Effect of intrafollicular indomethacin injection on gonadotropin surge-induced expression of select extracellular matrix degrading enzymes and their inhibitors in bovine preovulatory follicles

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Abstract

A growing body of evidence supports an obligatory role for intrafollicular prostanoids in the mechanism of ovulation. However, the prostanoid-dependent mediators of the follicular extracellular matrix degradation required for ovulation are unknown. The objectives of this study were to determine the cellular compartment(s) in which the gonadotropin surge-induced regulation of select extracellular matrix degrading enzymes and their cognate inhibitors occurs in bovine preovulatory follicles, and to test whether such regulation is blocked by intrafollicular administration of the prostanoid synthesis and ovulation inhibitor, indomethacin (INDO). Follicular fluid prostaglandin E₂ concentrations were elevated in diluent-treated follicles before ovulation (24 h after GnRH injection), but the increase was blocked in INDO-treated follicles. Real-time PCR analysis revealed the specific follicular cell types where gonadotropin surge-induced increases in mRNA abundance for members of the matrix metalloproteinase/tissue inhibitor of metalloproteinase and plasminogen activator families occurred. INDO treatment increased thecal cell mRNA for tissue inhibitor of metalloproteinase-4 and its protein abundance in the apex of preovulatory follicles before ovulation, but suppressed granulosa cell mRNA and activity for tissue plasminogen activator in follicular fluid and the follicle apex. Plasmin activity was also suppressed in the follicular fluid of INDO-treated follicles. Effects of INDO injection on select matrix metalloproteinases were not observed. The results suggest that gonadotropin surge-induced regulation of tissue inhibitor of metalloproteinase-4 and tissue plasminogen activator may be prostanoid dependent, and support a potential role for increased tissue plasminogen activator expression and decreased tissue inhibitor of metalloproteinase-4 expression in the mechanism of ovulation.

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Introduction

One of the hallmarks of ovulation is the extensive tissue remodeling that occurs at the follicular apex versus the base (Murdoch & McCormick 1992). The preovulatory luteinizing hormone (LH) surge initiates a cascade of proteolytic activity controlling the tissue-remodeling process during ovulation (Robker *et al.* 2000). Two principal families of proteolytic enzymes, the matrix metalloproteinases (MMPs) and the plasminogen activators (PAs)/plasmin, are implicated in the tissue remodeling accompanying the ovulatory process (Smith *et al.* 2002). The MMPs are a comprehensive family of at least 28

zinc-containing endopeptidases, which can be classified into collagenases, gelatinases, stromelysins, membrane type and other MMPs by their structure, substrate specificity and subcellular distribution (Nagase & Woessner 1999, Curry & Osteen 2003). The activity of MMPs in the extracellular milieu is regulated by their tissue inhibitors (TIMPs 1-4), which bind reversibly to MMPs in a 1:1 stoichiometry and inhibit their activity (Gomez *et al.* 1997, Brew *et al.* 2000, Murdoch & Gottsch 2003). The PA system consists of the ubiquitous zymogen plasminogen, which can be activated to the proteolytic enzyme plasmin by two distinct forms of PA (tissue-type PA (tPA) and urokinase-type PA (uPA)) (Tsafriri & Reich 1999,

Ny *et al.* 2002). PA activity is regulated by specific PA inhibitors (PAI-1 and PAI-2) and the urokinase receptor (uPAR), a cell-surface glycoprotein that binds both PAs (Plow *et al.* 1986, Ny *et al.* 2002) and focalizes plasmin activity to the cell surface or pericellular space. In addition to a direct role in extracellular matrix (ECM) degradation (Mignatti *et al.* 1986, Alexander & Werb 1991), plasmin can activate the pro-form of several MMPs (DeClerck & Laug 1996, Murphy *et al.* 1999), underscoring the potential for synergism/cooperativity between the two proteinase families during the ovulatory process.

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). The cyclooxygenase (COX)-2 enzyme, which catalyzes the rate-limiting step in intrafollicular prostanoid synthesis, is induced within the granulosa layer of rat (Sirois *et al.* 1992), equine (Sirois & Dore 1997) and bovine preovulatory follicles (Sirois 1994, Tsai *et al.* 1996) in response to the LH surge. Indomethacin (INDO), an inhibitor of the COX pathway, is a potent inhibitor of ovulation in numerous species (Tsafiri *et al.* 1972, Murdoch *et al.* 1986, Sogn *et al.* 1987), including cattle (De Silva & Reeves 1985). Mice with a targeted mutation in the COX-2 gene are infertile (Dinchuk *et al.* 1995) and anovulatory (Davis *et al.* 1999), potentially due to a proteoglycan synthesis-related alteration in cumulus expansion and impaired follicle rupture. Despite the well-established role of prostanoids in ovulation, the downstream mediators of prostanoid-stimulated follicle rupture remain largely undefined, especially in monotocous species such as cattle. However, several lines of evidence indicate that prostanoids are potent regulators of various MMP and PA system components in other cell types/model systems (Strickland & Beers 1976, Liu *et al.* 2001, Negoro *et al.* 2002, Ito *et al.* 2004a,b). For example, prostanoid regulation of MMP-13 was reported previously in mouse calvarial cultures, and prostaglandin (PG) E_2 -induced MMP-13 production was impaired in calvarial cultures from EP $_4$ (PGE $_2$ receptor subtype)-knockout mice (Miyaura *et al.* 2000).

