Evidence that the preovulatory rise in intrafollicular progesterone may not be required for ovulation in cattle

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Abstract

Despite ample evidence pointing to an obligatory involvement of progesterone in ovulation, the mechanisms responsible for the ovulation promoting effects of intrafollicular progesterone are unclear. The objectives of this study were to determine if ovulation, luteinization and the gonadotropin surge-induced regulation of select extracellular matrix-degrading enzymes and their inhibitors, and mRNAs for prostaglandin (PG) biosynthesis and metabolizing enzymes are blocked following suppression of the intrafollicular increase in progesterone. Bovine preovulatory follicles were injected with the 3 β -hydroxysteroid dehydrogenase inhibitor trilostane or diluent and collected at 0, 12, and 24 h after GnRH induction of the preovulatory LH surge. Intrafollicular trilostane administration blocked the preovulatory increase in follicular fluid progesterone resulting in concentrations similar to those observed at time 0 post-GnRH injection. The preovulatory increase in follicular fluid PGE₂ and PGF_{2 α} was reduced in trilostane-treated

follicles and accompanied by upregulation of prostaglandin dehydrogenase mRNA in the granulosa and thecal cells. However, follicle rupture was not blocked by inhibition of the preovulatory rise in intrafollicular progesterone, and normal serum progesterone concentrations were observed during subsequent luteal development. Effects of trilostane administration on preovulatory changes in mRNA abundance and protein/activity in preovulatory follicles for most regulators of extracellular matrix remodeling examined were distinct from changes previously observed following the inhibition of intrafollicular prostaglandin synthesis. Results suggest that the preovulatory increase in intrafollicular progesterone may not be obligatory for bovine follicle rupture, luteinization, or regulation of prominent matrix-degrading proteinases and their inhibitors associated with ovulation.

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Introduction

Ovulation, triggered by the preovulatory luteinizing hormone (LH) surge, is a prerequisite for fertilization and embryonic development. Preovulatory degradation of the extracellular matrix at the follicle apex is a hallmark of the ovulatory process (Fata *et al.* 2000, Ny *et al.* 2002). Available evidence indicates that local regulation of genes implicated in the tissue remodeling process, including members of two major families of proteolytic enzymes, the matrix metalloproteinase (MMP) and the plasminogen activator (PA)/plasmin system (Iwamasa *et al.* 1992, Tanaka *et al.* 1992, Chaffin & Stouffer 1999), and their cognate inhibitors, controls the targeted degradation of the follicular extracellular matrix required for ovulation.

We have previously demonstrated gonadotropin surge-induced regulation of expression of several key MMP and PA system components in bovine preovulatory follicles, including MMP-13 (Bakke *et al.* 2004), MMP-14 (Bakke *et al.*

2002), MMP-28 (Li *et al.* 2006), tissue inhibitor of metalloproteinases (TIMPs) 1–4 (Smith *et al.* 1996, Bakke *et al.* 2002, Li *et al.* 2004), tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR; Dow *et al.* 2002a), and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2; Dow *et al.* 2002b). A requirement of intrafollicular prostanoids for bovine follicle rupture (De Silva & Reeves 1985, Peters *et al.* 2004) and gonadotropin surge-induced regulation of specific MMP and PA system components (Li *et al.* 2006) has been demonstrated. However, increased intrafollicular progesterone is characteristic of the luteinization process accompanying bovine ovulation (Dieleman *et al.* 1983, Sirois 1994). It is not known whether the preovulatory increase in the intrafollicular progesterone *per se* mediates the gonadotropin surge-induced changes in the expression of above regulators of the follicular extracellular matrix in bovine follicles and if such effects are potentially mediated by progesterone-induced regulation of prostanoids, such as

prostaglandin (PG) E_2 . Thus, in view of the essential role of progesterone in promoting follicle rupture in other species (Espey *et al.* 1990, Hibbert *et al.* 1996), we hypothesized that the preovulatory increase in the intrafollicular progesterone of the dominant ovulatory follicle is required for ovulation and gonadotropin surge-induced regulation of PGE $_2$, PGF $_{2\alpha}$, and the above mentioned regulators of extracellular matrix remodeling in bovine preovulatory follicles. Thus, in the present study, intrafollicular injection of a 3 β -hydroxysteroid dehydrogenase (3 β -HSD) inhibitor (trilostane) to block the preovulatory rise in intrafollicular progesterone was used as a tool to investigate the requirement of increased intrafollicular progesterone for bovine ovulation and gonadotropin surge-induced intrafollicular regulation of mediators of the tissue remodeling process and other molecular, hormonal, and (or) biochemical indices of ovulation and luteinization.

