Localization and temporal regulation of tissue inhibitors of metalloproteinases 3 and 4 in bovine preovulatory follicles

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Abstract

The matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are potential regulators of the focalized extracellular matrix degradation required for ovulation. The objectives of the present study were to determine localization and temporal regulation of TIMP-3 and TIMP-4 mRNA and protein in bovine preovulatory follicles. Ovaries containing preovulatory follicles were collected at 0, 12 and 20 h after GnRH injection for real-time PCR quantification of TIMP-3 and TIMP-4 mRNAs and immunohistochemical localization studies. Additional samples collected at 0, 6, 12, 18 and 24 h post GnRH injection were subjected to Western analysis to determine temporal changes in TIMP-3 and TIMP-4 proteins in the apex and base of preovulatory follicles. Results indicate the gonadotropin surge regulates TIMP-3 and TIMP-4 expression. TIMP-3 and TIMP-4 mRNAs increased within 12 h after GnRH injection. TIMP-3 protein was localized to granulosa and thecal layers of preovulatory follicles and adjacent ovarian stroma, whereas TIMP-4 immunoreactivity was localized to granulosa and thecal cells and ovarian blood vessels. Amounts of TIMP-3 and TIMP-4 proteins in the follicular apex peaked within 12 h post GnRH injection and subsequently declined by 24 h. However, amounts of TIMP-3 and TIMP-4 proteins in the base were not elevated after GnRH administration. Results demonstrate that mRNA and protein for both TIMP-3 and TIMP-4 are increased in bovine preovulatory follicles following the gonadotropin surge. Coordinate expression of TIMPs and MMPs may help regulate the extracellular matrix remodeling characteristic of the ovulatory process.

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Introduction

Ovulation, triggered by the preovulatory gonadotropin (luteinizing hormone (LH)) surge, occurs only once per reproductive cycle in monotocous species such as the cow and human. Since ovulation is a prerequisite for fertilization and embryonic development, the mechanism of ovulation has been a subject of investigation for some time. Experimental evidence supports the theory that proteolytic degradation of the extracellular matrix (ECM) at the apex of ovulatory follicles prior to ovulation is a crucial step in the complex cascade of events initiated by the LH surge (Fata *et al.* 2000, Ny *et al.* 2002). Matrix metalloproteinases (MMPs) or matrixins, together with their tissue inhibitors (TIMPs) regulate ECM remodeling during both physiological and pathological conditions (Vu & Werb 2000, Coussens *et al.* 2002, Curry & Osteen 2003). Therefore, they are postulated to play important roles in regulation of the ECM remodeling characteristic of the ovulatory process.

The MMPs are a multigene family of zinc-dependent proteinases that digest specific components (e.g. collagens, laminin, fibronectin and proteoglycans) of the ECM.

At least 25 MMPs have been discovered to date (Sternlicht & Werb 2001, Visse & Nagase 2003). The activities of MMPs are tightly regulated by TIMPs, thus providing a homeostasis that prevents overproduction and unrestrained ECM proteolysis. The TIMP gene family consists of four members (TIMPs 1–4) that share structural similarities including the presence of 12 cysteine residues which form six intramolecular disulfide bonds (Nagase & Woessner 1999). Each of the TIMPs differs in molecular mass and degree of glycosylation, as well as solubility in the extracellular milieu (Brew *et al.* 2000). While TIMP-1, TIMP-2 and TIMP-4 are soluble within the extracellular milieu, TIMP-3 can bind to the ECM (Leco *et al.* 1994). TIMP-4, the newest TIMP family member, can inhibit numerous MMPs (Brew *et al.* 2000) and possesses pro-MMP-2 binding activity similar to that of TIMP-2 (Bigg *et al.* 1997).

We have previously demonstrated that expression of the fibrillar collagenases MMP-1 and MMP-13 (Bakke *et al.* 2004), the membrane type-1 MMP (MMP-14) (Bakke *et al.* 2002) and TIMP-1 and TIMP-2 (Smith *et al.* 1996, Bakke *et al.* 2002) are increased in bovine preovulatory follicles following the gonadotropin surge. A general role for

TIMPs in regulation of MMP activity is well established, but the regulation and regulatory role of TIMP family members, particularly TIMP-3 and TIMP-4, in control of preovulatory follicular ECM remodeling are not completely understood, especially in monotocous species. Thus, the objectives of the present study were to determine the localization and temporal regulation of TIMP-3 and TIMP-4 mRNA and protein in bovine preovulatory follicles following the gonadotropin surge. Differential regulation of TIMP-3 and TIMP-4 proteins in the apex vs the base of preovulatory follicles was also investigated.