We have previously reported increased expression of several key MMP and PA system components in bovine preovulatory follicles following the gonadotropin surge, including MMP-13 (Bakke *et al.* 2004), MMP-14 (Bakke *et al.* 2002), TIMPs 1-4 (Bakke *et al.* 2002, Li *et al.* 2004), tPA, uPA, uPAR (Dow *et al.* 2002a), PAI-1 and PAI-2 (Smith *et al.* 1996, Dow *et al.* 2002b). Furthermore, the preovulatory regulation of MMP-28 in bovine follicles remains undefined. MMP-28, or epilysin, is one of the most recently identified MMPs and belongs to the MMP-19 subfamily of the MMP superfamily (Lohi *et al.* 2001, Marchenko & Strongin 2001). MMP-28 has been implicated in several physiologic events including embryo implantation (Li *et al.* 2003), but its role in the ovulatory process is not known. In addition, the intrafollicular cell types where gonadotropin surge-induced regulation of the above enzymes and

inhibitors takes place are unclear. The above studies of changes in mRNA abundance after the gonadotropin surge were conducted with RNA isolated from whole follicles, and changes in mRNA abundance in individual cell types were not examined. Furthermore, in view of the obligatory involvement of prostanoids and ECM proteolysis in the ovulatory process, we hypothesize that the above regulators of ECM remodeling are potential downstream mediators of prostanoid-induced follicle rupture. The objectives of the current study were to determine:

- the cellular compartments (granulosa cells and/or thecal cells) in which the gonadotropin surge-induced regulation of the above select ECM degrading enzymes and their cognate inhibitors occurs in bovine preovulatory follicles
- whether the above regulation is blocked by intrafollicular administration of the prostanoid synthesis and ovulation inhibitor INDO in dairy cattle.

Materials and Methods

Animal care and experimental model

All animal procedures were approved by the All University Committee on Animal Use and Care at Michigan State University. The experimental model utilized in this study has been described elsewhere (Bakke *et al.* 2002, 2004). Follicular development and timing of the preovulatory gonadotropin surge were synchronized in nonlactating Holstein cows by the Ovsynch (GnRH-7d-PGF $_{2\alpha}$ -36-h-GnRH) procedure (Pursley *et al.* 1995, 1997) with a slight modification. Briefly, gonadotropin-releasing hormone (GnRH) was injected to start a new wave of follicular growth and thus a new dominant follicle. Then, PGF $_{2\alpha}$ was given to regress the corpus luteum 6.5 days later. A second GnRH injection was given 36 h later to induce a gonadotropin surge resulting in ovulation of the new dominant follicle. Synchronized ovulation of the dominant follicle occurs an average of 29 h after the second GnRH injection (Pursley *et al.* 1995). Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection to verify follicle synchrony and to exclude animals that exhibited a new follicular wave prior to the second GnRH injection (two animals were excluded) or follicles that collapsed after intrafollicular injection (no animals were excluded). INDO, in a volume of 100 μ l (200 μ g/ml final intrafollicular concentration), or the same volume of PBS (control; CON) was injected into preovulatory follicles immediately after the second GnRH injection by previously described ultrasound-mediated intrafollicular injection procedures (Peters *et al.* 2004). The dose of INDO selected was determined by the results of a preliminary dose-response experiment that compared the ability of various doses of INDO (0, 200 and 1000 μ g/ml) to block gonadotropin surge-induced increase in follicular fluid (FF) PGE $_2$ measured at 24 h after GnRH ($n = 5$ per treatment).

The concentration of INDO to administer intrafollicularly (in 100 μ l volume) in each individual follicle to achieve the desired FF concentration was calculated mathematically after ultrasonographic measurement of follicle diameter immediately before injection.

