Regulation of Angiogenesis-Related Prostaglandin F2alpha-Induced Genes in the Bovine Corpus Luteum

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ABSTRACT

We recently compared prostaglandin F2alpha (PG)-induced global gene expression profiles in PG-refractory, bovine corpus luteum (CL) collected on Day 4 of the estrous cycle, versus PG-responsive, Day 11 CL. Transcriptome analyses led us to study the regulation of angiogenesis-related genes by PG and their functions in luteal endothelial cells (ECs). We found that PG regulated angiogenesis-modulating factors in a luteal stage-dependent way. A robust increase in FGF2 expression (mRNA and protein) occurred in the PG-refractory Day 4 CL promoting CL survival and function. Inhibitors of FGF2 action, thrombospondins, and PTX3 were upregulated by PG specifically in Day 11 CL undergoing luteolysis. VEGF mRNA decreased 4 h post-PG in both Day 4 and Day 11 CL. The resulting destabilization of blood vessels in Day 11 CL is expected to weaken the gland and reduce its hormonal output. These genes were expressed in dispersed luteal ECs and steroidogenic cells; however, thrombospondin 1 and FGF2 were more abundant in luteal ECs. Expression of such genes and their ability to modulate FGF2 actions were investigated. Similar to its in vivo effect, PG, in vitro, stimulated the expression of thrombospondins and PTX3 genes in several luteal cell models. Importantly, these factors influenced the angiogenic properties of luteal ECs. FGF2 dose-dependently enhanced cell migration and proliferation, whereas thrombospondin 1 and PTX3 inhibited FGF2 actions in luteal ECs. Collectively, the data presented here suggest that, by tilting the balance between pro- and antiangiogenic factors, PG can potentially control the ability of the CL to resist or advance toward luteolysis.

angioinogenesis, corpus luteum, gonadal function, luteolysis, prostaglandins

INTRODUCTION

In mammalian species, including cattle, the corpus luteum (CL) is critically important for reproductive success [1, 2]. In the absence of an embryonic signal, the CL will regress. CL regression is necessary for initiating a new reproductive cycle, whereas extension of luteal life span and secretion of progesterone are absolutely required for maintenance of pregnancy. Prostaglandin F2α (PG) is the principal luteolytic hormone in cattle and other species with an estrous cycle [1, 2]; it is therefore widely used for synchronization of the reproductive cycle. In a nonfertile cycle, PG is secreted from the uterus at the late luteal phase and triggers CL regression [3, 4]. During luteolysis, progesterone secretion rapidly decreases, which is followed by a gradual structural demise of the CL [2]. Exogenously administered PG can initiate luteolysis only in the mature CL [5]. For instance, the bovine CL is resistant (or refractory) to the luteolytic actions of PG before Days 5–7 of the estrous cycle [6, 7]. The refractory period exists even though early bovine CL contain receptors for PG [8] and can respond to PG injection by changing hormone secretion and gene expression [7, 8]. Although the original observation by McCracken on the role of PG in luteal regression was made in the early 1970s, and despite its widespread use in laboratory and farm animals [4, 9], the underlying mechanisms associated with the stage-specific response to PG have remained elusive and are currently the subject of intense research.

We recently reported results of studies using functional genomics to compare PG-induced gene expression profiles in PG refractory (collected on Day 4 of the estrous cycle) versus PG-responsive bovine CL (collected on Day 11) [10]. The results indicated a more robust and distinct gene expression response to exogenous PG of Day 11 (PG-responsive) versus Day 4 (PG-refractory) bovine CL. The accentuated gene expression response in Day 11 CL was accompanied by specific enrichment of PG-regulated genes in distinctive gene ontology categories. One of the intriguing pathways that emerged from these analyses was a group of genes involved in angiogenesis (FGF2, PTX3, CD36, THBS1, THBS2): genes encoding both pro- and antiangiogenic proteins. We therefore hypothesized that the balance between pro- and antiangiogenic factors may control the ability of the CL to evade or undergo PG-induced luteolysis. To gain a better understanding of the relationship between luteolytic PG actions and angiogenesis, we investigated how these genes are regulated by PG in whole CL and in vitro, in several luteal cell models. Thereafter, their luteal cell localization was determined and the biological effects of PG-regulated gene products on the angiogenic response of luteal endothelial cells (ECs) were studied.
MATERIALS AND METHODS

