fig. 3D).

Expression of endothelial cell marker BS-1 was positively correlated with peripheral progesterone concentration ($R = 0.378$, $P < 0.069$), and mRNA expression for *VEGF*, *Flt*, *KDR*, *ANGPT2*, *NOS3* and *GUCY1B3* ($R = 0.672$, $P < 0.001$; $R = 0.550$, $P < 0.005$; $R = 0.576$, $P < 0.003$; $R = 0.360$, $P < 0.083$; $R = 0.505$, $P < 0.012$, and $R = 0.537$, $P < 0.007$ respectively); and negatively correlated with collagen type 1 expression and apoptotic cell number ($R = -0.685$, $P < 0.001$, and $R = -0.773$, $P < 0.001$ respectively) after PGF treatment.

Gene expression for five different angiogenic growth factor families including the *VEGF*, *ANGPT*, *FGF2*, *NO*

Table 3 Progesterone concentration in peripheral blood, CL weight at 0, 4, 8, 12 and 24 h after PGF administration, and CL number.

Parameter	0 h	4 h	8 h	12 h	24 h	P value
Progesterone (ng/ml)	5.35 ± 1.53 ^a	5.75 ± 2.54 ^a	1.73 ± 0.72 ^b	1.32 ± 0.22 ^b	1.32 ± 0.52 ^b	<0.08
Percentage change of progesterone to compare with 0 h (% ng/ml)	100 ^a	107 ± 47 ^a	32 ± 14 ^{bc}	24 ± 4 ^c	24 ± 10 ^c	<0.01
Progesterone (ng/ml per g of luteal tissue)	1.39 ± 0.40 ^a	0.60 ± 0.19 ^b	0.28 ± 0.08 ^b	0.33 ± 0.07 ^b	0.13 ± 0.01 ^b	<0.01
Percentage change of progesterone to compare with 0 h (% ng/ml per g of luteal tissue)	100 ^a	43 ± 14 ^b	20 ± 6 ^c	24 ± 5 ^{bc}	9 ± 0.5 ^c	<0.01
Mean CL weight (g)*	392 ± 14	440 ± 11	393 ± 13	399 ± 13	402 ± 12	>0.10
Number of CL (range)*	5–21	6–40	10–24	5–33	10–44	–

^{a,b,c} values with different superscripts differ within a row (P values provided in the table).

*Across the experiment, the number of CL per ewe ranged from 5 to 44 (mean 23 ± 1).

Figure 1 Representative micrograph of staining for BS-1 lectin (A, B), SMCA (C, D), collagen type 1 (E, F) and apoptotic cells (G, H) in luteal tissues from 0 h (left) and 24 h (right) after PGF-treatment. Control sections did not exhibit any positive staining (data not shown), magnification 400X.

Figure 2 Representative micrograph of staining for collagen type 1 (bright green) and cell nuclei (PI; red) in luteal tissue 24 h after PGF injection. Collagen type 1 was localized in cytoplasm of luteal cells (arrowheads) and extracellular matrix (arrows) in the CL, magnification 400 $\times$.