Sample collection and preparation

Ovaries containing preovulatory follicles were collected (under epidural anesthesia) by colpotomy (Drost *et al.* 1992) at 0 ($n = 5$), 12 (CON; $n = 3$) and 24 h (INDO and CON; $n = 5$ each) after the second GnRH injection. Because of the time course of induction of the COX-2 enzyme (Sirois 1994) and accompanying increase in FF PGE₂ (Peters *et al.* 2004), INDO-treated follicles were not collected at 12 h after GnRH injection. FF was harvested from each follicle by aspiration and centrifugation to remove possible blood cell contamination. INDO was then added to FF to achieve a final concentration of 3.6 μ g/ml before being frozen on dry ice and stored at -80°C . INDO was added, according to the manufacturer's recommendations for PGE₂ assay (see below), to prevent *ex vivo* generation of eicosanoids. To examine changes in mRNA abundance for genes of interest within specific follicular cell compartments during the preovulatory period, follicles were dissected as described elsewhere (Bakke *et al.* 2002, Li *et al.* 2004) and then sagittally cut into four portions (approximately 3–5 mm wide) containing both apex and base. Granulosa cells (GC) and thecal tissue (TC) were isolated from one portion. GCs were scraped and collected by centrifugation, while TC was isolated by dissection from a portion of the follicle. A volume of 1 ml TRIzol (Life Technologies, Gaithersburg, MD, USA) was added to the GC immediately after collection. Both GC and TC were snap-frozen in liquid nitrogen and stored at -80°C for total RNA isolation and real-time PCR assay. Since the ECM degradation characteristic of the ovulatory process occurs primarily in the follicle apex, we were interested to know whether regulation of protein/activity for select ECM degrading enzymes/inhibitors was specific to the follicle apex versus the base. A second portion of the same follicles was separated into two equal subportions (containing the follicle apex or base) as previously described (Dow *et al.* 2002a), snap-frozen separately and kept at -80°C for protein extraction.

FF PGE₂ assay

Concentrations of FF PGE₂ were analyzed by a previously described (Peters *et al.* 2004) PGE₂ EIA (Cayman Chemical, Ann Arbor, MI, USA). FF was diluted 1:50 in EIA buffer before analysis. Intra- and interassay coefficients of variation (CV) were 5.3% and 3.1% respectively. According to the manufacturer, the assay displays <18.1% cross-reactivity with PGE₁ and <0.1% cross-reactivity with other prostanoids.

RNA isolation and reverse transcription

Total RNA was extracted from GC and TC of bovine preovulatory follicles with TRIzol reagent. The yields of RNA were measured by spectrophotometry at 260 nm, and the integrity was evaluated by examination of intensity of 28 S and 18 S ribosomal RNA bands on a nondenaturing agarose gel. To eliminate possible genomic DNA contamination, 1 μ g total RNA from each sample was incubated for 15 min at 25°C with 1 U DNase I (Invitrogen) before reverse transcription. The protocol for reverse transcription using Superscript RNase H⁻ Reverse Transcriptase (Invitrogen) was described previously (Li *et al.* 2004). The concentration of the resulting cDNA in each sample was determined by spectrophotometry. Each sample was diluted to 100 and 10 ng/ μ l in nuclease-free water (Ambion, Austin, TX, USA) and kept at -20°C for real-time PCR analysis.

Real-time PCR

Real-time PCR primers were designed with Primer Express software (Primer Express, Applied Biosystems, Foster City, CA, USA). The optimal primer ratio and the amount of cDNA used were determined in a preliminary experiment. The primers used in this study are listed in Table 1. The amplifications were performed in a 96-well optical plate (Applied Biosystems) in 25 μ l reaction volume containing 12.5 μ l SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA samples, the appropriate amount of primers (Table 1) for each gene and nuclease-free water. Nontemplate controls (RNase-free water) for each primer set were included. The real-time PCR reaction was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the thermal cycler program consisting of 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantification was performed by the comparative threshold cycle (CT) method established by Livak and Schmittgen (2001). The amount of transcripts for each gene was normalized to the endogenous reference (RPL-19 mRNA). The mean CT of each gene analyzed and endogenous control was calculated for each sample from duplicate wells. ΔCT was produced by subtracting the mean CT of RPL-19 from the CT of each target gene. Fold changes in the relative mRNA abundance for target genes in 12- and 24-h samples relative to 0 h were determined by the following formula: $2^{-\Delta\Delta\text{CT}}$, where $\Delta\Delta\text{CT} = \Delta\text{CT}_{12\text{- or } 24\text{-h sample}} - \Delta\text{CT}_{0\text{-h sample}}$. A similar amplification efficiency of target genes relative to endogenous reference (RPL-19) was determined in preliminary validation experiments. Pooled samples of TC and GC cDNA were used. Five serial dilutions of cDNA from pooled samples (2.5, 5, 10, 20 and 40 ng) were assayed for individual target genes and for RPL-19, and parallel slopes for amplification profiles for target genes of interest relative to RPL-19 confirmed.

Table 1 Real-time RT-PCR primer sequences utilized for quantification of mRNA for select extracellular matrix degrading proteinases and their cognate inhibitors.

