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Expression of Key Prostaglandin Synthases in Equine Endometrium During Late Diestrus and Early Pregnancy[1]

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ABSTRACT

Luteolysis in domestic species is mediated by the release of luteolytic pulses of prostaglandin (PG) $F_{2\alpha}$ by the uterus at the end of diestrus, which must be suppressed by the conceptus to permit maternal recognition of pregnancy. In many species, including the horse, both the conceptus and the endometrium also synthesize PGE_2, which may antagonize $PGF_{2\alpha}$ by playing a luteotropic and/or antiluteolytic role. While the release of PGE_2 and $PGF_{2\alpha}$ by the equine endometrium in late diestrus and early pregnancy has been previously studied, the underlying prostaglandin synthase gene regulatory mechanisms remain poorly defined. To resolve this issue, cyclooxygenase-2 (COX-2), microsomal PGE_2 synthase (PGES), and $PGF_{2\alpha}$ synthase (PGFS) expression were examined in a series of endometrial biopsies obtained from cycling mares on Days 10, 13, and 15 postovulation, as well as from pregnant mares on Day 15. Quantification of COX-2 expression revealed significant ($P < 0.01$) increases in both mRNA and protein levels at Day 15 in cycling endometrium relative to other timepoints. Importantly, the level of COX-2 expression in Day 15 pregnant endometrium was found to be comparable with that observed in Day 10 and Day 13 cycling animals, suggesting that the presence of the conceptus blocks the induction of COX-2. Immunohistochemistry demonstrated that the induction of COX-2 expression on Day 15 occurs specifically in surface epithelial cells in cycling animals only. As equine PGFS had not been previously characterized, a 1380-base pair (bp) cDNA transcript was cloned by a combination of reverse transcription-PCR techniques and found to be highly homologous to bovine liver-type PGFS. The pattern of expression observed for the terminal PG synthases was distinct from that of COX-2, as PGES and PGFS mRNA and protein levels were found to be invariant throughout the timecourse and unaffected by pregnancy. Similar to COX-2, however, the PGES and PGFS proteins were found to localize mainly to the surface epithelium. Thus, this study describes for the first time the regulation and spatial distribution of COX-2, PGES, and PGFS expression in equine endometrium in late diestrus, with a marked induction of COX-2 but not of PGES and PGFS expression in uterine epithelial cells at Day 15. Furthermore, the presence of the conceptus was shown to block the induction of COX-2 expression at Day 15, suggesting an important mechanism by which it may suppress uterine $PGF_{2\alpha}$ release and prevent luteolysis during early pregnancy.

female reproductive tract, gene regulation, ovulatory cycle, pregnancy, uterus

INTRODUCTION

Luteolysis, defined as the functional and/or structural regression of the corpus luteum, is a key process in the ovarian cycle [1, 2]. In domestic species, luteolysis must be initiated to permit a new ovarian cycle to begin when conception does not occur. Conversely, the function and integrity of the corpus luteum are required to establish and maintain pregnancy. The conceptus must therefore somehow abrogate luteolysis, a process commonly referred to as the maternal recognition of pregnancy [1, 2]. Among domestic livestock species, luteolysis and maternal recognition of pregnancy have been most extensively studied in sheep. In this species, luteolysis is initiated in late diestrus as oxytocin is released in high-frequency bursts from the posterior pituitary, while the oxytocin receptor is simultaneously upregulated in the endometrium [1]. Pituitary oxytocin, supplemented with additional oxytocin released from the corpus luteum, then acts to stimulate the release of luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the uterus, which in turn binds its cognate receptor on luteal cells, thereby initiating luteolysis [1]. In the event of pregnancy, interferon τ (IFNτ) secreted by the ovine conceptus acts to prevent the up-regulation of endometrial oxytocin receptors [1]. While this does not appear to affect the overall production of $PGF_{2\alpha}$ by the uterus, the pulsatility of $PGF_{2\alpha}$ secretion is abolished, thus preventing luteolysis [3, 4]. Although the ovine model has greatly advanced our understanding of how the fate of the corpus luteum is controlled, it is clear that the mechanisms involved in the maternal recognition of pregnancy vary considerably from one species to another [1, 2, 5]. For instance, in the mare, $PGF_{2\alpha}$ secretion is diminished in early pregnancy, presumably due to the action of an as-yet unidentified product secreted by the conceptus [6, 7]. Unlike the ewe, the increase in uterine oxytocin receptor levels in late diestrus is not suppressed by the conceptus in mares [8], yet neither endogenous nor exogenous oxytocin can cause the release of $PGF_{2\alpha}$ by the

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uterus during early pregnancy [8, 9]. Thus, while it can be inferred that the equine conceptus acts downstream of the oxytocin receptor to block $PGF_{2\alpha}$ secretion, the mechanism by which this blockage is achieved remains unclear.

Aside from altering the expression of the oxytocin receptor, another potential means by which the conceptus could inhibit luteolysis is by altering the expression of the enzymes involved in prostaglandin (PG) synthesis. PGs are produced in response to a variety of physiological and pathological stimuli that activate phospholipase A_2, resulting in the hydrolytic release of arachidonic acid (AA) from membrane phospholipids [10–13]. AA is then metabolized to the unstable cyclic endoperoxide PGH_2 by the cyclooxygenase enzymes COX-1 and COX-2 (also known as prostaglandin G/H synthases PGHS-1 and PGHS-2). In cows and sheep, endometrial COX-2 expression was found to be transiently induced during late diestrus around the expected time of luteolysis, whereas COX-1 expression was found to be invariant (in sheep) or undetectable (in cows) throughout the estrous cycle [14, 15]. However, pregnancy did not block the induction of COX-2 in sheep but rather appeared to prolong its expression [14]. It thus seems unlikely that the conceptus targets COX-2 expression in vivo as a means to modulate $PGF_{2\alpha}$ release, as least in the ewe.