Materials and Methods

Animal care

All procedures described where animals were used were approved by the All University Committee on Animal Use and Care at Michigan State University (Protocol #04/98-056-00 and #03/01-051-00).

Experimental model and tissue collection

Collection of tissues for immunohistochemical and mRNA analyses was as follows. Crossbred beef heifers ($n = 21$) received two injections of prostaglandin (PG) $F_{2\alpha}$ (25 mg, 11 days apart) to synchronize estrus. Daily ultrasound analyses were performed from the time of the second PGF $_{2\alpha}$ injection until the time of follicle collection to map initiation of the first follicular wave and identify the dominant follicle. On day 6 post estrus, animals received two injections of PGF $_{2\alpha}$ (15 mg each, 12 h apart) to regress the corpora lutea (CL). Approximately 30 h after the first PGF $_{2\alpha}$ injection on day 6, animals were treated with 100 μ g gonadotropin-releasing hormone (GnRH) to induce a gonadotropin surge. Ovaries containing the ovulatory follicle were collected by colpotomy at 0, 12 and 20 h after GnRH injection and processed for RNA isolation ($n = 4-6$ each) or immunohistochemistry (0 and 20 h only; $n = 3$ per timepoint). Preovulatory follicles were isolated by cutting away all remaining ovarian stroma and small follicles. The follicles were then transversely cut in half. One half was used for total RNA isolation. Approximately 4–5 mm² pieces from the apex and base of the remaining half of the follicle were then fixed overnight in neutral buffered formalin and embedded in paraffin for immunohistochemistry.

Previously described preovulatory follicle samples collected at 0, 6, 12, 18 and 24 h after GnRH injection were utilized for Western blot analyses of TIMP-3 and TIMP-4 protein. The experimental model utilized for sample collection has been described previously (Bakke *et al.* 2002). Briefly, follicular development and timing of the preovulatory gonadotropin surge were synchronized in Holstein cows using the Ovsynch (GnRH-7d-PGF $_{2\alpha}$ -36h-GnRH) procedure (Pursley *et al.* 1995, 1997). Briefly, GnRH is injected to start a new wave of follicular growth and thus a new dominant follicle. Seven days later, PGF $_{2\alpha}$ is given

to regress the CL. A second GnRH injection is given to induce a gonadotropin surge resulting in ovulation of the dominant follicle an average of approximately 29 h later (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 turned over a new follicular wave prior to the second GnRH injection. Ovaries containing preovulatory follicles were collected by colpotomy at 0, 6, 12, 18 and 24 h after the second GnRH injection ($n = 4$ or 5 per timepoint). Follicles were dissected and processed as described above, except that samples of the follicular apex and base were snap frozen in liquid nitrogen and stored at -80°C until preparation of homogenates for Western blot analysis.

RNA isolation and reverse transcription

Total RNA was extracted from preovulatory follicles (collected at 0, 12 and 20 h after GnRH injection to induce the preovulatory gonadotropin surge) using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA). The RNA pellets were dissolved in nuclease free water (Ambion, Austin, TX, USA) and yields of RNA were measured by spectrophotometry at 260 nm. Integrity of RNA was checked by examination of intensity of 28S and 18S ribosomal RNA bands following agarose gel electrophoresis, and purity was estimated based on the ratio of absorbance at 260/280 nm. Before reverse transcription, 1 μ g total RNA per sample was incubated for 15 min at 25°C with 1 U DNase I (Invitrogen, San Diego, CA, USA) to eliminate possible genomic DNA contamination, followed by inactivation of the enzyme with 1 μ l 25 mM EDTA at 65°C for 10 min. Then, 1 μ g of the above RNA was mixed with 500 ng oligo (dT)₁₅ and incubated at 70°C for 10 min, followed by rapid cooling to 4°C . The RNA was then reverse transcribed into cDNA in 1 $\times$ reverse transcriptase buffer containing 10 mM dithiothreitol, 500 μ M deoxynucleotide triphosphates and 200 U Superscript RNase H⁻ Reverse Transcriptase (Invitrogen). The reaction was carried out for 60 min at 42°C and stopped by heating for 10 min at 70°C . The concentration of cDNA in each sample was measured by spectrophotometry and dilutions of each sample to 100 ng/ μ l and 10 ng/ μ l were made in nuclease free water (Ambion). All cDNA samples were stored at -20°C for subsequent quantitative real-time PCR analyses.