Gene name	GenBank accession no.	Primer sequence	*Primer ratio (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: GCACATCACCACTGCAGTT Reverse: CCGGCGCTGAGCAGAA	50/50
TIMP-2	AF144764	Forward: GGGCTGTGAGTGCAAGATCA Reverse: CTCGTCCGGAGAGGAGATGTAG	300/300
TIMP-3	U77588	Forward: GATGTACCGAGGATTACCAAGA Reverse: TTAAGGCCACAGAGACTTTCAGAA	300/900
TIMP-4	AF037273	Forward: GAAAGTCTGATCACCGCTACCA Reverse: CAGGGCACCGCATAGCA	900/300
tPA	X85800	Forward: ACGTCAGGTTGTACCCATCCA Reverse: GTTGTGGTGACGGTCTCTGTT	300/300
uPA	X85801	Forward: GCCGTGACTGTGCCATGA Reverse: GTGTTGATCCAGGGCAGGAA	300/300
uPAR	S70635	Forward: GCCGCGGCCCTATGA Reverse: GCTTGGGTTCCTCAGTCCTTT	300/300
PAI-1	X16383	Forward: TGGGCCCCGTGGAACAA Reverse: AGGTCCCCGTGCACGAA	300/300
PAI-2	AF416234	Forward: CCATATGCTGGCGATGTCAGT Reverse: GCCCGTAGAGGATTACGAA	300/300
RPL-19	XM_587778	Forward: CAGACGATACCGTGAATCTAAGAAGA Reverse: TGAGAATCCGCTTGTTTTGAA	300/300

* Forward/reverse primer ratio was determined by primer matrix analysis.

Protein extraction

Homogenates of follicle apex and base were prepared as described previously (Bakke *et al.* 2002). Briefly, the apical or basal portions of each follicle were homogenized in 300 µl homogenization buffer (10 mM calcium chloride and 0.25% Triton X-100). The homogenates were then centrifuged at 9000 *g* for 30 min at 4 °C. The supernatants were collected, and protein concentration was determined by Lowry's method (Lowry *et al.* 1951). Aliquots of proteins were frozen at –20 °C until analyzed.

Immunoblot analysis

Immunoblot analysis was conducted for quantification of TIMP-4 protein by our previously described protocol (Li *et al.* 2004). Membranes were stripped and probed with the actin antibody for normalization and quantification (Li *et al.* 2004). Rabbit antihuman TIMP-4 polyclonal antibody and mouse antihuman actin monoclonal antibody were obtained from Chemicon (Temecula, CA, USA), while donkey antirabbit and goat antimouse antibodies were purchased from Amersham Biosciences (Bucks, UK) and GenoTech (St Louis, MO, USA) respectively.

Casein zymography

The activity of tPA, uPA, and plasmin in the follicle apex, base and FF was measured by casein zymography, as described previously (Dow *et al.* 2002a). A homogenate

of bovine cerebellum served as a positive control for tPA activity and bovine ovarian surface epithelial cell-conditioned medium as a positive control for uPA activity (Dow *et al.* 2002a).

Statistical analysis

Differences in PGE₂ concentration, mRNA abundance, protein amounts and enzymatic activities among different time points/groups were determined by one-way analysis of variance (ANOVA) with the general linear model procedure of the Statistical Analysis System (Version 8, SAS Institute, Cary, NC, USA). When heterogeneity of variance was observed, data were log transformed before statistical analysis. Individual comparisons were performed with Tukey's test. Data are reported as least-square (LS) mean ± pooled s.e.m. *P* < 0.05 was considered statistically significant.

Results

Reduced FF PGE₂ after intrafollicular INDO injection

FF PGE₂ concentrations were low in 0-h preovulatory follicle samples collected before GnRH injection, but were markedly increased at 24 h after GnRH administration (*P* < 0.01) (Fig. 1). A dose of 200 µg/ml INDO was selected for intrafollicular injection, on the basis of the results of a preliminary dose–response experiment

Figure 1 Effect of intrafollicular indomethacin (INDO) injection on concentrations of follicular fluid PGE₂ in preovulatory follicles collected at 0 h (CON), 24 h (CON) and 24 h (INDO) relative to GnRH injection ($n = 5$ each). Each bar represents the least-square (LS) mean $\pm$ pooled S.E.M. Bars without a common superscript are different at $P < 0.01$. CON: control; diluent-treated follicles; INDO: indomethacin-treated follicles.

(diluent control = 72 ng/ml PGE₂; 200 μ g/ml INDO = 130 pg/ml PGE₂; 1000 μ g/ml INDO = 40 pg/ml PGE₂) comparing the ability of two doses of INDO to suppress the gonadotropin surge-induced increase in FF PGE₂ (measured as an index of intrafollicular prostanoid synthesis) at 24 h after GnRH injection. Intrafollicular injection of INDO blocked the gonadotropin surge-induced increase in FF PGE₂ at 24 h after GnRH injection ($P < 0.01$) (Fig. 1), resulting in concentrations similar to those observed at the 0-h time point.

