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Effect of the Preovulatory Gonadotropin Surge on Matrix Metalloproteinase (MMP)-14, MMP-2, and Tissue Inhibitor of Metalloproteinases-2 Expression Within Bovine Periovulatory Follicular and Luteal Tissue[1]

Leanne J. Bakke,[4] Mark P.D. Dow,[3,4] Carolyn A. Cassar,[3] Michael W. Peters,[3] J. Richard Pursley,[3] and George W. Smith[2,3,4]

Departments of Animal Science[3] and Physiology,[4] Michigan State University, East Lansing, Michigan 48824-1225

ABSTRACT

The matrix metalloproteinases (MMPs) have been implicated in the ovulatory process, but the specific roles of individual MMPs are unclear. This study examined the effect of the preovulatory gonadotropin surge on localization and regulation of MMP-2, MMP-14, and tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA and MMP-2 and TIMP-2 activity in bovine preovulatory follicles and new corpora lutea (CL). Ovaries containing ovulatory follicles or new CL were collected at approximately 0, 6, 12, 18, 24, and 48 h (CL) after a GnRH-induced gonadotropin surge. Messenger RNA for TIMP-2 and MMP-14 increased within 6 and 24 h of the gonadotropin surge, respectively, whereas MMP-2 mRNA was constitutively expressed. Activity for MMP-2 in follicular fluid and follicle homogenates was not changed, but follicular fluid TIMP-2 activity increased in response to the gonadotropin surge. Messenger RNA for MMP-2 was localized to the thecal layer of bovine preovulatory follicles, whereas MMP-14 mRNA was localized primarily to the thecal layer and adjacent ovarian stroma. Expression of MMP-14 was also observed in the granulosal layer after the gonadotropin surge. In contrast, TIMP-2 mRNA was localized predominantly to the granulosal layer with intense expression in the antral portion of the granulosal layer in response to the gonadotropin surge. These data support the hypothesis that increased expression of MMP-14 and TIMP-2 may help regulate follicle rupture and/or the ovulatory follicle-CL transition in cattle.

follicle, granulosa cells, ovary, ovulation, theca cells

INTRODUCTION

Rupture of a mature ovarian follicle and subsequent release of a viable oocyte are prerequisites for reproductive success. Numerous studies have implicated the matrix metalloproteinases (MMPs) as important mediators of ovulation and subsequent corpus luteum (CL) formation [1, 2]. The MMPs are a large gene family of >26 metal-dependent enzymes that digest specific components (collagens, laminin, fibronectin, and proteoglycans) of the extracellular matrix (ECM) and are noted for their role in cell remodeling, ECM degradation, and tissue repair [3, 4]. The collagenous layers in the theca externa, tunica albuginea, and surface epithelium at the follicle apex must be degraded for a follicle to rupture. Furthermore, breakdown of the basement membrane, which separates the granulosal and thecal cells and contains primarily type IV collagen, is required for release of the oocyte. Administration of synthetic MMP inhibitors blocks ovulation [5, 6]. However, the specific MMPs required for follicle rupture are not known.

The membrane-type MMPs have been termed as possible ''master switches'' that control ECM remodeling [7]. Membrane-type 1 MMP (MMP-14) is noted both for its ability to activate other MMPs (MMP-2, MMP-13) and focalize their activity to the cell surface and for its own capacity to degrade numerous ECM substrates [8–10]. MMP-14 has been shown to activate progelatinase A (MMP-2) and localize its activity in a complex with tissue inhibitor of metalloproteinases-2 (TIMP-2) at the cell surface [8]. MMP-14 may also play a direct role in degradation of the preovulatory follicle wall via its ability to cleave type I and III collagen, fibronectin, laminin, and proteoglycans [9]. However, the specific role of MMP-14 in mediating follicle rupture is unknown.

The specific functions of MMP-2 and TIMP-2 during the periovulatory period are also unclear. MMP-2 has been proposed to play an important role in degradation of the basement membrane that separates the granulosal cells from the thecal cells, and it also likely further hydrolyzes the denatured collagen fibrils in the follicle wall after their initial cleavage by a collagenase. In addition to its potential role in proMMP-2 activation and focalization of MMP-2 activity at the cell surface, TIMP-2 is most known for its ability to bind MMPs in a 1:1 stoichiometry and inhibit their activity.

The regulation of MMP-2 and TIMP-2 during the periovulatory period is also species-specific. MMP-2 mRNA and enzyme activity are increased in rat ovaries after an ovulatory dose of hCG [11, 12], whereas expression in mouse ovaries in response to an ovulatory stimulus is constitutive [13]. Expression of TIMP-2 is constitutive in rat [14] and mouse [13, 15] ovaries and sheep preovulatory follicles [16] after an ovulatory stimulus, but is increased in macaque granulosal cells [17].

As an initial step toward elucidating the physiologic roles of MMP-14, MMP-2, and TIMP-2 in the ovulatory process, we characterized the effect of the preovulatory gonadotropin surge on localization and regulation of MMP-2, MMP-14, and TIMP-2 mRNA and MMP-2 and TIMP-2 activity in bovine preovulatory follicles and new CL. Results presented here support the hypothesis that increased expression of MMP-14 and TIMP-2, but not MMP-2, may help regulate follicle rupture and/or the ovulatory follicle-CL transition in cattle.

MATERIALS AND METHODS

Animal Care

All procedures described in which animals were used were approved by the All University Committee on Animal Use and Care at Michigan State University (protocol 04/98-056-00).