Following its synthesis, PGH_2 is rapidly converted to more stable compounds by the terminal PG synthases, which are differentially expressed and regulated in particular cell types [13]. $PGF_{2\alpha}$ synthases (PGFSs) have been most extensively studied in the bovine species, in which at least three distinct enzymes have been characterized that are referred to as liver-type PGFS [16], lung-type PGFS [17], and PGFSII [18]. All are closely related members of the AKR1C subclass of aldo-keto reductases and can catalyze the reduction of PGH_2 to $PGF_{2\alpha}$ as well as PGD_2 to 9α-, 11β-PGF_2 [19]. To date, reports of PGFS regulation in the uterus have been limited to in vitro studies using cultured bovine endometrial cells. These have shown a down-regulation of lung-type PGFS by IFNτ, suggesting that the conceptus could perhaps target PGFS expression as a means to down-regulate $PGF_{2\alpha}$ release [20, 21]. However, a more recent study failed to detect any of the known PGFS enzymes in late diestrus bovine endometrium, calling into question the identity of the enzyme responsible for $PGF_{2\alpha}$ synthesis in the uterus [22]. The same group also showed a previously described 20α-hydroxysteroid dehydrogenase (AKR1B5) to possess potent PGFS activity, and further demonstrated that its expression is up-regulated in the endometrium during the second half of the estrous cycle [22]. Whether or not pregnancy alters endometrial PGFS activity therefore remains essentially unknown in any species.

In domestic species, the endometrium also secretes PGE_2 that, contrary to $PGF_{2\alpha}$, is thought to act in an antiluteolytic manner. Indeed, uterine PGE_2 production increases in early pregnancy in species such as sheep and pigs, and intrauterine infusions of PGE_2 in these species can transiently protect the corpus luteum against the luteolytic effects of endogenous or exogenous $PGF_{2\alpha}$, although nonphysiological amounts of PGE_2 are required [1]. Therefore, PGE_2 synthesis may be important during maternal recognition of pregnancy, a notion that is supported by the fact that compounds originating from the conceptus such as estrogen (in pigs) and interferon τ (IFNτ, in ruminants) are thought to increase the endometrial PGE_2:$PGF_{2\alpha}$ production ratio [5, 23]. One likely mechanism by which endometrial PGE_2 synthesis may be regulated is the modulation of microsomal PGE_2 synthase (PGES) expression, which has been found to increase in late diestrus in bovine endometrium and can be induced in vitro by IFNτ in cultured endometrial cells [15, 24]. In addition, the PLA_2, COX-2, and PGES proteins are believed to be functionally coupled, suggesting that the regulation of PGE_2 biosynthesis involves an intricate coordination of the requisite enzymes [13, 25, 26]. While there is little conclusive evidence presently available to show that PGE_2 plays a luteoprotective role in mares, appreciable amounts of PGE_2 have been shown to accumulate in the equine uterine lumen in late diestrus, and these levels continue to increase in pregnant animals until Day 20 after ovulation [7]. Whether the effects of pregnancy on PGE_2 release can be attributed to changes in endometrial PGES expression has yet to be determined in the mare or any other species.

As pregnancy does not appear to prevent the increase in endometrial oxytocin receptors in mares [8], the hypothesis for the present study was that the equine conceptus alters the expression of the enzymes involved in PG synthesis. To test this, the objectives of this study were 1) to clone and characterize an equine PGFS transcript; 2) to study expression of COX-2, PGES, and PGFS in equine endometrium in late diestrus; 3) to determine the effects of pregnancy on the expression of the PG synthases; and 4) to determine the cellular localization of the COX-2, PGES, and PGFS proteins within the endometrium.

MATERIALS AND METHODS

Materials

Sigma Fast DAB tablets were purchased from Sigma Chemical Co. (St. Louis, MO); [α-^{32}P]deoxycytidine 5′-triphosphate (dCTP) was obtained from Perkin Elmer Life Sciences (Woodbridge, ON, Canada); QuikHyb hybridization solution was purchased from Stratagene Cloning Systems (La Jolla, CA); hCG was obtained from The Buttler Co. (Columbus, OH); Prime-a-Gene labeling system, pGEM-T Easy Vector System I, and the Access RT-PCR kit were purchased from Promega Corp. (Madison, WI); synthetic oligonucleotides, the 5′-RACE System, Version 2.0 kit, and TRIzol total RNA isolation reagent were purchased from Invitrogen Life Technologies (Burlington, ON, Canada); dye-labeled hybridization probes were obtained from TIB Mol-Biol (Adelphia, NJ); Vectastain Elite ABC Kit was obtained from Vector Laboratories, Inc. (Burlingame, CA); QuantiTect Probe RT-PCR Kit was purchased from Qiagen Inc. (Mississauga, ON, Canada); Hybond-P polyvinylidene fluoride(PVDF) membrane, HRP-conjugated donkey anti-rabbit IgG antibody and ECL Plus Western Blotting Detection Reagents were obtained from Amersham Biosciences Corp. (Piscataway, NJ); Complete Mini Protease Inhibitor Cocktail Tablets were purchased from Roche Applied Science (Laval, PQ, Canada); and Antimicrosomal PGES antiserum was obtained from Cayman Chemical Co. (Ann Arbor, MI).

Cloning and Characterization of the Equine PGFS cDNA and Protein

The equine PGFS transcript was isolated in fragments using a multistep cloning strategy (Fig. 1). A 354-base pair (bp) reverse transcription-PCR product (RT-PCR) (Fig. 1Ab) was initially cloned from pooled equine ovarian RNA samples isolated from a corpus luteum (from cycle Day 8), a preovulatory follicle, and an ovulatory follicle (36 h post-hCG treatment). Ovarian tissues were isolated and RNA was extracted as previously described [27]. RT-PCR was performed using the Access RT-PCR kit (Promega) as directed by the manufacturer, using 500 ng of RNA and oligonucleotide primers designed by sequence alignments of known PGFS species homologues (Fig. 1B). Following agarose gel electrophoresis, the RT-PCR product was excised and ligated into the PGEM-T Easy plasmid vector (Promega), and proper recombinant plasmids were identified from transformed bacterial colonies using standard techniques [28]. Sequencing of the insert was performed by the Service de Séquençage de l'Université Laval (Québec, PQ, Canada) using vector-based T7 and Sp6 oligonucleotide primers and PGFS sequence-specific primers (Invitrogen).

Sequences obtained from the initial RT-PCR product served to design specific oligonucleotides for 5′- and 3′-Rapid Amplification of cDNA

Ends (RACE) procedures. The 3′-RACE was performed as previously described [29], except 5 μg of pooled ovarian tissue RNA (as described above) was used as a template for the initial RT reaction. Briefly, an RT reaction was performed using a poly-dT oligonucleotide with anchor sequences at its 5′ end (Fig. 1Ac). This was followed by nested PCR reactions using oligonucleotide primers that bound to the anchor sequence in conjunction with PGFS-specific forward primers. The product of the second PCR reaction (Fig. 1Ac) was isolated and sequenced as described above. 5′-RACE was performed using the 5′-RACE System, Version 2.0 kit (Invitrogen) as directed by the manufacturer, using 5 μg of pooled ovarian tissue RNA (as described above) and PGFS-specific primers for reverse transcription and PCR, along with forward primers supplied with the kit (Fig. 1). The longest product obtained (Fig. 1Aa) was isolated and sequenced as described above.