Changes in MMP and PA system component mRNAs in specific follicular cell compartments (GC and TC) after the preovulatory gonadotropin surge

Because the intrafollicular cell types responsible for increases in select MMP and PA system components in bovine preovulatory follicles are not known, we examined the effect of the preovulatory gonadotropin surge on mRNA abundance for select MMP and PA and their inhibitors specifically within GC and TC. MMP-13 transcripts were mainly detected in TC, while mRNAs for TIMP-2, tPA and PAI-2 were mainly in GC. Messenger RNAs for MMP-14, MMP-28, TIMP-1, TIMP-3, TIMP-4, uPA, uPAR and PAI-1 were detected in both GC and TC. In the GC compartment, TIMP-3, tPA, uPA, uPAR and PAI-1 mRNAs were increased at both 12 and 24 h after GnRH injection ($P < 0.05$) (Fig. 2F and H–K). In contrast, elevated transcript abundance for MMP-14, TIMP-1, TIMP-2 and TIMP-4 was observed only at 12 h ($P < 0.05$) (Fig. 2B, D, E and G); Abundance of GC MMP-28 and PAI-2 mRNAs was increased only at 24 h ($P < 0.05$) (Fig. 2C and L). In the TC compartment, MMP-13, uPA, uPAR and PAI-1 mRNAs were elevated at both time points examined ($P < 0.05$)

(Fig. 2A and I–K). TIMP-1 and TIMP-4 mRNAs in TC were upregulated only at 12 h ($P < 0.05$) (Fig. 2D and G), while a significant increase in TC mRNA abundance for MMP-14 was detected only at 24 h after GnRH injection ($P < 0.05$) (Fig. 2B). No changes in MMP-28 and TIMP-3 mRNAs in TC were observed after GnRH injection ($P > 0.05$) (Fig. 2C and F).

Effect of intrafollicular administration of INDO on GC and TC MMP and PA system component mRNA abundance

Among the seven MMPs and TIMPs examined (MMP-13, MMP-14, MMP-28 and TIMPs 1–4), only abundance of TIMP-4 transcripts was increased in TC of INDO-treated animals ($P < 0.05$) (Fig. 3A). Effects of INDO on mRNA abundance for other MMPs and TIMPs were not detected (data not shown). For the PA system components, intrafollicular administration of INDO significantly decreased tPA, PAI-1 and PAI-2 mRNA abundance in GC ($P < 0.05$) (Fig. 3B, E and F) and uPA, uPAR and PAI-1 ($P < 0.05$) (Fig. 3C–E) mRNA abundance in TC.

Effect of intrafollicular administration of INDO on amount of TIMP-4 protein in the follicle apex and base

Immunoblot analysis was utilized to determine whether changes in abundance of TIMP-4 protein mirror changes in mRNA abundance observed in INDO-treated follicles and whether regulation was specific to the follicular apex. The TIMP-4 antibody recognized a single band of 28 000 Da in bovine preovulatory follicles. Amounts of TIMP-4 protein were increased in the apex ($P < 0.05$) (Fig. 4), but not the base (data not shown), in INDO-treated versus control follicles.

Effect of intrafollicular INDO injection on follicular tPA, uPA and plasmin activity

Activities for both tPA (single band) and uPA (double bands; presumably corresponding to the single- and two-chain forms of uPA) were detected in the follicle apex and base (Fig. 5A, lanes 1 and 4). Plasmin and tPA activity was detected in FF (Fig. 5A, lane 5). Very minor activity for plasmin in follicle homogenates and uPA in FF was observed (data not shown). Activity for tPA, uPA and plasmin was not observed when plasminogen was omitted from gels (data not shown). Incubation of gels in the presence of amiloride, a specific uPA inhibitor, dramatically attenuated activity corresponding to uPA (Fig. 5A, lane 2), and incubation with aprotinin, the plasmin inhibitor, attenuated both PA activity in follicle homogenates (Fig. 5A, lane 3) and plasmin activity in FF (data not shown). In the follicle apex, tPA activity was significantly decreased in response to INDO treatment ($P < 0.05$) (Fig. 5B). In the follicle base, neither tPA nor uPA activities were significantly affected by INDO treatment (data not

Figure 2 Real-time PCR analysis of changes in MMP and PA system component mRNA abundance in GC and/or TC of bovine preovulatory follicles after the gonadotropin surge. Abundance of MMP-13 (A), MMP-14 (B), MMP-28 (C), TIMP-1 (D), TIMP-2 (E), TIMP-3 (F), TIMP-4 (G), tPA (H), uPA (I), uPAR (J), PAI-1 (K) and PAI-2 (L) mRNAs was normalized relative to that of RPL-19 mRNA. Relative mRNA abundance at 0 h ($n = 5$), 12 h ($n = 3$) and 24 h ($n = 5$) relative to GnRH injection was calculated by the $\Delta\Delta CT$ method. Data are shown as least-square (LS) mean $\pm$ pooled S.E.M. Time points without a common superscript (lower case for GC and upper case for TC) are different at $P < 0.05$. GC: granulosa cell; TC: theca cell.

shown). In FF, plasmin and tPA activity were both reduced in INDO-treated follicles versus control follicles ($P < 0.05$) (Fig. 5C).