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[2]Correspondence: George W. Smith, Michigan State University, Department of Animal Science, 1230D Anthony Hall, East Lansing, MI 48824-1225. FAX: 517 353 1699; e-mail: smithge7@msu.edu

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Experimental Model

Follicle development and timing of the preovulatory gonadotropin surge were synchronized in Holstein cows using the Ovsynch procedure (GnRH-7days-prostaglandin$F_{2\alpha}$[$PGF_{2\alpha}$]-36h-GnRH) [18]. Briefly, GnRH is injected to start a new wave of follicle growth and thus a new dominant follicle. Seven days later, $PGF_{2\alpha}$ is given to regress CL. A second GnRH injection is given to induce an LH surge resulting in ovulation of the dominant follicle an average of 29 h later. 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 turned over a new follicular wave before the second GnRH injection. Ovaries containing ovulatory follicles or new CL were collected by colpotomy at 0, 6, 12, 18, 24, and 48 h (CL) after the second GnRH injection (n = 5–8 per time point). Blood samples were collected at the time of $PGF_{2\alpha}$ injection and at the time of the second GnRH injection. Serum progesterone concentrations in these samples were measured by RIA (Diagnostic Products Corporation, Los Angeles, CA) to ensure that all animals responded to the $PGF_{2\alpha}$ injection. Assay of serum progesterone levels at the time of $PGF_{2\alpha}$ injection revealed levels indicative of a functional CL. At the time of the second GnRH injection, progesterone levels were below 1 ng/ml in all animals included in the study, indicative of a regressing CL. Intraassay and interassay coefficients of variation for the progesterone RIA were 5.6% and 9.1%, respectively. To exclude any animals that exhibited a preovulatory gonadotropin surge before the second GnRH injection, three blood samples at 15-min intervals were collected every 8 h beginning 16 h after $PGF_{2\alpha}$ injection until the time of ovariectomy or GnRH injection. To confirm that an LH surge was elicited by the second GnRH injection, blood samples were also collected every hour for 4 h after the second GnRH injection. Concentrations of serum LH in the aforementioned samples were measured by RIA [19, 20]. For all animals included in the study, no premature LH surge was detected in any of the animals in the 0 h (pregonadotropin surge) group. An LH surge was detected 1–2 h after GnRH injection for all animals in the postsurge groups (6, 12, 18, 24, and 48 h), verifying control of timing of the gonadotropin surge in our model system. Intraassay and interassay coefficients of variation for the LH RIA were 5.8% and 15.6%, respectively.

Tissue Collection

For mRNA quantification and enzyme activity assays, ovaries containing the ovulatory follicle or new CL were collected at 0, 6, 12, 18, 24, and 48 h (n = 5–6 each) after the second GnRH injection. After ovariectomy, the ovulatory follicle or new CL was isolated by cutting away all remaining ovarian stroma and small follicles so that the ultrastructure at the apex of the follicle remained intact. Follicular fluid was aspirated, centrifuged, aliquoted, and stored at −20°C until activity assays. Follicles were then sagittally cut in half. One half was used for total RNA isolation. For protein analysis, the remaining half was cut transversely into two equal pieces, one containing the follicle apex and one the base. New CL collected 48 h after GnRH injection were used only for mRNA analyses. Samples were frozen at −80°C within 15 min of ovariectomy. For in situ hybridization, ovaries containing the ovulatory follicles were collected at 0, 12, and 24 h (n = 3 each) after GnRH injection. Ovulatory follicles were dissected from the ovary, immediately immersed in embedding medium, frozen over liquid nitrogen vapors, and stored at −80°C until sectioned. Twelve-micrometer frozen sections were cut at −20°C and mounted onto positively charged slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA). Slides were then stored in an airtight box at −80°C until hybridization.

Generation of cDNAs

Bovine cDNAs for MMP-2 and MMP-14 were generated by the reverse transcription-polymerase chain reaction (RT-PCR) using bovine CL RNA and primers corresponding to 100% conserved sequences present in the nucleotide sequence of analogous human and rat cDNAs. A 416-base pair (bp) partial cDNA encoding MMP-2 (corresponding to nucleotides 1317–1733 of human MMP-2 cDNA; GenBank accession no. AJ243311.1) and a 360-bp partial cDNA encoding MMP-14 (corresponding to nucleotides 599–959 of human MMP-14 cDNA; GenBank accession no. XM033440.1) were ligated into pBluescript plasmids (Stratagene, La Jolla, CA) and subjected to fluorescent dye terminator sequencing to verify identity and determine orientation. A 289-bp fragment of an ovine TIMP-2 cDNA [16] was used for detection of bovine TIMP-2 mRNA in the present studies.

