

## Patterns of Gene Expression in the Bovine Corpus Luteum Following Repeated Intrauterine Infusions of Low Doses of Prostaglandin F2alpha<sup>1</sup>

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### ABSTRACT

Natural luteolysis involves multiple pulses of prostaglandin F2alpha (PGF) released by the nonpregnant uterus. This study investigated expression of 18 genes from five distinct pathways, following multiple low-dose pulses of PGF. Cows on Day 9 of the estrous cycle received four intrauterine infusions of 0.25 ml of phosphate-buffered saline (PBS) or PGF (0.5 mg of PGF in 0.25 ml of PBS) at 6-h intervals. A luteal biopsy sample was collected 30 min after each PBS or PGF infusion. There were four treatment groups: Control (n = 5; 4 PBS infusions), 4XPGF (4 PGF infusions; n = 5), 2XPGF-non-regressed (2 PGF infusions; n = 5; PGF-PBS-PGF-PBS; no regression after treatments), and 2XPGF-regressed (PGF-PBS-PGF-PBS; regression after treatments; n = 5). As expected, the first PGF pulse increased mRNA for the immediate early genes *JUN*, *FOS*, *NR4A1*, and *EGR1* but unexpectedly also increased mRNA for steroidogenic (*STAR*) and angiogenic (*VEGFA*) pathways. The second PGF pulse induced immediate early genes and genes related to immune system activation (*IL1B*, *FAS*, *FASLG*, *IL8*). However, mRNA for *VEGFA* and *STAR* were decreased by the second PGF infusion. After the third and fourth PGF pulses, a distinctly luteolytic pattern of gene expression was evident, with inhibition of steroidogenic and angiogenic pathways, whereas, there was induction of pathways for immune system activation and production of PGF. The pattern of PGF-induced gene expression was similar in corpus luteum not destined for luteolysis (2X-non-regressed) after the first PGF pulse but was very distinct after the second PGF pulse. Thus, although the initial PGF pulse induced mRNA for many pathways, the second and later pulses of PGF appear to have set the distinct pattern of gene expression that result in luteolysis.

*corpus luteum, gene expression, hormone action, luteolysis, mRNA, prostaglandins*

### INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland involved in establishment and maintenance of pregnancy due

to production of progesterone (P4). The new CL develops from cells that remain in the follicle following ovulation but is eventually composed of multiple, distinctive cell types including steroidogenic cells (small and large luteal cells) and non-steroidogenic cells (endothelial cells, pericytes, fibrocytes, and immune cells) [1–4]. In the absence of a viable embryo, functional and structural regression of the CL occurs. In many species, including the cow, regression of the CL is due to multiple pulses of prostaglandin F2α (PGF) released by the nonpregnant uterus [4–6]. There is substantial variability in the frequency and amplitude of PGF pulses associated with ruminant luteolysis, but typically, there are 4–8 discrete pulses that occur at 6- to 14-h intervals [7–9]. For example, in heifers evaluated at hourly intervals during the seventh day around luteolysis, there was complete luteolysis, as defined by a decrease in basal P4, after four distinct PGF pulses that occurred over ~30 h [7]. Recent studies have mimicked the normal pattern of PGF pulses by infusing low doses of PGF into the uteri of heifers [10, 11]. The PGF metabolite patterns were similar to the patterns observed during natural luteolysis. In addition, the changes in circulating P4 concentrations and luteal blood flow showed patterns similar to those that occurred during natural luteolysis with an increase in circulating P4 and blood flow after the initial PGF pulse and a subsequent decrease in P4 and blood flow after the second, third, and fourth infusions of PGF [10, 11].

Many studies have focused on understanding the molecular mechanisms underlying the luteolytic process. Some of those studies evaluated changes in gene expression during natural luteolysis by removing the CL at specific days of the estrous cycle [12–14]. These studies provided substantial insight into the molecular changes associated with the later stages of luteolysis but could not provide detailed information for the changes associated with specific pulses of PGF (first, second, third) or during precisely defined stages of the luteolytic process. Many studies [14–20] have also investigated luteal regression by using a single injection of a supraphysiological dose of PGF. A single high dose of PGF suppressed steady-state concentrations of mRNA for specific genes that might have been involved in “luteotropic” functions, whereas there was an induction of mRNA for genes that might have been involved in functional or structural luteolysis [15, 16, 20–23]. For example, treatment with PGF decreased *VEGFA*, *STAR*, *PTGFR*, and *LHCGR* mRNA concentrations, but the same PGF treatments increased *FAS*, *FASLG*, *TNF*, *IL1B*, *IFNG*, *EDN1*, *MMP1*, and *PGTS2* mRNA concentrations [15, 16, 20, 22–24]. Not surprisingly, molecular changes were not always consistent when results of studies of CL during natural luteolysis were compared to those of luteolysis induced by a single large PGF

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treatment. For example, patterns of luteal cytokine expression, such as CCL2, and luteal immune cell populations had quite different patterns during induced versus those during natural luteolysis [12, 14]. Recent studies have drawn particular attention to differences in luteal responses following a single supraphysiological dose of PGF compared to those after lower doses of PGF infused into the uterus, to more closely mimic natural luteal regression [10–12, 25–30]. Preliminary reports of the intraluteal changes that follow multiple low doses of PGF in the ovine CL have been presented [31–33], but, to our knowledge, no studies of the changes in gene expression that accompany multiple individual pulses of physiological doses of PGF in any species have been published.

This research used a 0.5-mg intrauterine infusion of PGF [11] in an attempt to mimic physiological pulses of PGF. In order to evaluate gene expression at multiple times after individual intrauterine PGF infusions, the previously described and validated method of ultrasound-guided luteal biopsy was used [34]. Three different treatment groups were evaluated after four different intrauterine infusions in this study, in order to provide information for the changes in concentrations of key mRNA that follow infusion of 4 pulses of PGF (4XPFG; expected full luteolysis), 2 pulses of PGF with two infusions of saline (2XPFG; expected partial luteolysis), and 4 infusions of saline (control, no luteolysis). Our main hypotheses were that an individual PGF pulse would induce a distinct pattern of gene expression that would vary by 1) whether the CL had been exposed to a previous PGF pulse (first vs. second vs. third vs. fourth) and 2) whether the CL underwent full or partial luteal regression (4XPFG vs. 2XPFG). Unexpectedly, some of the animals treated with two PGF infusions underwent complete luteal regression, whereas other animals did not undergo luteal regression after two PGF infusions, providing a more valid comparison for testing our second hypothesis that gene expression after individual PGF pulses would vary in CL that subsequently underwent complete luteal regression compared to CL that recovered after a similar PGF treatment. In order to test these two hypotheses, 18 different mRNA were evaluated in each luteal biopsy. Specific classes of mRNA were chosen to allow testing of these hypotheses including immediate early genes (jun proto-oncogene [*JUN*], FBI murine osteosarcoma viral oncogene homolog [*FOS*], nuclear receptor subfamily 4, group A, member 1 [*NR4A1*], and early growth response 1 [*EGR1*]), steroidogenic genes (steroidogenic acute regulatory protein [*STAR*], cytochrome P450, family 11, subfamily A, polypeptide 1 [*CYP11A1*], nuclear receptor subfamily 5, group A, member 1 [*NR5A1*, formerly known as *SF-1*], and luteinizing hormone/choriogonadotropin receptor [*LHCGR*]), prostaglandin-related genes (prostaglandin-endoperoxide synthase 2 [*PTGS2*], prostaglandin-F synthase [*PTGFS*], hydroxyprostaglandin dehydrogenase 15-[NAD] [*HPGD*], prostaglandin F receptor [*PTGFR*]), immune-related genes (interleukin 1, beta [*IL1B*], interleukin 8 [*IL8*], Fas [*FAS*], Fas-ligand [*FASLG*]), and angiogenesis-related genes (vascular endothelial growth factor A [*VEGFA*], fibroblast growth factor 2 [*FGF2*]). Moreover, we evaluated the cellular localization for two of these genes (*NR4A1* and *PTGFR*) and three of the proteins involved in P4 synthesis (*STAR*, *CYP11A1*, *HSD3B7*) by using in situ hybridization and immunohistochemistry, respectively, following intrauterine PGF infusions.

## MATERIALS AND METHODS

### Reagents

TRIzol was purchased from Invitrogen (Invitrogen Life Technologies Inc., Carlsbad, CA). GoScript reverse transcriptase system, RNase-free DNase I,

dNTP set, and *Taq* DNA polymerase for PCR were purchased from Promega (Madison, WI). Maxima SYBR quantitative PCR (qPCR) Master Mix (2×) for real-time PCR was obtained from Fermentas Life Sciences (Thermo Scientific, Foster City, CA). Gel extraction kit and RNeasy mini-kit were obtained from Qiagen (Frederick, MD). Digoxigenin (DIG)-11-UTP, RNase inhibitor, T7 RNA polymerase anti-DIG-AP antibody, blocking reagent, and BM Purple AP substrate were purchased from Roche Applied Science (Mannheim, Germany). Specific oligonucleotide primers were synthesized by the Biotechnology Center of the University of Wisconsin-Madison. Steroidogenic acute regulatory protein (*STAR*); cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*); and hydroxy- $\Delta^5$ -steroid dehydrogenase, 3  $\beta$ - and steroid  $\Delta^5$ -isomerase 7 (*HSD3B7*) antibodies (anti-rabbit) were generous gifts from Dr. Leang-Shin Wu and Chieh-Hsien Chiu from Taiwan. The PGF (Lutalyse) and intrauterine P4-releasing devices (Eazi-Breed CIDR) were gifts from Pfizer, Inc. (Pfizer Animal Health, New York, NY), and GnRH (Factrel, Fort Dodge, IA) was purchased. Other chemicals and reagents were obtained from Fisher Scientific (Kansas City, MO) or Sigma Chemical Company (St. Louis, MO).