Discussion

Despite the well-established role of prostanoids in ovulation, the downstream targets via which prostanoids help

promote the ovulatory process remain to be completely illustrated. The present studies tested the hypothesis that the gonadotropin surge-induced regulation of select ECM-degrading enzymes and their inhibitors potentially involved in ovulation is blocked by local administration of the prostanoid synthesis and ovulation inhibitor INDO. We showed for the first time that intrafollicular INDO injection *in vivo* resulted in increased TC mRNA

Figure 3 Real-time PCR analysis of the effect of INDO injection on MMP and PA system component mRNA abundance in GC and/or TC of bovine preovulatory follicles. Abundance of TIMP-4 (A), tPA (B), uPA (C), uPAR (D), PAI-1 (E) and PAI-2 (F) mRNAs was normalized relative to that of RPL-19 mRNA. Relative mRNA abundance at 24 h after GnRH injection for the CON ($n = 5$) and INDO-treated groups ($n = 5$) was calculated by the $\Delta\Delta\text{CT}$ method. Data are shown as least-square (LS) mean $\pm$ pooled S.E.M. Bars without a common superscript (lower case for GC and upper case for TC) are different at $P < 0.05$. CON: control; diluent-treated follicles; INDO: INDO-treated follicles; GC: granulosa cell; TC: thecal tissue.

abundance for TIMP-4 and increased TIMP-4 protein in the apex of bovine preovulatory follicles before ovulation, and suppressed GC mRNA and activity for tPA in FF and the follicle apex, and plasmin activity in FF. Given the key role of prostanoids in the ovulatory process (Murdoch *et al.* 1986, Dinchuk *et al.* 1995, Lim *et al.* 1997, Mikuni *et al.* 1998, Peters *et al.* 2004), results from the current study support a potential functional role for prostanoid-mediated suppression of TIMP-4 and maintenance of elevated tPA expression in the mechanism of ovulation.

We acknowledge that we cannot exclusively attribute the effects of INDO administration on expression of TIMP-4 and tPA specifically to absence of a specific prostanoid and/or to inhibition of COX-2 activity. Prostanoids are synthesized from arachidonic acid by the action of COX-1 and 2, and COX inhibitors, including INDO, inhibit ovulation both *in vivo* and *in vitro* (De Silva & Reeves 1985, Mikuni *et al.* 1998, Gaytan *et al.* 2002, Peters *et al.* 2004). Measurement of PGE_2 was used as an index of intrafollicular prostanoid synthesis in the present studies. Although FF concentrations of PGE_2 and $\text{PGF}_{2\alpha}$ both increase after the LH surge in cattle (Sirois 1994), the time course of induction of the $\text{PGF}_{2\alpha}$ receptor (FP receptor) is not consistent with a role in bovine ovulation (Tsai *et al.* 1996).

Thus, $\text{PGF}_{2\alpha}$ regulation of TIMP-4 and tPA seems unlikely, despite evidence of $\text{PGF}_{2\alpha}$ regulation of MMP expression in other systems (Weinreb *et al.* 2004). Furthermore, a predominant contribution of COX-1 inhibition to the suppression of PGE_2 observed in the present study seems unlikely. COX-1 immunoreactivity is low or undetectable in bovine preovulatory follicles (Sirois 1994), and FF concentrations of PGE_2 in INDO-treated follicles were not different from the pre-gonadotropin surge concentrations of PGE_2 observed at the 0-h time point.