Characterization of MMP-2, MMP-14, and TIMP-2 mRNA Abundance

Total RNA was isolated using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. To determine transcript size and number and to optimize specificity of hybridization conditions, approximately 15 μg of pooled RNA from each sample per time point was subjected to Northern analysis [16, 21]. For quantification of MMP-2, MMP-14, and TIMP-2 mRNA abundance, 5 μg of total RNA isolated from each sample was subjected to dot blot analysis [16, 21]. Northern and dot blot analyses were carried out using ^{32}P-labeled MMP-2, MMP-14, TIMP-2, and ribosomal protein L-19 (RPL-19) cDNA probes generated by PCR [22]. RPL-19 was used for normalization purposes. Membranes were prehybridized and hybridized as described previously [16, 21] with minor exceptions (concentration of sodium phosphate in hybridization buffer was 20 mM, and 10% dextran sulfate was added). Membranes were washed in 1× saline-sodium citrate (SSC), 0.1% SDS, 0.1% sodium pyrophosphate for 20 min at 42°C followed by one 20-min wash in 0.1× SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 42°C; one 20-min wash at 47°C; and one 20-min wash at 50°C. After the washes, filters were exposed to a phosphorimager cassette. After Northern analyses, the size of RNA transcripts was determined based on relative migration of RNA molecular weight markers (Roche, Indianapolis, IN). After hybridizations for MMP-2, MMP-14, or TIMP-2, the membranes were then stripped and reprobed with the ^{32}P-labeled RPL-19 cDNA. Preliminary experiments demonstrated that RPL-19 mRNA abundance in bovine preovulatory follicles and new CL was not regulated by the gonadotropin surge ($P > 0.05$; data not shown). Relative densitometric units for MMP-2, MMP-14, and TIMP-2 were quantitated and adjusted relative to RPL-19 mRNA expression using Molecular Analyst version 1.5 software (BioRad, Hercules, CA). Preliminary Northern blot experiments demonstrated that hybridization and washing conditions used in subsequent dot blot analyses were specific and yielded hybridization to single transcripts of the expected size for each mRNA of interest. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected after hybridization of each cDNA to increasing amounts of sample RNA (1–10 μg).

In Situ Hybridization

The intrafollicular localization of MMP-2, MMP-14, and TIMP-2 mRNAs in follicles collected at 0, 12, and 24 h after GnRH injection (n = 3 per time point) was determined by in situ hybridization according to our previously published procedure [16] using [^{35}S]antisense and sense (negative control) UTP-labeled cRNA probes. After the hybridization and washing [16], slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 4 days (MMP-2), 2 days (TIMP-2), or 2 wk (MMP-14) at 4°C before development and counterstaining. Digital bright- and dark-field images were acquired on a Leica research microscope equipped with a SPOT Model No. 1.1.0 camera and version 3.2.4 software (W. Nuhsbaum, McHenry, IL).

Preparation of Follicle Homogenates

Follicles were homogenized using procedures previously described by Murdoch and McCormick [23]. Briefly, the apical or basal sections of follicles were homogenized using a polytron homogenizer (Fisher Scientific, Chicago, IL) in 800 μl of homogenization buffer (10 mM $CaCl_2$, 0.25% Triton X-100), then centrifuged at 9000 × *g* for 30 min at 4°C. The supernatants were removed and are hereafter referred to as Triton extracts. Each pellet was resuspended in 200 μl of resuspension buffer (50 mM Tris pH 7.6, 100 mM $CaCl_2$, 0.15 M NaCl) and heated for 10 min at 60°C followed by centrifugation at 27 000 × *g* for 30 min at 4°C. Supernatants were removed and are hereafter referred to as heat extracts.

Gelatin Zymography/Reverse Zymography

Gelatin zymography was performed for semiquantitative analysis of gelatinolytic activity in follicular fluid and follicle extracts. Follicular fluid (0.25 μl) as well as proteins in Triton (80 μg per lane) and heat (20 μg per lane) extracts from the apex and base of each follicle were subjected to electrophoresis in duplicate on one-dimensional SDS-polyacrylamide gels containing 0.25% (w/v) gelatin. All samples in a given experimental group (i.e., Triton apex or heat base) were run together on a given day. The protein concentration for each sample was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Follicular fluid was treated as a bodily fluid and loaded by volume; however, follicular

fluid protein concentrations in each sample were measured, and variation in concentration was <10%. After electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 to remove SDS. Gels were rinsed and incubated overnight in 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, and 0.02% NaN_3 with or without 1,10-phenanthroline (final concentration 0.05 mM) at 37°C. Gels were then stained with Coomassie brilliant blue for 1 h to visualize clear bands of digested gelatin representing gelatinolytic activity. Gels were photographed using a Gel Documentation System (BioRad). The area of degradation was measured using Molecular Analyst version 1.5 software (BioRad) to derive individual estimates of MMP-2 activity. A pooled follicular fluid standard was run on each gel and used to calculate intraassay coefficients of variation (gel to gel variation) for analysis of each set of samples. Intraassay coefficients of variation were all ≤1.5%. All gelatinolytic activity was inhibited when 1,10-phenanthroline was included in the incubation buffer (data not shown). To confirm that gelatinolytic activity detected corresponded primarily to MMP-2, recombinant proMMP-2 as well as a combined MMP-2-MMP-9 standard (Oncogene Research Products, Cambridge, MA) were run with each group of samples analyzed. Activity for MMP-2 comigrated with the appropriate standards (data not shown). To further confirm that the majority of activity detected represented the latent form of MMP-2, selected samples and MMP-2 standards were subjected to organomercurial activation by incubation in 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, and 1 mM aminophenylmercuric acetate for 30 min at 37°C before gelatin zymographic analysis. Preliminary experiments established the optimal incubation time and demonstrated that increasing activity was detected when increasing amounts of sample protein were loaded on gels (data not shown).

Reverse zymography was performed to characterize effects of the preovulatory gonadotropin surge on TIMP-2 activity. Samples of Triton extracts (80 μg per lane), heat extracts (15 μg per lane), and follicular fluid (2 μl per lane) were subjected to electrophoresis in one-dimensional SDS polyacrylamide gels containing 0.6% gelatin and 400 μl of BHK-21 cell (American Type Culture Collection, Manassas, VA) conditioned media (source of gelatinases). Standards for TIMP-1, TIMP-2, and TIMP-3 (University Technologies International, Calgary, AB, Canada) were run with each set of samples analyzed. Activity corresponding to TIMP-2 migrated similarly to that of the TIMP-2 standard (data not shown). Preliminary experiments established optimal incubation times and demonstrated that increasing activity was detected when increasing amounts of sample protein were loaded on reverse zymography gels (data not shown).