### Animals

All procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison. This experiment was conducted from October 2010 to December 2010, using cows housed at the University of Wisconsin dairy facilities. Nonpregnant, cyclic, lactating Holstein cows with normal estrous cycles were used for this experiment. Animals had ovulation synchronized with the Ovsynch procedure that involves an initial treatment with GnRH followed 7 days later by a PGF treatment, followed at 56 h by a second treatment with GnRH. Day of ovulation was determined by ultrasonography and designated Day 1. Biopsies were performed on Day 9.

### Experimental Design

Cows with a mature CL on Day 9 were assigned randomly to three treatment groups, with unequal numbers in each group (1:2:1). All cows received four intrauterine infusions with either 0.25 ml of PBS or PGF (4XPFG; 0.5 mg of PGF in 0.25 ml of PBS) at 6-h intervals (PBS or PGF were infused into the greater curvature of the uterine horn ipsilateral to the CL). Control animals ( $n = 5$ ) received 0.25 ml of PBS four times, once every 6 h. Cows receiving two infusions of PGF (2XPFG;  $n = 10$ ) received two PGF treatments at first and third infusions, with PBS treatment at the other two infusions (PGF-6 h-PBS-6 h-PGF-6 h-PBS). It was assumed that the response by this group might be variable, and therefore, more cows were assigned to this treatment group. The cows receiving 4XPFG ( $n = 5$ ) received four PGF treatments at 6-h intervals. All cows had an ultrasonography-guided biopsy of the CL, collected 30 min after each intrauterine PBS or PGF infusion, and only cows that had at least 10 mg of tissue collected at each of these biopsy sessions were used in the experiment.

### Collection of Samples

Luteal biopsy samples were collected by the methods described by Tsai et al. [34]. Briefly, caudal epidural anesthesia was induced with 5 ml of lidocaine hydrochloride (Phoenix Pharmaceutical, Inc., St. Joseph, MO). An ultrasound probe with a needle guide was inserted in the vaginal fornix. The ovary containing the CL was positioned transrectally against the vaginal wall. The needle was then advanced through the vaginal wall and into the CL. The biopsy cutting blade was triggered with excised luteal tissue trapped in the specimen notch. The biopsy tool was removed, and the tissue was inspected to ensure that only luteal tissue was collected; the sample was then washed with PBS and weighed. Part of the biopsy sample was immediately frozen in dry ice and stored at  $-80^{\circ}\text{C}$  for later isolation of RNA. The remaining biopsy tissue was fixed in a solution of 4% paraformaldehyde overnight at  $4^{\circ}\text{C}$  and then dehydrated using serial dilutions of methanol (25%, 50%, 75%, and 100%). Dehydrated samples were stored in 100% methanol at  $-20^{\circ}\text{C}$  until evaluated by in situ hybridization or immunohistochemistry.

### P4 Analyses

To determine changes in P4 concentrations during and after treatment, blood samples were collected from coccygeal vessels before treatment and every 3 h after treatment for 24 h. Blood samples were then collected every 6 h from 24 to 48 h after treatment. Blood samples were allowed to clot and were then centrifuged at 3000 rpm for 20 min. Sera were stored at  $-20^{\circ}\text{C}$  until assayed for P4. Concentrations of P4 were quantified using a competitive ELISA method described by Tsai and Wiltbank [17].

### RNA Isolation, RT Reaction, and Quantitative PCR

Ten milligrams of luteal tissue were minced with a scalpel and homogenized in TRIzol. Total RNA was then extracted using the manufacturer's protocol (TRIzol; Invitrogen). Integrity of RNA was verified by agarose gel electrophoresis and by optical density at 260/280 nm of  $2 \pm 0.1$  with NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). Two micrograms of total RNA were treated with DNase I to eliminate genomic DNA contamination, and RNA was then reverse transcribed in the presence of both random hexamer and oligo(dT) primers in equal volumes, using GoScript kit according to the manufacturer's protocol. To clarify RT specificity, all components of the RT kit without RNA (RT negative) were also analyzed.

Sequences for primers were designed using Primer3 from the NCBI (<http://www.ncbi.nlm.nih.gov/>) gene database or were derived from published studies [16, 22, 35–37]. Primer pair sequences and product sizes are shown in supplemental Table S1 (available online at [www.biolreprod.org](http://www.biolreprod.org)). All PCR reactions were set up as follows: 5 µl of SYBR Green Master Mix (2×), 2.5 pmol of each primer, 0.5 µl of cDNA, and ddH<sub>2</sub>O to bring the final volume to 10 µl. Thermal cycling was done by initially incubating the mixture at 50°C for 2 min with subsequent denaturation at 95°C for 10 min. This was followed by 40 cycles of denaturation, annealing, and amplification (95°C for 30 sec, 60°C for 1 min, 72°C for 30 sec). All reactions were carried out on an Opticon2 real-time PCR system (Bio-Rad Life Science, Foster City, CA). Melting curve analysis was performed as follows: 95°C for 1 min, followed by fluorescence measurement performed at 1° increments between 55°C and 95°C. In each run, negative controls with no cDNA template and RT negative controls were included. To verify reaction specificity, amplification products were evaluated after separation on a 2% agarose gel. All samples were evaluated in duplicate for each cDNA. For the determination of the dynamic range and amplification efficiencies of real-time PCR assays for each gene product, amplifications were performed with specific primers in duplicate with a serial dilution of specific pooled cDNA. Efficiencies of qPCR for amplifications were between 95% and 105% for all genes.

### In Situ Hybridization and Immunohistochemistry

The first and fourth biopsy samples were evaluated from biopsies in the control and 4XPFG groups for *NR4A1* and *PTGFR* mRNAs by using in situ hybridization. Tissue sectioning and in situ hybridization were conducted as previously described [38] with modifications. Briefly, luteal tissue sections were embedded in a low-melting agarose and cut into 28-µm-thick sections by using a vibrating microtome. Primer sequences for *NR4A1* (forward: 5'-ggcatggtgaaggaagtgt-3'; reverse: 5'-cgatgttaatacagactcactatagg-catgctcgctgtgatgagg-3'; product size: 559 bp) and *PTGFR* (forward: 5'-ttgccaactggaagaa gacc-3'; reverse: 5'-cgatgttaatacagactcactataggctgagacctgctgtctgt-3'; product size: 666 bp) were used to generate DIG-11-UTP-labeled RNA probe templates. After hybridization with DIG-labeled riboprobes, sections were incubated with anti-DIG antibody that was coupled to alkaline phosphatase. To prevent nonspecific binding of anti-DIG-AP antibody, the antibody was preincubated with a protein powder prepared from bovine luteal tissue according to the method described by Abler et al. [38]. To visualize bound probes, BM Purple was used as an alkaline phosphatase chromagen to detect antibody-bound DIG-labeled riboprobes.

For immunohistochemistry, sections were deparaffinized in xylene, rehydrated, and boiled in 10 mM sodium citrate (pH 6.0) for 20 min. Tissues were washed with a solution containing 25 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20 (TBSTw), and nonspecific binding sites were blocked for 1 h in TBSTw containing 1% blocking reagent (Roche Diagnostics, Indianapolis, IN), 5% normal goat serum, and 1% bovine serum albumin fraction 5 (RGTW). Tissue samples were incubated overnight at 4°C with primary antibodies diluted in RGTW as follows: 1:60 dilution of rabbit anti-STAR, 1:80 dilution of rabbit anti-CYP11A1, and 1:80 dilution of rabbit anti-HSD3B7. After several washes with TBSTw, tissues were incubated for 1 h with RGTW containing 1:250 dilution of Dylight488-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA). The secondary antibodies were preincubated with luteal protein powder for at least 1 h to prevent nonspecific binding. Labeled tissue sections were counterstained with 4', 6-diamidino-2-phenylindole dilactate and mounted in anti-fade medium (PBS containing 80% glycerol and 0.2% *N*-propyl gallate).

Brightfield images were captured using Nikon Eclipse E80i. Fluorescent images were captured using a Nikon Eclipse E600 compound microscope and were merged using NIS elements imaging software (Nikon Instruments Inc., Melville, NY).

### Statistical Analysis

Glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) was used to normalize the expression of target genes. The use of *GAPDH* as a housekeeping

gene has been previously validated [36], and we further validated the consistency of steady-state concentrations for this gene in the CL in preliminary experiments (unpublished results). This preliminary experiment indicated similar expression of *GAPDH* after multiple biopsies of the same animal and similar concentrations in the different treatment groups and different collection times in the present experiment. Mean cycle threshold (Ct) values from the first biopsy of the control animals were used as the reference points, and Ct values for each biopsy were used to calculate the fold changes from the reference points for each biopsy (including original control values at first biopsy) using reference point Ct values according to the  $2^{-\Delta\Delta C_t}$  methods described by Livak and Schmittgen [39]. Fold-change values that reflected the steady-state mRNA concentrations were then analyzed by one-way ANOVA using the general linear model procedure of the Statistical Analysis System (version 9.2). Differences among the means were separated by Fischer least significant difference test. Data were considered statistically significant when *P* values were lower than 0.05. Data in text are expressed as fold change from controls (increased) or percentage decrease as compared to controls.