Animals and Sample Collection

Animals, treatment, and sample collection were described in detail in our previous publication [10]. All animal procedures were approved by the All University Committee on Animal Use and Care at Michigan State University. Briefly, heifers were assigned to one of six treatments (n = 5 per treatment) using a completely random design. Ovaries containing corpora lutea were collected under epidural anesthesia at 0 (control), 4 h after injection of saline (control) or PG on Day 4 or Day 11 of the estrous cycle. Upon ovariectomy, luteal tissue was dissected away from stroma and weighed. Following ovariectomy, aliquots of CL were diced and snap frozen in liquid nitrogen and stored at −80 °C for isolation of RNA for real-time RT-PCR and protein for Western blot analyses.

Microarray Data Analysis

Our previous dataset (accession no. GSE23348; Gene Expression Omnibus) of PG-regulated gene [10] was examined to look for angiogenic-related genes differentially regulated by PG on Day 4 versus Day 11.

CL Dispersion and Preparation of Luteal Cell Subpopulations

The procedure was carried out as we and others have described previously [11, 12]. Briefly, CL were collected at a local slaughterhouse, and the luteal stage was determined by macroscopic examination of the ovaries, according to criteria described by Fields and Fields [13]. CL of early (before Day 5) and mid- (Days 8–12) luteal stages were dispersed using collagenase IV (Worthington).

Dispersed luteal cells were either incubated with PG or used for further isolation using BS-1-coated magnetic tosylactivated beads to obtain luteal ECs. Adherent, BS-1-positive cells (endothelial) and nonadherent cells (enriched steroidogenic cell fraction) were immediately processed for RNA extraction. Dispersed luteal cells (10 000 cells/well) were incubated in 96-well plates at 37 °C in a humidified atmosphere (5% CO2 and 95% air) for 4 h in the presence of different doses of PG, as indicated. RNA was extracted from cells collected at the end of the incubation and processed for real-time PCR (see below).

Isolation and Culture of Luteinized Granulosa Cells

Ovaries bearing large follicles (>10 mm in diameter) were collected at a local slaughterhouse as previously described [14, 15]. Only follicles containing >4 million viable cells were included in these experiments. Granulosa cells were enzymatically dispersed and cultured overnight in Dulbecco modified Eagle medium (DMEM)-F12 containing 3% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Hemeek, Israel), 2 mM l-glutamine, and 100 μg/ml penicillin streptomycin solution (Biological Industries). The next day, the medium was replaced with luteinization medium (1% FCS, 2 μg/ml insulin and 10 μM forskolin; Sigma Aldrich, St. Louis, MO). On Day 6 of culture, the cells were washed and PG or its analog chloroprostol (Cayman Chemical Co., Ann Arbor, MI) was added in basal media and incubated for 4 h.

Isolation and Long-Term Culture of Luteal Microvascular ECs

The procedure used for the isolation and culture of luteal ECs from midcycle bovine CL was carried out as described previously by Spanel-
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18]. The glyceraldehyde 3-phosphate dehydrogenase (GPDH) gene was used as the housekeeping gene. Dissociation curves after each real-time run confirmed the presence of only one product and the absence of the formation of primer dimers. The threshold cycle number (Ct) for each tested gene X was used to quantify the relative abundance of the gene; arbitrary units were calculated as \(2^{-\Delta\Delta Ct} = 2^{-\Delta Ct \text{ target gene } X - \Delta Ct \text{ housekeeping gene}}\). The primer sequences used were as follows: VEGF forward, 5’-CCATGAACTTTCTGCTCTTGG-3’, reverse, 5’-TTCATGAACTCCACCCTCCTGG-3’ (NM_174216); GPDH (NM_001034034) forward, 5’-GGCTGGAACCCAGGAAAGTAT-3’, reverse, 5’-CGTGGGAGCGGTCACAATGG-3”; CD36 (NM_174010) forward, 5’-ACTGAGATGACGACCTTCA-3’, reverse, 5’-AATGGATTTGTA TAGGCCC-3’; PTX3 (NM_001076259) forward, 5’-AACAGACTTGGTCGCT GATGCT-3’, reverse, 5’-TTCCAGTTGCTGGCAACAGAT-3’; FGF2 (NM_174056) forward, 5’-TGGTCTCCCTCTACTCTGTA-3’, reverse, 5’-ACTCCCTGTAAAGAAGGTTGCT-3’; THBS1 (NM_174196) forward, 5’-ATCATGCTGATCTGACGAC-3’, reverse, 5’-TAAAGCCTGATGTCTCCA GAA-3’; THBS2 (NM_176872) forward, 5’-GCTGTCGCTCGTGTGACTAC-3’, reverse, 5’-TAGGTAGGTCAGGGTTG-3’.

