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Research Article

Mechanisms for rescue of corpus luteum during pregnancy: gene expression in bovine corpus luteum following intrauterine pulses of prostaglandins E_1 and $F_{2\alpha}$ †

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Abstract

In ruminants, uterine pulses of prostaglandin (PG) $F_{2\alpha}$ characterize luteolysis, while increased PGE_2/PGE_1 distinguish early pregnancy. This study evaluated intrauterine (IU) infusions of $PGF_{2\alpha}$ and PGE_1 pulses on corpus luteum (CL) function and gene expression. Cows on day 10 of estrous cycle received 4 IU infusions (every 6 h; n = 5/treatment) of saline, PGE_1 (2 mg PGE_1), $PGF_{2\alpha}$ (0.25 mg $PGF_{2\alpha}$), or $PGE_1 + PGF_{2\alpha}$. A luteal biopsy was collected at 30 min after third infusion for determination of gene expression by RNA-Seq. As expected, IU pulses of $PGF_{2\alpha}$ decreased ($P < 0.01$) P4 luteal volume. However, there were no differences in circulating P4 or luteal volume between saline, PGE_1, and $PGE_1 + PGF_{2\alpha}$, indicating inhibition of $PGF_{2\alpha}$-induced luteolysis by IU pulses of PGE_1. After third pulse of $PGF_{2\alpha}$, luteal expression of 955 genes were altered (false discovery rate [FDR] < 0.01), representing both typical and novel luteolytic transcriptomic changes. Surprisingly, after third pulse of PGE_1 or $PGE_1 + PGF_{2\alpha}$, there were no significant changes in luteal

gene expression (FDR > 0.10) compared to saline cows. Increased circulating concentrations of the metabolite of $PGF_{2\alpha}$ (PGFM; after $PGF_{2\alpha}$ and $PGE_1 + PGF_{2\alpha}$) and the metabolite PGE (PGEM; after PGE_1 and $PGE_1 + PGF_{2\alpha}$) demonstrated that $PGF_{2\alpha}$ and PGE_1 are entering bloodstream after IU infusions. Thus, IU pulses of $PGF_{2\alpha}$ and PGE_1 allow determination of changes in luteal gene expression that could be relevant to understanding luteolysis and pregnancy. Unexpectedly, by third pulse of PGE_1, there is complete blockade of either $PGF_{2\alpha}$ transport to the CL or $PGF_{2\alpha}$ action by PGE_1 resulting in complete inhibition of transcriptomic changes following IU $PGF_{2\alpha}$ pulses.

Summary Sentence

Treatment with $PGF_{2\alpha}$ induced dramatic changes in expression of 955 genes, based on RNA-Seq after the third $PGF_{2\alpha}$ pulse (FDR < 0.01), whereas simultaneous treatment with pulses of PGE_1 blocked luteolysis and gene expression induced by $PGF_{2\alpha}$.

Key words: corpus luteum, luteolysis, prostaglandin $F_{2\alpha}$, prostaglandin E_1.

Introduction

The corpus luteum (CL) is a transitory endocrine gland, which is essential for the establishment of pregnancy in cattle due to the production of progesterone (P4). In nonpregnant animals, or in the absence of the appropriate embryonic signal, function of the ruminant CL is terminated by the action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released by the uterus [1, 2]. During early pregnancy in the cow, interferon tau (IFNT) has been shown to be the primary signal from the ruminant embryo that results in maintenance of the CL [3, 4]. Nevertheless, it is still being debated whether IFNT rescues the CL by acting directly on the CL, inhibiting the $PGF_{2\alpha}$ pulse pattern, or stimulating release of a luteotropic substance such as prostaglandin E_2 or E_1 (PGE_2/PGE_1). Most early researchers advocated a primary effect of IFNT on inhibition of uterine production of luteolytic pulses of $PGF_{2\alpha}$ [5]. More recently, evidence that IFNT also exits the uterus and can act directly on peripheral tissues, including the CL, has been reported [6, 7]. However, there is also evidence from both early research and from recent research that IFNT alters PGE secretion by the endometrium [8–11], and this may also be a key factor in maintenance of the CL during early pregnancy, by acting as a luteoprotective agent [8, 9, 12, 13].

Prostaglandins are modified fatty acids containing 20 carbons that regulate many aspects of reproductive physiology [1, 14]. Both $PGF_{2\alpha}$ and PGE_2 are derived from the essential fatty acid, arachidonic acid (AA), which is released from membrane phospholipids through the action of phospholipases, such as phospholipase A2 [15, 16]. The resulting AA is converted to the unstable peroxide PGH_2 by the action of constitutive PTGS1 and inducible PTGS2 [17, 18]. The resulting PGH_2 can be converted into various prostaglandins including PGE_2 through the action of PGE synthase (mPGES-1) or $PGF_{2\alpha}$ through the action of PGF synthase (PTGFS) [8, 19, 20].

The PGs act through G-protein-coupled receptors that have seven transmembrane domains. For $PGF_{2\alpha}$, there is a single receptor, termed the prostaglandin F2α receptor (PTGFR) [21]. Activation of the PTGFRs leads to inositol phosphate accumulation, protein kinase C (PKC) activation, and increased free intracellular calcium concentrations [22–26], consistent with coupling of the PTGFR to the Gαq family of G-proteins [26, 27]. In contrast, PGE molecules bind to a family of PGE-binding receptors, termed EP receptors, that activate a multitude of G-proteins, with some activating similar pathways as $PGF_{2\alpha}$ and others activating opposing intracellular effector systems such as the cAMP/protein kinase A pathways [28, 29]. Binding studies with agonists of PG receptors in bovine luteal membranes have demonstrated the specificity and affinity of different PG agonists for the different luteal EP and PTGFRs [30]. $PGF_{2\alpha}$ and PGE_1 were found to bind with high affinity and specificity to the PTGFR or EP receptors, respectively. Similar to PGE_1, PGE_2 had high affinity for the EP receptors; however, it also had cross-reactivity with the PTGFR though the binding affinity was about 10-fold higher for $PGF_{2\alpha}$ than for PGE_2. Similarly, some studies have found that high concentrations of PGE_2 can produce effects that resemble $PGF_{2\alpha}$ actions in luteal cells [31]. This is important for the present studies as we chose to use PGE_1 as the PGE receptor agonist, in order to avoid any potential cross-reactivity with the PTGFR. Inactivation of all of these PGs occur through similar pathways by the action of 15-hydroxyprostaglandin dehydrogenase (HPGD) converting $PGF_{2\alpha}$, PGE_2, or PGE_1 into the inactivate metabolites PGFM and PGEM [32].

Regression of the CL in the cow, as well as in many other species, is mediated by production of $PGF_{2\alpha}$ by the uterus at the end of the luteal phase [1, 33, 34]. Functional and structural regression of the CL is characterized by the release of $PGF_{2\alpha}$ in pulses. The main $PGF_{2\alpha}$ metabolite is PGFM and evaluation of circulating PGFM has been used to monitor $PGF_{2\alpha}$ release during luteolysis in many studies [35]. Measurements of PGFM at the end of the estrous cycle have demonstrated that luteolysis, as measured by decreases in circulating P4, is associated with three to five pulses of PGFM that occur approximately every 12 h during the last 2–3 days of the luteal phase [35, 36]. The intermittent secretion of $PGF_{2\alpha}$ has been recently simulated in heifers by intrauterine (IU) infusions of low doses of $PGF_{2\alpha}$ that mirrored both the concentrations of PGFM pulses and the decline in circulating P4 and CL volume that occur during natural luteolysis [37]. A recent study [38] evaluated the patterns of gene expression in the bovine CL in response to four IU infusions of low doses of PGF. Interestingly, each of the four infused pulses of $PGF_{2\alpha}$ were followed by an increase in expression of early response genes (FOS, JUN, EGR-1). However, it was only after the second $PGF_{2\alpha}$ pulse that immune regulators, such as IL1β and IL8, had increased mRNA expression. The mRNA for enzymes involved in steroidogenesis were inhibited at varying times after $PGF_{2\alpha}$ pulses, with StAR, a key regulator of intracellular transport of cholesterol, inhibited after the second pulse of $PGF_{2\alpha}$; whereas CYP11A1, also known as cholesterol side chain cleavage enzyme, only showed significant inhibition following the fourth pulse. Consistent with these patterns, genes involved in the biosynthesis of $PGF_{2\alpha}$ (PTGS2, PTGFS) increased their expression after the first and second $PGF_{2\alpha}$ pulses, respectively, consistent with induction of an autoamplification pathway for $PGF_{2\alpha}$ production within the CL during luteolysis. Further, mRNA for the gene that metabolizes $PGF_{2\alpha}$ into PGFM, HPGD, decreased after the third pulse of $PGF_{2\alpha}$, perhaps allowing increased activity for $PGF_{2\alpha}$ within the CL. A number of other studies have also reported the effects of a single large dose of $PGF_{2\alpha}$ on the luteal transcriptome

[39, 40]; although global gene transcriptional changes in response to physiologically relevant pulses of $PGF_{2\alpha}$ have not yet been reported.