Quantitative real-time PCR

Effect of the gonadotropin surge on abundance of TIMP-3 and TIMP-4 mRNAs in bovine preovulatory follicles was investigated by real-time PCR using samples collected at 0, 12 and 20 h relative to GnRH injection ($n = 4-6$ per timepoint). The following primers for quantitative real-time PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA): TIMP-3 forward primer: 5'-GAT GTA CCG AGG ATT CAC

CAA GA-3'; TIMP-3 reverse primer: 5'-TTA AGG CCA CAG AGA CTT TCA GAA-3'. TIMP-4 forward primer: 5'-GAA AGT CTG AAT CAC CGC TAC CA-3'; TIMP-4 reverse primer: 5'-CAG GGC ACC GCA TAG CA-3'. Ribosomal protein L-19 (RPL-19) forward primer: 5'-ATC CGC AAG CCT GTG ACT GT-3'; RPL-19 reverse primer: 5'-TCG GGC CAG GGT GTT TTT-3'.

The real-time assay was performed on an ABI Prism 7000 Sequence Detection System thermal cycler (Applied Biosystems). The optimal primer ratios and amounts of cDNA used in the assays were determined in a preliminary experiment. Amplifications were performed in a 96-well plate (Applied Biosystems) in 25 µl reaction volume containing 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), TIMP-3 primers (7.5 pM each of forward and reverse primers) or TIMP-4 primers (22.5 pM of forward primer and 7.5 pM of reverse primer), diluted cDNA samples (20 ng for TIMP-3 and 100 ng for TIMP-4), and appropriate amount of nuclease free water (Ambion). Plasmids containing a 340bp TIMP-3 cDNA (Ricke *et al.* 2002), a 317 bp TIMP-4 cDNA or a 360bp RPL-19 cDNA (Dow *et al.* 2002) were used to generate standard curves for the real-time PCR assay. The bovine TIMP-4 cDNA was amplified from bovine follicular RNA by RT-PCR, subcloned and sequenced prior to use in standard curve generation and displayed 100% identity with available bovine TIMP-4 nucleotide sequence (Genbank Accession #AF037273.1). In each 96-well plate, a standard curve for TIMP-3 or TIMP-4 was incorporated including serial dilutions of plasmid DNA as template (10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag) along with appropriate unknown samples. RPL-19 was selected as an internal control for data normalization. Abundance of RPL-19 mRNA was determined in each sample, and the RPL-19 standard curve (a 6-point dilution series from 10 pg to 100 ag) was prepared as described above. RPL-19 was previously validated as an internal control for RNA analysis in bovine preovulatory follicles exposed to the gonadotropin surge (Bakke *et al.* 2002). The amounts of TIMP-3, TIMP-4 and RPL-19 mRNAs in each sample were determined using ABI Prism Sequence Detection System Software (Applied Biosystems) by comparison of cycle threshold for each sample against that of the respective standard curve. Each experimental sample and point on the standard curves was run in duplicate. Results are shown as the ratios of TIMP-3 or TIMP-4 mRNA to RPL-19 mRNA.

Immunohistochemistry

Paraffin-embedded sections of preovulatory follicular tissue (apex and base) collected at 0 and 20 h relative to GnRH injection ($n=3$ follicles per timepoint) were subjected to immunohistochemical analyses to localize TIMP-3 and TIMP-4 proteins. A total of 18 sections per TIMP per timepoint were examined for both the follicular apex and base. Immunohistochemistry was performed using a VECTASTAIN Elite ABC Kit (Vector Labs,

Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, approximately 5 µm serial sections were prepared and mounted onto 3-aminopropyltriethoxysilane coated microscope slides. Paraffin sections were deparaffinized in xylene and then rehydrated in graded alcohol. Antigen retrieval was performed by boiling the sections in antigen retrieval *citra* solution (BioGenex Laboratories, San Ramon, CA, USA) for 15 min, and allowing the slides to cool for 30 min at room temperature. The slides were then treated for 10 min with 3% (v/v) hydrogen peroxide in deionized water to eliminate endogenous peroxidase activity. The sections were serially incubated with normal goat serum for 30 min at room temperature, mouse anti-human TIMP-3 monoclonal antibody (2 mg/ml) (MAB 3318; Chemicon, Temecula, CA, USA) at 1:1000 dilution or rabbit anti-human TIMP-4 polyclonal antibody (1 mg/ml) (AB19168; Chemicon) at 1:200 dilution in 1.5% (v/v) normal goat serum for 1 h at room temperature, and biotinylated anti-mouse/rabbit IgG for 30 min at room temperature, followed by incubation with ABC reagent for 30 min at room temperature. Intervening washes were performed after each antibody incubation. The sections were developed using a DAB Substrate kit (Vector Labs) for 2–10 min, and were then counterstained with Vector Hematoxylin QS (Vector Labs) and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Parallel controls included (i) omission of the primary antibody, (ii) omission of the biotinylated secondary antibody and (iii) replacement of the primary antibody by equivalent amounts of normal rabbit or mouse IgG (Vector Labs).