Statistical Analyses

Differences in mRNA abundance and enzyme activity were determined by one-way ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS version 8.0; SAS Institute Inc., Cary, NC). Individual comparisons of mean RNA concentrations were performed using the Fisher protected least significant differences test, and results are reported as mean ± SEM.

RESULTS

Effect of the Preovulatory Gonadotropin Surge on MMP-14 mRNA Abundance and Localization

Relative abundance of MMP-14 mRNA in bovine periovulatory follicular and luteal tissue was regulated by the preovulatory gonadotropin surge. The MMP-14 cDNA hybridized specifically to a 3.1-kilobase (kb) transcript present within bovine periovulatory follicles and new CL (Fig. 1). Expression of MMP-14 mRNA was increased in preovulatory follicles within 24 h of the gonadotropin surge and remained elevated in new CL (24 and 48 h versus 0 h; $P < 0.01$). MMP-14 mRNA was localized primarily to the thecal layer and adjacent ovarian stroma of bovine preovulatory follicles. Differential expression of MMP-14 mRNA within the apex versus the base of preovulatory follicles was not detected. However, significant hybridization was also observed in the granulosal layer at the 12 and 24 h time points (Fig. 2).

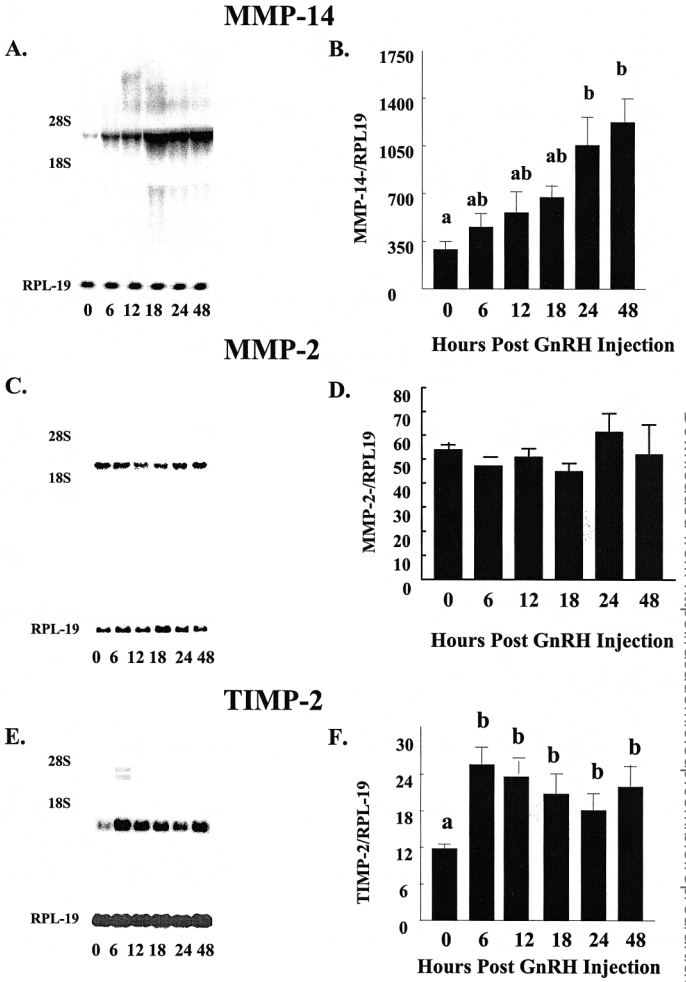

FIG. 1. Effect of a GnRH-induced gonadotropin surge on MMP-14, MMP-2, and TIMP-2 mRNA abundance in bovine periovulatory follicular and luteal tissue. **A**) Northern analysis of MMP-14 mRNA expression. **B**) Effect of the preovulatory gonadotropin surge on relative levels of MMP-14 mRNA in bovine periovulatory follicles and new CL. **C**) Northern analysis of MMP-2 mRNA expression. **D**) Effect of the preovulatory gonadotropin surge on relative levels of MMP-2 mRNA in bovine periovulatory follicles and new CL. **E**) Northern analysis of TIMP-2 mRNA expression. **F**) Effect of the preovulatory gonadotropin surge on relative levels of TIMP-2 mRNA in bovine periovulatory follicles and new CL. Data (**B**, **D**, **F**) are expressed as relative units of MMP-14, MMP-2, or TIMP-2 mRNA per unit of RPL-19 mRNA. Data are shown as mean ± SEM (n = 5–6 per time point). Time points without a common superscript are different at $P < 0.05$.