and angiotensin systems changed during CL regression (Fig. 4). The mRNA for *VEGF* and *Flt* increased ($P<0.05$) at 4 h after PGF injection compared to the 0 h control ($P<0.05$), decreasing ($P<0.05$) at 8 h to 0 h levels (Fig. 4A). At 24 h post-PGF injection, expression of *VEGF* and *Flt* mRNA was lower ($P<0.05$) than 0 h control (Fig. 4A). The mRNA for *KDR* did not change at 4 h post PGF. However, at 8, 12 and 24 h, levels of expression were less than ($P<0.05$) that of the 0 h control. Expression of *KDR* mRNA was lower ($P<0.05$) at 12 h and lower ($P<0.05$) at 24 h compared with 4 h post PGF (Fig. 4A). Expression of *NP-1* mRNA was not affected by PGF treatment. While there was no change in *ANGPT-1* expression, *ANGPT-2* mRNA increased ($P<0.05$) at 8 h post-PGF injection compared to 0 h (Fig. 4B). At 24 h post-PGF injection, *ANGPT-2* mRNA expression decreased ($P<0.05$) compared to the 8 h. The ANGPTs receptor *Tie-2* mRNA increased ($P<0.05$) at 4 and 12 h compared with 0 h. Further, mRNA expression of *Tie-2* at 24 h post PGF was less ($P<0.05$) than at 4, 8, and 12 h (Fig. 4B). Basic *FGF* mRNA expression increased ($P<0.05$) from 0 h to 8 and 12 h post-PGF injection. At 24 h post-PGF injection, *FGF2* mRNA expression returned to control level and was less than ($P<0.05$) at 4, 8, and 12 h post PGF. The expression of *FGF2-R* mRNA decreased ($P<0.05$) at 4 and 24 h post-PGF injection, with the expression returning to control level at 8 and 12 h (Fig. 4C). Expression of *NOS3* mRNA increased ($P<0.05$) 4 h post-PGF injection compared to the 0 h control, but then decreased ($P<0.05$) to 0 h expression level at 8, 12, and 24 h (Fig. 4D). In addition, expression of *NOS3* mRNA decreased ($P<0.05$) from 12 to 24 h post-PGF injection (Fig. 4D). Expression of

Figure 3 Changes in BS-1 lectin (A), SMCA (B), collagen type 1 (C) staining of luteal tissues and changes in number of apoptotic cells (D) in luteal tissues after PGF administration. Data are expressed as a percentage change of 0 h. ^{a,b,c,d}Means $\pm$ S.E.M. differ, $P\leq 0.01$; * value differs from 0 h; $P<0.01$; For 0 h of PGF treatment, percentage of total area that exhibited positive staining for BS-1, SMCA and for collagen type 1 were $4.53 \pm 1.52\%$, $10.31 \pm 0.95\%$, and $0.34 \pm 0.04\%$ respectively; the number of apoptotic cells per area was 0.70 ± 0.06 .

GUCY1B3 mRNA at 4, 8, 12 and 24 h was similar to 0 h control; however, at 12 and 24 h, post-PGF injection expression of *GUCY1B3* mRNA was less ($P<0.05$) than at 4 h post-PGF injection (Fig. 4D). While the mRNA expression for *AT2* did not change over time, *AT1* mRNA

Figure 4 Changes in VEGF (A), angiopoietins (B), FGF (C), and nitric oxide (D) systems and their related receptor mRNA in CL after PGF-induced regression. Within a specific gene, ^{a,b,c} means $\pm$ S.E.M. differ, $P \leq 0.05$; *value differs from 0 h; $P < 0.05$.

expression decreased ($P < 0.05$) at 8 h from 0 h controls (Table 4). Expression of *AT1* mRNA was lower ($P < 0.05$) at 8 and 12 h than at 4 h post-PGF treatment (Table 4). Further, there was no difference in the *AT1:AT2* ratio during luteal regression (Table 4).

Expression of *VEGF*, *Flt* and/or *KDR* mRNA was positively correlated with peripheral progesterone concentration, expression of BS-1 and expression of *ANGPT2*, *Tie2*, *FGF2*, *NOS3*, *GUCY1B3* and/or *AT1* mRNA, and negatively correlated with collagen type 1 expression and apoptotic cell number (Table 5), but did not correlate with *ANGPT1*, *FGF2-R*, *AT2* or *NP-1* mRNA expression after PGF treatment.