Western blot analysis

To gain a comprehensive understanding of temporal and spatial regulation of TIMP-3 and TIMP-4 protein abundance in bovine preovulatory follicles, Western blot analysis of samples of the apex and base of bovine preovulatory follicles collected 0, 6, 12, 18 and 24 h after GnRH injection was conducted ($n=4$ or 5 per timepoint). Homogenates from the apex and base of preovulatory follicles were prepared separately as described previously (Bakke *et al.* 2002). The optimal protein amount for quantification was determined by Western analysis of increasing protein concentrations of pooled follicle homogenates from each timepoint. For each antibody used, an increase in signal intensity was obtained with increasing protein concentrations. Thirty micrograms of protein (determined by spectrophotometry at 280 nm) from each individual sample were separated on 15% (w/v) SDS-polyacrylamide gels and then electro-blotted to polyvinylidene fluoride membranes (Bio-Rad, Richmond, CA, USA) at 100 V for 1 h in 192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3. Membranes were blocked in 5% w/v BLOT-QuickBlocker (GenoTech, St Louis, MO, USA) with 0.1% Tween-20 in TBST (150 mM NaCl, 50 mM Tris, 0.1% Tween-20, pH 7.5) for 1 h at room temperature, followed by incubation with primary antibody in

1 × femto TBST (GenoTech) with 0.5% BLOT-Quick-Blocker overnight at 4°C. The concentration of primary antibody used for TIMP-3 (AB802, rabbit anti-human polyclonal TIMP-3 antibody; Chemicon), TIMP-4 (AB19168, rabbit anti-human TIMP-4 polyclonal antibody; Chemicon), and actin (MAB3318, mouse anti-human actin monoclonal antibody; Chemicon) were 0.2 µg/ml, 0.2 µg/ml and 1:500 000 (v/v) respectively. After primary antibody incubation, membranes were washed five times for 5 min each in TBST, followed by incubation with goat anti-rabbit (1:2500) (Amersham Biosciences UK, Bucks, UK) or goat anti-mouse (1:10 000) (GenoTech) peroxidase-conjugated antibodies for 1 h at room temperature. After four TBST washes, immunoreactive proteins were visualized using a chemiluminescence horseradish peroxidase immuno-detection system (GenoTech). After incubation with TIMP-3 or TIMP-4 antibodies, membranes were stripped using GenoTech Re-Probe buffer and then reprobed with the actin antibody. Relative molecular masses of immunoreactive proteins detected were determined based on relative migration of protein standards (Broad Range SDS PAGE Standards; Bio-Rad). No signal was detected when duplicate membranes were incubated with equal amounts of rabbit IgG or mouse IgG. After development, films were scanned, and the density of each band analyzed using computer-aided densitometry. Amounts of TIMP-3 and TIMP-4 proteins were normalized relative to amounts of immunoreactive actin detected in each sample.

Statistical analysis

Differences in mRNA and protein abundance were determined by one-way ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS; Version 8.0). When heterogeneity of variance was observed, data were log transformed prior to statistical analysis and are reported as least square mean ± average S.E.M. Individual comparisons of mean RNA and protein concentrations were performed using Fisher's protected least significant differences test.