Cell Proliferation Assay

Cultured luteal ECs were seeded in 96-well dishes and cultured overnight in DMEM-F-12 medium containing 10% FCS. Cells were then starved for 24 h in media containing 0.5% bovine serum albumin and 0.1% FCS. The following day, fresh basal media containing FGF2 (1–25 ng/ml; PeproTech, Inc., NJ), thrombospondin 1 (ProSpec-Tany TechnoGene Ltd., Ness-Zion, Israel), small molecule 27 (previously shown to mimic the FGF2 binding sequence present in TSP-1; obtained from the Developmental Therapeutics Program, NCI National Institutes of Health, Rockville, MD), or recombinant human PTX3 (kindly provided by A. Inforzato, Istituto Clinico Humanitas, IRCCS, Rozzano, Italy) were added, and incubation was continued for another 2 days. Cell numbers were estimated as previously described [18] using the XTT kit (Biological Industries), which measures the reduction of a tetrazolium component (XTT) into a soluble formazan product by the mitochondria of viable cells. The intensity of the dye obtained is proportional to the number of metabolically active cells. On the day of measurement, cells were washed and XTT was added according to the manufacturer’s instructions. Plates were incubated at 37°C for 1–3 h. The absorbance was read at 450 nm (reference absorbance, 630 nm).

Readings of blank wells (without cells) were subtracted and changes in cell numbers were determined by dividing the mean of treated wells by the mean of controls (four replicates for each treatment or control group). Each experiment was accompanied by a calibration curve of cells (1500–10000). Only optical density readings in the linear range of the calibration curves were used.

Western Blot Analyses

Western blot analysis was conducted using procedures described previously [10, 17]. Protein samples were mixed with sample buffer (×5) containing β-mercaptoethanol and separated by 7.5%-12.5% SDS-PAGE, and subsequently transferred to nitrocellulose membranes. Membranes were blocked overnight in 5% nonfat milk at 4°C and then incubated for 2 h with rabbit anti-FGF2 antisera (diluted 1:1000; kindly provided by D. Schams, Technical University of Munich, Munich, Germany) or with anti-PTX3 antisera (diluted 1:2000; kindly provided by A. Inforzato) in 1% nonfat milk. Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in 1% nonfat milk at room temperature for 1 h. A chemiluminescent signal was generated with SuperSignal (Thermo Fisher Scientific, Rockford, IL), and the membranes were exposed to x-ray film. Membranes were stripped and reprobed with actin antibody for normalization prior to quantification of effects of PG injection on protein abundance.

Scratch/Wound Healing Assay

Approximately 100,000 cultured luteal ECs/well were seeded in 24-well plates and incubated in complete medium until the culture became confluent. Cells were rinsed with PBS and starved (as above) for 24 h. Using sterile 200-ml pipette tips, 2 vertical lines were scratched on bottom of wells. Cells were washed and treatments (10 ng/ml FGF2 with or without small molecule 27) were added at specific time intervals. Cells were photographed at time 0 and 16 h later using a phase-contrast microscope. Cell coverage of scratched area was analyzed with NIH image software.

Statistical Analyses

Data are presented as means ± SEM; experiments were repeated at least three times. Data were analyzed by either one-way ANOVA or Student t-test.
Differences were considered significant at $P < 0.05$. All data were assessed for heterogeneity of variance using a Bartlett test, and were found to be nonsignificant.