## RESULTS

### Concentrations of Circulating P4 after Treatments

Circulating P4 concentrations were similar in all groups before treatments, although numerically, the control group was lowest, and the 2XPFG-non-regressed and 2XPFG-regressed groups were highest (Fig. 1). Circulating concentrations of P4 in control animals did not change significantly during or by 48 h after the intrauterine PBS treatments. P4 concentrations in the 4XPFG group decreased significantly compared to those in the control group by 9 h after first PGF infusion and at all subsequent time points ( $P < 0.05$ ). Cows in the 2XPFG group could be divided into two distinct groups. One group of 2XPFG cows ( $n = 5$ ) had luteal regression that was similar to that in the 4XPFG group with decreasing P4 by 9 h after first PGF treatment and at all subsequent times ( $P < 0.05$ ). Another group of 2XPFG cows ( $n = 5$ ) could be classified as not regressing during the experiment. Despite initial decreases in circulating P4 in this group, P4 concentrations returned to normal and were not significantly different from those of controls at 30 to 48 h after initial PGF treatment. Nevertheless, both 2XPFG groups showed a numerical decline in P4 after the saline infusion at 6 h, suggesting that endogenous PGF may have been released at this time due to the initial PGF infusion. The 2XPFG-regressed cows continued the downward trend for P4 from 9 to 12 h, even though no further PGF treatments were given, whereas the 2XPFG-non-regressed cows recovered P4 levels between 9 and 12 h after the first PGF infusion. The 2XPFG-non-regressed group remained intermediate between the control and two groups of cows that regressed following PGF infusion throughout the remainder of the sampling period.

### Effect of PGF on mRNA for Immediate Early Genes (*JUN*, *FOS*, *NR4A1*, *EGR1*)

Steady-state concentrations of mRNA for immediate early genes (*JUN*, *FOS*, *NR4A1*, *EGR1*) in the biopsy samples taken during the treatments are shown in Figure 2. Concentrations of mRNA for *JUN*, *FOS*, *NR4A1*, and *EGR1* increased significantly at the first biopsy (30 min after PGF infusion) for all three treatments groups that received PGF infusion. The increase after the initial PGF treatment generally exhibited the greatest magnitude compared to subsequent increases. All three PGF treatments exhibited similar increases for *JUN* ( $6.2 \pm 1.2$ -fold for 4XPFG,  $8.6 \pm 1.9$ -fold for 2XPFG-regressed,  $4.3 \pm 1.4$ -fold for 2XPFG-non-regressed), *FOS* ( $21.5 \pm 4.7$ -fold for 4XPFG,  $36.7 \pm 5$ -fold for 2XPFG-regressed,  $17.5 \pm 7.5$ -fold for 2XPFG-non-regressed), and *EGR1* ( $7.1 \pm 1.6$ -fold for 4XPFG,  $7.7 \pm 2.05$ -fold for 2XPFG-regressed,  $5.1 \pm 2.6$ -fold for 2XPFG-non-regressed); however, *NR4A1* had a greater



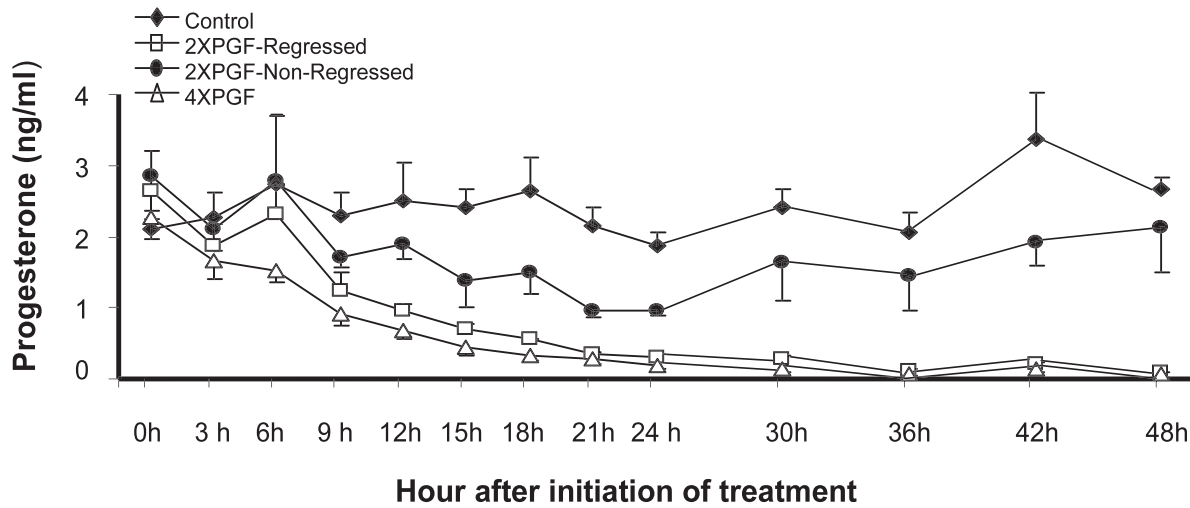


FIG. 1. Effect of intrauterine PGF or PBS treatment on circulating P4 concentration in cows. Data are presented as means  $\pm$  SEM.

increase in 4XPGF ( $15.5 \pm 4.2$ -fold) than 2XPGF-non-regressed ( $7.6 \pm 1.8$ -fold), with 2XPGF-regressed being intermediate ( $14.2 \pm 2.3$ -fold). At the second luteal biopsy only the 4XPGF group had increased mRNA concentrations

for *JUN*, *FOS*, *NR4A1*, and *EGR1*, while the other three groups that were all treated with PBS at 30 min before this biopsy showed no differences in concentrations for any of these mRNA. At the third biopsy, the 4XPGF and 2XPGF-regressed

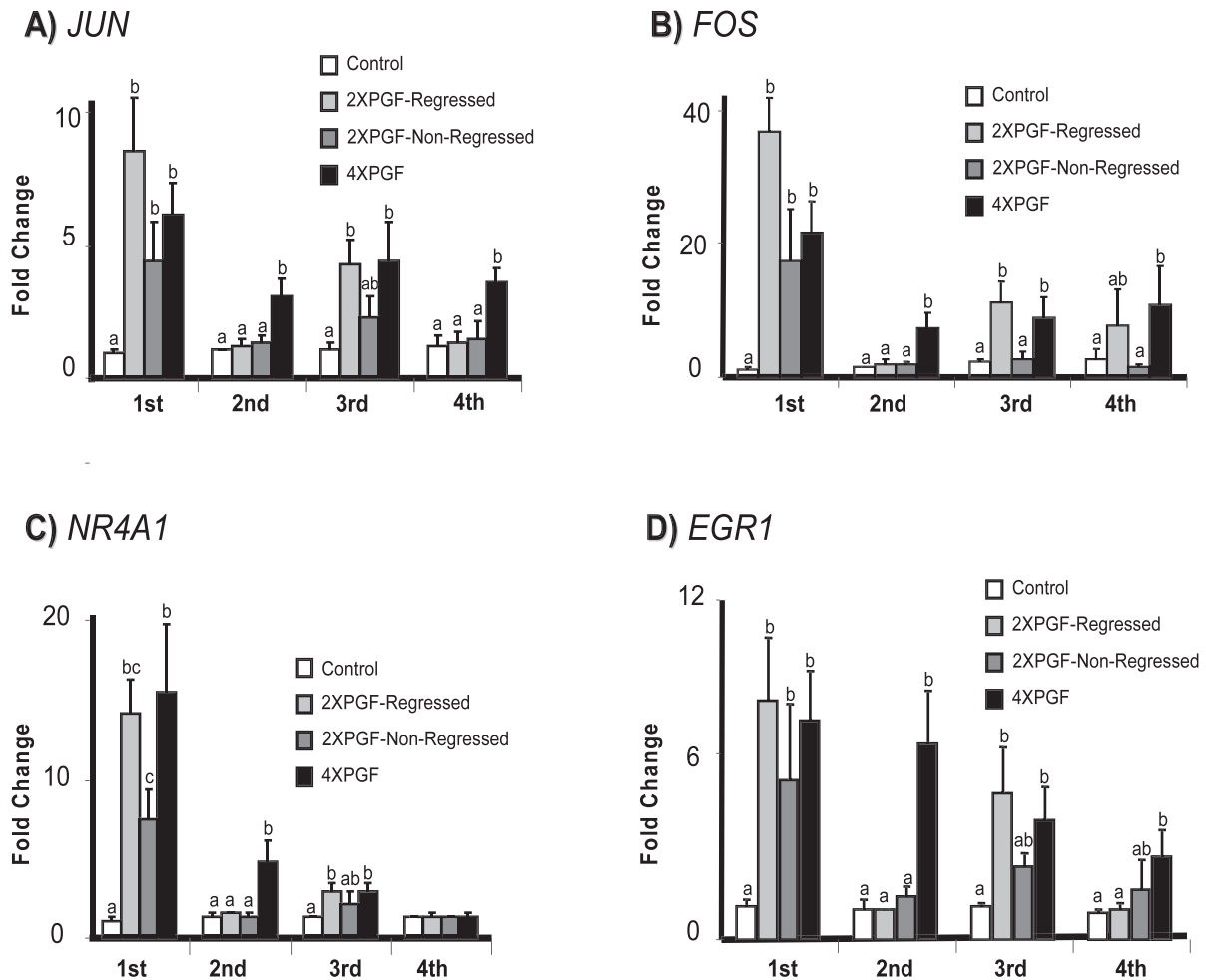


FIG. 2. Effect of intrauterine PGF or PBS treatment on steady-state mRNA concentrations for **A)** *JUN* (jun proto-oncogene), **B)** *FOS* (FBJ murine osteosarcoma viral oncogene homolog), **C)** *NR4A1* (nuclear receptor subfamily 4, group A, member 1), and **D)** *EGR1* (early growth response 1). Data are shown as fold changes  $\pm$  SEM. Columns with different letters (a, b, c) at each biopsy indicate differences;  $P < 0.05$ . First, second, third, and fourth indicate biopsy times.