Results

Effect of the preovulatory gonadotropin surge on TIMP-3 and TIMP-4 mRNA abundance

Results indicate that TIMP-3 and TIMP-4 mRNA abundance in bovine preovulatory follicles was regulated by the gonadotropin surge. Dissociation curves for each PCR product demonstrated a single specific peak (data not shown). Representative standard curves for TIMP-3, TIMP-4 and RPL-19 are depicted in Fig. 1a. Abundance of mRNA for TIMP-3 and TIMP-4 was normalized relative to expression of RPL-19 mRNA. Abundance of RPL-19 mRNA was not affected by GnRH injection in the present experiments (data not shown). Abundance of TIMP-3 mRNA (Fig. 1b) was increased in preovulatory follicles

collected 12 h following GnRH injection, then declined by 20 h post GnRH injection to pre-gonadotropin surge levels ($P < 0.05$). Relative abundance of TIMP-4 mRNA (Fig. 1c) also was increased in bovine preovulatory follicles collected 12 h after GnRH injection ($P < 0.05$), but a significant decrease at 20 h vs the 12 h timepoint was not detected.

Intrafollicular localization of TIMP-3 and TIMP-4 proteins in bovine preovulatory follicles

Given the gonadotropin surge-induced regulation of TIMP-3 and TIMP-4 mRNA abundance observed, the intrafollicular cell types that express TIMP-3 and TIMP-4 were determined. The intrafollicular cell types to which TIMP-3 and TIMP-4 were localized were consistent between the two timepoints. Therefore data for the 0 h timepoint only is depicted. We did not attempt to quantify changes in intensity of staining in the various cell types in response to the gonadotropin surge. Immunoreactive TIMP-3 was localized to the granulosa and thecal cell layers of bovine preovulatory follicles, as well as adjacent ovarian stroma (Fig. 2a and c). Immunoreactivity for TIMP-4 was detected in the granulosa and thecal layers, with a detectable level of immunoreactivity also observed in adjacent ovarian stroma (Fig. 2b and d). Blood vessels in the ovarian stroma were also positive for TIMP-4 (Fig. 2b). No significant staining was detected when sections were incubated in the absence of primary antibodies (data not shown), absence of biotinylated secondary antibody (data not shown) or when sections were incubated with appropriate concentrations of normal mouse or rabbit IgGs (negative control; Fig. 2e and f). Similar cellular localization for TIMP-3 and TIMP-4 proteins was observed in sections of the follicular apex (Fig. 2) and base (data not shown).

Differential regulation of TIMP-3 and TIMP-4 proteins in the apex vs the base of bovine preovulatory follicles

Results indicate that the abundance of TIMP-3 and TIMP-4 proteins in bovine preovulatory follicles was temporally and spatially regulated in response to the gonadotropin surge. Amounts of TIMP-3 and TIMP-4 proteins were normalized relative to amounts of immunoreactive actin detected in each sample. Abundance of actin in homogenates of bovine preovulatory follicles did not change in response to the gonadotropin surge (data not shown).

For TIMP-3, two immunoreactive bands of approximately 46 and 30 $M_r \times 10^{-3}$ were detected in bovine preovulatory follicular homogenates and an increase in signal intensity was obtained with increasing sample protein concentrations (Fig. 3a). Proteins of approximately 46 and 30 $M_r \times 10^{-3}$ were also detected in bovine preovulatory follicular homogenates when the TIMP-3 monoclonal antibody utilized in immunohistochemistry experiments was used for Western blot analysis (data not shown). In the follicular apex, amounts of the 46 $M_r \times 10^{-3}$ form of

Figure 1 Quantitative real-time PCR analysis of the effect of a GnRH-induced gonadotropin surge on TIMP-3 and TIMP-4 mRNA abundance in bovine preovulatory follicles. (a) Representative standard curves for TIMP-3, TIMP-4 and RPL-19. The x axis (Log C0) represents the log value of standard DNA (fg), and the y axis denotes threshold cycle (Ct). (b) Quantification of TIMP-3 mRNA abundance in bovine preovulatory follicles collected at 0, 12 and 20 h post GnRH injection ($n = 4-6$ per timepoint). (c) Quantification of TIMP-4 mRNA abundance in bovine preovulatory follicles collected at 0, 12, and 20 h post GnRH injection ($n = 4-6$ per timepoint). Expression of TIMP-3 and TIMP-4 mRNAs was normalized relative to expression of RPL-19 mRNA. Data are shown as LS mean $\pm$ average S.E.M. Timepoints without a common superscript are different at $P < 0.05$.

TIMP-3 were increased at 12 h following GnRH injection (relative to the 0 h timepoint) and then returned to pre-gonadotropin surge levels by 24 h ($P < 0.05$; Fig. 3b and d). However, no changes in abundance of the lower M_r immunoreactive form of TIMP-3 were detected in samples of the follicular apex (Fig. 3b and d). Furthermore, the increase in abundance of the larger M_r form of TIMP-3 was restricted to the follicular apex. No significant changes in abundance of either form of TIMP-3 were detected in samples of the base of bovine preovulatory follicles (Fig. 3c and e).