Effect of the Preovulatory Gonadotropin Surge on MMP-2 mRNA and Enzyme Activity

In contrast to the temporal regulation of MMP-14 mRNA observed, expression of MMP-2 mRNA was not regulated by the preovulatory gonadotropin surge. The bovine MMP-2 cDNA hybridized specifically to a 2.7-kb transcript (Fig. 1). Expression of MMP-2 mRNA was not different at any of the time points evaluated ($P > 0.05$). MMP-2 mRNA was localized primarily to the thecal layer of bovine preovulatory follicles (Fig. 3). Differential localization of MMP-2 mRNA within the apex versus the base of preovulatory follicles was not observed. Likewise, bovine follicular MMP-2 activity was not regulated by the preovulatory gonadotropin surge. Relative levels of MMP-2 activity in follicular fluid (Fig. 4) and follicle extracts (Triton and heat extracts, apex and base; data not shown) were not

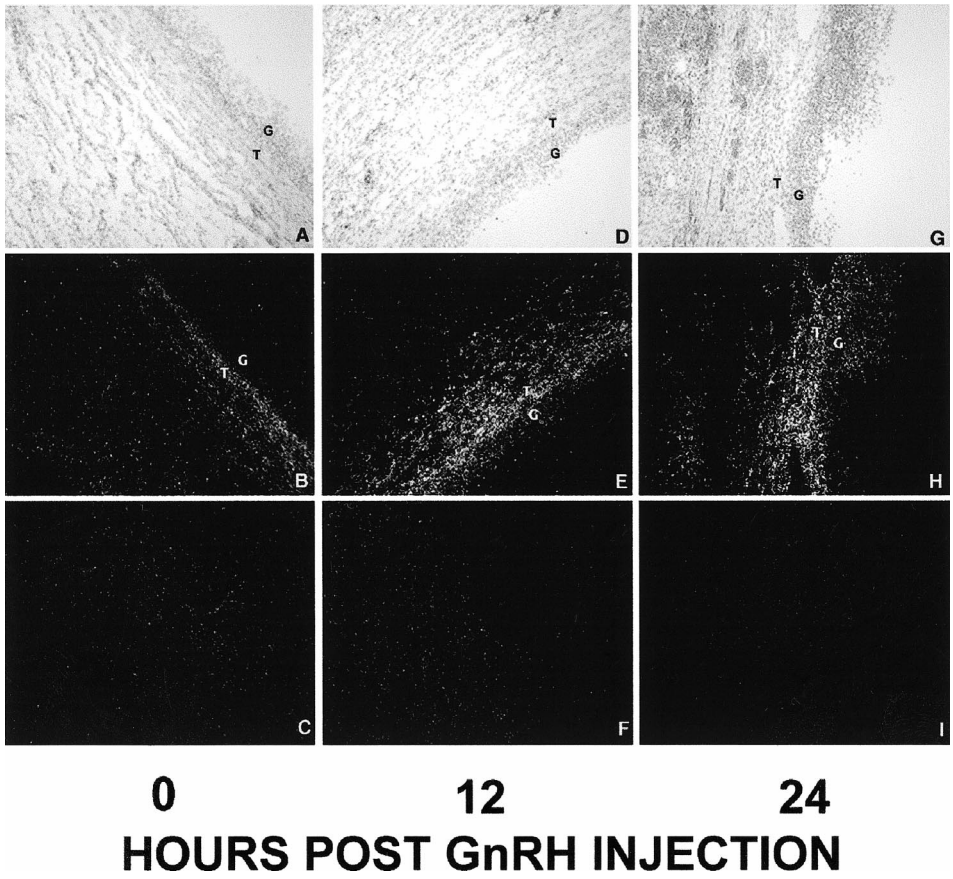

FIG. 2. In situ localization of MMP-14 mRNA within bovine periovulatory follicles collected at 0, 12, and 24 h after GnRH injection (n = 3 per time point). **A**) Bright-field micrograph of a preovulatory follicle collected at the 0 h time point and stained with hematoxylin and eosin. **B**) Dark-field micrograph of the same section hybridized with an ^{35}S antisense MMP-14 cRNA **C**) Dark-field micrograph of adjacent serial section of the same follicle hybridized with an ^{35}S sense (negative control) MMP-14 cRNA. **D**, **G**) Similar to **A** except that preovulatory follicles were collected at 12 and 24 h, respectively. **E**, **H**) Similar to **B** except that preovulatory follicles were collected at 12 and 24 h, respectively. **F**, **I**) Similar to **C** except that preovulatory follicles were collected at 12 and 24 h, respectively. T, Thecal layer; G, granulosal layer. Magnification ×120.