Discussion

The present study demonstrates dramatic changes in blood progesterone levels, vascular composition and apoptosis in the CL and describes the profile of angiogenic factors and their receptors' mRNA during PGF-induced luteal regression in superovulated sheep. While concentrations of peripheral progesterone were quickly reduced, the weight of the CL was not impacted

up to 24 h after PGF treatment. A similar decrease in systemic progesterone at 4–5 h after PGF_{2α} or PGF analogue treatment was observed for non-superovulated ewes (Nett *et al.* 1976). Also, similar to our study, Silva & Niswender (1984) did not observe changes in luteal weight after PGF treatment in sheep. However, a decrease in CL weight, 16 or 24 h after PGF injection was demonstrated by others (Ji *et al.* 1991, Juengel *et al.* 2000). These discrepancies may be due to some differences in PGF treatment (e.g., doses and type of PGF compound), and FSH treatment vs. no FSH treatment among these studies.

The luteolytic process, which is under the control of PGF, consists of two phases, functional and structural luteolysis, which are reflected predominantly by a decrease in progesterone and a decrease in luteal size respectively (Niswender & Nett 1994). Davis & Rueda (2002) discuss that PGF exerts both direct and indirect effects on luteal steroidogenesis and immune cells as well as indirectly altering endothelial cell function by many intracellular mechanisms. In the present study, we specifically investigated how capillary endothelial cells and pericytes, and fibroblasts may be differentially responsive to PGF treatment.

Table 4 Percentage change in gene expression of angiotensin receptors (AT1 and AT2) from 0 h (100%) and the AT1:AT2 ratio in PGF-treated ewes.

Gene of interest	4 h	8 h	12 h	24 h
AT1	-2.18 ± 9.69^a	$-48.75 \pm 6.66^{b*}$	-12.86 ± 26.98^b	$-37.37 \pm 1.80^{a,b}$
AT2	26.77 ± 34.30	-22.79 ± 20.55	46.23 ± 46.89	-36.36 ± 20.74
AT1:AT2	0.03 ± 0.01	0.02 ± 0.01	0.08 ± 0.01	0.06 ± 0.02

^{a,b} $P < 0.05$; values with different superscripts differ within a row.

* $P < 0.05$; differs from 0 h PGF levels.

Table 5 Correlation coefficients (R) between expression of mRNA for *VEGF*, *Flt* and *KDR* and specific variables after PGF treatment.

Variable	Peripheral progesterone (ng/ml)	BS-1	VEGF	Flt	KDR	ANGPT2	Tie2	FGF2	NOS3	GUCY1B3	AT1
VEGF	0.521 <i>P</i> <0.009	0.672 <i>P</i> <0.001	–	0.819 <i>P</i> <0.001	0.562 <i>P</i> <0.004	0.403 <i>P</i> <0.051	0.562 <i>P</i> <0.004	0.466 <i>P</i> <0.022	0.606 <i>P</i> <0.002	NS	NS
Flt	NS	0.550 <i>P</i> <0.005	0.819 <i>P</i> <0.001	–	0.613 <i>P</i> <0.001	0.387 <i>P</i> <0.061	0.400 <i>P</i> <0.053	NS	0.637 <i>P</i> <0.001	0.484 <i>P</i> <0.016	NS
KDR	NS	0.576 <i>P</i> <0.003	0.562 <i>P</i> <0.004	0.613 <i>P</i> <0.001	–	0.344 <i>P</i> <0.099	0.441 <i>P</i> <0.031	NS	0.449 <i>P</i> <0.028	0.480 <i>P</i> <0.017	0.529 <i>P</i> <0.008

NS=not significant.