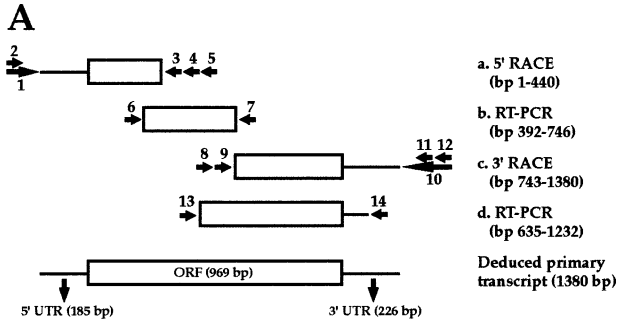
A second RT-PCR product (Fig. 1Ad) was generated that spanned the region of overlap between the initial RT-PCR product and the 3′-RACE product. RT-PCR primers were designed to amplify a 598-bp PGFS cDNA product, and the reaction was performed as described for the initial RT-PCR procedure except the RNA template was obtained from granulosa cells isolated from a preovulatory follicle at 39 h post-hCG. The sequence of the resulting product was found to be identical to corresponding regions of the RT-PCR and 3′-RACE products and overlapped both by over 100 bp, clearly indicating that all products were derived from the same transcript. As further confirmation, a clone encompassing the entire coding region was isolated by RT-PCR (not shown) and found to correspond with the deduced primary PGFS transcript reported herein (Fig. 1A). Nucleotide analyses and protein sequence comparisons were performed using MacDNASIS software version 2.0 (Hitachi, Hialeah, FL), and comparisons to GenBank databases were performed online by standard BLAST analyses [30] at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). Protein sequence alignments (Fig. 2) were performed using the CLUSTALW v1.7 software [31] online at the Protein Information Resource of the National Biomedical Research Foundation (pir.georgetown.edu/pirwww)[32].

Isolation of Equine Endometrial Biopsies

Adult cycling Standardbred and Thoroughbred mares were acquired from a local supplier and fed hay and concentrates. Mares were teased regularly with a stallion for detection of estrus, and ovarian follicular development was monitored by transrectal real-time ultrasonography. When preovulatory follicles reached 35 mm in diameter, the ovulatory process was induced by injection of hCG (2500 IU, i.v.) and ovulation was confirmed by ultrasonography, taking place between 36 and 48 h post-hCG. Certain mares were artificially inseminated with freshly collected semen immediately before hCG injection, using standard techniques [33]. Biopsies were obtained on Days 10, 13, and 15 of the 21- to 22-day equine estrous cycle (d0 = day of ovulation, n = 5 mares/timepoint) using Jackson uterine biopsy forceps as described [34]. In addition, inseminated mares were examined ultrasonographically on Day 15, and uterine biopsies were obtained as described above when the embryonic vesicle could be unequivocally identified (n = 5 mares). Biopsies were obtained once only from each individual animal. Certain biopsy samples were fixed for immunohistochemical analyses (described below), while others were frozen and stored at −70°C until RNA and protein extractions could be performed. All animal procedures were approved by the institutional animal care and use committee (Comité de Déontologie Animale de l'Université de Montréal) and were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Northern Blotting

Total RNA was isolated from frozen biopsy samples using TRIzol reagent (Invitrogen) according to manufacturer's instructions, using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, PQ, Canada) and quantified by spectrophotometry according to standard protocols [28]. RNA samples (10 μg) were processed, separated electrophoretically, and transferred to a nylon membrane as previously described [27]. Probes were synthesized using the Prime-a-Gene labeling system (Promega) using a previously described cDNA library clone for COX-2 [27], the 3′-RACE product (Fig. 1Ac) for PGFS, and a purified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR product generated by a real-time RT-PCR analysis described below. Probes were hybridized successively to the same membrane, with stripping after each round of hybridization performed by immersion in boiling 0.1× saline-sodium citrate with 0.1% SDS for 10 min. Hybridizations were performed using



B

1.	Abridged anchor primer
2.	Abridged universal amplification primer
3.	5'- CGGGT CGAAG GAAAG TCAGC CA -3'
4.	5'- GCTGG CCGGA CCAAC TCGGG -3'
5.*	5'- CCCCT GGCTT CAGCG CTATT GG -3'
6.	5'- GGACT GGCCA TCCGA AGCAA GAT -3'
7.**	5'- CGGAT GACAT TCTAC CTGGT TGCA -3'
8.	5'- CGCAG GCAGC TGGAG ATGAT CC -3'
9.	5'- TGTGT GCAAC CAGGT GGAAT GTC -3'
10.*	5'- GTACC GGATC CTCTA GAGAG CTCGT CGACC TCGAG GAATT CAAGC TTTTT TTTTT TTTTT TTTT -3'
11.	5'- GTCGA CCTCG AGGAA TTCAA GCTT 3'
12.	5'- GTACC GGATC CTCTA GAGAG CTC -3'
13.	5'- TCTCT GTGCC ACATG GGAGG C -3'
14.**	5'- CATGC AGATG TGTCT GATCA CC -3'

FIG. 1. Cloning strategy for equine PGFS. **A**) Cloned cDNA fragments. Each fragment is schematically represented, with its identity indicated on the right and its position in the deduced transcript sequence indicated in parentheses. Lines indicate untranslated regions (UTRs); open boxes designate the open reading frame (ORF). Lengths of the deduced transcript and its structural elements are indicated in base pairs (bp). Arrows indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. **B**) Oligonucleotides used in the various cloning procedures. All primers were employed only in PCR reactions except those noted with a single asterisk (which were used for RTs) or a double asterisk (which were used for both RT and PCR). Oligonucleotides 1 and 2 are components of the 5′-RACE kit (Invitrogen).