RESULTS

Stage-Specific Regulation of Angiogenesis-Related Gene Expression by PG Administration

Results presented in Figure 1 demonstrate increased mRNA ($P < 0.05$) for the antiangiogenic factors PTX3, thrombospondin 1 (gene symbol, THBS1; protein abbreviation, TSP-1), and THBS2 in Day 11, but not in Day 4, CL at 4 h post-PG injection relative to the 0-h time point. In addition, the expression of the CD36 molecule (thrombospondin receptor) was more profoundly elevated in the mature CL 24 h post-PG injection (11.3-fold vs. 2.1-fold on Day 4). The two proangiogenic factors, VEGF and FGF2, exhibited inverse patterns of expression; VEGF was significantly decreased 4 h post-PG in Day 4 CL and tended to decrease ($P = 0.07$) on Day 11. On the other hand, FGF2 mRNA levels was elevated. The increase in FGF2 following PG for Day 4 CL was dramatic: its mRNA levels were 24-fold higher relative to the 0 time point, compared with 6-fold on Day 11. Furthermore, a significant elevation (approximately 10-fold) in FGF2 protein levels in response to PG was only observed in Day 4, but not in Day 11 CL (Fig. 2).

Stage and Cellular Distribution of PG-Regulated Genes That Modulate Angiogenesis

We next examined which luteal cell types expressed angiogenic genes that were shown to be induced by PG. We found that these genes were expressed by both luteal endothelial and enriched steroidogenic cells derived from early or midcycle CL (Fig. 3). This is in contrast to cell-specific gene markers, such as CD31, EDN1, and SELE (for luteal ECs), or STAR and CYP11A (for steroidogenic cells), which were exclusively expressed in these respective cell types (data not shown). THBS1 was more abundant in luteal ECs (both from early and midluteal stages), and THBS2 was expressed at much lower levels than THBS1 and did not exhibit preferential expression between steroidogenic and luteal cells. FGF2 mRNA levels were higher in ECs derived from early-stage CL as compared with their steroidogenic counterparts or with dispersed luteal ECs derived from midcycle CL. PTX3 expression did not differ between cells and luteal stages (Fig. 3).

While cells isolated by the magnetic beads method reflect the CL from which they were obtained, they quickly lose their
characteristics in culture [19, 20]. Therefore, for the next experiments, other luteal cells were used.

In Vitro Regulation of Angiogenesis-Related Gene Expression by PG in Luteal Cell Models

We then examined whether angiogenesis-related genes, shown to be upregulated by PG in vivo (Fig. 1), can also be induced in vitro in response to PG or its analog, cloprostenol, in several luteal cell models (Fig. 4). Luteinized granulosa cells incubated with varying doses of PG exhibited dose-dependent increases in FGF2 and the two THBSs. The highest stimulation (2.8-fold over control) was observed for THBS2 in these cells (Fig. 4A). Similar effects were recorded with cloprostenol in luteinized granulosa cells (data not shown). Exposure of luteal ECs to PG analog also elevated the expression of FGF2, THBS1, and THBS2, with FGF2 being the gene induced most notably in these cells (Fig. 4B). PTX3 could not be examined in these two cell preparations, because it was downregulated during longer culture periods. However, using freshly dispersed mixed luteal cells, we could show that PG elevated PTX3 mRNA in a dose-dependent manner (Fig. 4C). In both luteinized granulosa cells and mixed luteal cells, the maximal response was observed with 0.1 μg/ml of PG.

Effects of Angiogenesis Modulating Proteins on Luteal EC Functions

The biological effects of FGF2, TSP-1, and PTX3 were studied in cultured luteal ECs. FGF2 dose dependently elevated luteal EC numbers after 2 days of incubation. It was found that 10 ng/ml was the most effective dose for augmenting viable cell numbers (~2-fold; Fig. 5). Cell photographs (Fig. 5, lower panel) confirmed that cells cultured in the presence of 5 and 10 ng/ml FGF2 became more populated than cells grown under basal conditions, indicating that cell proliferation had occurred. The effects of PTX3, another PG-regulated gene, on luteal EC proliferation are shown in Figure 6. Addition of rhPTX3 to