Treating ewes with PGF resulted in a decreased area of positive BS-1 lectin staining in luteal tissues, indicating that the endothelial cell component of the vascular bed was decreased, which was accompanied by a high rate of apoptosis observed during early regression. For ovine CL, Sawyer *et al.* (1990) demonstrated that after PGF treatment, the number of endothelial cells decreased first, followed by decrease of other cell types. However, SMCA expression remained high during luteal regression in the present study, indicating continued presence of SMC and pericytes throughout the induced luteolysis. The increase in the expression of markers for endothelial cells and SMC/pericytes at 4 h after PGF treatment, followed by a later decrease is likely to be associated with increased blood flow observed just after injection of PGF or PGF analogue in cows (Miyamoto *et al.* 2005). Furthermore, it has also been shown that increased blood flow within 6 h activates endothelial cells, which was manifested by lumen protrusions, increases in cytoplasmic organelles, sproutings in the capillaries, and/or other changes, comparable to angiogenesis (Masuda *et al.* 2003). Therefore, we hypothesize that the initial increase in positive staining of endothelial cells is associated with increased blood flow and tissue remodeling induced by PGF. In addition, in our study, 4 h post-PGF treatment, we have observed enhanced expression of *NOS3* mRNA, indicating an increase in NO production. Therefore, NO is likely to have caused vasodilatation of luteal blood vessels, which may be reflected by the enhanced area of positive staining of endothelial cells and pericytes/SMC at 4 h post-PGF treatment in our study.

Pericytes are regulators of endothelial function and have been shown to produce angiogenic factors (Bergers & Song 2005). Redmer *et al.* (2001) confirmed VEGF localization to pericytes/vascular SMC in the CL. VEGF is recognized as a potent angiogenic factor, but it can also play a role in vascular permeability and vascular bed maintenance (Stacker & Achen *et al.* 1999). It is possible that during early regression, pericytes and SMC may serve to regulate tissue remodeling and maintain integrity of larger blood vessels, allowing normal luteolysis to occur.

Interestingly, expression of collagen type 1 increased as luteolysis progressed. This indicates that during CL regression numbers of fibroblasts and/or ability of

fibroblasts to produce collagen type 1 are enhanced. Comparing non-regressed and regressed CL on the same day 16 of the estrous cycle, Farin *et al.* (1986) demonstrated that while the percentage of luteal cells remained somewhat constant, the percentage of fibroblasts tended to increase, and capillary endothelial cells/pericytes tended to decrease. Similarly, Sawyer *et al.* (1990) reported an increase in fibroblasts and a decrease in endothelial cells in the CL from PGF-treated ewes. Progesterone concentrations decrease soon after PGF treatment (present study; Nett *et al.* 1976). These data clearly demonstrate that PGF treatment not only affects the function of the steroidogenic cells but also directly affects both the composition and function of non-steroidogenic cells in CL. Further, as collagen type 1 is involved with fibrosis, an understanding of fibroblast function in the regressing CL may aid in the understanding of fibroid diseases (Ghosh 2002). These data suggest that luteal regression is, in part, a fibrosis response to luteolytic mechanisms. Initiation of fibrosis can be mediated by TGF- β produced by pericytes (Antonelli-Orlidge *et al.* 1989), and later maintained by FGF2, as demonstrated before in skin fibrotic disorders (Takehara 2000). However, elucidation of fibrosis mechanisms during luteal regression requires additional study.

The number of cells undergoing apoptosis increased at 12 h and 24 h after PGF treatment, which was associated with the decrease in the number of endothelial cells in the present study. The initial hours following PGF treatment do not appear to decrease the total number of cells within the CL or CL weight, but rather seems to affect cell function since progesterone declines very early (4 h) after induction of luteolysis but total cell numbers start to decline at about 24 h post-PGF treatment (Nett *et al.* 1976, Farin *et al.* 1986, Sawyer *et al.* 1990, present study). It seems that during functional luteolysis, when endothelial cells begin to undergo cell death, fibroblast numbers start to rise, likely negating any change in the total weight of the CL. Sawyer *et al.* (1990) postulated that during luteal regression, endothelial cell numbers are reduced first, followed by a decline of parenchymal cell numbers, and lastly the number of fibroblasts decrease. Data from the present and other studies (Azmi *et al.* 1984a,b) support this observation.