Materials and Methods

Animal model

Mature Holstein cows (*Bos taurus*; ≥ 2 years old, 449–680 kg) were fed a balanced corn silage diet and housed at the Michigan State University Beef Cattle Research Center during the course of the experiments. Experimental procedures were approved by the All University Committee on Animal Use and Care at Michigan State University. The model utilized in this study has been described previously (Bakke *et al.* 2002, 2004). Briefly, follicular development and timing of the preovulatory gonadotropin surge were synchronized using the Ovsynch procedure established by Pursley *et al.* (1995, 1997) with slight modifications. In brief, the first gonadotrophin-releasing hormone (GnRH) injection was given to initiate a new wave of follicular growth resulting in a new dominant follicle. PGF $_{2\alpha}$ was then injected to regress the corpus luteum 6.5 days later. A second dose of GnRH was given 36 h later to trigger the gonadotropin surge and ovulation of the dominant follicle. Synchronized ovulation of the dominant follicle occurs an average of 29 h after the second GnRH injection (Pursley *et al.* 1995). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrosterane-2-carbonitrile; Sanofi-Synthelabo Research, Malvern, PA, USA), an inhibitor of 3 β -HSD (10 μ M final concentration in follicular fluid), or diluent (PBS; control) was injected into preovulatory follicles immediately after the second GnRH injection using previously described ultrasound-mediated intrafollicular injection procedures (Peters *et al.* 2004). Dose of trilostane administered was selected based on the results of a preliminary dose response experiment, comparing effects of different trilostane concentrations (1, 2, 5, and 10 μ M) on follicular fluid progesterone levels 24 h after GnRH injection (data not shown).

Effects of intrafollicular trilostane administration on follicular fluid hormone concentrations and granulosa and thecal cell mRNA and (or) protein for regulators of extracellular matrix remodeling and prostaglandin biosynthesis/metabolism

Experiment 1 For investigation of the effects of intrafollicular injection of trilostane on changes in mRNA and (or) protein/activity for matrix-degrading proteinases and their inhibitors, mRNA for cyclooxygenases 1 and 2 (COX-1 and COX-2; enzymes responsible for the conversion of arachidonic acid to prostaglandins), the NAD $^{+}$ -dependent 15-hydroxy prostaglandin dehydrogenase (PGDH; the key enzyme that metabolizes PGE $_2$ and PGF $_{2\alpha}$ to biologically inactive 15-keto derivatives) and oxytocin, and follicular fluid progesterone, estradiol, PGE $_2$ and PGF $_{2\alpha}$ concentrations, ovaries containing preovulatory follicles were collected at 0 ($n=5$ animals), 12 ($n=3$ and 5 for controls and trilostane treated respectively), and 24 h ($n=3$ and 6 for controls and trilostane treated respectively) after the second GnRH injection. Follicular fluid was aspirated from each follicle and centrifuged to remove potential blood cell contamination. Indomethacin was then added to follicular fluid (to prevent *ex vivo* generation of eicosanoids) to achieve a final concentration of 3.6 μ g/ml (Li *et al.* 2006) before it was frozen in liquid nitrogen and stored at -80°C . Dissection of preovulatory follicles was described previously (Bakke *et al.* 2002, Li *et al.* 2004). The dissected follicles were sagittally cut into several portions for granulosa cells (GC) and thecal tissue (TC) isolation and for protein extraction. GC were scraped from the theca interna of preovulatory follicles and collected by centrifugation, while TC was isolated from a portion of the same follicles. TRIzol (Life Technologies, Inc., Gaithersburg, MD, USA) was added to the GC immediately after collection. Follicle apex and base containing GC and TC were separated from another portion of the same follicles for protein extraction. All the above samples were snap frozen in liquid nitrogen and stored at -80°C until use.

Effects of intrafollicular trilostane administration on follicle rupture and subsequent luteal function

Experiment 2 To examine the effect of trilostane on bovine follicle rupture and subsequent luteal function, cows were injected (in two replicates) with trilostane ($n=13$ total) or diluent ($n=17$ total) immediately after the second GnRH injection and follicle rupture was monitored (beginning immediately after intrafollicular injection) by ultrasonography every 12 h for 2 days. Blood samples were collected at 2 to 3-day intervals (days 0, 2, 4, 7, 9, and 11) after intrafollicular trilostane injection and sera were separated from animals ($n=7$ and 9 for trilostane treated and control respectively) in the second replicate of the above experiment. Sera were frozen at -20°C until progesterone assay.

Steroid hormone radioimmunoassays

Concentrations of progesterone in follicular fluid and serum were measured by RIA using a commercially available kit (Diagnostic Products Corp., Los Angeles, CA, USA) as described previously (Peters *et al.* 2004). Intra- and interassay coefficients of variation (CV) were 5 and 9% respectively. Concentrations of estradiol-17 β in follicular fluid samples were measured by RIA (Diagnostic Products Corporation) in a single assay (Jimenez Krassel & Ireland 2002). Intraassay CV was 5.4%.