In contrast, during pregnancy, the CL remains steroidogenically active, as measured by luteal P4 production and circulating P4 concentrations, and structurally intact, as evidenced by luteal volume and cellular histology [35, 41–43]. Similar to the local effects of $PGF_{2\alpha}$ during CL regression, the protective effect of the embryo appears to be mediated by local pathways, as evidenced by elegant vascular anastomoses experiments showing that the uterine venous effluent from the gravid uterine horn contains a small molecule that is transferred to the ipsilateral ovarian artery and this blocks the normal luteolytic process [44, 45]. A number of reports indicate that this locally active, luteoprotective factor is likely to be PGE [8, 9, 12, 13, 46]. During pregnancy, the bovine or ovine uterus produces much greater amounts of PGE_2 than during a similar time period in nonpregnant animals [47, 48]. In addition, ovine [49, 50] and bovine [51] embryos also produce PGE_2 during early pregnancy. There are also numerous studies demonstrating that treatment with PGEs can inhibit the luteolytic actions of $PGF_{2\alpha}$ in the ovine and bovine CL [9, 12, 52–54]. In addition, studies have demonstrated that PGE_2 [9] or PGE_1 [12] can diffuse through the utero-ovarian plexus and thereby could provide a luteoprotective effect on the CL during establishment of pregnancy in sheep. Nevertheless, no previous studies have reported the transcriptomic changes in the CL in response to IU treatment with PGE.

This study uses our previous model of CL regression by pulsatile IU infusions of low doses of $PGF_{2\alpha}$ (0.25 mg), but it extends this model by simultaneously infusing low doses of PGE_1 (2 mg) to mimic physiological concentrations of PGE that are secreted into the uterus during pregnancy [9, 55]. To eliminate any confounding effects of PGE_2 binding to the PTGFR, PGE_1 was utilized as the PGE agonist in this study. Dynamic changes in CL structure and function during the IU treatments were measured by evaluating changes in circulating P4 concentrations and CL volume, using ultrasound. Circulating concentrations of PGFM and PGEM were assessed to determine if infusions of PGE_1 altered the transport of $PGF_{2\alpha}$ from the uterus to the uterine vein. In addition, CL biopsies were obtained following the third $PGF_{2\alpha}$ pulse to allow quantitative evaluation of changes in mRNA concentrations in the CL in response to treatments with $PGF_{2\alpha}$ and/or PGE_1. By the time of third $PGF_{2\alpha}$ pulse, many of the luteolytic processes, such as activation of the immune system and prostaglandin synthesis, could be clearly identified by differential gene expression [38]. Thus, this study focused on three specific hypotheses. Our first hypothesis was that IU pulses of low doses of $PGF_{2\alpha}$ would induce CL regression and a gene expression pattern in the CL that would be typical of luteolysis, as previously reported [38, 39]. Our second hypothesis was that IU pulses of low doses of PGE_1 would not alter the size or P4 production by the CL but would induce a distinct pattern of gene expression that would be typical of PGE actions. Finally, our third hypothesis was that simultaneous infusion of $PGF_{2\alpha}$ and PGE_1 would maintain the CL, both structurally and functionally, and would produce distinctive gene expression patterns that would signify lack of CL regression, through inhibition of specific pathways that are normally activated by physiological $PGF_{2\alpha}$ pulses.

Materials and methods

Reagents

Purchase of $PGF_{2\alpha}$ (Lutalyse) and intravaginal P4 inserts (Eazi-breed controlled internal drug release, CIDR) was from Zoetis, Inc. (Zoetis Animal Health, Kalamazoo, MI). The Gonadotropin releasing hormone, GnRH (GONAbreed) was a gift from Parnell Veterinary Pharmaceuticals (Parnell US 1 Inc. Leawood, KS). Specific primers for target genes were synthesized by the Biotechnology Center of the University of Wisconsin-Madison. RNeasy Plus Universal Mini Kit was purchased from QIAGEN (Qiagen Inc. Valencia, CA, USA). The iScript cDNA Synthesis Kit (catalog # 1708891) and SsoFast EvaGreen Supermix (catalog # 1725200) were purchased from Bio-Rad (Bio-Rad Laboratories Inc. Hercules, CA). Secondary antibody (Goat anti-Rabbit IgG, catalog ab6702.) was purchased from Abcam (Abcam Inc. Boston, MA). PGFM (catalog # 16670), PGEM enzyme-linked immunosorbent assay (ELISA) kit (Item N° 514531) and PGE_1 (Item N° 13010) were purchased from Cayman (Cayman Chemical Company, Ann Arbor, MI). Primary antibody (Rabbit anti-PGFM) was a generous gift from Dr W.W. Thatcher, University of Florida.

Animals: housing and estrus synchronization

This experiment was performed from August 2015 to October 2015, using multiparous nonpregnant dry cows housed at the University of Wisconsin dairy facilities. Holstein cows with normal estrous cycles were used for this experiment. A cow was not used if there was an indication of uterine or ovarian abnormality based on ultrasonic scanning. Cows had ovulation synchronized with a CIDR-synch modified protocol consisting of an initial treatment with GnRH and insertion of a CIDR, followed by a PGF treatment on days 6 and 7, followed by CIDR withdrawal on day 8, and 24 h later (day 9) a second treatment with GnRH. Day of ovulation was determined by ultrasonography and designated day 1. All procedures used in this experiment were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison.

Experimental protocols

Cows with a mature CL on day 10 of the estrous cycle were assigned randomly to one of four treatment groups, with equal numbers in each group. All cows received four IU infusions at 6-h intervals followed by a luteal biopsy 30 min after the third IU infusion. Cows in saline group (n = 5) received IU infusions of 0.1 ml of saline and 0.1 ml of dimethyl sulfoxide (DMSO). Cows in group PGE (n = 5) received IU infusions of 0.1 ml of saline and 2 mg of PGE_1 diluted in 0.1 ml of DMSO. Cows in group PGF (n = 5) received IU infusions of 0.25 mg of $PGF_{2\alpha}$ diluted in 0.1 ml of saline and 0.1 ml of DMSO. Cows in group PGE + PGF (n = 5) received IU infusions of 2 mg of PGE_1 in 0.1 ml of DMSO and 0.25 mg of $PGF_{2\alpha}$ in 0.1 ml of saline. The infused dose (2 mg) of PGE_1 was calculated based on an approximate concentration of 30 ng/ml of PGE that has been reported in the uterine vein of pregnant sheep [9], and a rate of blood flow in the uterine vein of 200 ml/min [55] during a 6-h period ((30 ng/ml × 200 ml/min) × (360 min) = 2.16 mg every 6 h). All treatments were infused into the greater curvature of the uterine horn ipsilateral to the CL, using an embryo transfer gun. All cows had an ultrasonography-guided biopsy of the CL 30 min after the third IU infusion. A total of 25 experimental periods were performed, but five periods were removed due to either inapropriate biopsies or lack of synchronization to the protocol. Therefore, 20 experimental periods were analyzed (n = 5/treatment group).

Ovarian ultrasound imaging

The ovaries of synchronized cows were evaluated by transrectal ultrasonography once per day from the day of the second GnRH

(day 0), and on days 2, 10, 11, 12, and 13 of the estrous cycle. Serial ultrasound videos of the ovary containing the CL were recorded using a B-mode, portable ultrasound fitted with a 7.5 MHz linear-array transducer (Ibex Pro; E. I. Medical Imaging, Loveland, CO) to determine day of ovulation and changes in volume of the luteal tissue on days 10, 11, 12, and 13. The ultrasound settings (focus position, field gain, total gain, and frequency) were configured and maintained for all the replicates. Videos of the CL were recorded for 16 s (241 frames) by a single technician. Analyses of ultrasonographic videos were performed using the open-source image processing software, Image J 1.49v (National Institute of Health, Bethesda, MD; http:rsb.info.nih.gov/ij/index.html).

Luteal volume determination

Videos were analyzed frame by frame to select the cross-sectional area in which the CL size was maximal. Images including a central cavity were taken into account. To determine the volume of the CL, electronic calipers were used to trace the perimeter of the entire CL and the perimeter of any central cavity for the CL. A scale of 5 pixels/mm was used to measure the length in each image. The area was used to calculate the radius by the formula: $r^2 = \text{area}/\pi$, and the radius was used to calculate the total CL volume ($V = 4/3\pi r^3$) minus the volume of the central cavity in cm^3. Values in cm^3 were calculated for each animal and the percentage volume, relative to day 10, was determined for each day.

Transvaginal ultrasound-guided biopsy of the CL

Procedures to collect luteal biopsies were done in a similar manner as previously described [56]. Cows were given caudal epidural anesthesia using 5 ml of lidocaine hydrochloride (Phoenix Pharmaceutical, Inc., St. Joseph, MO). A 7.5-MHz convex array ultrasound transducer (Aloka SSD 900, Hitachi Aloka Medical, Japan) was adapted with a needle guide to allow a 48-cm, 16-gauge biopsy needle (US Biopsy, Division of Promex Inc, Indianapolis, IN) to be inserted through the needle guide. The transducer face was applied to the wall of the vaginal fornix and 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 and luteal tissue was trapped within the specimen notch. After removing the biopsy device, the tissue was inspected to ensure that only luteal tissue was removed from the ovary. Only biopsies that had at least 20 mg of tissue collected were analyzed for this experiment. Biopsies were rinsed with PBS, weighed, and immediately frozen in liquid nitrogen and stored at –80°C for later evaluation of mRNA expression.