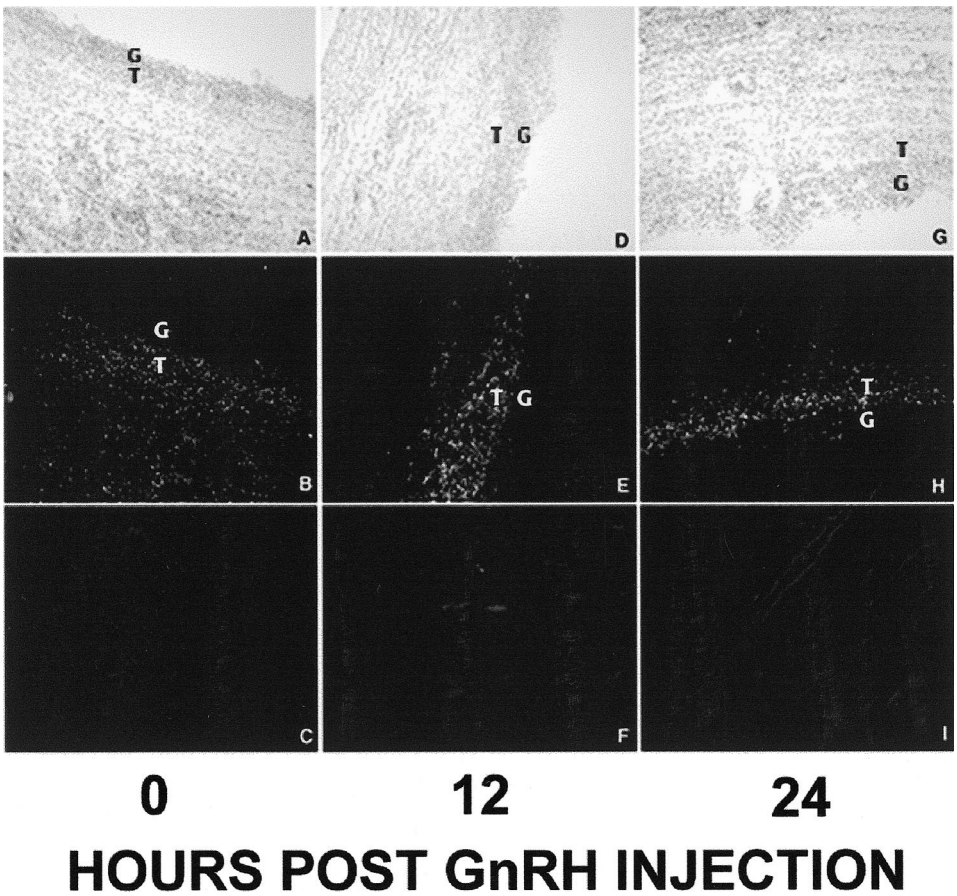

FIG. 3. In situ localization of MMP-2 mRNA within bovine periovulatory follicles collected at 0, 12, and 24 h after GnRH injection (n = 3 per time point). **A**) Bright-field micrograph of a preovulatory follicle collected at the 0 h time point and stained with hematoxylin and eosin. **B**) Dark-field micrograph of the same section hybridized with an ^{35}S antisense MMP-2 cRNA. **C**) Dark-field micrograph of adjacent serial section of the same follicle hybridized with an ^{35}S sense (negative control) MMP-2 cRNA. **D**, **G**) Similar to **A** except that preovulatory follicles were collected at 12 and 24 h, respectively. **E**, **H**) Similar to **B** except that preovulatory follicles were collected at 12 and 24 h, respectively. **F**, **I**) Similar to **C** except that preovulatory follicles were collected at 12 and 24 h, respectively. T, Thecal layer; G, granulosal layer. Magnification ×120.

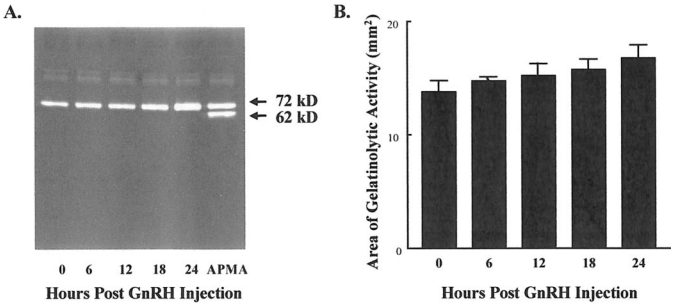

FIG. 4. Gelatin zymographic analysis of MMP-2 activity in bovine periovulatory follicular fluid. **A**) Representative zymogram demonstrating MMP-2 activity in follicular fluid of bovine periovulatory follicles. **B**) Semiquantitative analysis of MMP-2 activity in follicular fluid of bovine periovulatory follicles collected at 0, 6, 12, 18, and 24 h after GnRH injection (individual samples). Data are expressed as mean ± SEM (n = 6 per time point).

different at any of the time points examined (0, 6, 12, 18, or 24 h after GnRH injection; $P > 0.05$). The latent form of MMP-2 was the predominant form detected in both follicular fluid (Fig. 4) and follicle homogenates (data not shown). Addition of 1,10-phenanthroline to incubation buffer inhibited all gelatinase activity (data not shown).

Effect of the Preovulatory Gonadotropin Surge on TIMP-2 mRNA and Activity

Localization and abundance of TIMP-2 mRNA and TIMP-2 activity were regulated by the preovulatory gonadotropin surge. The TIMP-2 cDNA hybridized specifically to a 1.1-kb transcript (Fig. 1). Messenger RNA abundance for TIMP-2 was increased within 6 h of the preovulatory gonadotropin surge and remained elevated throughout the entire periovulatory period (6–48 h; $P < 0.05$). Messenger RNA for TIMP-2 was localized specifically to the granulosal layer at all time points (0, 12, and 24 h) examined (Fig. 5). Differential expression of TIMP-2 mRNA within the apex versus the base of preovulatory follicles was not detected. However, in follicles collected before the gonadotropin surge, expression of TIMP-2 mRNA was prominent in the portion of the granulosal layer proximal to the basement membrane. At subsequent time points, expression was increased in the distal (antral) portion of the granulosal layer. Expression in the thecal layer was nondetectable in comparison to the high level of expression detected in the granulosal layer (Fig. 5). No obvious differences in TIMP-2 activity were detected in follicle homogenates (Triton and heat extracts, apex and base) collected before versus after the gonadotropin surge (data not shown). However, TIMP-2 activity was clearly increased in periovulatory follicular fluid samples collected after the gonadotropin surge (Fig. 6).