Follicular fluid PGE₂ and PGF_{2 α} assays

Concentrations of follicular fluid PGE₂ were analyzed using a PGE₂ enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI, USA) as described previously (Peters *et al.* 2004). Concentrations of follicular fluid PGF_{2 α} were analyzed using a PGF_{2 α} EIA (Cayman Chemical). Follicular fluid was diluted in EIA buffer before analysis in separate single assays for PGE₂ and PGF_{2 α} . Intraassay CV were 3.1 and 5.5% for PGE₂ and PGF_{2 α} respectively.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from GC and TC of bovine preovulatory follicles using TRIzol reagent according to the manufacturer's instructions. One microgram of total RNA from each sample was incubated for 15 min at 25 °C with 1 U DNase I (Invitrogen) to eliminate possible genomic DNA contamination before reverse transcription which was detailed elsewhere (Li *et al.* 2004). Real-time PCR was applied to quantify mRNA levels for genes of interest in GC/TC of trilostane-versus diluent-treated control follicles. Real-time PCR primers were designed using Primer Express program (Primer Express, Applied Biosystems, Foster City, CA, USA) and the primer information is indicated in Table 1. The PCRs (25 μ l volume) contained 12.5 μ l SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and primers (Table 1) for each gene and were run using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with a program consisting of 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Li *et al.* 2004). The reactions were set up in duplicate in a 96-well plate (Applied Biosystems) and the mean cycles to threshold (CT) were

Table 1 Primers utilized for quantification of mRNA abundance by real-time PCR

Gene name	Genbank accession no.	Primer sequence	Primer ratio ^a (nM/nM)
MMP-13	AF072685	Forward: GCAGAGAGCTACCTGAAATCATACTACT; Reverse: AATCACAGAGCTTGCTGCAGTTT	300/300
MMP-14	AF290429	Forward: CCTCAACCCAGGACCACTTC; Reverse: GGCCCGTAGGTGGGATTTT	300/300
MMP-28	XM_583321	Forward: CAGGGAAGGCGTCCAGAA; Reverse: CAGTGACGGCATCGAAGGA	300/900
TIMP-1	M60073	Forward: GCACATCACCACCTGCAGTT; Reverse: CCGGCGCTGAGCAGAA	50/50
TIMP-2	AF144764	Forward: GGGCTGTGAGTGCAAGATCA; Reverse: CTCGTCCGGAGAGGAGATGTAG	300/300
TIMP-3	U77588	Forward: GATGTACCGAGGATTCACCAAGA; Reverse: TTAAGGCCACAGAGACTTTCAGAA	300/900
TIMP-4	AF037273	Forward: GAAAGTCTGAATCACCGCTACCA; Reverse: CAGGGCACCGCATAGCA	900/300
tPA	X85800	Forward: ACGTCAGGTTGTACCCATCCA; Reverse: GTTGTTGGTGACGGTCTCTGTT	300/300
uPA	X85801	Forward: GCCGTGACTGTGCCATGA; Reverse: GTGTTGATCCAGGGCAGGAA	300/300
uPAR	S70635	Forward: GCCGCGGCCCTATGA; Reverse: GCTTGGGTTCTCAGTCCTTT	300/300
PAI-1	X16383	Forward: TGGGCCCGTGGAACAA; Reverse: AGGTCCCGCTGCACGAA	300/300
PAI-2	AF416234	Forward: CCATATGCTGGCGATGTCACT; Reverse: GCCCGTAGAGGATTCAGCAA	300/300
Oxytocin	X00950	Forward: CCGGCCCCCGATACC; Reverse: TCATTGTCATAATTCCTAGGGATGATT	50/300
COX-1	AF004943	Forward: CAAGATGGGTCTCTGGCTTCA; Reverse: CCATAATGTGGCCGAGGTCTA	300/50
COX-2	AF004944	Forward: TTGATTGACAGTCCACCACTTATAAT; Reverse: GAGCTCTGGTATAATAAGACAGGTTAGAAA	300/150
PGDH	BE237549	Forward: GAGGCGCTGCTGCTCAAG; Reverse: TGACACCTGCTTCGAGATTCC	50/300
RPL-19	XM_587778	Forward: CAGACGATACCGTGAATCTAAGAAGA; Reverse: TGAGAATCCGCTTGTTTTGAA	300/300

^aForward/reverse primer ratio was determined by a primer matrix analysis.

calculated for each sample. The comparative CT method (Livak & Schmittgen 2001) was used for quantification. Expression of each gene of interest was normalized relative to that of ribosomal protein L-19 (RPL-19) mRNA. Δ CT was produced by subtracting the mean CT of RPL-19 from the CT of each target gene. Fold changes in the relative mRNA expression of target genes (except COX-2 in 12- and 24-h samples) relative to 0 h were determined using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT_{12 \text{ or } 24 \text{ h sample}} - \Delta CT_{0 \text{ h sample}}$. COX-2 mRNA abundance was normalized relative to 12-h timepoints. Control reverse transcription reactions, where reverse transcriptase or template was omitted from reactions, were also analyzed to verify the lack of genomic DNA contamination.