In this study, we investigated 13 different angiogenic factors and receptors, including members of the VEGF, FGF, ANGPT, NO, and angiotensin systems. Prostaglandin F_{2α} treatment appeared to have impacted the VEGF system most dramatically with *VEGF*, *Flt* and *KDR* mRNA expression declining below control levels. While the patterns of *VEGF* and *Flt* mRNA expression mirrored each other, *KDR* mRNA decreased earlier after PGF treatment but changes in mRNA expression for VEGF, Flt and KDR were positively correlated. The rise in *VEGF* mRNA at 4 h after PGF treatment may be a result of acute hypoxia (Shweiki *et al.* 1992) and/or NO stimulatory effects (Reynolds *et al.* 2000). Since VEGF acts through KDR receptor to increase endothelial cell proliferation (Shalaby *et al.* 1995), we postulate that KDR may protect endothelial cells against apoptosis and maintain endothelial cell function, however, this hypothesis requires further investigation. Nevertheless, this hypothesis is supported by the observation that *KDR* mRNA expression was positively correlated with a decrease in endothelial cell expression after PGF treatment, although a decrease in *KDR* mRNA expression appeared earlier (8 h) compared to the decrease in endothelial cell area (12 h). When VEGF acts via Flt, endothelial cell migration and regulation of vessel integrity occurs (Shibuya 2001). The expression of *Flt* mRNA was not reduced from the 0 h control until 24 h post-PGF treatment. While it seems unlikely that angiogenesis occurs during luteolysis, vessel integrity needs to be maintained early to ensure the removal of cells from the regressing CL (Azmi *et al.* 1984a,b). However, for the cow, a decrease in *VEGF* mRNA occurred at 12 h, *Flt* and *KDR* mRNA at 48 h, while VEGF protein decreased at 2 h post-PGF treatment (Neuvians *et al.* 2004b). The difference between our study and Neuvians *et al.* (2004b) is likely to be due to the different species and PCR procedures used for mRNA detection. Interestingly, after PGF treatment, VEGF could stimulate other biological responses including non-angiogenic functions in vascular permeability, allowing for the increase in neutrophils necessary for luteal regression (Pate 1996). Further, we have demonstrated that VEGF is localized to the pericytes in the corpus luteum, and that cultured luteal pericytes express *VEGF* mRNA (Redmer *et al.* 2001, Beckman *et al.* 2006). In this study, at 24 h post-PGF, we observed a decrease in *VEGF* mRNA expression. We hypothesize that the decrease of VEGF expression initiates the loss of capillary endothelial cells followed by the loss of pericytes during luteal regression, since in fully regressed ovine CL, expression of SMCA is greatly reduced and restricted to larger vessels only (Redmer *et al.* 2001). This hypothesis is supported by the observation that suppression of VEGF causes sharp decline in endothelial cell number in primate CL (Fraser & Duncan 2005).

In the present study, there was no change in *ANGPT-1* mRNA expression, while *ANGPT-2* mRNA increased

at 8 h post-PGF when *VEGF* mRNA started to decline. Lack of changes in *ANGPT-1* and *ANGPT-2* mRNA expression from mid- and late-luteal phases were reported for human and macaque CL (Hazzard *et al.* 2000, Wulff *et al.* 2000). In contrast, in bovine CL, *ANGPT-1* but not *ANGPT-2* mRNA declined during luteal regression (Tanaka *et al.* 2004). In fact, *ANGPT-2* mRNA expression increased in the CL, 2 h after PGF treatment (Tanaka *et al.* 2004). These data indicate that expression of ANGPT is species specific. Hypoxia, along with VEGF, stimulates ANGPT-2 production (Oh *et al.* 1999). While ANGPT-1 acts synergistically with VEGF (Koblizek *et al.* 1998) to stimulate angiogenesis, ANGPT-2 is an antagonist of ANGPT-1, and in the absence of VEGF, it is thought to destabilize or regress blood vessels (Maisonpierre *et al.* 1997). Therefore, ANGPT-2 may have a role in initiation of vascular regression in sheep and cows. This is supported by results of Sugino *et al.* (2005) who reported enhanced expression of *ANGPT2* mRNA in regressing human CL to compare with differentiated CL from the mid-luteal stage of the menstrual cycle.