DISCUSSION

Successful ovulation is necessary for fertilization and reproductive success. The MMPs have been implicated as important mediators of the ovulatory process because of their ability to degrade specific components (collagens, laminin, fibronectin, and proteoglycans) of the follicle wall and regulate ECM remodeling. However, the effect of the preovulatory gonadotropin surge on the intrafollicular localization and regulation of mRNA expression for individ-

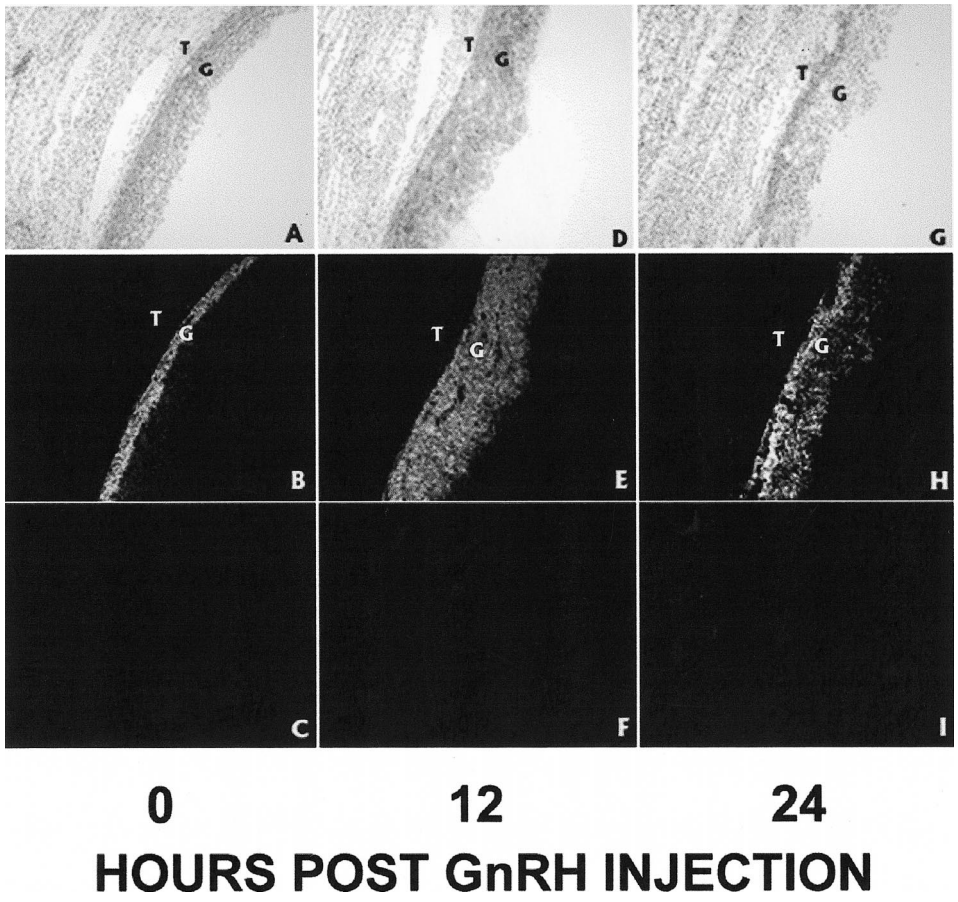

FIG. 5. In situ localization of TIMP-2 mRNA within bovine periovulatory follicles collected at 0, 12, and 24 h after GnRH injection (n = 3 per time point). **A**) Bright-field micrograph of a preovulatory follicle collected at the 0 h time point and stained with hematoxylin and eosin. **B**) Dark-field micrograph of the same section hybridized with an ^{35}S antisense TIMP-2 cRNA. **C**) Dark-field micrograph of adjacent serial section of the same follicle hybridized with an ^{35}S sense (negative control) TIMP-2 cRNA. **D**, **G**) Similar to **A** except that preovulatory follicles were collected at 12 and 24 h, respectively. **E**, **H**) Similar to **B** except that preovulatory follicles were collected at 12 and 24 h, respectively. **F**, **I**) Similar to **C** except that preovulatory follicles were collected at 12 and 24 h, respectively. T, Thecal layer; G, granulosal layer. Magnification ×120.

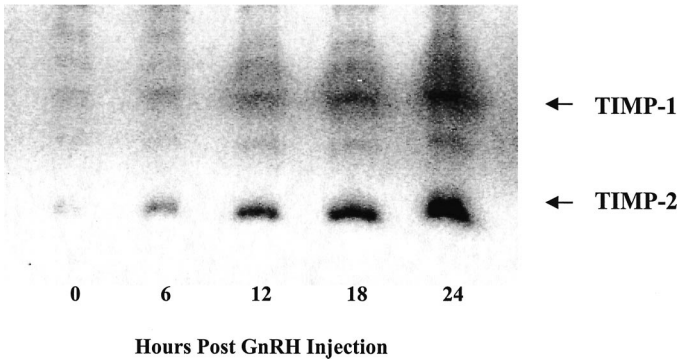

FIG. 6. Reverse zymographic analysis of TIMP-2 activity in bovine periovulatory follicular fluid. Representative zymogram demonstrating TIMP-2 activity in follicular fluid (2 μl per sample) collected at 0, 6, 12, 18, and 24 h after GnRH injection (n = 6 per time point).

ual MMPs and their specific requirement for follicle rupture are not well understood.

In the present study, MMP-14 mRNA was increased in bovine preovulatory follicular and luteal tissue after exposure to a gonadotropin surge. Previous studies of MMP-14 mRNA expression in mice (by Northern analysis) demonstrated no change in expression in the ovary after an ovulatory stimulus (hCG injection) [13]. The spatial regulation of MMP-14 expression observed in bovine preovulatory follicles after the LH surge also was distinct. Expression of MMP-14 mRNA was detected primarily in the thecal layer and adjacent ovarian stroma, with expression also detected in the granulosal cells at the 12 and 24 h time points. In rats [24] and mice [13], MMP-14 mRNA is also localized to the thecal layer of preovulatory follicles. In mouse and rat preovulatory follicles, abundant MMP-14 mRNA expression is also evident in the granulosal cells, but expression is down-regulated in the granulosal layer and increased in the thecal layer in response to hCG injection [13, 24]. The reasons for the prominent species differences in the cell-specific periovulatory follicular regulation of MMP-14 mRNA are not known.