Blood sampling and hormonal assays

To determine changes in P4 concentrations coccygeal blood was collected just before each IU infusion (Hour 0—first infusion; Hour 6—second infusion; Hour 12—third infusion; Hour 18—fourth infusion). Thereafter, starting at 24 h after the first infusion, P4 was assayed every 12 until 72 h. Blood samples were stored on ice, allowed to clot, and centrifuged at 3000 rpm for 20 min. Sera were stored at –20°C until the assay. For P4 determination, samples were analyzed using an antibody-coated tube RIA kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) with intra-assay and interassay coefficients of variation (CV) of 3.37% and 7.28%, respectively.

In a subset of cows (n = 16), additional samples were taken just before and 10 min after the first and second infusions, to determine circulating concentrations of PGFM and PGEM. These samples were collected into heparinized tubes, centrifuged at 3000 rpm for 20 min, and stored at –20° C until assayed. The plasma samples were assayed for PGFM by an ELISA assay that was previously validated for use in bovine and plasma [57] with some modifications. Briefly, the ELISA plates were coated with 100 μl of secondary antibody (2 μg/ml) in coating buffer (0.05 M sodium carbonate [pH 9.6]) overnight. Standards were prepared by serial dilution (1250–4.88 pg/ml) of PGFM in prostaglandin-free (banamine-treated) bovine plasma. Prostaglandin-free plasma was obtained from two cows treated at 12-h intervals with three intravenous injections of a prostaglandin synthase inhibitor (1.1 mg/kg of flunixin meglumine. Banamine; Intervet International B.V.). Blood was collected in heparinized tubes 1 h after the last injection. Aliquots of 250 μl of standards (B_0, serial dilutions and nonspecific binding [NSB]), quality control samples, and unknown samples were transferred to glass extraction tubes. The pH was adjusted to 3.0 with diluted hydrochloric acid and vortexed immediately. Two milliliter of diethyl ether were added to all the samples and mixed using a vortex for 3 min. The tubes were then placed in a bath of dry ice and methanol for at least 1 min. Unfrozen, ether extracts were transferred to new glass culture tubes and dried overnight. On the day of the assay, 250 μl of ELISA assay buffer (0.04 M 3-(N-morpholino)propanesulfonic acid (MOPS), 0.12 M sodium chloride, 0.01 M EDTA, 0.05% Tween 20, 0.005% chlorhexidine digluconate [pH 7.4], 0.1% gelatin) was added to all dried extracts and vortexed for 2 min. The tubes were incubated for 90 min at room temperature and vortexed in the middle and the end of incubation. The ELISA plates were coated with secondary antibody, washed four times with wash buffer, and 100 μl of primary antibody (diluted 1:4000 in ELISA assay buffer) added to all wells, except the NSB wells, which received 100 μl assay buffer. The plate was incubated for 1.5 h at room temperature and washed again four times with wash buffer. Reconstituted extracts of standard, control, and unknown samples in duplicate were transferred (100 μl/well) to the respective wells in duplicate. After incubating the plate for 25 min at room temperature, without washing the plate, 50 μl of a conjugate of PGFM and horseradish peroxidase (PGFM-HRP) conjugate [57], diluted in assay buffer (1:1000), was added to all wells, and the plate was incubated for 1 h at room temperature. The plate was washed four times, and 125 μl of substrate solution was added and incubated for 20 min at 37°C. To stop the reaction, 50 μl of stop solution (0.5 M H_2SO_4) was added to all wells and the optical density was measured at a dual wavelength of 450 and 600 nm. A pool of samples collected from 25 pregnant and nonpregnant cows on day 19 of the estrous cycle was used as a quality control in all assays. The intra-assay and interassay CV were 9.8% and 10.1%, respectively. The samples collected to determine PGFM concentrations, were also assayed for PGEM using a commercially available kit (Cayman Chemicals) according to instructions described by the manufacturer. The kit specifications report a 100% cross-reactivity with 13, 14-dihydro-15-keto PGE_1 and 13,14-dihydro-15-keto PGE_2 and a minimum detection limit of 0.39 pg/ml. All PGEM samples were analyzed in one assay with an intra-assay CV of 6.62%.

Statistical analysis of CL volume and hormonal data

Differences between variables prior to treatments were calculated by Levene test for homogeneity of variance. The data obtained for the response variables of concentration of P4 (ng/ml) and luteal volume (cm^3) for each one of the four treatment groups were normalized

to 100% compared to time 0 (Hour 0 of treatment for P4 concentrations and day 0 for luteal volume) and expressed as percentage change in each cow relative to time 0. The values were analyzed for differences between treatments using the Proc Mixed procedure of SAS and differences between means at specific time points were assessed using Fisher LSD. Data for PGFM and PGEM concentrations were not normally distributed and therefore were transformed to natural logarithms. Differences between treatments were analyzed by one-way ANOVA. Assumptions of normality and homogeneity of variance were evaluated and transformations (natural logarithm) performed, when appropriate.

RNA Isolation and cDNA Preparation

Ten milligrams of luteal tissue were minced with a scalpel and homogenized in QIAzol Lysis Reagent with a Bio-gen PRO200 Homogenizer Motor Unit (PRO Scientific, CT, US). Total RNA from each sample was extracted using RNeasy Plus Universal Kit (RNeasy; QIAGEN), according to manufacturer's protocol. All individually homogenized samples were treated with gDNA Eliminator Solution (QIAGEN, USA) to reduce genomic DNA contamination. Concentrations of RNA from each sample were determined by optical density at OD_{260nm}/OD_{280nm} ratio using NanoDrop 2000 spectophometer (Thermo Scientific, DE, USA). One microgram of total RNA from each sample was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit and diluted (1:5) in deionized water. All cDNA samples were stored at –20°C until analyzed.

RNA-Seq analysis

To compare the effect of the third IU infusion of PGE1, PGF, and PGE1 + PGF on gene expression of the CL, luteal biopsies (n = 5/treatment) were analyzed using RNA-seq. A total of 50 ng of RNA from each luteal biopsy was used to prepare sequencing libraries following Illumina's mRNASeq protocol. Libraries were sequenced with Illumina's HiSeq 2000 at the Biotechnology Center of the University of Wisconsin-Madison. The 20 libraries (i.e., five libraries per treatment) were barcoded, multiplexed, and sequenced. A read was defined as a 100 bp cDNA fragment sequenced from a single end. Approximately 30 million reads were sequenced from each library.

The mapping of sequence reads and subsequent assembly of transcripts was performed as described in detail by Peñagaricano et al. [58, 59]. Briefly, raw sequencing reads were mapped to the bovine reference genome UMD3.1 using the software package Tophat (v2.0.13) [60, 61]. The resulting alignments were used to reconstruct and infer transcript models using the software Cufflinks (v2.2.1) [62]. Furthermore, the computational tool *cuffmerge* was used for merging together each of the sample assemblies with the reference annotation file to combine novel transcripts with known annotated transcripts. Finally, the number of reads that mapped to each gene in each sample was calculated using the tool *htseq-count* [63] (Supplemental Table 4).

Differentially expressed genes between treatments were detected using the *R* package *edgeR* (v.3.14.0) [64]. This *R* package combines the application of the trimmed mean of M-values as the normalization method of the sequencing data, an empirical Bayes approach for estimating genewise negative binomial dispersion values, and finally, generalized linear models and likelihood ratio tests for detecting differentially expressed genes between treatments of interest [65].

The enrichment of Gene Ontology (GO) and Medical Subject Headings (MeSH) functional terms with significant genes was tested using Fisher exact test [66, 67]. Differentially expressed genes with false discovery rate (FDR) ≤ 0.05 and Ensembl annotations were tested against the background set of all genes with Ensembl annotations. These gene set analyses were performed using *goseq R* package [68] and *meshr R* package [69].

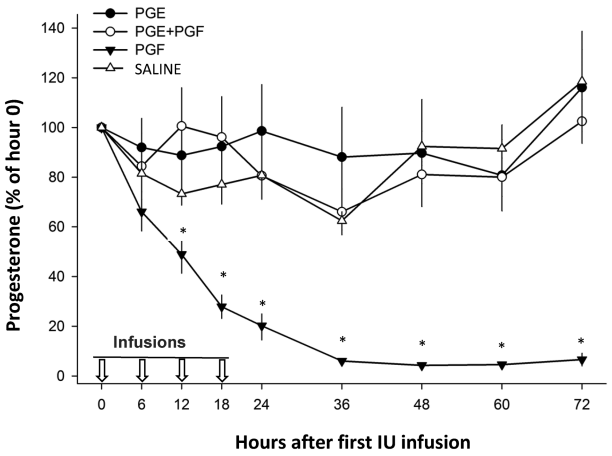

Figure 1. Effect of four IU infusions (shown with arrows) of saline, PGF, PGE, and PGE + PGF treatments (n = 5/treatment) on circulating P4 concentrations in cows. Data are presented as means ± SEM. Asterisks indicate significant decreases in plasma P4 concentrations ($P < 0.05$).