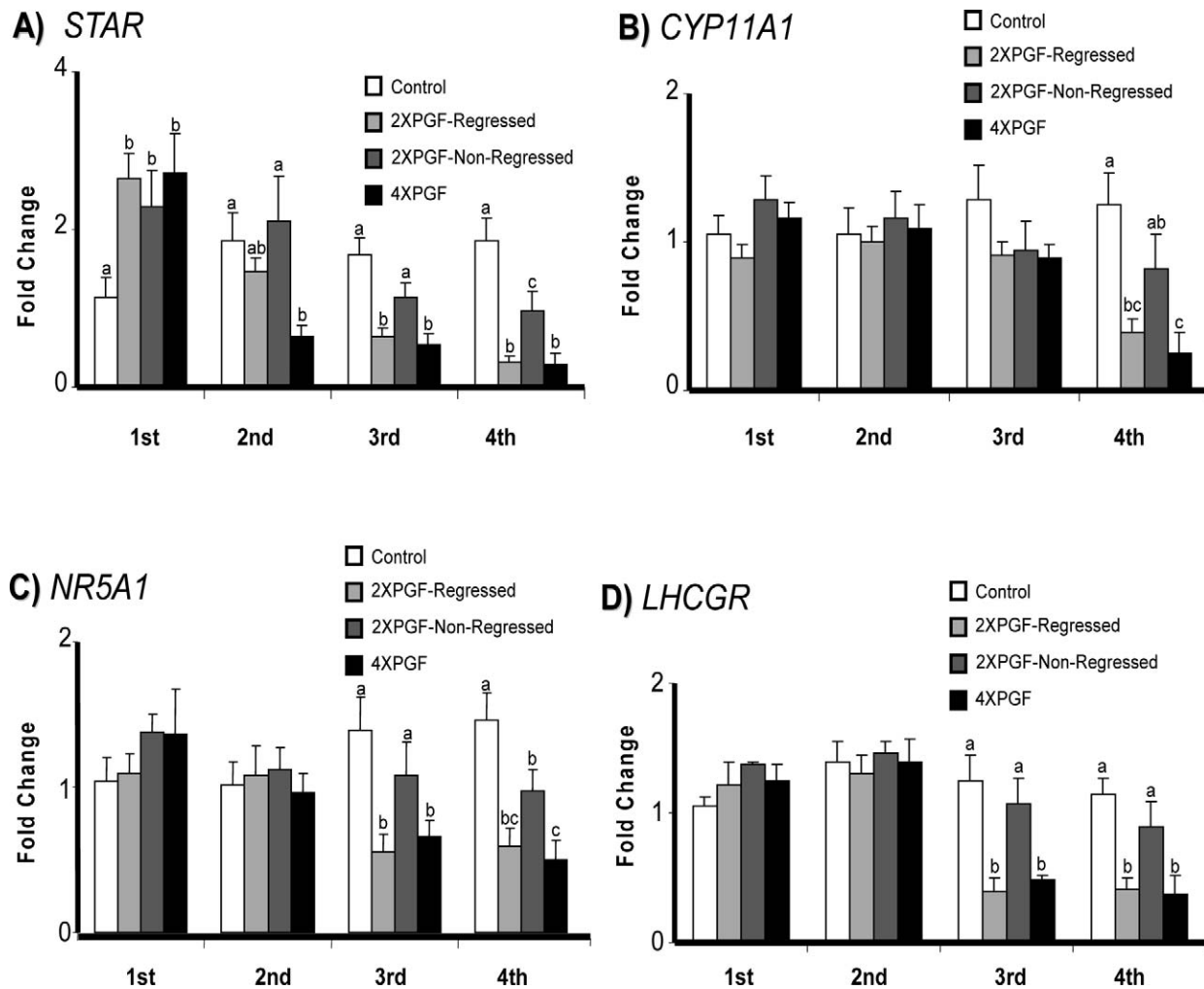


FIG. 3. Effect of intrauterine PGF or PBS treatment on steady-state mRNA concentrations for **A)** *STAR* (steroidogenic acute regulatory protein), **B)** *CYP11A1* (cytochrome P450, family 11, subfamily A, polypeptide 1), **C)** *NR5A1* (nuclear receptor subfamily 5, group A, member 1), and **D)** *LHCGR* (luteinizing hormone/choriogonadotropin receptor). Data are shown as fold changes  $\pm$  SEM. Columns with different letters (a, b, c) at each biopsy indicate differences;  $P < 0.05$ . First, second, third, and fourth indicate biopsy times.

groups had increased mRNA for all four immediate early genes; whereas the 2XPGF-non-regressed group, despite receiving PGF prior to this biopsy, similar to the 4XPGF and 2XPGF-regressed cows, appeared to have a reduced response to this treatment. At the fourth biopsy, there were increased mRNA for *JUN*, *FOS*, and *EGR1* in the 4XPGF group but not in the other three groups. Surprisingly, there was no increase in *NR4A1* after the fourth PGF treatment (Fig. 2C).

#### Effect of PGF on mRNA for Steroidogenic Genes (*STAR*, *CYP11A1*, *NR5A1*, *LHCGR*)

The steady-state concentrations of luteal mRNA for various steroidogenic genes (*STAR*, *CYP11A1*, *NR5A1*, and *LHCGR*) are shown in Figure 3. Surprisingly, *STAR* mRNA expression was increased at the first biopsy for all three treatments groups that received PGF infusion. In contrast, expression of *STAR* was decreased (66%) at the second biopsy in the 4XPGF group and decreased in both the 4XPGF (69%) and 2XPGF-regressed (63%) groups at the third biopsy. At the fourth biopsy, there was a decreased expression of *STAR* mRNA for the 4XPGF (84%) and 2XPGF-regressed (82%) groups, with the 2XPGF-non-regressed group having an intermediate value for *STAR* mRNA (49%). Messenger RNA concentrations for *NR5A1* and

*LHCGR* were decreased at the third and fourth biopsies for 2XPGF-regressed and 4XPGF compared to those of control. At the fourth biopsy, the 2XPGF-non-regressed group had decreased mRNA concentration for *NR5A1* but no change in *LHCGR* and *CYP11A1* mRNA. Meanwhile, mRNA expression for *CYP11A1* was decreased only at the fourth biopsy in the two groups that had luteal regression, 2XPGF-regressed and 4XPGF.

#### Effect of PGF on mRNA for Prostaglandin-Related Genes (*PTGS2*, *PTGFS*, *HPGD*, *PTGFR*)

The steady-state concentrations of mRNA for prostaglandin-related genes (*PTGS2*, *PTGFS*, *HPGD*, and *PTGFR*) are shown in Figure 4. There were dramatic increases in mRNA concentrations for *PTGS2* at all four biopsies taken from 2XPGF-regressed and 4XPGF groups. The 2XPGF-non-regressed CL also had an increase in *PTGS2* mRNA at the first and third biopsies but not at the second and fourth biopsies. Expression of *PTGFS* increased at the second biopsy and remained at higher concentrations in both 2XPGF-regressed and 4XPGF groups; however, *PTGFS* increased only at the third biopsy for 2XPGF-non-regressed group.