QuikHyb solution (Stratagene) under the most stringent conditions suggested by the manufacturer, and the membrane exposed to a PhosphorScreen imaging plate (Molecular Dynamics-Amersham Biosciences, Sunnyvale, CA) for 16–48 h. Visualization and quantification of radioactive signal was performed by scanning the imaging plate using a Storm 840 Phosphorimager (Molecular Dynamics) and analyzing the resulting image using IQMac v 1.2 software (Molecular Dynamics).

Real-Time RT-PCR Analysis

Quantification of PGES transcripts, which could not be detected by Northern blotting, was performed by real-time RT-PCR analysis using the LightCycler system (Roche Diagnostics Corp.) and the QuantiTect Probe RT-PCR Kit (Qiagen), following the manufacturer's instructions. GAPDH mRNA was also quantified in separate real-time RT-PCR reactions and served as a control. RT and PCR reactions were conducted sequentially in a single capillary reaction tube using 10 ng of each RNA sample and the equine gene-specific oligonucleotide pairs 5′-GGAAC GACAT GGAGA CCATC TAC-3′ and 5′-GAAGG GATGC CCAAT CCCCT AG-3′ for PGES and 5′-ATCAC CATCT TCCAG GAGCG AGA-3′ and 5′-GTCTT CTGGG TGGCA GTGAT GG-3′ for GAPDH, resulting in the generation of 311- and 341-bp products, respectively. Cycling conditions were one cycle of 55°C for 20 min, one cycle of 95°C for 15 min, 45 cycles of (95°C for 5 sec, 59°C for 30 sec, and 72°C for 30 sec), and one cycle of 40°C for 30 sec. The accumulation of PCR products was quan-

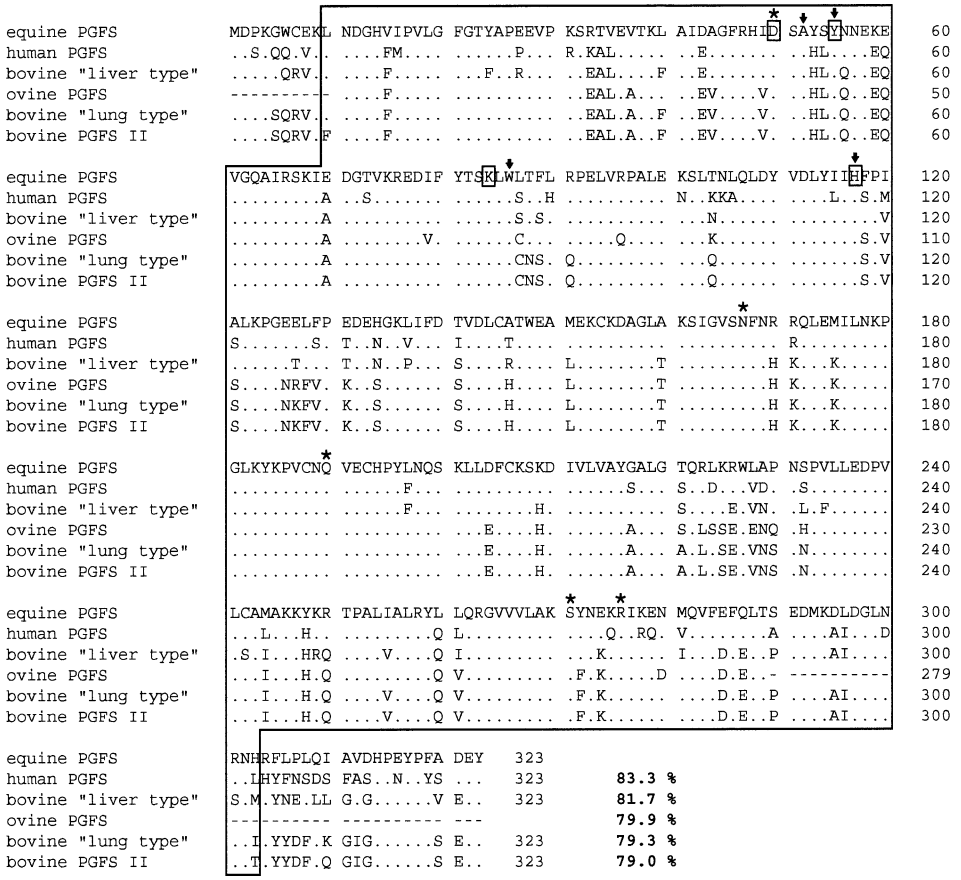


FIG. 2. Structure of the equine PGFS protein and comparison with known homologues. The predicted amino acid sequence of the equine PGFS protein is aligned with human (GenBank accession number AB018580) and ovine (AF257738), as well as with three bovine PGFS enzymes known as lung type (J03570), liver type (D88749), and PGFS II (M86544). The boxed region delimits the aldo-keto reductase domain. Identical residues are indicated by a printed period, numbers on the right refer to the last amino acid residue on that line, and percentages show the degree of homology between a given protein and equine PGFS. Boxed residues are components of the active site, inverted arrows indicate highly conserved amino acids with a proposed role in substrate binding, and residues marked with asterisks are thought to be involved in NADP+ cofactor binding [38].

tified at every cycle by use of the dye-labeled, gene-specific oligonucleotide hybridization probes 5′-CTGTG ACCGC CAGCT GAGAC CTC (fluorescein)-3′ and 5′-(LC red 640) TTGCC GCCAG ACCGC TGACC GTG (phosphate)-3′ for PGES and 5′-CCAAG GTCAT CCATG ACCAC TTTG (fluorescein)-3′ and 5′-(LC red 640) CATCG TGGAG GGACT CATGA CCAC (phosphate)-3′ for GAPDH. Fluorescence versus cycle number data curves were compared with standard curves generated by the PCR amplification of plasmid DNA samples of known concentration, thereby permitting the calculation of target RNA molecule copy numbers in each sample.

Western Blotting

Protein extracts were prepared from frozen biopsy samples as previously described [35]. Briefly, tissues were homogenized on ice in a buffer containing 50 mM Tris (pH 8.0), 10 mM EDTA, 1 mM diethyldithiocarbamic acid (DEDTC), and 2 mM octyl glucoside and centrifuged at 30 000 $\times$ *g* for 1 h at 4°C. The resulting pellets (containing membranes, nuclei, and mitochondria) were then resuspended in a buffer containing 20 mM Tris (pH 8.0), 50 mM EDTA, 0.1 mM DEDTC, 32 mM octyl glucoside, and protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, 1 tablet per 10 ml of buffer; Roche) and disrupted using a Sonifier 450 sonicator (Branson Ultrasonics Corp., Danbury, CT) at 30% frequency: intensity level 1 for 8 cycles. Samples were then centrifuged at 16 000 $\times$ *g* for 15 min at 4°C, and the protein content of the supernatants (referred to as the membrane fractions) was quantified by the Bradford method.