Preparation of protein homogenates

Homogenates of follicle apex and base were prepared as described previously (Bakke *et al.* 2002). Protein concentration was determined using Lowry *et al.* (1951) method and aliquots of protein samples stored at -20°C for western blot and zymography analyses.

Western blot analysis

Western blot analysis of TIMP-4 protein expression in trilostane-treated versus control follicles was performed according to our previously published protocol (Li *et al.* 2004).

Casein zymography

Casein zymography was conducted to measure the activity of tPA, uPA, and plasmin in the follicle apex, base, and follicular fluid of the trilostane-treated versus control follicles using our previously established protocols (Dow *et al.* 2002a).

Statistical analysis

Differences in the concentrations of follicular fluid progesterone, estradiol, PGE_2 , $\text{PGF}_{2\alpha}$, abundance of protein and (or) mRNA for genes of interest, and activity of PA and plasmin among timepoints or treatment groups were assessed by ANOVA using the General Linear Model procedure of the Statistical Analysis System (SAS; Version 8, SAS Institute, Cary, NC, USA). Differences between individual groups were further analyzed with Tukey's test. When heterogeneity of variance was observed, data were log-transformed before statistical analysis. Analysis of the serum progesterone concentrations in animals following intrafollicular trilostane and control injections was performed using the Proc Mixed function of SAS for repeated measures with treatment, time, and interaction of treatment by time as main effects and cow within treatment as the error term. Data are presented as least square (LS) mean $\pm$ S.E.M. and $P < 0.05$ was considered to be statistically significant.

Results

Effect of intrafollicular trilostane injection on follicular fluid steroid concentrations

Mean follicular fluid progesterone concentrations were 94 ng/ml in 0-h preovulatory follicle samples collected prior to GnRH injection, and were significantly increased in control follicles 24 h after GnRH administration to induce the preovulatory gonadotropin surge ($P < 0.05$; Fig. 1A). Follicular fluid progesterone concentrations were lower in trilostane-treated versus control follicles at 12 and 24 h after GnRH injection (16 and 25% of those observed in control follicles respectively; $P < 0.05$; Fig. 1A) and were not significantly different from those observed in control follicles prior to GnRH injection. Relative to controls, follicular fluid estradiol concentrations were also lower in trilostane-treated follicles collected at 12 and 24 h after GnRH injection ($P < 0.05$; Fig. 1B).

Figure 1 Concentrations of progesterone and estradiol in follicular fluid after GnRH injection and the effect of intrafollicular trilostane (TRI) injection. (A) Progesterone concentrations (ng/ml) in follicular fluid of control (CON) animals at 0 ($n=5$), 12 ($n=3$), and 24 h ($n=5$) after GnRH injection and TRI-treated animals at 12 ($n=5$) and 24 h ($n=6$) post-GnRH-injection. (B) Estradiol concentrations (ng/ml) in follicular fluid of CON animals at 0 ($n=5$), 12 ($n=3$), and 24 h ($n=5$) after GnRH injection and TRI-treated animals at 12 ($n=5$) and 24 h ($n=6$) post-GnRH-injection. Each bar represents the LS mean $\pm$ pooled S.E.M. (A) or mean $\pm$ S.E.M. (B), and bars without a common letter are different at $P < 0.05$.

Effect of intrafollicular injection of trilostane on follicle rupture and luteal development in cattle

As determined by ultrasonography, all 17 diluent-treated control animals and 13 trilostane-treated animals ovulated within 48 h after intrafollicular injection and no difference between control and trilostane-treated animals in timing of ovulation was observed. Oxytocin mRNA increased in response to GnRH injection ($P < 0.05$; Fig. 2A), but no effect of trilostane treatment on oxytocin mRNA abundance was detected (Fig. 2A). Moreover, intrafollicular trilostane administration did not affect amounts or pattern of progesterone production during the timepoints examined (Fig. 2B).