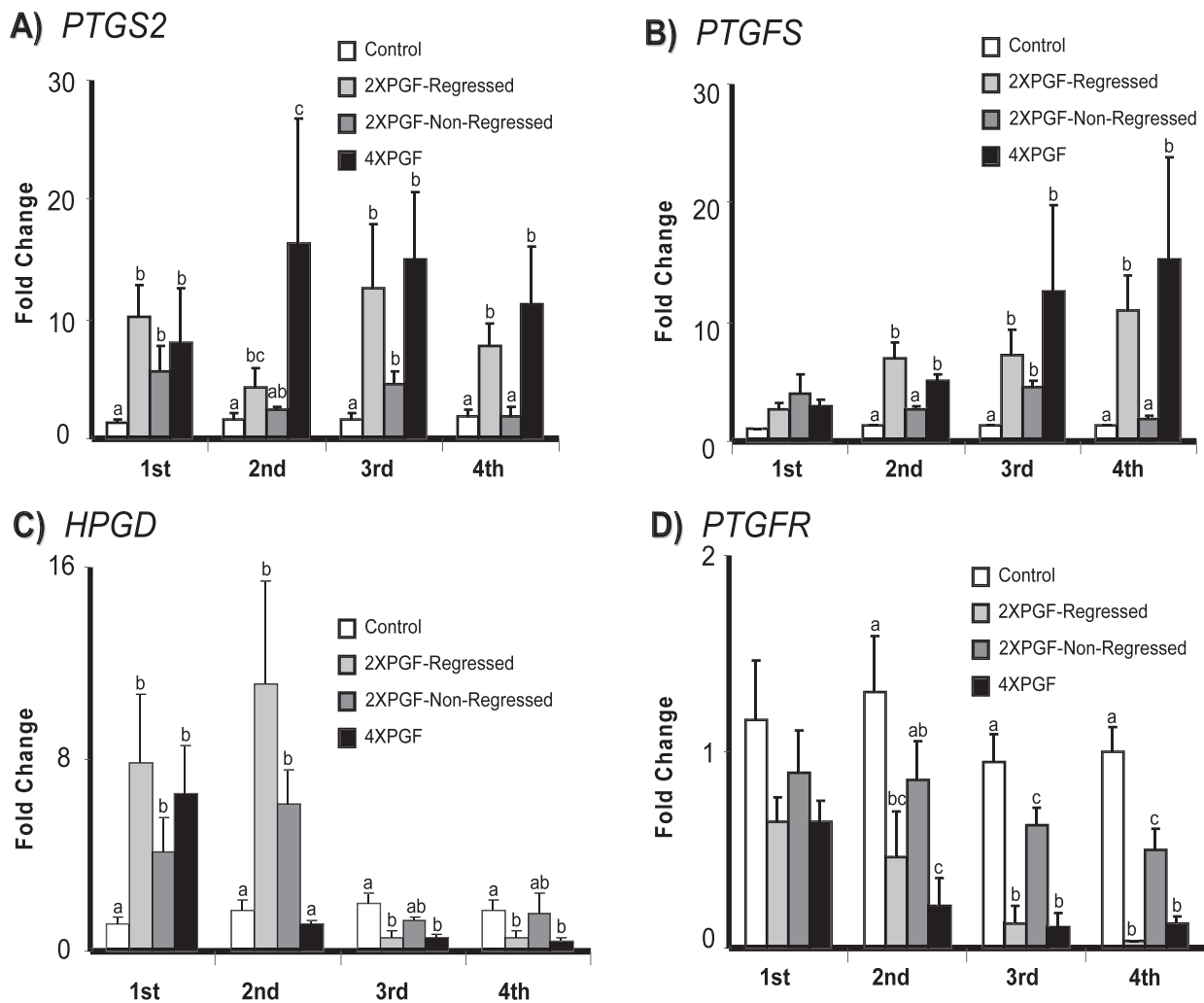


FIG. 4. Effect of intrauterine PGF or PBS treatment on steady-state mRNA concentrations for **A)** *PTGS2* (prostaglandin-endoperoxide synthase 2), **B)** *PTGFS* (prostaglandin F synthase), **C)** *HPGD* (hydroxyprostaglandin dehydrogenase 15-[NAD]), and **D)** *PTGFR* (prostaglandin F receptor). Data are shown as fold changes  $\pm$  SEM. Columns with different letters (a, b, c) at each biopsy indicate differences,  $P < 0.05$ . First, second, third, and fourth indicate biopsy times.

The pattern for *HPGD* expression was very different from our expectations (Fig. 4C). At the first biopsy, there was a similar increase in steady-state mRNA concentrations for *HPGD* in all three treatment groups ( $6.5 \pm 2.05$ -fold for 4XPGE,  $7.9 \pm 2.8$ -fold for 2XPGE-regressed,  $4.1 \pm 1.5$ -fold for 2XPGE-non-regressed). However, at the second biopsy, mRNA concentrations for *HPGD* remained elevated in the 2XPGE-regressed and 2XPGE-non-regressed groups, but *HPGD* mRNA decreased to control concentration levels for the 4XPGE group. At the third and fourth biopsies, *HPGD* mRNA concentrations were much lower than those of controls in the 2XPGE-regressed and 4XPGE groups but not in the 2XPGE-non-regressed group.

Concentrations of mRNA for *PTGFR* showed numerical decreases at the first biopsy (44% and 45%) and significant decreases compared to controls at the second (65% and 84%), third (87% and 89%), and fourth (97% and 90%) biopsies in the regressed groups (2XPGE-regressed and 4XPGE, respectively). The 2XPGE-non-regressed group had decreased *PTGFR* mRNA only at the third and fourth biopsies, and the decreases were much less dramatic than those observed in the regressed groups (34% at third, 52% at fourth biopsies).

#### Effect of PGF on mRNA for Immune-Related Genes (*IL1B*, *IL8*, *FAS*, *FASLG*)

The concentrations of mRNA for immune-related genes (*IL1B*, *IL8*, *FAS*, and *FASLG*) showed steady increases after PGF treatments in treatment groups that resulted in regression of the CL, as shown in Figure 5. mRNA concentrations for all four immune-related genes were increased at the second, third, and fourth biopsies for the regressed groups (2XPGE-regressed and 4XPGE). At the fourth biopsy, there was maximal up-regulation of *IL1B* ( $25.4 \pm 12.9$ -fold for 4XPGE,  $18.4 \pm 3.8$ -fold for 2XPGE-regressed), *IL8* ( $36 \pm 9.7$ -fold for 4XPGE,  $28.6 \pm 12.7$ -fold for 2XPGE-regressed), *FAS* ( $7.9 \pm 2.7$ -fold for 4XPGE,  $6.6 \pm 1.5$ -fold for 2XPGE-regressed), and *FASLG* ( $11.9 \pm 2.6$ -fold for 4XPGE,  $7.5 \pm 1.4$ -fold for 2XPGE-regressed). In addition, the regressed groups had a slight but significant increase in *IL1B* at the first biopsy ( $3.4 \pm 0.9$ -fold for 4XPGE,  $4.1 \pm 1.1$ -fold for 2XPGE-regressed). In the 2XPGE-non-regressed group there was no significant increase in mRNA for *IL1B* or *IL8* in any of the biopsy samples and only a significant increase in mRNA for *FAS* and *FASLG* in the second biopsy and not in any of the other biopsy samples.

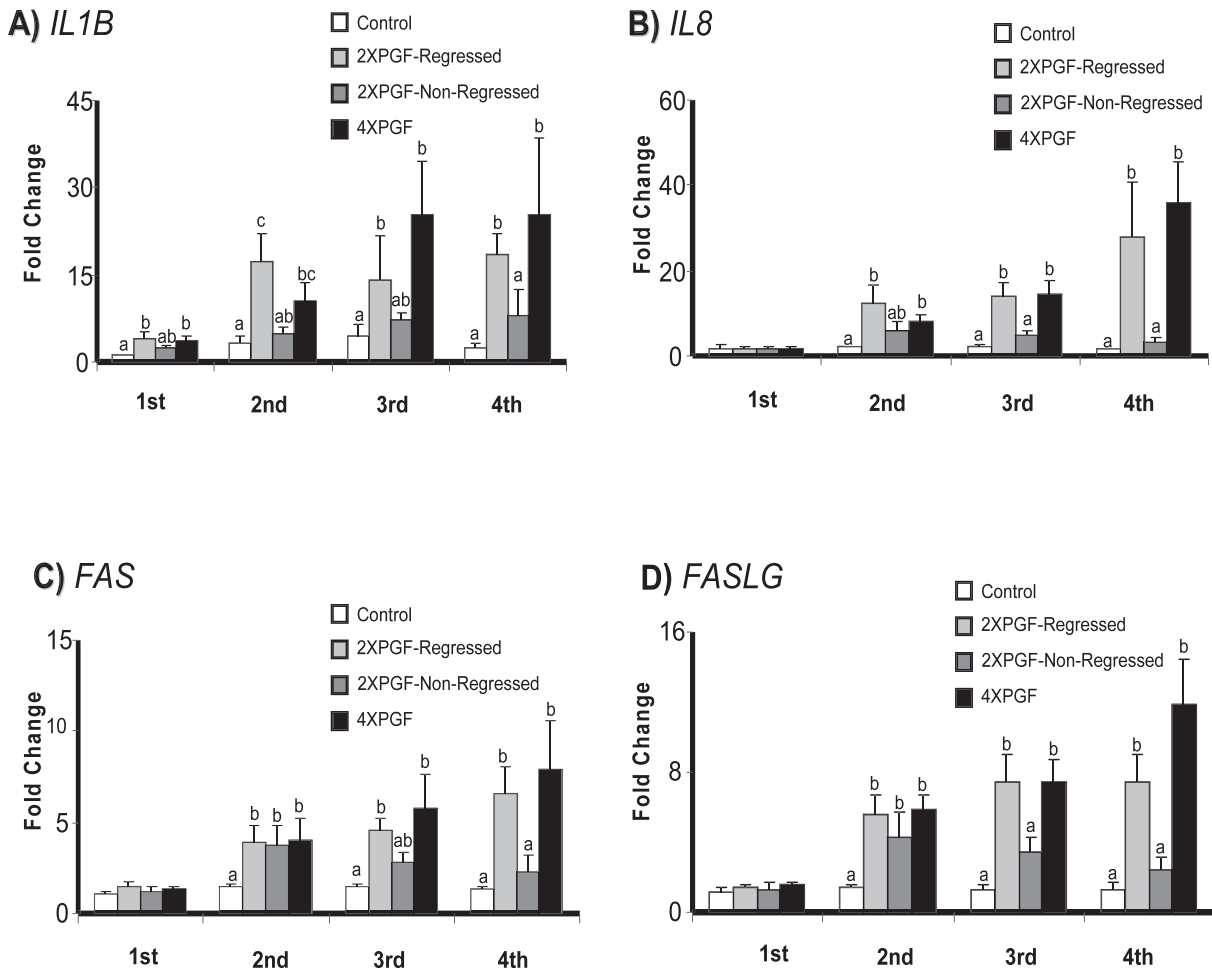


FIG. 5. Effect of intrauterine PGF or PBS treatment on steady-state mRNA concentrations for **A) IL1B** (interleukin 1, beta), **B) IL8** (interleukin 8), **C) FAS** (Fas, TNF receptor superfamily, member 6), **D) FASLG** (Fas ligand). Data are shown as fold changes  $\pm$  SEM. Columns with different letters (a, b, c) at each biopsy indicate differences,  $P < 0.05$ . First, second, third, and fourth indicate biopsy times.