Results

Concentrations of circulating P4 after treatments

Circulating concentrations of P4 for the four treatment groups are shown in Figure 1. Circulating P4 concentrations were somewhat variable between the four treatment groups prior to treatments, although they were not significantly different ($P = 0.21$): saline—5.0 ± 0.6, PGF—7.1 ± 0.9, PGE—6.0 ± 1.5, PGE + PGF—4.15 ± 0.4 ng/ml (mean ± SEM). To normalize for variation in pretreatment circulating P4, the P4 value for each cow was standardized, using the pretreatment P4 as 100%, and all subsequent circulating P4 concentrations in that cow were calculated as a percentage of this pretreatment value.

There was a significant effect of hour ($P < 0.0001$), treatment ($P < 0.0001$), and an hour by treatment interaction ($P < 0.0001$) for circulating P4 concentrations (Figure 1). The source of these effects was the PGF group. There were no differences between groups or within hours in circulating P4 concentrations during the experimental period (0–72 h) for control, PGE1, or PGE1 + PGF groups. In contrast, the PGF-treated group was different from each of the other three groups ($P < 0.0001$) during the entire experimental period, starting at 12 h after the first infusion of PGF and at all subsequent time points ($P < 0.05$). For example, at 12 h after treatment there were clear differences between the PGF group and the saline group ($P = 0.0314$), the PGE1 + PGF group ($P = 0.0008$), and the PGE1 group ($P = 0.0042$). At all subsequent times, the PGF group maintained this difference when compared to each of the other treatments ($P < 0.0001$), whereas the other three groups were similar at all of the evaluated times ($P > 0.05$; Figure 1).

Luteal volume

There was some variability in luteal volume between the four treatment groups prior to treatments, although there was no significant

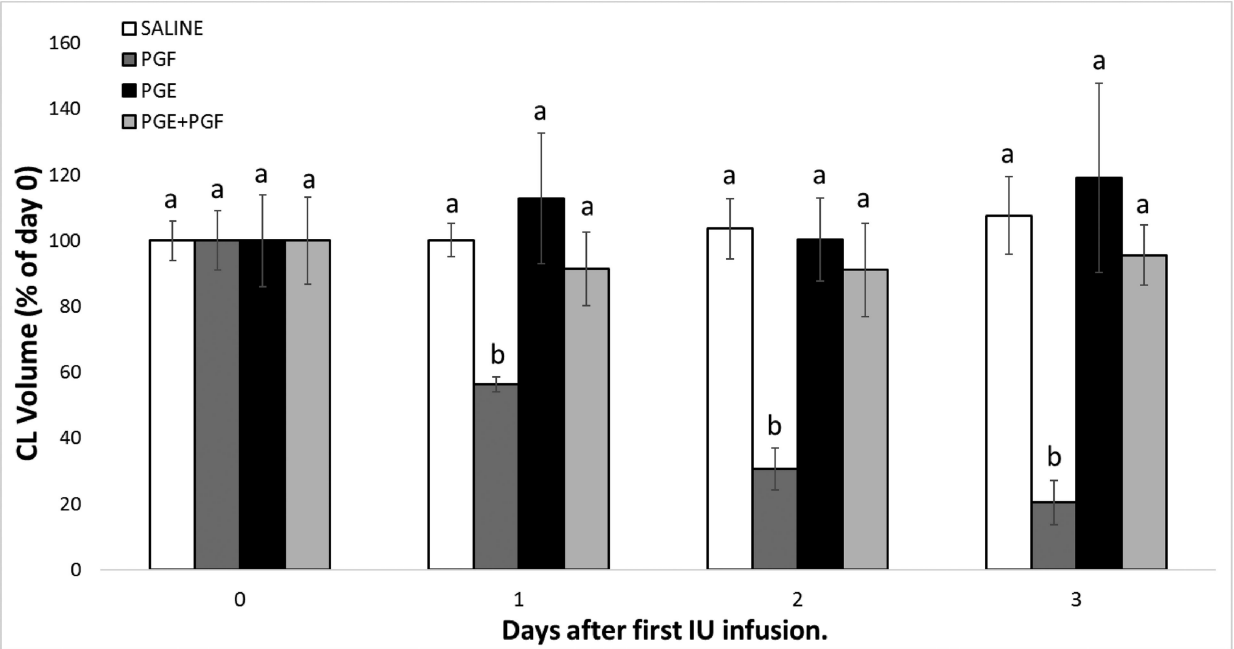

Figure 2. Effect of four IU infusions of saline, PGF, PGE, and PGE + PGF treatments (n = 5/treatment) on luteal volume in cows. Data are presented as means ± SEM. Days 0, 1, 2, and 3 after first infusion correspond to days 10, 11, 12, and 13 of the estrous cycle. Columns with different letters (a, b) indicate statistical differences among treatments within a day ($P < 0.05$).

difference between treatments ($P = 0.45$): saline—8.33 ± 0.50, PGF—11.62 ± 1.05, PGE—7.82 ± 1.08, PGE + PGF—9.67 ± 1.27 cm^3 (mean ± SEM). To normalize for variation in pretreatment luteal volume, the value in cm^3 for each cow was standardized, using the pretreatment volume as 100%, and all subsequent values for volume in that cow were calculated as a percentage of this pretreatment value (Figure 2).

Similar to circulating P4, the luteal volume was different due to the effect of treatments ($P = 0.0002$), time (day, $P = 0.0018$) and the treatment by Day interaction ($P < 0.0001$). Again, the only source of significance was the PGF treatment group, which was different from the PGE group ($P < 0.0001$), PGE + PGF group ($P = 0.0004$), and the saline group ($P < 0.0001$). The three other groups were not different from each other. The decrease in luteal volume between the PGF group and the other three groups began to be evident from day 1 onwards and the differences increased until day 3 (Figure 2). No differences were detected between any of the treatments PGE, PGE + PGF, and saline for any of the days during which luteal volume was evaluated.

Concentrations of circulating PGFM and PGEM

Circulating PGFM concentrations were low and did not differ among treatments prior to IU infusions. Concentrations of PGFM in the saline group were similar before and after the first two infusions (Figure 3). Cows in the PGE group showed low concentrations before and 10 min after the two pulses. However, there was a small but significant ($P = 0.015$) increase in PGFM in pulse 2 (3.1 ± 1.7 pg/ml at min 0 vs 11.1 ± 2.7 pg/ml at min 10). Concentrations of PGFM increased dramatically at 10 min after infusion of PGF for either the first (6.5 ± 1.9 pg/ml at min 0 vs 97.1 ± 34.6 pg/ml at min 10; $P < 0.001$) and second (5.7 ± 1.9 pg/ml at min 0 vs 86.8 ± 30.6 pg/ml at min 10; $P = 0.062$) IU infusions of PGF. Similarly, cows in the group PGE + PGF had increased concentrations of PGFM after pulse 1 (5.0 ± 2.4 pg/ml for min 0 vs 139.1 ± 54.5 pg/ml for min 10; $P = 0.006$) and pulse 2 (4.3 ± 2.2 pg/ml for min 0 vs 132.9 ± 61.1 pg/ml for min 10; $P = 0.037$). At 10 min after IU infusions, cows enrolled in groups saline and PGE, had lower concentrations of PGFM than cows in the groups PGF and PGE + PGF during pulse 1 ($P = 0.001$) and pulse 2 ($P = 0.001$).

Circulating concentrations of PGEM were low and did not vary among treatments before IU infusions (Figure 4). Cows in the saline group showed low concentrations of PGEM before and 10 min after first and second pulse. Nevertheless, there was an increase ($P = 0.05$) after the first saline infusion (32.6 ± 6 pg/ml at min 0 vs 83.1 ± 10.1 pg/ml at min 10). Concentrations of PGEM in the PGE group increased significantly after the first (55.5 ± 12.5 pg/ml at min 0 vs 323.2 ± 92.5 pg/ml at min 10; $P = 0.029$) and second (50.1 ± 11.2 pg/ml at min 0 vs 417.4 ± 98.4 pg/ml at min 10; $P = 0.01$) IU infusions. In cows that received IU infusions of PGF, concentrations of PGEM did not increase after pulse 1 ($P = 0.83$) or pulse 2 ($P = 0.79$). In contrast, cows receiving IU infusions of PGE + PGF showed a marked increase in PGEM levels after pulse 1 (49.9 ± 7 pg/ml for min 0 vs 476.9 ± 118.5 pg/ml for min 10; $P < 0.001$) and pulse 2 (47.1 ± 4.3 pg/ml for min 0 vs 434.1 ± 61.2 pg/ml for min 10; $P < 0.001$).