FIG. 4. Effect of varying doses of PG or cloprostenol on gene expression in luteal cell types. A) Luteinized granulosa cells were incubated with varying doses of PG for 4 h. B) Cultured ECs were incubated with cloprostenol 1 (μg/ml) for 4 h. FGF2, THBS1, and THBS2 genes were measured in both A and B. C) Mixed luteal cells were incubated with varying doses of PG for 4 h and PTX3 mRNA was measured. Data were normalized relative to the abundance of GPDH in the same samples. *P < 0.05.
luteal ECs reduced FGF2-stimulated luteal EC numbers. PTX3 did not, however, alter cell number under basal conditions. Similarly, conditioned media of PTX3 overexpressing cells also reduced cell numbers as compared with luteal ECs incubated with conditioned media harvested from cells overexpressing GFP (data not shown).

Addition of small molecule 27 (mimicking FGF2 binding domain in TSP-1 [21]), reduced luteal EC proliferation induced by FGF2 in a dose-dependent manner (Fig. 7A). These molecules were ineffective under basal conditions (Fig. 7A, inset). The presence of the small molecule (20 and 100 \( \mu \)g/ml) also inhibited luteal EC migration (as determined by scratch/wound healing assay) stimulated by FGF2 (Fig. 7B). Addition of recombinant TSP-1 (whole molecule) reduced cultured luteal EC numbers under basal or FGF2-stimulated conditions (Fig. 8A). Contrary to FGF2, TSP-1 visibly induced cell death (Fig. 8B, left panel) and the appearance of apoptotic 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (Fig. 8B).

**DISCUSSION**

Our findings show luteal-stage-dependent regulation of angiogenesis modulating genes by PG. While proangiogenic FGF2 was markedly elevated in the Day 4 refractory CL, inhibitors of FGF2 action, particularly THBS1, THBS2, and PTX3, increased specifically in the Day 11, but not the Day 4, gland. Furthermore, pronounced inhibition of FGF2-induced proliferation and/or migration of luteal ECs was observed in vitro in response to TSP-1 and PTX3 treatment. These genes were expressed by dispersed steroidogenic and luteal ECs alike, but THBS1 and FGF2 were more abundant in luteal ECs. Reduced angiogenic support, due to lower levels of FGF2 and VEGF, along with increased expression of antiangiogenic factors in Day 11 PG-responsive CL observed here, is expected to destabilize luteal vasculature and reduce its hormonal output characteristic of luteal regression. The increased ANGPT2 mRNA and protein soon after PG administration [22, 23] would also contribute to vessel destabilization. These events, along with increased blood vessel constriction by endothelin 1 elevated by PG only in the mature gland [5, 24], would further reduce progesterone secretion [25–27] and promote regression of the mature CL.

We found that the two main luteal proangiogenic factors, VEGF and FGF2, were inversely affected by PG. VEGF mRNA was reduced or tended to be reduced 4 h post-PG on both Days 4 and 11. In contrast, a robust increase in FGF2 mRNA and protein occurred in the immature, Day-4 gland. The changes induced by PG in FGF2 in bovine CL were more pronounced at the mRNA level as compared with its respective protein. This difference could result from the higher sensitivity of the RT-PCR technique compared with the Western blot, or possibly from PG differently affecting transcription versus translation of FGF2. Shirasuna et al. reported an increase in luteal FGF2 mRNA within 30 min post-PG administration on Day 4 of the cow’s estrous cycle [28, 29]. Consistent with our findings, an inverse expression profile for VEGF and FGF2 was reported by Neuvians et al. [30] in cows injected with PG at midcycle. They also found that, along with VEGF, its type 2 receptor decreased during luteolysis, further diminishing VEGF function, but effects of PG on the early CL were not examined in that study.

Both VEGF and FGF2 levels are elevated during luteinization, and both are involved in angiogenesis, which accompanies the development of the CL [31–33]. However, their profiles are different: high levels of FGF2 were evident mostly during the very early luteal phase, whereas VEGF
increased after ovulation, but remained high throughout midcycle [34, 35].