#### Effect of PGF on mRNA for Angiogenesis-Related Genes (VEGFA, FGF2)

The steady-state concentrations of mRNA for the two genes related to angiogenesis (*VEGFA*, *FGF2*) showed distinct patterns of changes, as shown in Figure 6. The *VEGFA* mRNA

concentrations increased at the first biopsy for all three treatment groups ( $4.2 \pm 0.9$ -fold for 4XPGF,  $4.6 \pm 1.6$ -fold for 2XPGF-regressed,  $5.3 \pm 1$ -fold for 2XPGF-non-regressed). At the second biopsy, *VEGFA* mRNA decreased in the 4XPGF group (48%) but not in other groups. At the third biopsy, there were decreased *VEGFA* mRNA concentrations for the 4XPGF

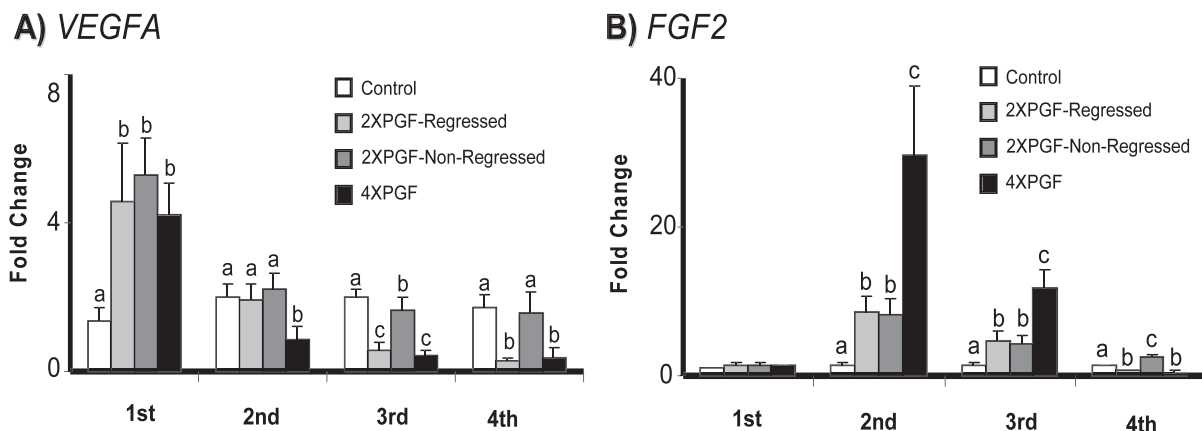


FIG. 6. Effect of intrauterine PGF or PBS treatment on steady-state mRNA concentrations for **A) VEGFA** (vascular endothelial growth factor A), **B) FGF2** (fibroblast growth factor 2). Data are shown as fold changes  $\pm$  SEM. Columns with different letters (a, b, c) at each biopsy indicate differences,  $P < 0.05$ . First, second, third, and fourth indicate biopsy times.



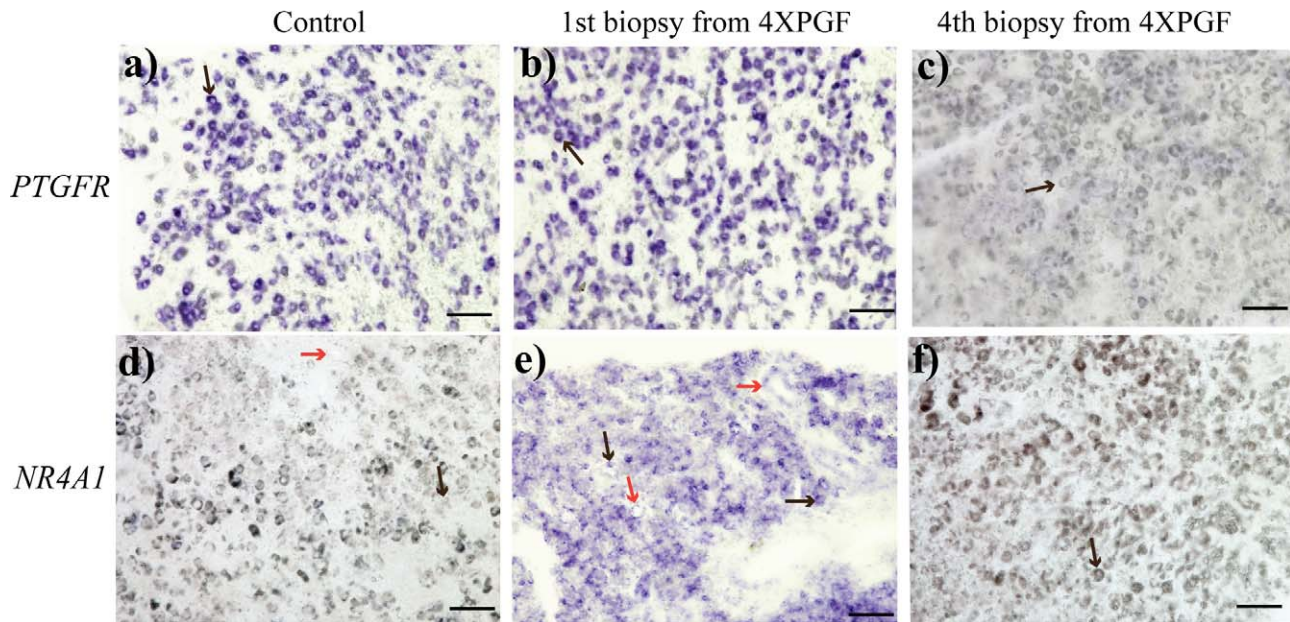


FIG. 7. Representative images of in situ hybridization staining for *PTGFR* (prostaglandin F receptor, **a, b, c**) and *NR4A1* (nuclear receptor subfamily 4, group A, member 1, **d, e, f**) in bovine CL in control and at first, and fourth biopsies from 4XPGF group. Large cells (black arrow) and vascular endothelial cells (red arrow) are shown. Bars = 100  $\mu$ m.

(80%) and 2XPGF-regressed (72%) groups with continued decreases in *VEGFA* mRNA concentrations at the fourth biopsy in the regressing groups. In the 2XPGF-non-regressed group the *VEGFA* mRNA concentrations were similar to those of controls, except at the third biopsy when a slight but significant decrease was observed (20%).

The *FGF2* mRNA concentrations were not altered after PGF treatment at the first biopsy; however all three treatment groups had increased mRNA at the second biopsy, with the 4XPGF group having a greater increase ( $29.5 \pm 9.2$ -fold) than the other two groups. Similarly, at the third biopsy, all three treatment groups had greater *FGF2* mRNA concentrations than controls, with the 4XPGF greater than the other two groups. By the fourth biopsy, the *FGF2* mRNA concentrations had decreased dramatically in the 2XPGF-regressed (53%) and the 4XPGF (65%) groups but remained elevated in the 2XPGF-non-regressed group ( $2.4 \pm 0.5$ -fold).

#### *In Situ Hybridization and Immunohistochemistry*

The mRNA for *PTGFR* was clearly localized to large luteal cells in the control biopsy sample (Fig. 7a). At 0.5 h after PGF, there was no difference in *PTGFR* mRNA with clear localization in the large luteal cells (Fig. 7b). After the fourth PGF treatment, there was no *PTGFR* mRNA detectable by in situ hybridization (Fig. 7c).

There was no detectable *NR4A1* mRNA in control tissues that were not treated with PGF (Fig. 7d). At 0.5 h after PGF treatment, there was substantial *NR4A1* mRNA localized throughout the luteal tissue. Although localization to large luteal cells was most dramatic, there also appeared to be localization in some capillary endothelial cells (Fig. 7e). After the fourth PGF treatment, *NR4A1* mRNA was not detectable by in situ hybridization (Fig. 7f).

The STAR, CYP11A1, and HSD3B7 proteins could be readily visualized in cells of the CL (Fig. 8). The STAR protein was particularly apparent in large luteal cells and also appeared to be present in small luteal cells but was not visualized in endothelial cells (Fig. 8, a and b). After the fourth PGF

treatment, there was very little STAR protein that could be visualized in the CL (Fig. 8c). The CYP11A1 and HSD3B7 proteins were localized in both large and small luteal cells but not in capillary endothelial cells. There were no changes in CYP11A1 or HSD3B7 protein expression levels that could be grossly observed by visual inspection of immunohistochemistry after the fourth PGF infusions (Fig. 8, f and i).

#### DISCUSSION

This study used intrauterine infusion of low doses of PGF to mimic the pulsatile secretion of PGF that accompanies normal luteolysis. The intrauterine infusion of 0.5 mg of PGF produced hormonal patterns similar to natural PGF pulses observed during luteolysis in previous experiments [11], although it remains to be determined whether the timing of PGF infusion used in the current experiment mimics natural luteolysis. The four luteal biopsy samples that were collected during this experiment allowed us to carefully monitor luteal gene expression following each pulse of PGF. Clearly there were distinct patterns of gene expression induced by each individual PGF pulse. The control group had the same quantity of PBS infused into the uterus and a biopsy schedule similar to that of the treatment groups. The observation that there were no substantial changes in any of the 18 mRNAs during any of the four biopsies provides some confidence that the biopsy and uterine treatments alone do not induce the observed luteal responses, consistent with previous results [11, 34]. We included a group of cows that received only two doses of PGF in order to observe responses to PGF that did not result in complete luteal regression. Some of the cows in this group reacted as expected with a partial decrease in P4 concentrations at 9 to 24 h after the first PGF treatment and a subsequent rebound in circulating P4 to near control concentrations. Unexpectedly, some of the cows treated with two doses of PGF underwent complete luteal regression of P4 concentrations from 12 to 48 h after the first PGF that were similar to those of cows receiving four doses of PGF. Thus, our experimental design allowed us to determine the temporal pattern of luteal