RNA-seq

Comparisons were made to determine changes in gene expression among treatments after the third IU treatment. An overall evaluation of gene expression between treatment groups was performed using multidimensional scaling (MDS) analysis, a multivariate technique that allows exploration of the relative similarities of the samples under study. The MDS plot shows that dimension 1 clearly separates the luteal biopsies of PGF-treated animals from the other three

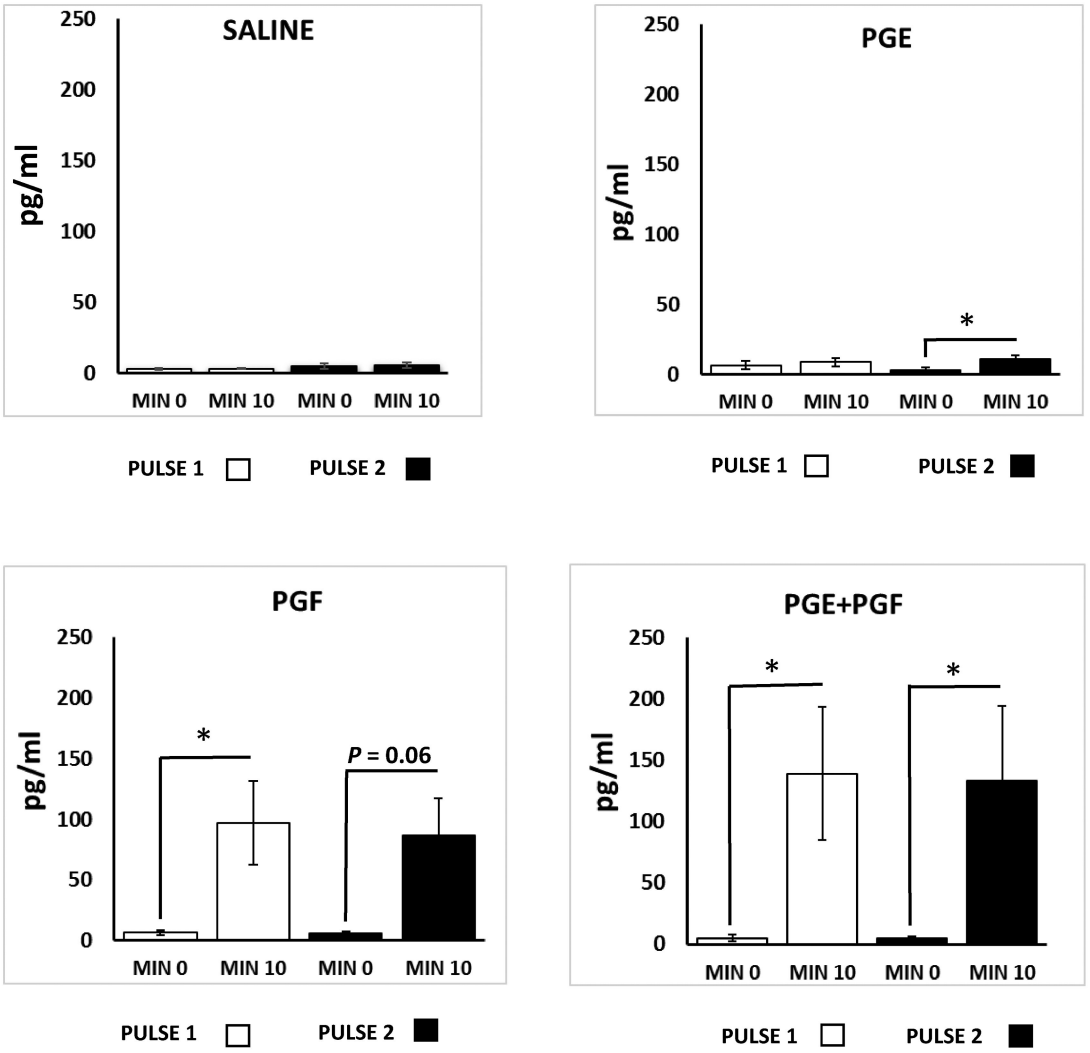

Figure 3. Effect of IU infusions of saline, PGF, PGE, and PGE + PGF treatments (n = 4/treatment) on circulating PGFM concentrations in cows. Data are presented as means ± SEM. MIN 0 and MIN 10 correspond to moments of sampling before and 10 min after the first (pulse 1) or second (pulse 2) IU infusion for each treatment. Asterisks indicate significant differences ($P < 0.05$) between MIN 0 and MIN 10 within a pulse.

treatment groups (Figure 5). On the other hand, samples from saline (CNT), PGE, and PGF + PGE were dispersed in the plot without any clear grouping.

A total of 13 612 genes were evaluated for differential expression between the four treatment groups. Controlling FDR at 1%, numerous genes showed significant differential expression between PGF vs. CNT (n = 572), PGF vs. PGE (n = 581), and PGF vs. PGF + PGE (n = 552). No genes were differentially expressed comparing CNT to PGE, PGE to PGE + PGF, or CNT to PGE + PGF (FDR > 10%). A Venn diagram was constructed to evaluate the overlap between the groups in differential gene expression (Figure 6). There were 265 genes that were differentially expressed (FDR < 0.01) in PGF compared to all of the other three treatment groups and 470 genes that were differentially expressed in PGF compared to one of the other groups (Figure 6). Therefore, a total of 955 genes were differentially expressed (FDR < 0.01) in PGF compared to at least one other treatment group (see Supplemental Table 1 for PGF vs. CNT list of top genes).

In order to dissect the pathways and biological processes that were differentially expressed in the luteal biopsies from cows that received PGF, compared to the other three groups, gene set enrichment analyses were performed using either GO or MeSH databases. These databases define functional terms (gene sets) that can be considered as group of genes that share some particular properties, typically their involvement in the same biological pathway or molecular process. In this study, genes that showed differential expression (FDR < 0.05) in PGF compared to all the other three treatments and had ENSEMBL and GO/MeSH annotations were tested against the background set of all expressed genes with ENSEMBL and GO/MeSH annotations. These gene set analyses included 578 and 543 significant genes, and 12 800 and 11 662 background genes, for GO and MeSH, respectively.

There were 37 GO biological process terms that were significantly enriched (FDR < 0.10) with differentially expressed genes (Supplemental Table 2). These functional terms included execution phase of apoptosis, growth, metabolic, catabolic, or biosynthetic processes for sulfur compounds, acetyl CoA, tetrapyrrole, heme, isoprenoid, alpha amino acids, small molecules, cholesterol, and steroids. There were also 14 GO molecular function terms that were significantly enriched (FDR < 0.10) with differentially expressed

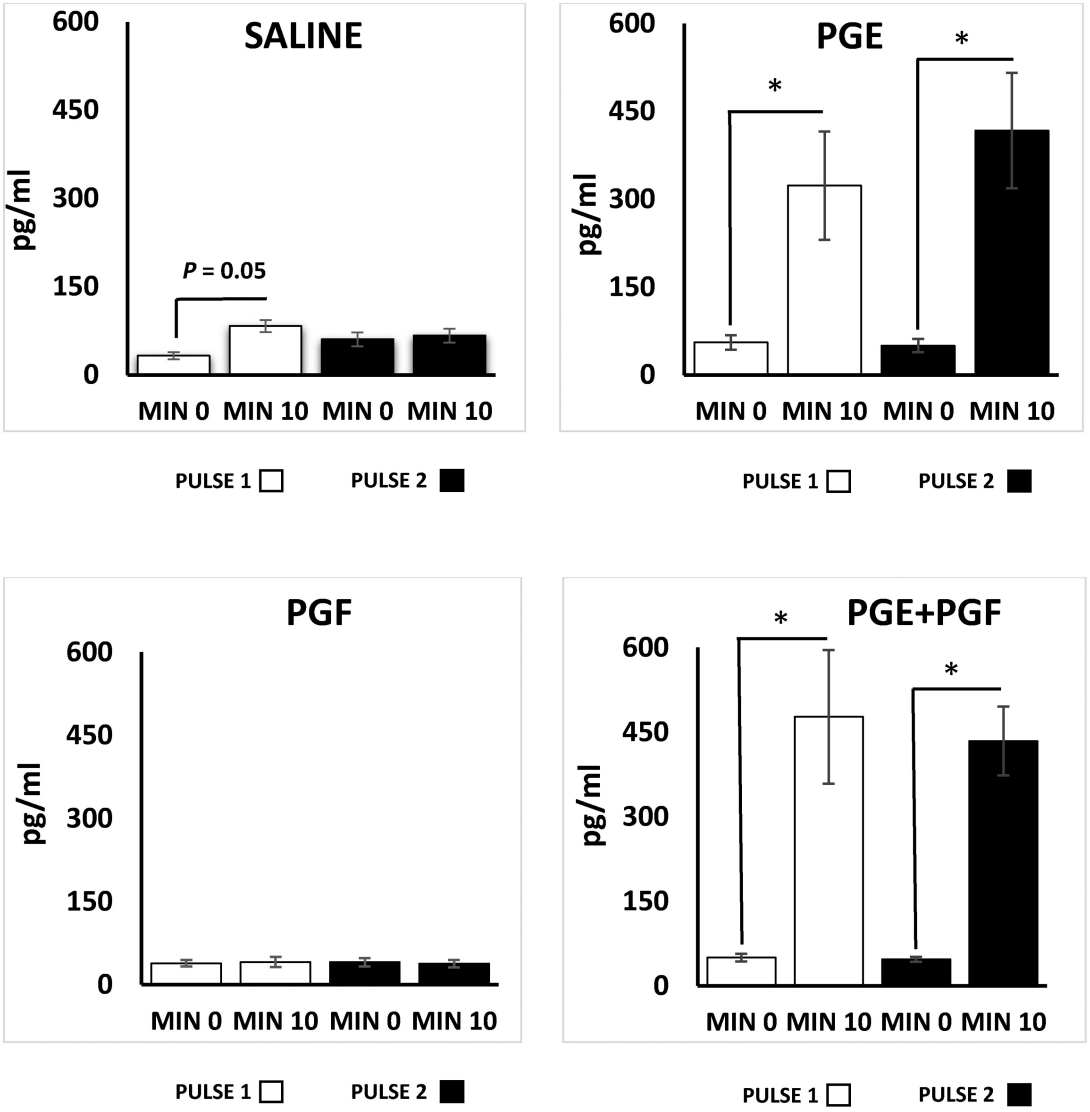

Figure 4. Effect of IU infusions of saline, PGF, PGE, and PGE + PGF treatments (n = 4/treatment) on circulating PGEM concentrations in cows. Data are presented as means ± SEM. MIN 0 and MIN 10 correspond to moments of sampling before and 10 min after the first (pulse 1) or second (pulse 2) IU infusions of each treatment. Asterisks indicate significant differences ($P < 0.05$) between MIN 0 and MIN 10 within a pulse.

genes (Supplemental Table 3). These categories included alcohol binding; transferase activity for nitrogenous, alkyl, and glutathione; oxidoreductase and steroid dehydrogenase activity; cyclin-dependent protein serine/threonine kinases; inhibitor activity for enzymes, peptidases, and endopeptidases; and growth factor binding.