In general, FGF2 is known to be more potent at inducing endothelial cell proliferation than VEGF; indeed, treatment with an FGF receptor signaling inhibitor almost completely blocked luteal endothelial network formation. Effects of FGF receptor signaling inhibitor occurred even in the presence of exogenous VEGF, which suggests that FGF2 is critical for the formation of luteal endothelial networks [36]. Here, too, we showed that FGF2 was a potent inducer of angiogenic characteristics in luteal ECs.

The mRNA for PTX3, THBS1, and THBS2 was strongly upregulated by PG on Day 11 of the cycle. The increased CD36, which is expected to further promote the biological functions of TSPs, was also observed in Day 11 CL. TSP-1 and TSP-2, as well as PTX3, have been implicated in antiangiogenic activities (as detailed below). FGF2 activity in the extracellular milieu is controlled by its interaction with various extracellular matrix proteins and binding factors. PTX3 and TSPs are two prominent examples of such factors [37]. Although their effects on ECs were examined before, studying their effects specifically on CL-derived ECs in the present study is necessary, because the endothelium exhibits remarkable diversity based on vessel size and tissue of origin [38–40]. Recent observations have shown that PTX3 binds FGF2 with high affinity and specificity [41, 42]. This interaction prevents the binding of FGF2 to its cognate tyrosine kinase receptors, leading to inhibition of the angiogenic activity of the growth factor [37, 43]. PTX3 is a prototypic member of the long-pentraxin family [37], which plays a role under various pathophysiological conditions, and may serve as a mechanism for amplifying inflammation and immune responses that are relevant to luteolysis as well [44]. We found here that both dispersed luteal endothelial and steroidogenic cells expressed PTX3. More importantly, PG upregulated PTX3 (mRNA and protein [10]) levels in CL and in dispersed luteal cells from midcycle. These findings suggest that PTX3 might be a novel physiological regulator of CL function associated with luteolysis. When cultured with luteal ECs here, purified hPTX3 protein and conditioned media of overexpressing cells (data not shown), hPTX3 inhibited the FGF2-induced increase in luteal EC numbers. However, the effect was somewhat smaller than that reported for human skin ECs [45] or murine aortic ECs [46]. The reason for this is currently unclear, but
factors, such as species specificity and extracellular matrix compositions, are expected to play a role in determining the interaction between FGF2 and PTX3.

Another potent angiogenesis inhibitor is TSP-1. TSPs are a family of extracellular matrix proteins, the inhibitory effects of which on angiogenesis have been established in several experimental models [47, 48]. Among thrombospondin family members, TSP-2 has a domain structure equivalent to TSP-1 and shares most TSP-1 functions [49]. TSP-1 is a multimodular protein [48], the N-terminal domain of which contains a high-affinity binding site for heparin and heparan sulfate proteoglycans; TSP-1 can exert its antiangiogenic activity through multiple mechanisms involving different active sequences in different domains. The core of the TSP-1 contains three thrombospondin type I repeats, and is the most studied TSP-1 antiangiogenic epitope [48]. Type I repeats and their peptide mimetics have been successfully used to block angiogenesis and tumor growth in preclinical models [50], and, in ovarian follicles, TSP-1 suppressed angiogenesis and promoted follicular atresia [51]. TSP-1 can also activate latent TGFβ; this ability indirectly or directly impinges on angiogenesis. TGFβ enhances recruitment of smooth muscle cells to the vasculature [52], and it was shown to disrupt the angiogenic potential of microvascular ECs of the bovine CL [53].

Recently, using recombinant portions of TSP-1, Colombo et al. identified an antiangiogenic site in the type III repeats (mimicked by small molecule 27) of TSP-1, and demonstrated that binding of FGF2 to this site inhibits angiogenesis by sequestration of FGF2 [21]. We found that small molecule 27 inhibited FGF2-induced luteal EC proliferation and migration in vitro. Unlike small molecule 27 (and PTX3), the inhibitory effects of TSP-1 were also evident in the absence of exogenous FGF2. Moreover, it was more effective in reducing luteal endothelial cell numbers than were the small molecules; this was expected, since the whole molecule can exhibit the full repertoire of TSP-1 antiangiogenic properties. In phase-contrast microscopy and DAPI staining, cell appearance in the presence of TSP-1 was consistent of apoptotic cell death. Regarding the endogenous origin of TSP-1, our data indicate that both luteal endothelial and steroidogenic cells express ample amounts of its mRNA. Interestingly, it was reported that TSP-1 has antiangiogenic effects on rat follicles and induces apoptosis of granulosa cells in vitro [54]; however, whether TSP-1 also affects luteal steroidogenic cells remains unknown. The contribution of the endogenous compounds to the effects observed by FGF2, PTX3, and TSP1 proteins added to EC culture is currently unknown and requires further research.