Proteins (100 μg/sample) were separated by SDS-PAGE and transferred to PVDF membranes (Amersham) as previously described [35]. Membranes were then probed with specific antisera against either COX-2 [36], PGES (Cayman Chemical Co.), or liver-type PGFS [16]. Detection was performed using an HRP-conjugated donkey anti-rabbit IgG antibody and the ECL Plus Western Blotting Detection Reagents (Amersham) as directed by the manufacturer. Autoradiograph images were digitized using a ScanMaker 4 flatbed scanner (Microtek, Redondo Beach, CA), and signal strength was quantified for each sample using IQMac v 1.2 software (Molecular Dynamics). Western blotting experiments using a specific anti-lung-type PGFS antibody [17] revealed either no or very low levels of lung-type protein in a small subset of biopsies (not shown). In these samples, immunohistochemistry showed lung-type PGFS to localize to the endometrial stroma and not the epithelium (not shown).

Immunohistochemistry

Biopsy samples were formalin-fixed and embedded in paraffin according to standard protocols before preparation of 3-μm sections. Immunohistochemical detection was then performed by an immunoperoxidase method using the antibodies detailed above and the Vectastain Elite ABC Kit (Vector Laboratories) essentially as directed by the manufacturer. Staining was performed by a 15-min incubation in a 3,3′-diaminobenzidine (DAB) peroxidase substrate solution, prepared by suspending a 10-mg Sigma Fast DAB tablet (Sigma) in 20 ml of 50 mM Tris (pH 7.6) with 0.045% hydrogen peroxide. Slides were counterstained with hematoxylin prior to mounting.

Statistical Analysis

One-way ANOVA was used to test the effect of time on COX-2, PGES, and PGFS signals. For the mRNA data, GAPDH was used as a covariate in the analysis to correct for differences in loading. When ANOVAs indicated significant differences ($P < 0.05$), Tukey honestly significantly difference test was used for multiple comparisons of the individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC). Results are expressed as means ± SEM (n = 5 biopsies [i.e., mares]/timepoint).

RESULTS

Cloning and Characterization of the Equine PGFS Transcript and Protein

To clone the equine PGFS transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of known PGFS species homologs. The resulting cDNA fragment (Fig. 1Ab) was sequenced and found to be highly homologous to all PGFS

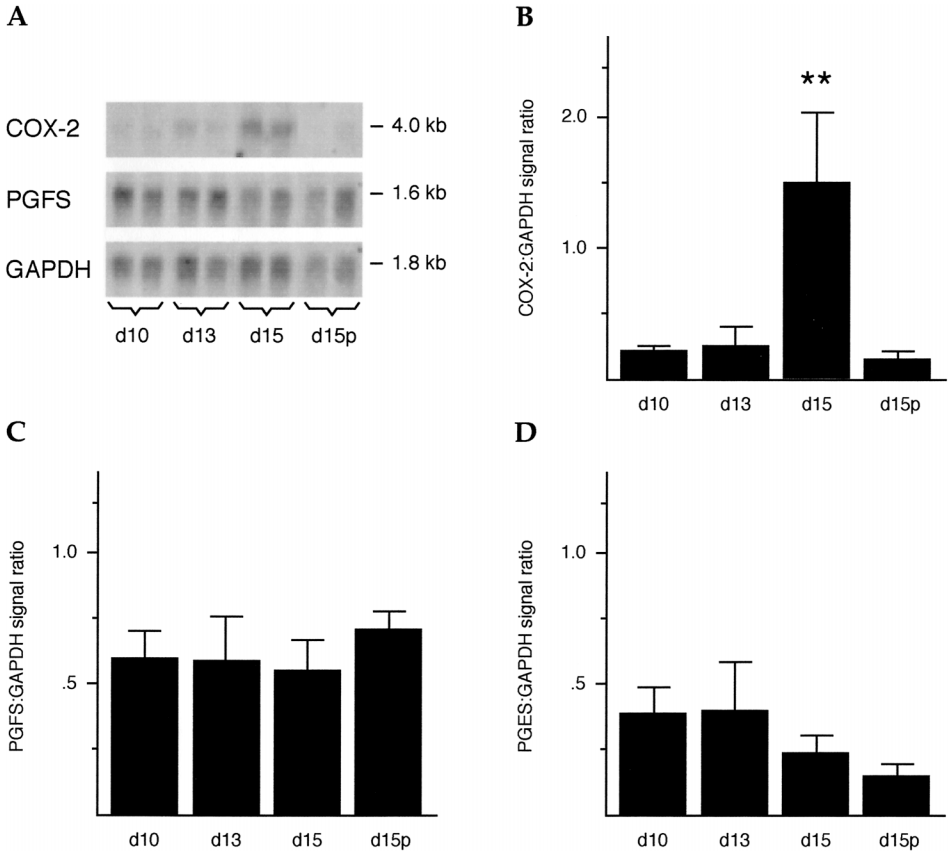


FIG. 3. Expression of prostaglandin synthase mRNAs in equine endometrium during late diestrus and early pregnancy. **A**) Steady-state COX-2, PGFS, and GAPDH mRNA levels in endometrial biopsy samples were evaluated by Northern blotting. Representative images (n = 2/timepoint) generated by the PhosphorImager are shown. Numbers below the images indicate the days of the ovarian cycle when the biopsies were collected (d15p = Day 15, pregnant), and numbers on the right show the approximate sizes of the transcripts (kb = kilobases). **B**) Quantification of the COX-2 mRNA data (n = 5/timepoint). Data were normalized by dividing the COX-2 signal values by their corresponding GAPDH values and are expressed as means (columns) ± SEM (error bars). Statistically significant difference ($P = 0.01$) from the control (Day 10) is indicated with a double asterisk. **C**) Quantification of the PGFS mRNA data (n = 5/timepoint). **D**) Quantification of the PGES mRNA data (n = 5/timepoint) by real-time RT-PCR using the LightCycler system. Data were normalized by calculating PGES:GAPDH copy number ratios.

transcripts identified thus far. A combination of 5′- and 3′-RACE reactions yielded cDNA products corresponding to all remaining coding regions, the complete 3′-UTR, as well as 185 bp of 5′-UTR (Fig. 1, Aa and Ac). As the RT-PCR and 3′-RACE products overlapped each other by only 4 bp, a second RT-PCR product was generated to extend the region of overlap, thereby confirming that both products were derived from the same transcript (Fig. 1Ad). The deduced 1380-bp primary transcript encoded a 969-bp open reading frame (Fig. 1A, GenBank accession number AY304536), which predicted a protein of 323 amino acid residues.