Using the MeSH methodology, there were 26 gene sets for the category Phenomena and Processes that were significantly enriched ($P < 0.01$) in differentially expressed genes. As shown in Table 1, MeSH terms included cell proliferation, genetic transcription and regulation, luteolysis, apoptosis, protein processing and binding, and signal transduction. There were also 44 MeSH terms included in the category Chemicals and Drugs that showed a significant ($P < 0.01$) over-representation of differentially expressed genes. Many of these terms were expected such as LH, 3-hydroxy dehydrogenases, DNA-binding proteins, cytochrome P-450 Enzyme System, and progesterone (Table 2).

Table 3 provides a functional characterization of some of the genes that were differentially expressed in the PGF group as compared to the other treatment groups. As emphasized in this table, signal transduction pathways were differentially regulated by PGF. For example, some cell surface receptors, such as GPR155, FZD1, SUCNR1, and BMPR1B were upregulated, while other receptors were downregulated such as PTGFR, NTRK1, ADORA2A (adenosine receptor), and GHR. Likewise, there was differential regulation of both serine/threonine kinases and tyrosine kinases with upregulation of MAPK8 (serine/threonine) and JAK1 (tyrosine) kinases but downregulation of PIM2, PIM3 (serine/threonine), and NTRK1 (tyrosine). Nuclear transcription factors were also regulated with upregulation of apotosis-related transcription factors such as factors in the P53 pathway (TP53INP1, PIDD1, PDRG1), SOX4, and ARID5B but downregulation of some transcription factors associated with steroid synthesis and action such as NR5A1, NR5A2, and ESRRA. Mitochondrial-associated proteins and apoptosis-related genes were generally upregulated such as CASP3, ATG8, and AMIGO2 as well as ceramide-related genes (HPCAL4, ORMDL3, SPHK1).

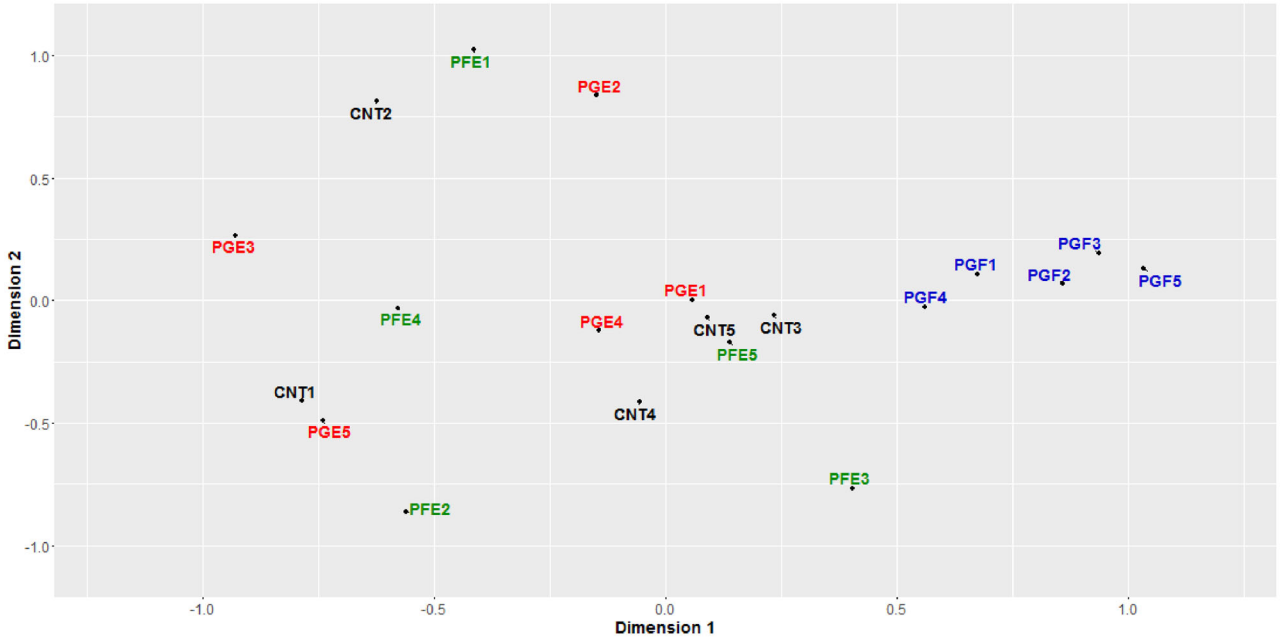

Figure 5. MDS plot showing the relative similarities between samples from the four treatment groups. Distance between samples is based on the common dispersion of the top 1000 mRNA that best distinguished that pair of samples. Samples were from saline (Control; CNT1–5), PGE-treated (PGE1–5), PGF-treated (PGF1–5), or PGF + PGE-treated (PGFE1–5).

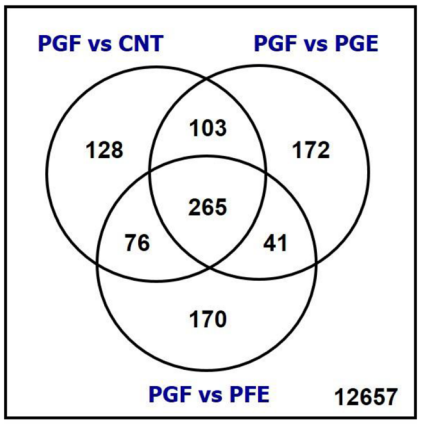

Figure 6. Venn diagram of differentially expressed genes between luteal biopsies from PGF-treated animals (PGF) compared to saline-treated (Control; CNT), PGE-treated (PGE), and PGE + PGF-treated (PFE) animals.

Discussion

This study used a model of IU infusions of $PGF_{2\alpha}$ to simulate the pulsatile secretion of $PGF_{2\alpha}$ that characterizes luteolysis in ruminants. The dose of 0.25 mg/infusion was used in this experiment, based on a previous report in Holstein heifers [37] and on a preliminary study that we performed in Holstein dry cows (unpublished results) that demonstrated that this dose was consistently luteolytic. This dose was lower than the dose of 0.5 mg of $PGF_{2\alpha}$ that was utilized in our previous study [66]; however, intervals between infusions (6 h) and the interval between infusions and biopsies (0.5 h) were the same. The previous study found that four IU pulses were needed to induce complete regression of the CL in all cows, although some cows had complete luteolysis with only two IU pulses of 0.5 mg of $PGF_{2\alpha}$. This study observed a similar complete CL regression in cows treated with four IU doses of 0.25 mg. Extremely low doses of $PGF_{2\alpha}$ are sufficient to regress the CL when it is administered in the uterine horn ipsilateral to the CL due to the local transport of prostaglandins through the utero-ovarian pathway [37, 70, 71]. The same model of IU infusions of PGE had not been previously utilized to mimic the mechanisms involved in rescue of the CL and therefore we utilized this method to mimic endometrial production of high amounts of PGE during pregnancy. The preferred use of PGE_1 instead of PGE_2 as a luteoprotective agent was based on previous experiments in which the use of PGE_2 was associated with a cross-reactivity with PTGFRs [30, 31]. The infused dose (2 mg) of PGE_1 was calculated based on an approximate concentration of 30 ng/ml of PGE that has been reported in the uterine vein of pregnant sheep [9] and a rate of blood flow in the uterine vein of 200 ml/min [55] during a 6-h period ((30 ng/ml × 200 ml/min) × (360 min) = 2.16 mg every 6 h).

Blood samples before and 10 min after the IU infusions allowed us to monitor circulating concentrations of PGFM and PGEM after IU treatment with $PGF_{2\alpha}$ or PGE_1 [32, 35]. The interval of 10 min between infusions and sampling for PGFM has been recommended in cattle to detect maximum concentrations after IU infusions with doses of $PGF_{2\alpha}$ ranging from 0.25 to 4 mg [37]. As expected, IU infusions of saline and PGE_1 did not induce a considerable increase in PGFM concentrations after first and second pulses. In contrast, infusions of $PGF_{2\alpha}$ and PGE_1 + $PGF_{2\alpha}$ resulted in a large increase in circulating PGFM concentrations at 10 min after infusion. Collectively, these results reflect not only the rapid absorption rate of $PGF_{2\alpha}$ from the uterus but also that simultaneous infusion of PGE_1 apparently did not prevent the absorption and transport of $PGF_{2\alpha}$ from the uterus to the uterine vein. Maximum mean (139 ± 54.5 pg/ml) or individual (288 ± 61 pg/ml) concentrations of PGFM from this experiment fall within the physiological concentrations for natural PGFM pulses observed in other studies conducted in non-pregnant cows [72] or heifers [35] during the luteolytic period. The

Table 1. Examples of MeSH terms that were significantly ($P < 0.001$) enriched with differentially expressed (DE) genes related to specific Phenomena and Processes.