The fact that many angiogenesis-modulating genes (FGF2, VEGF, THBS1, THBS2, CD36, and PTX3) were dynamically and stage-specifically regulated by PG may not be surprising. Another gene, CYR61, an integrin ligand that plays a role in extracellular matrix production and angiogenesis, was lately shown to be upregulated in the CL soon after PG administration to cows [55]. The CL is a highly vascular gland; the short period of extensive angiogenesis in the CL (until Day 5 of the cycle in the cow) results in development of an elaborate

FIG. 8. Effects of recombinant TSP-1 on viable luteal endothelial cell numbers and their morphology. A) Serum-starved cells were incubated with control medium, FGF2, TSP-1 (1–500 ng/ml), or FGF2 + varying doses of TSP-1, for 48 h. On the day of measurement, cell number was determined as detailed in the legend for Figure 5. Changes in the number of viable cells (percentage of control) are presented. Data represent the means ± SEM from three independent experiments. *Significant (P < 0.05) difference from control or FGF2. B) Representative microphotographs of cells incubated with FGF2 only or with FGF2 + TSP-1: left, phase-contrast images; right, DAPI staining insets (higher magnification). Arrows, apoptotic nuclei. Original magnification ×10; bars = 200 μm.
network of capillaries [1, 56, 57] that endows this gland with one of the highest blood flows per unit mass in the body [58]. Furthermore, the CL undergoes dynamic changes in its vasculature; the angiogenesis in the developing CL is later followed by either controlled regression of the microvascular tree in the nonfertile cycle during luteolysis or maintenance and stabilization of the blood vessels, such as occurs during pregnancy. Therefore, factors affecting vascular fate are likely to play a major role in regulating luteal function.

This study, as well as others [29, 55], point out the importance of angiogenesis-modulating agents in determining the ability of PG to induce luteolysis. The robust elevation of FGF2 by PG at an early stage (particularly when no FGF2 inhibitors exist) may act as a survival signal for both luteal ECs (here and [36]) and steroidogenic cells [33, 59]. Support of blood vessel growth and its stabilization is expected to enhance the supply of nutrients and hormones to the gland, promoting its survival and contributing to its ability to become resistant to luteolysis. Which cell type is responsible for this protective FGF2 surge in the early CL? Several lines of evidence suggest that these cells are luteal ECs. We showed that, functionally, these cells readily respond to PG with elevated FGF2 expression. Additionally, Schams et al. [60] reported that, during the early luteal phase, intensive immunostaining of FGF2 was exclusively observed in vascular cells (capillary ECs and vascular smooth muscle cells). In agreement with their findings, we show here that ECs derived from the early CL had high levels of FGF2. If ECs produce the most FGF2 at the early luteal stage, it would be interesting to study why these cells are so responsive to PG in terms of FGF2, whereas another PG-regulated endothelial gene, EDN1, is suppressed in the developing CL, and is only elevated in the mature gland [7].

In summary, we identify in the current study a series of angiogenesis-modulating genes that are regulated by PG at specific luteal developmental stages. These genes (FGF2, PTX3, THBS1, and THBS2) were also induced by PG in isolated luteal cell types in vitro. Our data also imply that pro- and antiangiogenic factors, expressed in the CL by endothelial and steroidogenic luteal cells, may act in a paracrine/autocrine manner to modulate the functions of the resident endothelial cells. These findings, therefore, suggest a potential functional relationship between angiogenesis and the luteolytic response to PG. Stage-specific regulation of FGF2 or its modulators by PG may help tilt the balance between pro- and antiangiogenic processes, controlling the ability of the CL to resist or advance toward luteolysis. This knowledge may lead to development of means to confer sensitivity of immature CL to the luteolytic actions of PG that will result in CL regression.

REFERENCES