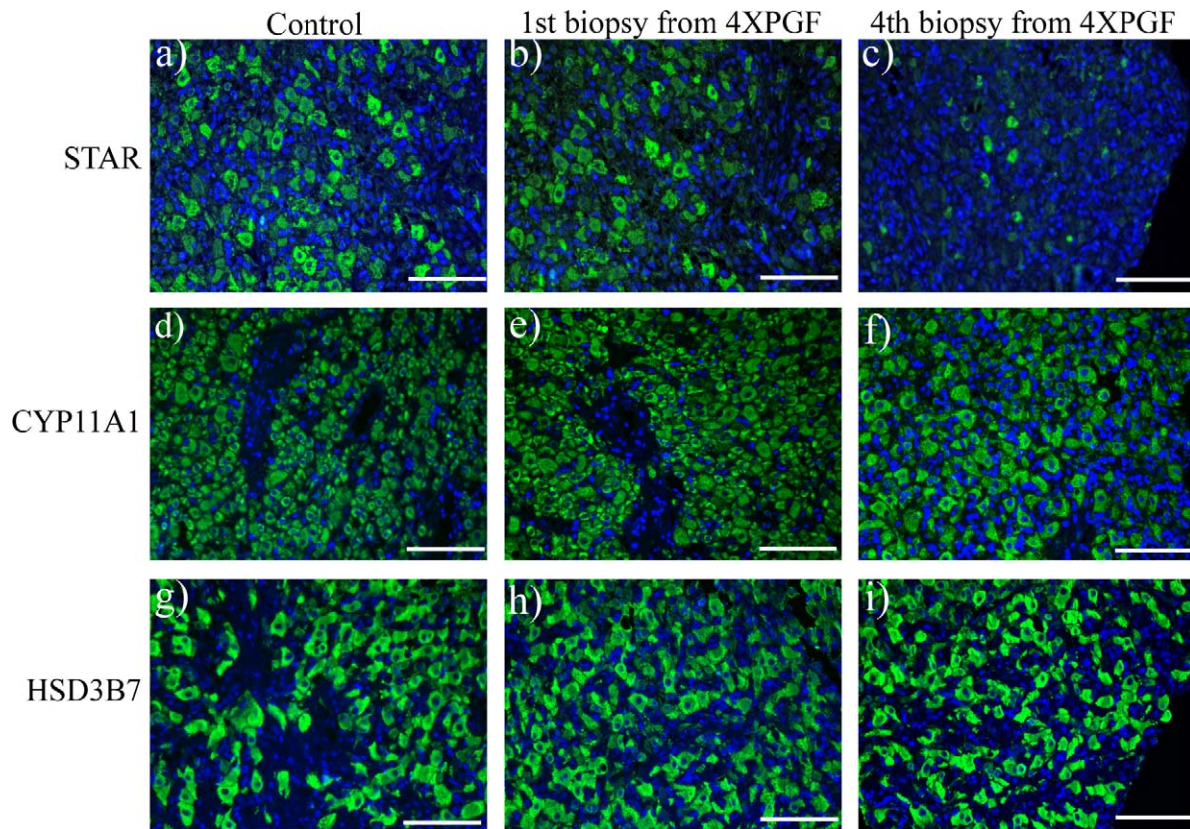


FIG. 8. Representative images of immunofluorescent staining for STAR (steroidogenic acute regulatory protein **a, b, c**), CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1, **d, e, f**), and HSD3B7 (hydroxy- $\delta$ -5-steroid dehydrogenase, 3  $\beta$ - and steroid  $\delta$ -isomerase 7, **g, h, i**) in bovine CL at control (30 min after saline), and at first and fourth biopsies from 4XPFG group. Labeled tissue sections were counterstained with 4', 6-diamidino-2-phenylindole dilactate (blue staining). Bars = 100  $\mu$ m.

gene expression associated with each specific PGF pulse and to differentiate PGF pulse-induced changes that were associated or not associated with complete luteal regression. We have summarized our findings using a physiological model that can guide a discussion of potential mechanisms that accompany natural luteolysis (Fig. 9).

After the first treatment with PGF (Fig. 9, first biopsy), there were similar responses in all three of the treatment groups. A single receptor for PGF has been identified, and the initial actions of PGF appear to be due to binding of PGF to this specific G-coupled receptor (PTGFR) on the plasma membrane of luteal cells [40, 41]. Use of in situ hybridization (Fig. 8) indicated that the *PTGFR* was primarily, if not exclusively, localized to large luteal cells. The concentration of *PTGFR* mRNA and protein are extremely high in CL of all species that have been evaluated, and binding of PGF to this receptor is likely to initiate the cascade of events resulting in altered expression of multiple genes. Binding of PGF to the PTGFR increases free intracellular calcium and activity of phospholipase C, protein kinase C (PKC), and mitogen-activated protein (MAP) kinase, with subsequent activation of multiple transcription factors [42–45]. These signaling pathways, particularly activated ELK1 and ERK signaling, are involved in inducing a rapid and dramatic increase in the immediate early genes such as *FOS*, *JUN*, *NR4A1*, and *EGR1* [46–49] that were evaluated in this study. Multiple hormones and other signaling molecules are known to rapidly induce these immediate early genes, with proposed involvement in a remarkable diversity of cellular responses [50, 51]. For example, immediate early genes have been proposed as critical mediators of apoptotic

signaling cascades [52] as well as growth and mitosis pathways [53]. In the CL, it has been reported that *EGR1* has an important role in expression of genes whose cognate proteins regulate luteal regression (e.g., *EGR1* [54]). *FOS* and *JUN* proteins, as constituents of the activator protein-1 (AP-1) transcription factor complex, appear to be involved in programming gene expression during PGF-induced luteolysis [47]. Other studies have previously shown that these immediate early genes increase after a supraphysiological dose of PGF [54, 55], and our study confirmed the fact that these genes are also induced by low doses of PGF delivered by intrauterine infusion. After the second infusion (Fig. 9), only the group (4XPFG) that was treated with PGF had an increase in these immediate early genes. Similarly, at the third and fourth biopsies, only cows that had been treated with PGF immediately before the biopsy had an increase in immediate early gene concentrations, indicating the acute role of PGF in induction of these genes. The magnitude of the increase in immediate early genes was generally greatest at the first PGF treatment and was reduced after subsequent PGF treatments. This may be due to the clear reduction in expression of *PTGFR* following PGF treatment or due to other intracellular mechanisms that were down-regulated by prior PGF treatments. Reduction in *PTGFR* expression levels after PGF treatment is consistent with findings from other reports that used supraphysiological doses of PGF [16, 17]. However, the observation that even low doses of PGF decrease *PTGFR* expression is in apparent conflict with the idea that prior exposure to low doses of PGF can increase subsequent luteolytic responses to a pulse of PGF. In contrast, the CL of

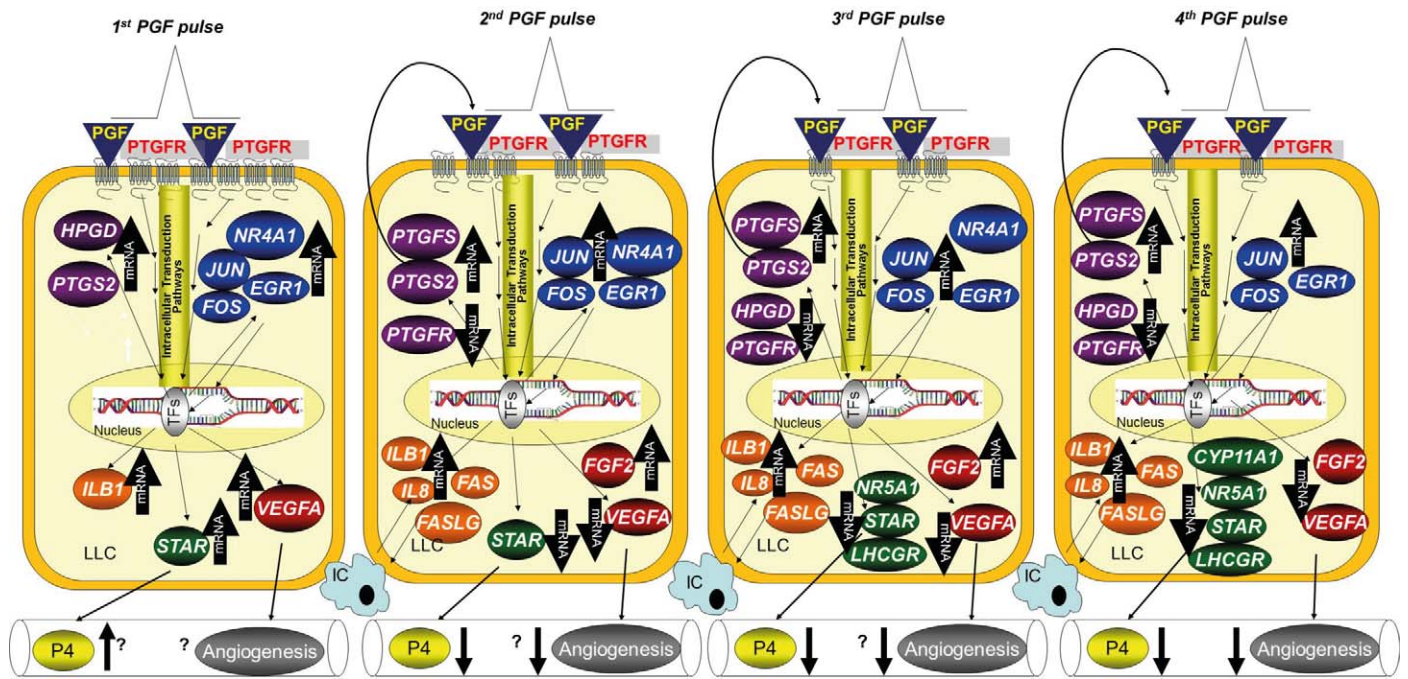


FIG. 9. Schematic model shows changes in mRNAs following the four PGF pulses associated with luteolysis. Following the first PGF pulse, there were increases in mRNA for specific genes in all five pathways that were examined: immediate early genes (blue), PGF-related genes (purple), immune-related genes (orange), steroidogenic genes (green), and angiogenesis genes (red). Increases were not specific for luteal regression as similar increases were observed in CL that subsequently underwent or did not undergo regression. The second PGF pulse also increased the immediate early genes, but this pulse induced decreases in *VEGFA* and *STAR*. These changes were specific for the second PGF pulse, as they did not happen in CL that were only exposed to a single PGF pulse. After the third PGF pulse, there were dramatic changes in mRNA, consistent with a course that would result in luteolysis. Most of these changes did not happen in CL that were not destined for luteolysis, even though they also received the second PGF pulse at 30 min prior to the third biopsy. The fourth biopsy continued the luteolytic cascade with increases or decreases in essentially all genes in a luteolytic direction that was specific for CL that were undergoing the full luteolytic cascade. Names of genes are defined in the text. An up arrow (↑) indicates up-regulation; a down arrow (↓) indicates down-regulation; IC, immune cell; LLC, large luteal cells; TFs, transcription factors; P4, progesterone. The question marks indicate possible or unknown outcomes such as increased P4 following *STAR* or changes in angiogenesis after changes in *VEGFA* or *FGF2*.