Although MMP-14 is most often noted for its ability to localize and activate MMP-2, it may contribute to follicular ECM degradation through other mechanisms. MMP-14 may be directly involved in degradation of types I and III collagen as well as fibronectin, laminin, and proteoglycans [9, 25], ECM components present within the bovine follicle wall. Espey and Lipner [26] postulated that proteinases localized on the cell surface promote cell dissociation and breakdown of the surrounding collagen fibers within the theca externa and tunica albuginea during ovulation. In addition, MMP-14 may indirectly help promote degradation of types I and III collagen in the bovine follicle wall by its ability to activate procollagenase 3 (MMP-13) [10]. MMP-13 mRNA is up-regulated in bovine preovulatory follicles after the gonadotropin surge (unpublished data). Furthermore, MMP-14 is not inhibited by TIMP-1, allowing follicular ECM degradation to occur in the presence of high concentrations of inhibitor [27]. This may be especially significant, given that TIMP-1 is dramatically increased in preovulatory follicles of several species in response to the gonadotropin surge [13, 17, 28–30].

The localization and regulation of TIMP-2 mRNA observed in the present studies in cattle are markedly different from those that have been previously reported for other species. In the present study, TIMP-2 mRNA and activity were increased after the preovulatory gonadotropin surge. In contrast, TIMP-2 mRNA is constitutively expressed in ovine, mouse, and rat follicles after an ovulatory stimulus [14, 15, 28]. We have previously observed increased expression of TIMP-2 mRNA in preovulatory follicular and luteal tissue collected from beef heifers within 8 and 48 h (CL) of the preovulatory gonadotropin surge [21], but the localization of TIMP-2 mRNA and changes in TIMP-2 activity were not determined. Furthermore, the localization of TIMP-2 mRNA observed in bovine follicles also was unique. Messenger RNA for TIMP-2 was localized exclusively to the thecal layer of ovine follicles [16], and a similar localization has been observed in rats and mice [13, 31]. In contrast, TIMP-2 mRNA was localized specifically to the granulosal layer of bovine preovulatory follicles. The physiologic significance of the observed increase in TIMP-2 mRNA expression after the gonadotropin surge in the antral portion of the granulosal layer is not known. Differences in gene expression in specific components of the granulosal layer have been observed previously [32–34].

In the present study, MMP-2 mRNA expression and enzyme activity were unchanged after exposure of bovine follicles to a gonadotropin surge. The effect of an ovulatory stimulus on MMP-2 expression during the periovulatory period appears species-specific. As in the cow, expression of MMP-2 mRNA and activity is constitutive during the periovulatory period in the mouse [13]. These results are in contrast to those of previous studies that have reported an increase in MMP-2 mRNA and gelatinolytic activity within rat ovaries after exposure to an ovulatory stimulus [11, 12]. MMP-2 mRNA is also increased in preovulatory macaque granulosal cells in response to hCG administration in vivo [17]. The localization of MMP-2 mRNA to the thecal layer observed in the present studies is consistent with results of previously published studies in rats and mice [11, 24]. Consistent, significant expression of MMP-2 mRNA in the granulosal layer was not detected in the present studies.

The potential absolute requirement of MMP-2 for follicle rupture and subsequent reproductive success may also be species-specific. Gene-targeting experiments have revealed that a functional MMP-2 gene is not required for successful pregnancy [35]. Potential direct effects of the MMP-2 null mutation on ovulation rate in the MMP-2 knock-out mice have not been reported. In contrast, results in sheep indicate that MMP-2 may play a key role in the ovulatory process. The LH surge increases MMP-2 activity in sheep follicular extracts [36], and intrafollicular injection of MMP-2 antibodies can block ovulation [37]. In addition, ewes immunized against the N-terminal peptide of the 43-kDa subunit of α-N inhibin show decreased MMP-2 activity in follicular fluid and an impairment of the ovulatory process [38]. Results of the present studies indicate that increased expression of MMP-2 is not obligatory for follicle rupture in cattle. However, in the bovine, MMP-9 may be the primary mediator of follicular basement membrane breakdown before ovulation. Messenger RNA for MMP-9 is increased in bovine preovulatory follicles in response to the gonadotropin surge (unpublished data).

In this study we have examined three potential regulators of follicle wall degradation after the LH surge. Considerable differences were found in terms of localization and regulation of MMP-2, MMP-14, and TIMP-2 in cattle versus previously reported results for other species. Thus, the potential role of or absolute requirement for aforementioned regulators in the ovulatory process and/or the ovulatory follicle-CL transition may be distinct.

In summary, we have shown that the gonadotropin surge results in increased MMP-14 mRNA and TIMP-2 mRNA and activity in bovine preovulatory follicles, whereas MMP-2 mRNA and enzyme activity are constitutively expressed. The intrafollicular localization of MMP-14, MMP-2, and TIMP-2 mRNA expression in bovine preovulatory follicles is distinct and does not strongly support a potential direct role for TIMP-2 in proMMP-2 activation and localization of MMP-2 activity at the cell surface. However, a role for TIMP-2 of granulosal cell origin in proMMP-2 activation and localization at the cell surface in the thecal layer cannot totally be discounted. Our results support the hypothesis that increased expression of MMP-14 and TIMP-2, but not MMP-2, may help regulate follicle rupture and/or the ovulatory follicle-CL transition in cattle.

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