When compared with various PGFS isoforms isolated from other species, the amino acid sequence of equine PGFS was found to be 79% or more identical to all known mammalian homologs (Fig. 2). Among the three known bovine PGFS isoforms, equine PGFS showed the highest degree of homology to the liver type, also known as dihydrodiol dehydrogenase 3 (DD3 or DDBX)[22, 37]. Structurally, equine PGFS was found to be essentially identical to its mammalian counterparts, consisting of a single conserved aldo-keto reductase domain that is predicted to adopt a $(\alpha/\beta)_8$-barrel three-dimensional structure [38]. In addition, critical amino acid residues that are required for catalytic activity, NADP+ cofactor binding, and that form the substrate pocket were found to be conserved in equine PGFS (Fig. 2).

COX-2, PGFS, and PGES Expression in Late Diestrus and Early Pregnancy

The expression of key enzymes involved in uterine prostaglandin biosynthesis was then examined in a series of endometrial biopsies obtained during late diestrus and in early pregnancy. Because the onset of luteolysis and maximal endometrial $PGF_{2\alpha}$ release occur around Day 14 postovulation in mares [7, 39], biopsies were obtained on Day 10 (diestrus), Day 13 (late diestrus), and Day 15 (during luteolysis) as well as from Day 15 pregnant mares, a time when the conceptus acts to greatly diminish $PGF_{2\alpha}$ release [7]. Steady-state endometrial COX-2 and PGFS mRNA levels were then quantified by Northern blotting (Fig. 3). For COX-2, a significant ($P = 0.01$) 6-fold induction in mRNA levels was observed on Day 15. Interestingly, biopsies collected from Day 15 pregnant mares contained basal COX-2 mRNA levels, comparable with those detected in the Day 10 or Day 13 nonpregnant samples. For PGFS, no concomitant increase in mRNA levels was observed on Day 15, and they were not found to be affected by time or pregnancy status (Fig. 3C). As PGES mRNA was found not to be readily detectable by Northern blotting (not shown), a quantitative real-time RT-PCR assay was employed to study its expression. As seen for PGFS, no significant variations in PGES mRNA levels were found throughout the timecourse nor were they affected by pregnancy at Day 15 (Fig. 3D).

To study the expression and regulation of the PG synthase proteins, Western blotting was performed using the same endometrial biopsy samples (Fig. 4). A significant ($P = 0.01$) 4.5-fold increase in COX-2 protein was observed at Day 15 only in nonpregnant mares, mirroring what was observed for its mRNA (Fig. 3B). Likewise, PGFS and PGES protein levels did not vary significantly throughout the timecourse and were unaffected by pregnancy, as had been observed for their transcripts (Fig. 3, C and D).

Immunohistochemical Localization of Endometrial COX-2, PGFS, and PGES Expression

To study the localization of the PG synthases within the endometrium, immunohistochemistry was performed using

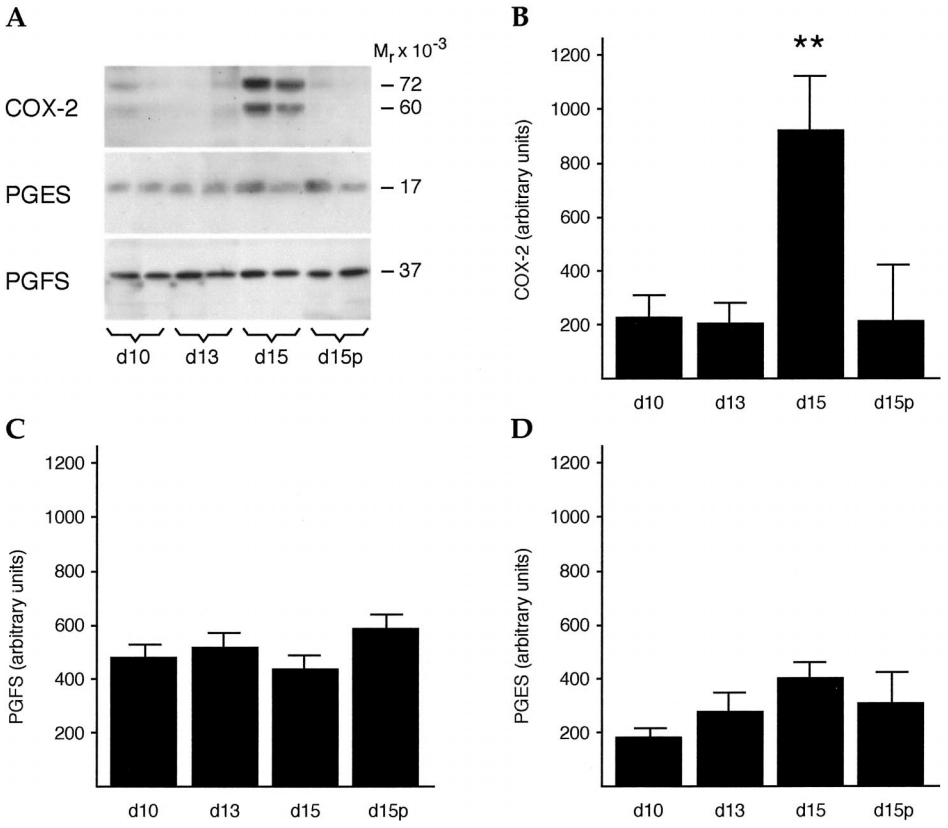


FIG. 4. Expression of prostaglandin synthase proteins in equine endometrium during late diestrus and early pregnancy. **A**) COX-2, PGES, and PGFS protein levels in endometrial biopsy samples were evaluated by Western blotting. Representative digitized autoradiograph images are shown (n = 2/timepoint). Numbers below the images indicate the days of the ovarian cycle when the biopsies were collected (d15p = Day 15, pregnant), and numbers on the right show the apparent molecular weights of the proteins. **B**) Quantification of the COX-2 expression data (n = 5/timepoint). Data are expressed as means (columns) ± SEM (error bars), and statistically significant difference ($P = 0.01$) from the control (Day 10) is indicated with a double asterisk. **C**) Quantification of the PGFS protein data (n = 5/timepoint). **D**) Quantification of the PGES protein data (n = 5/timepoint).