other species have either an increase [56, 57] or no change [58] in luteal *PTGFR* expression after PGF treatment. Somewhat surprisingly, in our study, the cows that did not regress after PGF treatment (2XPGF-non-regressed) had much less of a reduction in *PTGFR* mRNA (Fig. 4D) and generally a reduced magnitude of immediate early gene induction after PGF infusion. This may indicate that less PGF reached the CL after intrauterine PGF infusion in these cows or that these cows were less responsive to PGF that reached the CL. Although the precise role for these immediate early genes in luteolysis could not be determined in our experiment, our results are consistent with a clear induction of these genes after each PGF pulse and a role for these genes, probably due to their role as transcription factors, in PGF-induced luteolysis.

Five other genes were also found to have an increase in their mRNA at 0.5 h after PGF infusion. Three of these mRNA (*STAR*, *HPGD*, and *VEGFA*) were increased at the early times after PGF, with a later reduction in their mRNA following subsequent PGF pulses. This was a surprising and unexpected pattern that suggests complex regulatory mechanisms for these genes during multiple PGF pulses. Induction of these mRNA at only 0.5 h after PGF infusion suggests involvement of regulatory mechanisms similar to those found for the acute induction of the immediate early genes discussed above. Nevertheless, acute PGF-induced inhibition of degradation of these specific mRNA is another mechanism that could also produce an acute increase in these mRNA. The subsequent profound reduction in these three mRNA indicates activation of other regulatory mechanisms down-regulating these mRNA. It is noteworthy that these reductions occurred only after a second

PGF treatment (second biopsy for 4XPGF group or third biopsy for 2XPGF group). Furthermore, the CL that did not undergo subsequent regression had either little or no reduction in these mRNA following the second PGF treatment, although the first PGF treatment produced similar increases in *STAR*, *HPGD*, and *VEGFA* mRNA in all three groups.

The *STAR*, *HPGD*, and *VEGFA* genes encode proteins that are critical for maintenance of luteal function. For example, the *STAR* protein mediates cholesterol transport from the outer to the inner mitochondrial membrane, the key rate-limiting step for production of most steroid hormones, including P4 [59]. Similarly, it has been reported that PGF treatment causes a transient increase in circulating P4 followed by a decrease [25, 29, 60]. While it seems unlikely that the acute induction of *STAR* mRNA is involved in the acute increase in circulating P4 (translation of mRNA to functional protein would be expected to take a longer period), it seems possible that similar mechanisms may mediate both increases. The dramatic decrease in *STAR* mRNA after the second PGF pulse (second biopsy in 4XPGF or third biopsy in 2XPGF-regression) is consistent with observations from other research [61] and with the decrease in *STAR*-mediated cholesterol transport that is a key rate-limiting step in the decreased P4 production during luteolysis. The mRNA for other steroidogenesis-related mRNA were not decreased until the later times, with *CYP11A1* lower only at the fourth biopsy and *NR5A1* and *LHCGR* lower only at third and fourth biopsies. Nevertheless, the lack of a decrease in these mRNA in the 2XPGF-non-regressed CL suggests that decreases in these mRNA may have a role in luteolysis.



The product of the *HPGD* gene (formerly known as *PGDH*) metabolizes PGF into 13,14-dihydro-15-keto-PGF $_{2\alpha}$  (PGFM), thought to be an inactive metabolite. Thus, intraluteal PGF could be inactivated by HPGD and thus protect normal luteal function. A previous study [62] reported the greatest HPGD activity in the early CL and the pregnant CL, which are relatively resistant to PGF action. Thus, the initial induction of *HPGD* followed by a dramatic inhibition may produce an initial luteal protection followed by an increase in luteal sensitivity to PGF due to lack of intraluteal PGF inactivation.

Finally, the third mRNA with a distinct induction followed by inhibition was *VEGFA*, a growth factor that is critical for induction of angiogenesis. More than 50% of the cells in a mature CL are endothelial cells [1], and inhibition of VEGF leads to inadequate CL microvasculature and inadequate luteal function [63]. Again, the initial induction of *VEGFA* is perplexing. Previous studies using a single, high dose of PGF have reported either an increase [64] or decrease [16] in *VEGFA* mRNA at earlier times after PGF. The later reduction in *VEGFA* mRNA is consistent with a previous report [18]. The *FGF2* mRNA encodes another protein that is involved in angiogenesis, and the pattern of expression following PGF also demonstrates a pattern of initial induction followed by inhibition. Nevertheless, the initial induction of *FGF2* was not observed at 0.5 h after PGF and was observed only at the second biopsy (6.5 h after initial PGF). Intriguingly, the second PGF infusion acutely stimulated a greater induction of *FGF2* (Fig. 6, compare 2XPGF to 4XPGF), implying that the second PGF regulates immediate regulatory mechanisms involved in *FGF2* regulation. The subsequent inhibition, while profound, was not observed until the fourth biopsy. Similar profiles for *FGF2* mRNA were previously reported in sheep and cow CL during luteolysis [18, 64].

The other two mRNA that were induced at 0.5 h after PGF (*PTGS2* and *IL1B*) continued to be induced by PGF at all biopsies following the initial PGF pulse. Both of these mRNA were also less profoundly induced in animals that did not undergo luteal regression during the treatments (2XPGF-non-regressed) than in the other two PGF treatment groups. Induction of *PTGS2* mRNA in CL by higher doses of PGF has been demonstrated in every species that has been evaluated [24, 65–67]. It has been proposed that PGF-induced *PTGS2* expression represents an intraluteal autoamplification pathway that increases intraluteal PGF following exposure to the low amounts of PGF that reach the CL during the PGF pulses associated with luteolysis [68]. Our results are clearly consistent with the luteal PGF autoamplification concept due to the induction *PTGS2*. Furthermore, the induction of *PTGFS* mRNA, albeit at later times after PGF treatment, would also tend to increase intraluteal PGF synthesis. Again, the CL that did not undergo luteolysis following the two doses of PGF had a greatly reduced induction of *PTGFS* mRNA. Thus, the induction of *PTGS2* and *PTGFS* combined with the reduction in *HPGD* would all lead to increased intraluteal PGF and would amplify the PGF signal at later PGF pulses associated with natural luteolysis.

All of the immune-related mRNA increased after PGF pulses, although *IL1B* was the only mRNA that was increased at 0.5 h after PGF. All of the immune-related mRNAs that we examined were increased by 6.5 h after the first PGF pulse, with increasing magnitude of expression with increasing time. Most investigators have found an increase in lymphocytes and macrophages in the CL at the time of luteolysis [14, 69–71]. Thus, increases in immune-related mRNA may be the result of increased numbers of immune cells in the CL or greater induction of these mRNA in immune cells or other cells that

are already present in the CL. Recent research shows findings consistent with activation of the immune system during luteolysis [14, 72] and with a functional role for the immune system in luteal regression [73]. Further studies are needed to define the precise cellular localization and physiological role of each of the immune-related molecules in luteal regression. Consistent with a role for these molecules in luteal regression, there was a reduced induction of immune-related mRNA in CL that did not undergo luteolysis. This difference is particularly stark at the later times with all four immune-related mRNA concentrations similar in CL from the 2XPGF-non-regressed and control groups (Fig. 5).

Thus, our results are consistent with changes in four key regulatory pathways in the CL being increasingly shunted into a luteolytic direction with increasing numbers of low dose PGF pulses. This is illustrated in Figure 9. For example, the P4 synthesis pathway is increasingly inhibited at later times after the initial PGF treatments, probably primarily due to changes in STAR and cholesterol transport. The lack of substantial decreases in the two key proteins involved in P4 production, CYP11A and HSD3B7, as determined by immunohistochemistry, indicates the CL still has functional capacity for P4 synthesis, even at the time of the fourth luteal biopsy (18.5 h after initial PGF) when circulating P4 is low. The dramatic decrease in the two key angiogenic mRNA by the fourth biopsy is consistent with multiple PGF pulses causing regression of the luteal vasculature. Although the decrease in *PTGFR* would tend to reduce luteal PGF sensitivity, all of the other genes involved in PG pathways that were examined would tend to increase intraluteal PGF concentrations and move this pathway in a luteolytic direction. Similarly the increase in immune-related mRNA is consistent with multiple PGF pulses activating this luteolytic pathway. For almost all of the mRNA, PGF responses were absent or less profound after the second PGF treatment in CL that recovered normal function after PGF pulses compared to CL that underwent regression after two or four PGF pulses. Generally, most of these intracellular mechanisms would tend to prevent luteal regression following a single exposure to a low dose PGF pulse with the surprising finding of acute and dramatic increases in a multitude of mRNA after a single PGF pulse even in CL that are not destined for regression. However, this initial PGF pulse appeared to amplify the luteolytic effects of subsequent low amplitude PGF pulses in CL that subsequently underwent regression. These results are likely to have important implications for the mechanisms involved in normal luteolysis.

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