MeSH term ID	MeSH term name	DE genes/total genes	*P* value	Upregulated	Downregulated
D049109	Cell proliferation	17/128	5.1e-05	ERBB1, IGF1R, JNK1, CLLP, BMP2A, P27KIP1, TIMP1, MMP1, SPP1, ORA1, FGF2, TIAM1	G1/S-specific Cyclin D1, NR5A1, StARD1, HSC
D014158	Transcription, genetic	22/200	8.2e-05	AHSG, INHBA, GAL, HMGB3, ERBB1, JAK1, SPP1, CTGF, INBB, TUFT1, FGF2, GGTA1, EGFR	Cyclin D1, NR5A1, StARD1, TAU, FDXR, PTGFR, UMPS, GCSH, MAPT
D003341	Luteolysis	6/20	0.00017	SPP1, SerpinA14, FGF2, Caspase3	StARD1, SOD1
D004789	Enzyme activation	22/222	0.00037	CTNNAL1, ATG8, ERBB1, MAPK8, ITGAV, CASP3, TIMP1, MMP1, GALNT1, ENPP1, FGF2, TIAM1	MGST1, G6PD, StARD1, FDXR, SOD1, CALM3, TBXA2R, PRKAG1, EZR, SLC25A10
D002113	Calcification, physiologic	4/10	0.00066	AHSG, SPP1, A2M	ANX6
D018507	Gene expression regulation, developmental	18/174	0.00074	INHBA, A2M, IGF1R, CASP3, KDM3A, TIMP1, THBS2, PLIN2, FGF2, mir21	G6PD, NR5A1, NR5A2, RAMP3, HSD3B, GHR, SLC2A8, APLNR
D017209	Apoptosis	15/133	0.00083	THBS2, IGF1R, MAPK8, FGF2, BMPR1B, CASP3, CFLAR, BMP2	G6PD, TBXA2R, FDXR, APLNR, SOD1, ENDOG, PHB
D011499	Protein processing, post-translational	14/122	0.001	GATE-16, AHSG, HMGB3, EGFR, SPP1, GALNT1, FGF-2, TUFT-1, PTPRN	NR5A1, ATP5G1, TBXA2R, EZR, GCSH
D015533	Transcriptional activation	8/51	0.0017	MMP1, CTGF, HMGB3, EGFR, mir21	GHR, STAR, HEXIM1
D011485	Protein binding	36/487	0.0019	GATE-16, AHSG, INHBA, MYBPC3, A2M, SNX31, MAPK8, ITGAV, GNAQ, SPP1, SERPINA14, AHCYL2, TUFT1, CD46, FGF-2, GABARAPL2, STRA6	CLTB, FDXR, SOD1, PTGFR, TUBA1A, ITPR1, GHR, HSPA8, NR5A1, STAR, TAU, RFC2, ANXA6, CALM3, SLC25A10, CRABP1, TOM40A, ENDOG, ATP5G1
D050296	Microbial viability	2/2	0.002	SPP1	FDXR
D015398	Signal transduction	27/334	0.002	INHBA, GAL, A2M, EGFR, IGF1R, MAPK8, ITGAV, BMP2, JAK1, GNAQ, TIMP1, SMAD9, MMP1, BMPR1B, FGF2, TIAM1	G6PD, CCND1, FHIT, NR5A1, TAU, CAM, UNC119, PTGFR, GHR, TBXA2R, EZR
D006863	Hydrogen-ion concentration	10/77	0.0021	A2M, GALNT1, ENPP1, FGF-2	ITPR1, CMAS, HSPA8, ANXA6, SOD1, ENDOG

peak of the PGFM pulse at 10 min after a 0.25 mg IU injection or infusion of $PGF_{2\alpha}$ has been reported to be either greater than or less than the peak concentration during a natural PGFM pulse [37]. Thus, our pulsatile pattern and dose of IU $PGF_{2\alpha}$ mimicked the pulsatile pattern that is characteristic of luteolysis.

Our first hypothesis was supported since we observed a consistent and profound decrease in concentrations of P4 that was significant by 12 h after the first infusion of $PGF_{2\alpha}$ or 6 h after the second PGF treatment (Figure 1). The decrease in circulating P4 coincided with a mean loss of 43.6% in the luteal volume 24 h after the first PGF infusion (Figure 2) with subsequent decreases over the next 2 days resulting in complete CL regression by 72 h after the first $PGF_{2\alpha}$ treatment. The initial actions of $PGF_{2\alpha}$ in the CL are mediated by the binding of $PGF_{2\alpha}$ to specific G-protein-coupled receptors, termed PTGFRs [73, 74], which induce a rapid increase in concentrations of free intracellular calcium [26], and activation of PKC and mitogen-activated protein (MAP) kinase [75]. Activation of multiple intracellular signal transduction pathways induce transcription of a number of early response genes [76, 77] in response to supraphysiologic [78, 79] and low doses of IU $PGF_{2\alpha}$ [38]. In addition, treatment with $PGF_{2\alpha}$ dramatically reduces steady-state mRNA concentrations for PTGFR after a single [80] or sequential [38] treatments with $PGF_{2\alpha}$. Consistent with these results, we observed a dramatic decrease in mRNA for PTGFR in this experiment, based on the RNA-seq analysis after the third $PGF_{2\alpha}$ treatment. Other genes that have been observed to be changed by $PGF_{2\alpha}$ treatment in previous studies [38, 39, 81] were also altered in this experiment and numerous novel genes were found to be dramatically upregulated or downregulated following treatment with physiological pulses of $PGF_{2\alpha}$ into the uterus.

The use of RNA-Seq after physiological pulses of $PGF_{2\alpha}$ provides substantial insight into the changes in gene expression that follow the third $PGF_{2\alpha}$ pulse during luteolysis. All five of the $PGF_{2\alpha}$-treated animals had gene expression that was relatively similar using MDS. The expression of specific genes involved in steroidogenesis were

Table 2. Examples of MeSH terms that were significantly ($P < 0.001$) enriched with differentially expressed (DE) genes related to specific chemicals and drugs.

MeSH term ID	MeSH term name	DE genes/total genes	*P* value	Upregulated	Downregulated
D007986	LH	11/37	3.6e-07	THBS2, MMP1, SerpinA14, FGF2	HSD3B, NR5A1, STAR, APLNR, PTGFR, GSTA2, MGAT1
D015096	3-hydroxy dehydrogenases	4/7	0.00012	None	HSD3B, NR5A1, STAR, NR5A2
D004268	DNA-binding proteins	19/167	0.00016	INHBA, HMGB3, MAPK8, PAX8, KDM3A (lysine demethylase), SPP1, PLIN2, INHBB	G6PD, CLTB, NR5A1, HSPA8, STAR, RFC2, SOD1, GHR, PLIN3, ENDOG, FOXL2
D003577	Cytochrome P-450 enzyme system	5/13	0.00016	PGIS	HSD3B, TBXA2R, GCSH, FDXR
D011257	Pregnancy proteins	10/64	0.00048	PLIN2, SPP1, SERPINA14, FGF-2, BMP2A, IGFBP1, MMP1	PLIN3, ANXA6, FOXL2
D011374	Progesterone	11/76	0.00051	EGFR, SERPINA14, IGF1R, FGF-2, CASP3, IGFBP1	NTRK1, NR5A1, STAR, PTGFR, FOXL2
D050656	AP-2 transcription factors	3/5	0.000082	INHBA, INHBB	CLTB
D019869	Phophatidyl-inositol 3-kinases	10/68	0.00079	EGFR, IGF1R, SNX31, MAPK8, ITGAV, TIMP1, CTGF, TIAM1	PRKAG1, EZR
D050993	GATA-6 transcription factor	3/5	0.00082	None	NR5A1, STAR, NR5A2

decreased (STAR, HSD3B1, and NR5A1) consistent with the dramatic decrease in circulating P4 concentrations associated with the $PGF_{2\alpha}$ pulses. The decrease in luteal volume was associated with upregulation of numerous genes involved in apoptosis such as FBXO32, which regulates ubiquitination, ATG8, an autophagy-related ubiquitin modifier, CASP3, and KDM3A, which is involved in the anoikis process. Specific ligands were upregulated by the third pulse of PGF such as THBS2 (Thrombospondin 2), FGF2, NPTX2, AHSG, and IL33 or downregulated such as VEGFA suggesting that these ligands may be involved in the intercellular communication that occurs during the luteolytic process. A number of serine-threonine kinases were also upregulated, notably MAPK8 and MAP3K9 as well as RIPK4, SIK2, and TESK1, and tyrosine kinases such as JAK1, MERTK, and FGR suggesting the involvement of these kinases in specific intracellular signal transduction pathways that are regulated by physiological $PGF_{2\alpha}$ pulses. Numerous transcription factors were upregulated (SOX4, ARID5B, GREB1, SMAD9, and NFATC3), whereas others were downregulated (NR5A1, NR5A2, NROB2, ESRRA, and NUPR2), highlighting the potential gene transcription pathways that mediate $PGF_{2\alpha}$ action. Finally, the downregulation of essentially all mitochondria-related proteins suggests that mitochondria are being eliminated or inhibited during the luteolytic process. Many of these changes are expected, based on previous research, and provide a transcriptional signature to evaluate $PGF_{2\alpha}$ action during in vivo, physiological changes that are associated with luteolysis. Thus, our results provide important new information on global gene expression during luteolysis.