The current study was built on our previous identification of select MMP and PA system components that are increased in bovine preovulatory follicles after the gonadotropin surge and thus are potential regulators of ovulation. Increased expression of select MMP and PA system components in bovine preovulatory follicles after the LH surge was observed in our previous studies (Smith *et al.* 1996, Bakke *et al.* 2002, 2004, Dow *et al.* 2002a,b, Li *et al.* 2004), primarily using RNA isolated from whole follicles. Cellular localization of expression was determined by qualitative *in situ* hybridization or immunohistochemistry procedures. However, previous studies did not directly quantify the gonadotropin surge-induced regulation of mRNA for select MMP and PA system components

Figure 4 Effect of intrafollicular INDO injection on TIMP-4 protein abundance in homogenates of the apex of bovine preovulatory follicles. Upper panel: representative immunoblots demonstrating a single band of immunoreactivity for TIMP-4 (28 000 Da) in homogenates of follicle apex collected 24 h after GnRH injection. Lower panel: relative mean abundance of TIMP-4 protein derived from analysis of individual samples of homogenates of the follicle apex of INDO-treated versus control follicles ($n = 5$ per time point). Data are shown as least-square (LS) mean $\pm$ pooled S.E.M. Bars without a common superscript are different at $P < 0.05$. CON: control; diluent-treated follicles; INDO: indomethacin-treated follicles.

specifically in the two steroidogenic cell populations of preovulatory follicles that can respond both directly and indirectly to the LH surge. Thus, real-time PCR analysis was utilized in the present studies to examine regulation of MMP and PA system component mRNA abundance specifically in the GC and TC. As a whole, cellular source and the observed temporal changes in mRNA expression in GC and TC agree with previously published data for whole follicles with only a few exceptions (such as increased MMP-13 and PAI-1 mRNAs 12 h after GnRH injection and predominant localization of PAI-1 to the thecal layer). Such discrepancies are not easily explained, but they are probably attributable to the more sensitive and quantitative nature of the real-time PCR procedure used here than that of the dot-blot (Dow *et al.* 2002b) and semiquantitative RT-PCR procedures (Bakke *et al.* 2004) used previously, to differences in the nature of samples collected (whole follicles versus isolated GC and TC in present studies), to differences in the experimental model used in previous studies (Smith *et al.* 1996, Li *et al.* 2004) and degree of follicle synchrony at time of induction of the LH surge, and to the qualitative nature of procedures used previously for determination of cellular localization of mRNA and relative abundance in GC versus TC (Dow *et al.* 2002b).

Among the seven MMPs and TIMPs examined here (MMP-13, MMP-14, MMP-28 and TIMPs 1-4), only mRNA

for TIMP-4 changed significantly in response to INDO treatment. The results of our previous study (Li *et al.* 2004) demonstrated that the gonadotropin surge-induced increase in TIMP-4 mRNA and protein abundance is transient and maximal at 12 h after GnRH injection. We further demonstrated here that abundance of TC TIMP-4 mRNA and amounts of TIMP-4 protein in the follicular apex, but not the base, were increased in response to INDO treatment of preovulatory follicles, suggesting a potential key role for intrafollicular prostanoids in down-regulation of TIMP-4 expression during the window of time immediately before ovulation. Prostanoid-dependent regulation of MMP-14 and MMP-28 has not been reported. Furthermore, the effects of INDO administration on gonadotropin surge-induced expression of mRNA for TIMP family members in the present studies were specific to TIMP-4. An effect of INDO administration on TIMP-1 mRNA in rat ovaries was not observed in previous studies (Curry *et al.* 2000), but an inhibitory effect of $\text{PGF}_{2\alpha}$ on LH-stimulated TIMP-1 mRNA expression in macaque GC *in vitro* has been documented (Duffy & Stouffer 2003). To date, the effects of INDO administration on ovarian TIMP-2, TIMP-3 and TIMP-4 mRNA during the ovulatory process have not been reported in other species.

Unlike TIMPs 1-3, the TIMP-4 promoter region has no AP-1 motif, but contains consensus motifs for Sp1 and an inverted CCAAT box upstream of an initiator-like element that is in close proximity to a transcription start site, both of which have functional importance in TIMP-4 gene expression (Young *et al.* 2002). These unique features of the TIMP-4 promoter might have implications for selective TIMP-4 regulation by prostanoids, but the factors that bind specifically to the TIMP-4 initiator-like element and functional complexes that bind the putative CCAAT and Sp1 motifs are not known (Young *et al.* 2002).

Although coordinate upregulation of both MMPs and TIMPs is common during ECM remodeling (Curry & Osteen 2003), the balance is tipped in favor of MMP activity before ovulation (Curry & Osteen 2003). To our knowledge, the effects of intrafollicular INDO injection *in vivo* on preovulatory ovarian/follicular MMP inhibitor activity have not been reported. We propose that a potential key role for prostanoids in the mechanism of ovulation is to promote downregulation of gonadotropin surge-induced TIMP-4 expression in the follicular apex, in order to shift the MMP/TIMP ratio in favor of MMPs and help regulate, temporally and spatially, ovarian ECM degradation and follicle rupture. Unlike data reported in rodents (Simpson *et al.* 2003), TIMP-4 mRNA and protein are readily detectable in bovine preovulatory follicles, and TIMP-4 mRNA appears to be of similar abundance as mRNAs for TIMP 1-3 (Li *et al.*, unpublished). However, the relative abundance of TIMP-4 protein relative to the other TIMPs and hence the contribution of TIMP-4 to total MMP inhibitor activity in bovine preovulatory follicles remain to be determined.