the same antibodies that were employed for Western blotting to probe fixed histological sections of endometrial biopsies. High levels of COX-2 protein were detected in the surface epithelium in nonpregnant mares at Day 15, but not in pregnant animals (Fig. 5). Some COX-2 staining could also be observed in glandular epithelial cells, but only in a few of the most superficial endometrial glands (not shown). Interestingly, vascular endothelial cells also often stained quite strongly for COX-2 in both Day 15 pregnant and Day 15 nonpregnant equine endometrium, as well as at other timepoints (not shown). The distribution of PGFS and PGES proteins was found to overlap that of COX-2, as they localized almost exclusively to the surface epithelium, with limited staining present in the most superficial glandular epithelium (Fig. 5). Unlike COX-2, however, little PGES or PGFS staining could be associated with the vascular endothelium. Furthermore, comparable levels of PGES and PGFS staining could be detected in both the Day 15 pregnant and Day 15 nonpregnant surface epithelial cells, as predicted by our immunoblotting analyses.

DISCUSSION

While the secretion of $PGF_{2\alpha}$ by the uterus during late diestrus is a key event in the ovarian cycle in many domestic species, the underlying regulation of the requisite PG synthase genes remains poorly defined. To date, the best insights into in vivo endometrial PG synthase regulation have come from studies in ruminants, which have shown transient increases in COX-2 expression during late diestrus in cows and ewes that coincide with the expected time of luteolysis [14, 15]. However, this increase in COX-2 levels was also found to occur in pregnant ewes, and its expression was found to be prolonged by pregnancy [14]. This could reflect the fact that, unlike species such as the horse, pregnancy does not appear to affect the overall amount of $PGF_{2\alpha}$ released by the uterus in the ewe, but it does seem to affect the pulsatility of its secretion [3, 4]. In the present study, we used the equine model to examine the regulation of endometrial COX-2 mRNA and protein in late diestrus and to assess the effects of pregnancy on its expression. Our findings show that COX-2 expression is strongly induced at the expected time of luteolysis (i.e., Day 15) and that, unlike in the ewe, this induction is not seen in the pregnant mare. A previous study has shown $PGF_{2\alpha}$ levels present in the uterine lumen to be much higher in nonpregnant versus pregnant mares at Days 14–16, whereas PGE_2 levels were found to be comparable [7]. Our findings thus establish a potential correlation between $PGF_{2\alpha}$ (but not PGE_2) release and COX-2 expression in late diestrus and strongly suggest that the equine conceptus blocks endometrial $PGF_{2\alpha}$ synthesis at least in part by repressing the induction of COX-2 expression. As in the cow and sheep, the oxytocin stimulation of $PGF_{2\alpha}$ secretion seen in cyclic mares is attenuated in the pregnant animal [9]. However, contrary to what is observed in cows and sheep, oxytocin receptor number remains high in the pregnant mare at the expected time of luteolysis [8]. Thus, prevention of luteolysis in the pregnant mare appears to depend more on the conceptus inhibiting the up-regulation of COX-2 than inhibiting the up-regulation of the oxytocin receptor. This indicates that there are fundamental differences in the molecular mechanisms underlying maternal recognition of pregnancy in mares and ewes.

Unlike COX-2, PGFS expression was not found to change in late diestrus, and its expression was unaffected by pregnancy. This would indicate that PGFS does not play a rate-limiting role in endometrial $PGF_{2\alpha}$ synthesis and that the repression of the induction of COX-2 by the conceptus