Figure 2 Effect of preovulatory intrafollicular trilostane (TRI) injection on granulosa cell (GC) oxytocin mRNA abundance and serum progesterone concentrations during days 1–9 of the subsequent luteal phase. (A) GC oxytocin mRNA abundance in diluent-treated control (CON) and TRI-treated follicles collected at 0, 12, and 24 h after GnRH injection to induce the preovulatory gonadotropin surge. Data are shown as LS means $\pm$ pooled S.E.M., and bars without a common letter are different at $P < 0.05$. (B) Serum progesterone concentrations in animals subjected to intrafollicular TRI injection versus CON animals. Serum progesterone concentrations were measured on days -1.5 , 0.5 , 2.5 , 5.5 , 7.5 , and 9.5 after the second GnRH injection of the Ovsynch procedure, which corresponds to approximately days -3 to 9 of the subsequent luteal phase following intrafollicular TRI injection. A pronounced time effect ($P < 0.0001$) was detected, while a significant effect of treatment (TRI injection) and treatment by time interaction were not observed ($P > 0.05$).

Effect of intrafollicular trilostane injection on follicular fluid PGE₂ and PGF_{2 α} concentrations and mRNA abundance for enzymes involved in prostanoid biosynthesis and metabolism

Follicular fluid PGE₂ and PGF_{2 α} concentrations were increased at 24-h post-GnRH injection and intrafollicular trilostane administration reduced the GnRH-induced increase in follicular fluid PGE₂ and PGF_{2 α} ($P < 0.05$; Fig. 3). Although COX-1 mRNA was detected in GC and TC at all timepoints examined, its abundance did not change in response to GnRH injection and trilostane administration (data not shown). COX-2 mRNA was undetectable in GC samples collected prior to GnRH injection (0-h control), but was detectable at 12 and 24 h and increased at 24 h relative to the 12-h timepoint ($P < 0.05$; Fig. 4A; data normalized relative to 12-h samples). However, treatment of preovulatory follicles with trilostane had no effect on GC COX-2 mRNA abundance (Fig. 4A). Cell-specific effects of GnRH injection on PGDH mRNA abundance in GC and TC were observed (Fig. 4B and C). PGDH mRNA abundance was increased in GC at both 12 and 24 h following GnRH injection ($P < 0.05$; Fig. 4B), but suppressed in the TC at the 12-h timepoint

Figure 3 Effect of intrafollicular trilostane (TRI) injection on concentrations of follicular fluid PGE₂ (A) and PGF_{2 α} (B) in 0-h control (CON; $n = 5$ and 4 for PGE₂ and PGF_{2 α} respectively), 24-h CON ($n = 5$ and 4 for PGE₂ and PGF_{2 α} respectively), and 24-h TRI ($n = 6$ and 5 for PGE₂ and PGF_{2 α} respectively)-treated follicles. Each bar represents the LS mean $\pm$ pooled S.E.M., and bars without a common letter are different at $P < 0.05$.

Figure 4 Quantitative real-time PCR analysis of the effect of trilostane (TRI) injection on cyclooxygenase-2 (COX-2) and prostaglandin dehydrogenase (PGDH) mRNA abundance in granulosa cells (GC) and thecal tissue (TC) of bovine preovulatory follicles. Abundance of GC COX-2 mRNA (A) and PGDH mRNA in GC (B) and TC (C) was normalized relative to that of RPL-19 mRNA. Relative mRNA abundance at 12 h ($n=3$ and 5 for CON and TRI respectively) and 24 h ($n=5$ and 6 for CON and TRI respectively) was calculated using $\Delta\Delta C_T$ method. Due to undetectable COX-2 mRNA at 0-h timepoint, COX-2 mRNA abundance at the 12-h timepoints was used as calibrator. Data are shown as means $\pm$ S.E.M. Bars without a common letter are different at $P<0.05$.

($P<0.05$; Fig. 4C). In response to intrafollicular trilostane injection, PGDH mRNA abundance was increased (compared with controls) in GC and TC at 24 and 12 h respectively, following GnRH injection ($P<0.05$; Fig. 4B and C).

Effect of intrafollicular administration of trilostane on MMP and TIMP expression in preovulatory follicles

Since intrafollicular trilostane administration suppressed intrafollicular progesterone and PGE₂ concentrations, effects of trilostane administration on protein/activity and (or) mRNA for MMPs and TIMPs associated with bovine follicle rupture were determined. Among the seven MMPs and TIMPs examined (MMP-13, MMP-14, MMP-28, and TIMPs 1–4), only abundance of TIMP-4 mRNA was affected by trilostane injection (data not shown for MMP-13, MMP-14, MMP-28, and TIMPs 1–3). Trilostane treatment blocked the transient gonadotropin surge-induced increase in GC and TC TIMP-4 mRNA observed 12 h after GnRH injection ($P<0.05$; Fig. 5A and B). TIMP-4 protein was detected as a single band of 28 000 M_r in bovine follicle homogenates, but no significant differences were detected in the amount of TIMP-4 protein in trilostane-treated versus control follicles in both the apex and the base (Fig. 5C and D).