Our second hypothesis was also somewhat supported, since treatment with physiological doses of IU PGE_1 did not alter circulating P4 concentrations or the volume of the CL. To our knowledge, this is the first experiment using IU infusions of PGE_1 in cows as a model to study protective effects of PGE on the CL. As determined by peripheral blood samples collected before and 10 min after infusions, circulating concentrations of PGEM showed a rapid increase in cows treated with PGE_1 and PGE_1 + PGF after IU infusions (Figure 5). This indicates that PGE_1 was absorbed from the uterine lumen, transported through the uterine vein to the systemic circulation, and metabolized to the PGE metabolite in the lungs or other part of the circulatory route. An elegant experiment conducted in ewes [9] reported that PGE_2 production was 32.3-fold greater in pregnant compared to cycling ewes (day 15) with efficient transport of PGE_2 from the uterine lumen to the uterine vein (92.1% efficiency on day 16 of pregnancy), and from the uterine vein to the ovarian artery (12.2% transported). Although the scope of this experiment did not allow determination of the precise efficiency of PGE_1 transport from the uterus to the uterine vein, it does show that high PGE_1 amounts exit the uterus and are subsequently detected as PGE metabolite in the circulation. The PGEM concentrations (~400 pg/ml) were ~4-fold greater than PGFM concentrations (~100 pg/ml) reflecting the 8-fold greater amounts of PGE_1 that were infused compared to $PGF_{2\alpha}$. We also expected efficient transport of PGE_1 from the uterine vein to the ovarian artery and that we would be able to then detect a pattern of gene expression in the CL that would be distinct for PGE_1 action in the CL. A previous study in pregnant ewes found distinctive changes in proteins involved in the production (PTGES), metabolism (PGDH), and signaling (PTGER) of PGE_2 [9], and we anticipated that IU treatment with PGE might regulate these same genes. Somewhat surprisingly in our study, luteal mRNA from PGE-treated cows did not exhibit a distinctive pattern of expression in the CL, even when the complete transcriptome was evaluated using RNA-seq in this study. We must speculate that either PGE_1 does not regulate luteal gene expression or that active PGE_1 does not reach

Table 3. Functional characterization of some of the most significant genes (FDR < 0.001) that were differently regulated in the PGF group compared to the other three groups.

Functional category	Differentially expressed mRNA (underlined genes were downregulated in PGF treatment)
Signal transduction—tyrosine kinases	NTRK1, JAK1, MERTK, FGR,
Steroid pathways—synthesis	HSD3B1, SRD5A2, STAR,
Signal Transduction—ligands	NPTX2, FGF2, AHSG, PTHLH, IL33
Signal Transduction—receptors	ANTXR2, PLXDC2, APLNR, PTGFR, INSRR, NTRK1, GHR, SUCNR1, GPR155, BMPR1B, FZD1, IGF1R, ADORA2A,
Signal transduction—serine/threonine kinases	PIM3, MAPK8, PRKAG1, RIPK4, STK38L, SIK2, TESK1, MAP3K9, PIM2
Signal transduction—phosphatases	DUSP15, PTPRU,
RNA-binding translation control	Caprin2, LARP1,
Apoptosis	FBXO32 (ubiquitination), LARP1, AMIGO2, ATG8 (autophagy-related ubiquitin modifier), CASP3, KDM3A (anoikis), PIDD1
Nucleotide regulation	DCTPP1,
Intracellular signal transduction	DLG5, KLHL40, AHCYL2, MID1IP1, PNKD, ORMDL3 (ceramide),
Transcription factors	SOX4, ARID5B, GREB1, SMAD9, NR5A1, NR5A2, NR0B2, NRIP1, NUPR2, TP53INP1, ARNTL, NFATC3, ESRRA,
Cell proliferation	CCND3, CDKN1B (Kip1), CCNG2, CCND1
Intracellular transport/orientation	KIF1B, PDZ ligand, CRABP2
Angiogenesis	PLXDC2, FGF-2, VEGFA
Receptor tyrosine kinases	MUSK, JAK1,
Scaffolding	AMIGO2, PPFiBP1
Ceramide	HPCAL4, ORMDL3, SPHK1
Mitochondria-associated proteins	FDXR, TOMM5, SLC25A3, SLC25A10, PPOX, TOMM40

Underlined genes were downregulated in PGF treatment.

the CL after the third pulse of PGE_1. Our experimental design does not allow us to distinguish between these two possibilities. However, we currently favor the second possibility based on our bias that activation of EP receptors in the CL is likely to alter expression of at least some genes.

The third and most important hypothesis of the present study related to whether IU infusion of low doses of PGE_1 could block the luteolytic effects of IU infusions of low doses of $PGF_{2\alpha}$. The doses of $PGF_{2\alpha}$ and PGE_1 were chosen to fall within a physiological concentration and pattern that may be present during normal bovine pregnancy. One major finding of the study is the dramatic and complete inhibition of both the functional and structural effects of $PGF_{2\alpha}$ pulses by simultaneous infusion of PGE_1. This is consistent with our third hypothesis that physiological concentrations of PGE can block the effect of physiological concentrations of $PGF_{2\alpha}$. These results are consistent with a great deal of other research that has demonstrated an inhibition of $PGF_{2\alpha}$ action by simultaneous treatment with PGE [9, 12, 52, 53]. Results from this experiment indicate efficient transport of PGE_1 and $PGF_{2\alpha}$ from the uterus to the uterine vein, based on circulating PGFM and PGEM after IU infusions. In this regard, $PGF_{2\alpha}$, in the absence of PGE_1, is being efficiently transported to the ovary through the utero-ovarian plexus since there was a clear luteolytic response in cows treated with pulses of only $PGF_{2\alpha}$. However, the transcriptomic changes that were differentially regulated by $PGF_{2\alpha}$ pulses, alone, in our study and typically regulated by $PGF_{2\alpha}$ in other studies [38, 39] were completely prevented by simultaneous infusions of PGE + PGF. Again, there are two possible explanations for these results. First, PGE_1 action on the CL may completely inhibit the transcriptomic actions of $PGF_{2\alpha}$. This seems possible, but unlikely, given the numerous early responses and diverse pathways that are activated by a $PGF_{2\alpha}$ pulse, as demonstrated in this study and in a previous study [38]. A second possibility is that simultaneous infusion of PGE_1 completely inhibits the transport of $PGF_{2\alpha}$ to the ovarian artery and subsequently to the CL. Previous studies in sheep have demonstrated that PGE has a greater affinity for the PG transporter and that $PGF_{2\alpha}$ transport to the ovarian artery is reduced during pregnancy. Thus, it seems possible that PGE_1 may be inhibiting the transport of $PGF_{2\alpha}$ from the uterine vein to the ovarian artery, and this results in blockade of the action of $PGF_{2\alpha}$. Another related idea is that PGE may stimulate blood flow in the uterine vein to such an extent that transport from the uterine vein to the ovarian artery is extremely inefficient. Previous studies have clearly demonstrated increases in uterine blood flow in the gravid horn during early pregnancy, and it seems possible that this is mediated by increased PGE action causing vasodilation. Future studies are needed to investigate this novel, potential mechanism by which PGE may inhibit $PGF_{2\alpha}$ action during maternal recognition of pregnancy.

In summary, the results of the present study indicate that PGE exerts a luteoprotective effect even when it is delivered through IU infusions. This model may mimic some of the key events of early pregnancy in cows and further support a role for PGE in rescue of the CL during early pregnancy. These results are likely to have important implications for the underlying mechanisms involved in the rescue of the CL in cows.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Table 1. List of top 565 identified genes that are differentially expressed comparing PGF treatment to control animals. Genes are ordered by *P* value.
Supplemental Table 2. List of GO Biological Process terms that were significantly enriched (FDR < 0.10) with differentially expressed genes (DEG). For each term, these results shows GO ID, GO Name, brief description of the term, the total number of genes in the term, the total number of significant genes (DEG) in the term, the number and list of DEG upregulated in PGF treatment, the number and list of DEG downregulated in PGF treatment, and the nominal *P*-value from the Fisher exact test.
Supplemental Table 3. List of GO Molecular Function terms that were significantly enriched (FDR < 0.10) with differentially expressed genes (DEG). For each term, these results shows GO ID, GO Name, brief description of the term, the total number of genes in the term, the total number of significant genes (DEG) in the term, the number and list of DEG upregulated in PGF treatment, the number and list of DEG downregulated in PGF treatment, and the nominal *P* value from the Fisher exact test.
Supplemental Table 4. Summary of sequencing read alignments to the reference genome.

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