Figure 5 Casein zymographic analysis of the effect of intrafollicular INDO injection on tPA, uPA and plasmin activity in the apex, base and follicular fluid (FF) of bovine preovulatory follicles collected 24 h after GnRH injection. (A) Representative pictures depict the bands of activity for tPA, uPA and plasmin in follicular apex (lane 1), base (lane 4) and FF (lane 5). Specificity of tPA and uPA activity was confirmed by incubating the gels in the absence or presence of amiloride (uPA specific inhibitor; 1 mM; $\pm$ AM; lanes 1 and 2) or 2 μ g/ml of the plasmin inhibitor aprotinin (+AP; lane 3). Effects of INDO injection on the activity of tPA, uPA and/or plasmin in follicle apex (B) and FF (C) were analyzed by densitometry ($n = 5$ per time point). Data are shown as least-square (LS) mean $\pm$ pooled S.E.M. Bars without a common superscript (panel B: lower case for tPA and upper case for uPA; panel C: upper case for plasmin and lower case for tPA) are different at $P < 0.05$. CON: control; diluent-treated follicles; INDO: indomethacin-treated follicles.

In contrast to MMP system components analyzed, where effects of INDO administration were limited, mRNAs for all the PA system components examined were decreased in GC and/or TC compartments of INDO-injected follicles. Messenger RNA abundance for tPA in GC was decreased. Interestingly, we previously reported differential regulation of tPA activity in the follicle apex versus the base (Dow *et al.* 2002a). Activity for tPA was increased in the follicle apex but remained unchanged in the base 24 h after GnRH injection (Dow *et al.* 2002a). Therefore, it seems that the gonadotropin surge-induced regulation of preovulatory tPA activity is specific to the follicle apex. In the current study, we further demonstrated that tPA activity in the follicle apex, but not the base, was attenuated by INDO injection, and both tPA and plasmin activities in FF were decreased in INDO-treated follicles. The mechanisms responsible for differential effects of INDO administration on tPA activity in the apex versus the base of bovine preovulatory follicles are unclear.

As stated above, given the temporal changes in tPA mRNA and activity previously observed in response to the gonadotropin surge in samples collected before the 24-h time point (Dow *et al.* 2002a), the results of the present studies support a potential role for intrafollicular prostanoids in maintenance of the gonadotropin surge-induced increase in tPA mRNA and activity and FF plasmin activity in bovine preovulatory follicles immediately before ovulation. Our data agree with previous studies from other systems that demonstrated a role for prostanoids in

stimulating tPA mRNA and generation of plasmin activity by glomerular mesangial cells (Coffman *et al.* 1998). Moreover, PGE₁ and PGE₂ stimulated rat GC to produce PA *in vitro* (Strickland & Beers 1976), the predominant enzyme of which migrates with an apparent molecular mass of 75 000 Da (Strickland & Beers 1976), presumably corresponding to tPA.

Another interesting observation derived from the present studies was the selective effects of INDO on mRNA for MMP and PA system components within the two follicular cell compartments (GC and TC) of bovine preovulatory follicles. For example, TIMP-4 mRNA expression in TC, but not GC, was affected by INDO administration, yet GC expression of tPA mRNA was influenced by INDO administration. Due to the lack of knowledge of prostanoid signaling pathways activated during ovulation, and lack of information on prostanoid receptor subtype expression patterns in different follicular cell compartments (TC and GC) during the preovulatory period, we cannot determine from the present studies whether both TC and GC of bovine preovulatory follicles can respond to prostanoids directly, or whether the differential regulation observed is attributable to direct versus indirect effects of prostanoids and/or differential sensitivity of various PG-responsive genes to the signal transduction pathways involved. Furthermore, although the best-established function of INDO is to inhibit COX activity, other roles independent of prostanoid inhibition, including induction of Hsc70 nuclear

translocation (Lagunas *et al.* 2004) and activation of PPAR γ (Tegeder *et al.* 2001), have been demonstrated.

In summary, we have determined the effect of intrafollicular INDO administration on LH surge-induced regulation of select ECM degrading enzymes and their cognate inhibitors previously implicated in the ovulatory process. The present studies clearly demonstrated that gonadotropin surge-induced regulation of TIMP-4 and tPA mRNAs, TIMP-4 protein and tPA activity in the follicle apex, and FF tPA and plasmin activity is blocked by intrafollicular INDO administration in cattle. Collectively, the results illustrate a potential obligatory prostanoid-dependent mechanism for regulation of ovulation in cattle, but further investigation will be required to establish directly an essential role for these mediators of ECM remodeling in the ovulatory process.

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