Effect of intrafollicular administration of trilostane on GC and TC plasminogen activator system component mRNA abundance and activity

Gonadotropin surge-induced regulation of mRNA abundance for tPA and uPAR was not influenced by intrafollicular trilostane administration (data not shown). At 12 h after GnRH injection, uPA mRNA abundance in both GC and TC ($P<0.05$; Fig. 6A and B) was transiently increased in trilostane-treated versus control follicles. Relative to controls, trilostane treatment blocked the gonadotropin surge-induced increase in GC PAI-1 and PAI-2 mRNAs in follicles collected 24-h post-GnRH injection ($P<0.05$; Fig. 6C and D). In contrast to the observed effects of trilostane administration on uPA, PAI-1, and PAI-2 mRNAs, no effects of intrafollicular trilostane administration on tPA, uPA, and plasmin activities in the follicular apex, base, or follicular fluid were observed (Fig. 7).

Discussion

A role for increased intrafollicular progesterone in ovulation has been established in several species, including rat (Snyder *et al.* 1984, Brannstrom & Janson 1989, Espey *et al.* 1990), sheep (Murdoch *et al.* 1986), and monkey (Hibbert *et al.* 1996, Chaffin & Stouffer 2002). Furthermore, a requirement of the progesterone receptor for ovulation in mice has been demonstrated using gene targeting technology (Lydon *et al.* 1995, 1996). Although data in the mouse support a potential

Figure 5 Changes in tissue inhibitor of metalloproteinases-4 (TIMP-4) mRNA abundance in granulosa cells (GC) and thecal tissue (TC) and TIMP-4 protein in the apex and base of bovine preovulatory follicles following intrafollicular trilostane (TRI) injection. Abundance of TIMP-4 mRNA in GC (A) and TC (B) was normalized relative to that of RPL-19 mRNA. Relative mRNA abundance at 12 h ($n=3$ and 5 for CON and TRI respectively) and 24 h ($n=5$ and 6 for CON and TRI respectively) was calculated using $\Delta\Delta CT$ method. Amounts of TIMP-4 protein in follicle apex (C) and base (D) were normalized relative to amounts of actin and quantified by densitometry. Bars without a common letter are different at $P<0.05$.

role for progesterone-mediated regulation of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) (Robker *et al.* 2000, Mittaz *et al.* 2004) and cathepsin L proteinases in the ovulatory process (Robker *et al.* 2000), the intrafollicular targets of progesterone action that mediate the follicular extracellular matrix remodeling leading to follicle rupture are not completely understood. Using the bovine model system and ultrasound-mediated intrafollicular injection procedures, we determined the effect of inhibition of the preovulatory increase in intrafollicular progesterone (through intrafollicular trilostane injection) on follicle rupture, luteinization/luteal development, and expression of select regulators of follicular extracellular matrix remodeling, and prostaglandin biosynthesis and metabolism in bovine preovulatory follicles. Surprisingly, results of the present studies suggest that the preovulatory increase in intrafollicular progesterone *per se* is not obligatory for ovulation or gonadotropin surge-induced regulation of mRNA and activity/protein for the majority of the matrix-degrading proteinases and their inhibitors previously linked to the ovulatory process in cattle. Results, however, do suggest that

increased follicular fluid progesterone may play a role in preovulatory regulation of intrafollicular PGE₂ and PGF_{2 α} concentrations and mRNA for the PGDH enzyme involved in prostanoid metabolism (Tai *et al.* 2002).

There is significant evidence supporting progesterone responsiveness of GC from bovine preovulatory follicles. Progesterone receptor mRNA is transiently upregulated in bovine follicles within 6 h following GnRH injection to induce the preovulatory gonadotropin surge (Cassar *et al.* 2002, Jo *et al.* 2002) and was localized primarily to the GC layer of preovulatory follicles (Cassar *et al.* 2002). Oxytocin mRNA is induced in the GC layer of bovine follicles following the LH surge and is correlated with hormonal events of the preovulatory period associated with luteinization (Voss & Fortune 1992). Induction of oxytocin mRNA expression by luteinized bovine GC can be blocked by progesterone receptor antagonists *in vitro* (Lioutas *et al.* 1997, Jo *et al.* 2002). Given widespread reports of a role for progesterone/progesterone receptor in the ovulatory process in multiple species (Snyder *et al.* 1984, Murdoch *et al.* 1986, Brannstrom & Janson 1989, Espey *et al.* 1990,

Figure 6 Quantitative real-time PCR analysis of the effect of trilostane (TRI) injection on urokinase plasminogen activator (uPA) and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) mRNA abundance in granulosa cells (GC) and thecal tissue (TC) of bovine preovulatory follicles. Abundance of uPA (A and B), PAI-1 (C), and PAI-2 (D) mRNA was normalized relative to that of RPL-19 mRNA. Relative mRNA abundance at 12 h ($n=3$ and 5 for CON and TRI respectively) and 24 h ($n=5$ and 6 for CON and TRI respectively) was calculated using $\Delta\Delta CT$ method. Data are shown as the LS mean $\pm$ pooled S.E.M. Bars without a common letter are different at $P<0.05$.