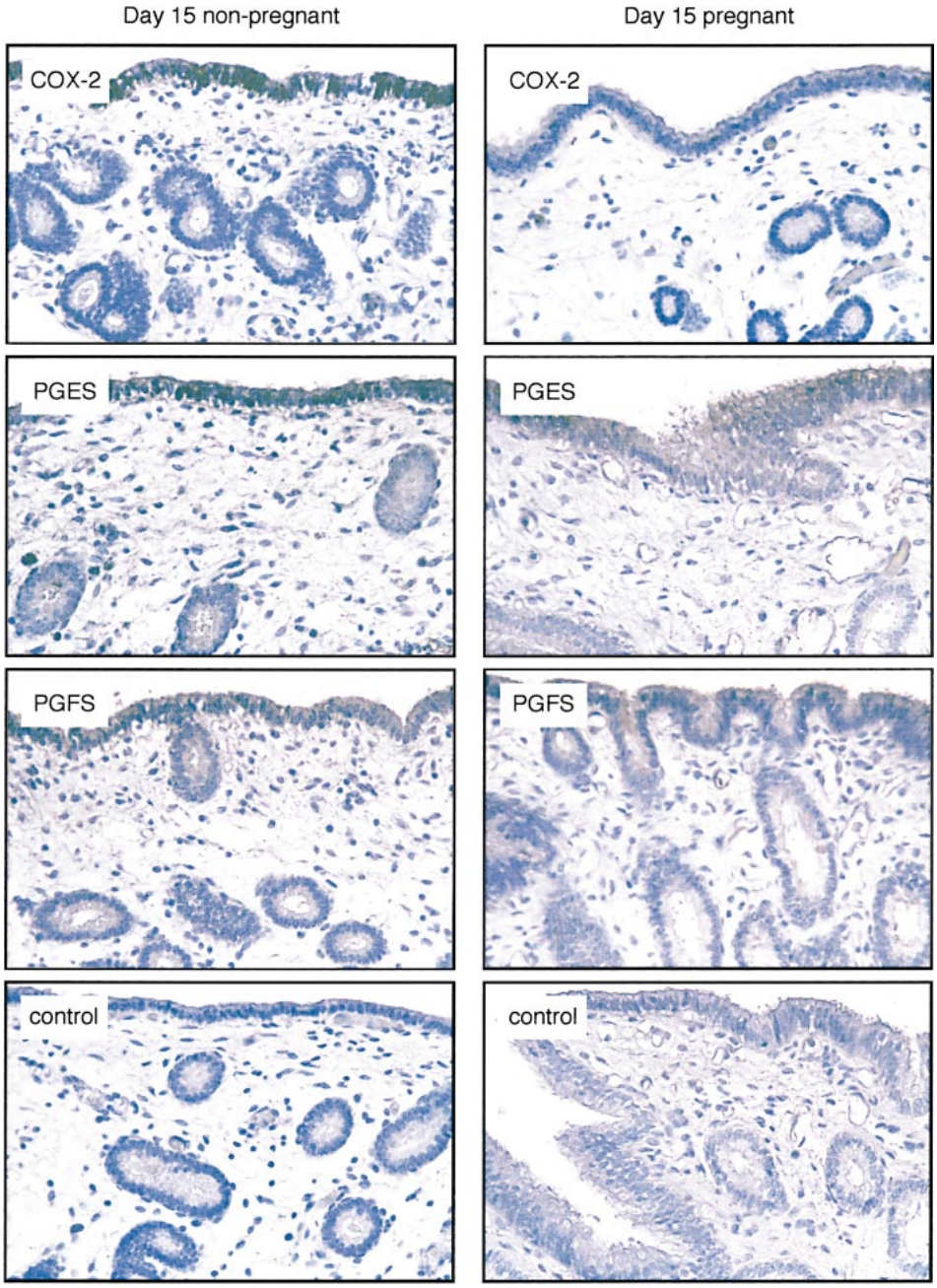


FIG. 5. Immunohistochemical analyses of COX-2, PGFS, and PGES protein distribution within the endometrium in cycle Day 15 pregnant and nonpregnant mares. Sections of fixed, paraffin-embedded, Day 15 and Day 15 pregnant endometrial biopsies were probed with either specific anti-COX-2, anti-PGES, or anti-PGFS antisera. Detection was performed using a peroxidase system with DAB as a substrate, and slides were counterstained with hematoxylin prior to mounting. The control panels show sections that were processed in the same fashion except that primary antibody was omitted. Magnification ×400.

could be sufficient in itself to block $PGF_{2\alpha}$ synthesis during early pregnancy. Previous in vitro studies have shown that IFNτ could down-regulate lung-type PGFS mRNA in bovine endometrial cells, suggesting that the conceptus could interfere with $PGF_{2\alpha}$ synthesis by targeting PGFS gene expression [20, 21]. A more recent study, however, has called into question the identity of the mRNA transcript that was detected in the aforementioned studies, given the high homology between the five known bovine AKR1C family members and the low stringency Northern blotting wash conditions that had been used [22]. In any event, no in vivo studies have been performed to demonstrate if the expression of any of the bovine PGFS enzymes is altered by pregnancy, and it therefore remains unknown if our results show a PGFS-regulatory pattern that is unique to the equine species. Recently, a study has shown that none of the known bovine PGFS enzymes is significantly expressed in the endometrium, and the luteolytic synthesis of $PGF_{2\alpha}$ by the endometrium was attributed to a previously uncharacterized PGFS activity of a 20α-hydroxysteroid dehydrogenase enzyme (AKR1B5). Interestingly, it was also shown that AKR1B5 expression is up-regulated in the endometrium during the second half of the estrous cycle [22]. Our results certainly cannot preclude the existence of an equine AKR1B5 homologue that could contribute to endometrial $PGF_{2\alpha}$ synthesis. However, unlike what was shown in the cow, we were able to detect the presence of abundant equine PGFS mRNA and liver-type PGFS immunoreactivity at all time points examined under high wash stringency conditions. This could indicate that different enzymes direct endometrial $PGF_{2\alpha}$ synthesis in mares and cows.

Another interesting finding reported herein is the lack of apparent regulation of equine PGES. PGES expression increases in bovine endometrium in parallel with COX-2 during late diestrus and can be induced by IFNτ in cultured endometrial cells, and therefore has been proposed to play

a potential role in the establishment of pregnancy [15, 24]. In mares, however, it should be noted that there is little change in PGE_2 levels present in the uterine lumen during diestrus, and pregnancy increases these only marginally [7]. In addition, intrauterine infusions of PGE_2 in mares do not seem to prolong the lifespan of the corpus luteum, and PGE_2 is not believed to produce a luteotropic effect on equine luteal cells in culture [7, 40, 41]. Our endometrial PGES expression results could therefore reflect important differences in the biological roles of PGE_2 in the equine versus ruminant reproductive tract. Indeed, the equine conceptus itself produces large amounts of PGE_2, and this has been proposed to stimulate myometrial contractions that occur during cycle Days 10–16 [6]. These contractions appear to serve to move the conceptus to all areas of the uterus, presumably allowing it to distribute an antiluteolytic signaling molecule that is yet to be identified [6]. Perhaps PGE_2 produced by the endometrium during this period serves to supplement PGE_2 produced by the conceptus rather than playing a luteotropic/antiluteolytic role as proposed for ruminants. It is also interesting to note that COX-2 and PGES do not appear to be functionally coupled in the equine endometrium as they are in other PG synthetic cells [25, 26] because the induction of COX-2 at Day 15 does not appear to lead to a meaningful increase in PGE_2 levels in the uterine lumen [7]. Perhaps a lack of COX-2/PGES coupling could somehow favor conversion of available PGH_2 to $PGF_{2\alpha}$ by PGFS at the expected time of luteolysis.

Predictably, our immunohistochemistry data demonstrated the colocalization of COX-2, PGES, and PGFS in the epithelial cells of the endometrium, confirming that this cell type is responsible for the bulk of endometrial PG synthesis. In addition, substantial amounts of COX-2 immunoreactivity also localized to vascular endothelial cells at all timepoints examined. This may account for much of the basal levels of COX-2 mRNA and protein detected in Day 10, Day 13, and Day 15 pregnant endometrial samples. If so, this would suggest that the induction of COX-2 expression that occurs at Day 15 in the surface epithelium could be even more pronounced than our Northern and Western blotting data would tend to indicate.

In summary, this study shows for the first time the expression of the key PG synthase genes in equine endometrium during late diestrus and early pregnancy. The period of luteolytic $PGF_{2\alpha}$ synthesis in late diestrus was linked to the induction of the COX-2 gene at Day 15, which did not occur in pregnant mares. As the expression of PGES and PGFS were not found to change during late diestrus or in pregnant animals, it would appear that the equine conceptus may modulate endometrial PG synthesis primarily by targeting the expression of the COX-2 enzyme. Further studies will be required to define how the conceptus blocks COX-2 expression, which could in turn provide insight into the nature and mechanism of action of the antiluteolytic factor that it secretes to permit the maternal recognition of pregnancy.

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