In the present experiment, *FGF2* mRNA expression increased after induction of regression and then decreased. Similar data for *FGF2* mRNA expression were reported for bovine CL during PGF-induced luteolysis (Neuvians *et al.* 2004a,b). However, different patterns of *FGFR* mRNA expression were observed for sheep (present study) and cows (Neuvians *et al.* 2004b). The increase in *FGF2* mRNA may have resulted from a skew in the ratio and/or activity of fibroblasts present in the CL during luteolysis since enhanced expression of collagen type 1 protein was observed in this study. On the other hand, enhanced *FGF2* mRNA could have caused increased luteal fibroblasts activity and/or number. Fibroblasts are known to increase in number in the regressing CL of several species including sheep (Farin *et al.* 1986, Sawyer *et al.* 1990) and the horse (van Niekerk *et al.* 1975). Basic FGF may not regulate endothelial cell function during the initial steps of luteolysis, as a decrease in endothelial cell staining was not seen until 12 h post-PGF injection, a time when *FGF2* mRNA expression was still elevated over control levels.

The NO system plays an important role in luteal regression in several species (Skarzynski *et al.* 2003, Jaroszewski *et al.* 2003, Boiti *et al.* 2003, Miyamoto *et al.* 2005). During luteolysis, *NOS3* mRNA decreases, which can be attributed to the decline in endothelial cell number (Bioti *et al.* 2003). In the present study, the initial increase in expression levels of *NOS3* mRNA may be explained by the initial increase in the area occupied by endothelial cells at 4 h post-PGF injection. In fact, *NOS3* mRNA expression was positively correlated with endothelial cell marker expression. Further, we did not look at other NOS isoforms including neuronal or inducible NOS. However, we do hypothesize that one or both synthases may play a role in progesterone decrease, as NO has been proposed to regulate luteal steroidogenesis

(Van Voorhis *et al.* 1994, Jaroszewski & Hansel 2000, Motta *et al.* 2001).

The specific role of GUCY1B3 in luteal regression has not been fully investigated. However, GUCY1B3 was immunolocalized to the blood vessels of the CL and in luteal tissue, stimulated with human chorionic gonadotropin in rats (Shi *et al.* 2004). The pattern of GUCY1B3 mRNA expression followed the pattern of NOS3 mRNA expression in the present study. Since GUCY1B3 serves as a receptor for NO, such a close relationship is not surprising.

In summary, we have demonstrated with immunohistochemical staining that during early regression, initial maintenance of endothelial cells and pericytes may serve to regulate tissue remodeling allowing normal luteolysis to occur. After an initial increase, the endothelial component of the vascular bed decreases during PGF-induced luteal regression. However, SMCA expression remained high during luteal regression, potentially indicating a role for pericytes and vascular smooth muscle in luteolysis, the regulation of tissue remodeling and for maintenance of the larger blood vessel integrity. Further, it appears that luteal regression is, in part, a fibrosis response. In the present study, the VEGF-receptor system was greatly impacted by PGF-induced luteal regression. Changes in mRNA expression for VEGF, Flt and/or KDR were positively correlated with changes in ANGPT2, Tie-2, FGF2, NOS3 and/or GUCY1B3 during PGF-induced luteolysis, indicating a functional relationship. The role of angiogenic factors, particularly VEGF and other VEGF-induced systems, but also FGF2, ANGPT, NO and renin-angiotensin systems, is likely to coordinate endocrine, immune and structural changes that are occurring during this rapid decline in the progesterone-producing capacity of the CL. It is obvious that pericyte-endothelial cell and other cell type interactions are necessary for both luteal growth (Redmer *et al.* 2001, Davis & Rueda 2002), and luteal regression.

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