Lydon *et al.* 1995, 1996, Hibbert *et al.* 1996, Chaffin & Stouffer 2002), it is unlikely that progesterone plays no role in the ovulatory process in cattle. Instead, it seems plausible that while significantly reduced below control levels, concentrations of progesterone present in trilostane-treated bovine follicles were still sufficient to transactivate the progesterone receptor and stimulate expression of progesterone responsive genes. Concentrations of progesterone detected in follicular fluid of trilostane-treated follicles are orders of magnitude greater than progesterone concentrations in serum during the luteal phase, which are sufficient to stimulate progesterone-responsive genes in target tissues. The observed lack of an effect of trilostane administration on GC oxytocin mRNA abundance in the present studies would support this conclusion, given the above-described negative effect of progesterone receptor antagonists on GC oxytocin mRNA (Lioutas *et al.* 1997, Jo *et al.* 2002). Moreover, it has been shown that the LH surge induces both an early and a late increase in progesterone in bovine follicular fluid (Dieleman *et al.* 1983). Although trilostane blocked/delayed the presumed second rise in intrafollicular progesterone, we cannot conclude that the potential first rise in intrafollicular progesterone was efficiently blocked in the present study. Furthermore,

we cannot discount potential unknown confounding effects of trilostane administration in animals induced to ovulate in response to an LH surge triggered by exogenous GnRH administration (in the present study) versus an endogenous LH surge. Nevertheless, results strongly indicate that the preovulatory increase in intrafollicular progesterone may not be required for ovulation in cattle.

Results in primates indicate that periovulatory progesterone production is required for normal corpus luteum function. Mid-cycle oral administration of trilostane to rhesus monkeys (days 0–2 relative to ovulation induction with human chorionic gonadotropin (hCG)) blocks ovulation and reduces serum progesterone concentrations through day 6 following hCG administration (Hibbert *et al.* 1996). However, progesterone receptor antagonist treatment of cultured bovine luteal cells does not support a role for progesterone in regulation of its own biosynthesis in cattle (Rueda *et al.* 2000). In the present studies, no effect of intrafollicular trilostane administration on GC oxytocin mRNA abundance and serum progesterone concentrations through approximately day 9 of the subsequent luteal phase was noted, indicating that increased intrafollicular progesterone is likely not required for normal luteinization/luteal development in cattle.

Figure 7 Zymographic analysis of the effect of intrafollicular trilostane injection on tPA, uPA, and plasmin activity in follicle apex, base, and follicular fluid of bovine preovulatory follicles collected at 12-h post-GnRH injection. Effects of TRI injection on the activity of tPA, uPA, and/or plasmin in follicle apex (A), base (B), and follicular fluid (C) were analyzed by densitometry ($n=3$ and 5 for CON and TRI respectively). Data are shown as LS mean $\pm$ S.E.M. Bars without a common letter (A and B: lower case for tPA and upper case for uPA; C: lower case for tPA and upper case for plasmin) are different at $P<0.05$. CON, diluent-treated follicles; TRI, trilostane-treated follicles.

Prostanoids play an obligatory role in the ovulatory process in numerous species (Murdoch *et al.* 1986, Dinchuk *et al.* 1995, Lim *et al.* 1997, Mikuni *et al.* 1998) including cattle (De Silva & Reeves 1985, Peters *et al.* 2004). A reduction in follicular fluid PGE₂ and PGF_{2 α} concentrations was observed in trilostane-treated follicles in the present studies, suggestive of a role for progesterone in the regulation of preovulatory prostanoid levels. Intrafollicular administration of the progesterone synthesis inhibitor isoxazol in sheep results in the maintenance of elevated PGE₂ concentrations, but a

reduction in PGF_{2 α} concentrations in the follicle wall within the last 4 to 8-h of anticipated time of follicle rupture (Murdoch *et al.* 1986). However, in contrast to results of the present studies, isoxazol treatment of bovine follicles caused a pronounced inhibition of ovulation (Murdoch *et al.* 1986). Our results in cattle support a potential role for increased intrafollicular progesterone in promoting maintenance of elevated intrafollicular PGE₂ and PGF_{2 α} concentrations prior to ovulation.

Observed reductions in follicular fluid PGE₂ and PGF_{2 α} concentrations in response to intrafollicular trilostane injection may be due to modulation of mRNA abundance for genes regulating prostaglandin biosynthesis and (or) metabolism. Abundance of mRNA for COX-1 and COX-2 was not affected by intrafollicular trilostane administration. Unlike the effect of trilostane, the progesterone receptor antagonist mifepristone inhibited LH-induced expression of cyclooxygenase-2 mRNA and PGE₂ and PGF_{2 α} secretion by granulosa cells (Bridges *et al.* 2006). However, intrafollicular trilostane administration increased TC mRNA abundance for PGDH at the 12-h timepoint to levels similar to those observed at time 0 and caused a further increase in GC PGDH mRNA abundance at the 24-h timepoint. Hormonal and cell-specific regulation of PGDH has been examined previously in a primate model, with results distinct from those observed in the present studies. A transient increase in PGDH mRNA was observed in monkey granulosa cells collected 12 h after hCG administration and PGDH protein was localized exclusively to the granulosa layer (Duffy *et al.* 2005). *In vitro* progesterone treatment of cultured monkey granulosa cells for 24 h did not change PGDH mRNA levels, but 48-h exposure resulted in reduced PGDH mRNA abundance (Duffy *et al.* 2005). Collectively, results of the present studies suggest that increased expression of PGDH, but not COX-1 and COX-2 mRNAs, in GC and TC could contribute to the observed reduction in intrafollicular PGE₂ and PGF_{2 α} in trilostane-treated follicles.

It is unclear why the observed decrease in intrafollicular prostaglandins in trilostane-treated follicles was not sufficient to block ovulation in the present studies. In the present study, follicular fluid PGE₂ concentrations at 24-h post-GnRH injection were >20 -fold higher in trilostane-treated follicles than observed in indomethacin-treated follicles (at the same timepoint) in a previous study where dynamic changes in protein abundance/activity and (or) mRNA for multiple MMP and PA system components were observed (Li *et al.* 2006). Although more variable, the concentrations of PGE₂ in follicles treated with the selective COX-2 inhibitor NS-398 (where a reduction in ovulation rate was noted) were similar (De Silva & Reeves 1985, Peters *et al.* 2004) to those observed following intrafollicular trilostane administration in the present studies. It is possible that the precise timing of the reduction in intrafollicular PGE₂, potentially mediated through the regulation of PGDH metabolism, may have been insufficient to inhibit ovulation in the present studies. Consistent with this hypothesis, the effects of intrafollicular

trilostane administration did not mimic changes in protein abundance/activity and (or) mRNA for MMP and PA system components observed in previous studies where intrafollicular synthesis of prostanoids was inhibited by intrafollicular indomethacin administration (Li *et al.* 2006) and discordant effects of trilostane on mRNA versus protein/activity were observed in some instances (e.g. TIMP-4).

While inhibition of the preovulatory increase in intrafollicular progesterone did not block follicle rupture, subsequent luteal development or gonadotropin surge-induced regulation of mRNA and (or) protein/activity for the majority of the matrix-degrading proteinases and their inhibitors potentially linked to the ovulatory process in cattle, paradoxical effects of intrafollicular trilostane administration on follicular fluid PGE₂ and PGF_{2α} concentrations, and abundance of mRNA for PGDH, TIMP-4, uPA, PAI-1, and PAI-2 were observed. The mechanisms responsible for the above-observed effects of trilostane administration, and lack of an effect of trilostane administration on established progesterone receptor-mediated pathways in bovine follicles (e.g. oxytocin expression) are unclear. While observed responses are most likely attributed to the inhibition of the intrafollicular increase in progesterone, trilostane administration also enhanced the rate of the preovulatory decrease in follicular fluid estradiol concentrations, presumably by an enhanced reduction in the availability of TC precursors for GC estradiol biosynthesis. It is unlikely that the observed effects of trilostane administration are due to accelerated depletion of intrafollicular estradiol. Follicular fluid estradiol concentrations in trilostane-treated bovine follicles were still over 1000-fold greater than circulating estradiol concentrations measured at similar timepoints (Sartori *et al.* 2004), which are known to trigger the preovulatory GnRH/LH surge. Observed effects of blocking the preovulatory increase in intrafollicular progesterone could also be due to inhibition of nonclassical progesterone-mediated responses requiring high concentrations of progesterone that are independent of the classical progesterone receptor. Microgram quantities of progesterone can induce expression of steroidogenic acute regulatory protein mRNA and protein in MA-10 Leydig tumor cells, which lack the classical form of the progesterone receptor (Schwarzenbach *et al.* 2003).

In summary, results of the present studies indicate that the preovulatory increase in intrafollicular progesterone may not be required for follicle rupture, subsequent luteal development, or gonadotropin surge-induced regulation of the majority of the matrix-degrading proteinases, and their inhibitors previously linked to the ovulatory process in cattle. Future studies will be required to determine the mechanisms responsible for observed paradoxical effects of inhibition of the preovulatory increase in progesterone and to determine the effects of *in vivo* antagonism of intrafollicular progesterone receptor-mediated pathways on follicle rupture, luteinization, and regulation of intrafollicular mediators linked to the ovulatory process in cattle.

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