For TIMP-4, a single immunoreactive protein of approximately $28 M_r \times 10^{-3}$ was detected in preovulatory follicular homogenates (Fig. 4a). An increase in signal intensity for TIMP-4 was obtained with increasing sample protein concentrations (Fig. 4a). Amounts of TIMP-4 protein in the apex of bovine preovulatory follicles were also regulated by the preovulatory gonadotropin surge. As observed for the larger form of TIMP-3, amounts of TIMP-4 protein were increased in the apex at 12 h following GnRH injection (relative to the 0 h timepoint) and then returned to pre-gonadotropin surge levels by 24 h ($P < 0.05$; Fig. 4b and d). However, no significant changes

in abundance of TIMP-4 protein were detected in samples of the base of bovine preovulatory follicles (Fig. 4c and e).

Discussion

Mammalian ovulation is an intricately controlled process initiated by the preovulatory gonadotropin (LH) surge. Within preovulatory follicles, the gonadotropin surge stimulates expression of proteolytic enzymes which cause follicular ECM degradation resulting in weakening of the follicular wall and ultimately extrusion of the oocyte (Tsafiri & Reich 1999). Accumulating experimental evidence indicates the MMPs are important mediators of the ovulatory process (Brannstrom *et al.* 1988, Butler *et al.* 1991). The relative abundance of MMPs vs their endogenous proteinase inhibitors (TIMPs) dictates net ECM deposition vs degradation (Fata *et al.* 2000). Results of the present studies demonstrated that mRNA and protein for TIMP-3 and TIMP-4 were increased in bovine preovulatory follicles following the gonadotropin surge, suggestive of a potential role for both TIMPs in regulation of preovulatory MMP activity.

Figure 2 Immunohistochemical localization of TIMP-3 and TIMP-4 within bovine preovulatory follicles. (a) Representative micrograph of a section through the apex of a bovine preovulatory follicle collected prior to injection of GnRH to induce the preovulatory gonadotropin surge (0 h timepoint) and incubated with anti-human TIMP-3 antibody, magnification $\times 200$. Note localization of TIMP-3 to granulosa and thecal layers as well as the adjacent ovarian stroma of bovine preovulatory follicle. (b) Representative micrograph of a section through the apex of a bovine preovulatory follicle collected prior to injection of GnRH to induce the preovulatory gonadotropin surge (0 h timepoint) and incubated with anti-human TIMP-4 antibody, magnification $\times 200$. Note localization of TIMP-4 to the granulosa and thecal layers and some ovarian blood vessels. (c) Higher magnification ($\times 400$) of a selected region from panel (a). (d) Higher magnification of a selected region from panel (b). (e) Serial section incubated with normal mouse IgG as negative control for TIMP-3. (f) Serial section incubated with normal rabbit IgG as negative control for TIMP-4. All sections are counterstained with hematoxylin. GC, granulosa cell; TC, thecal cell; BV, blood vessel. Bar = $100\ \mu\text{m}$ (a, b, e and f) and bar = $50\ \mu\text{m}$ (c and d).

In the present studies, mRNA and protein for TIMP-3 and TIMP-4 were increased in bovine preovulatory follicles following the gonadotropin surge. As far as we know, up-regulation of TIMP-3 and TIMP-4 mRNAs in response to an ovulatory stimulus has not been reported previously. Ovarian TIMP-3 mRNA is high at proestrus in mice (Inderdeo *et al.* 1996) and rats (Simpson *et al.* 2001), but in equine chorionic gonadotropin (eCG)-primed mice (Hagglund *et al.* 1999) and rats (Curry & Nothnick 2000), TIMP-3 expression is unchanged following administration of an ovulatory stimulus (human chorionic gonadotropin (hCG) injection). Although there was an increase in TIMP-4 mRNA in rat ovaries 48 h after eCG administration, TIMP-4 mRNA tended to decline after administration of an ovulatory stimulus (hCG treatment) (Simpson *et al.* 2003).

The observed intrafollicular localization of TIMP-3 and TIMP-4 proteins is in agreement with reports in other species. In the present studies, immunoreactive TIMP-3 protein was localized to the thecal and granulosa layers and adjacent ovarian stroma of bovine preovulatory follicles. Localization of TIMP-3 to granulosa and thecal cells and ovarian stroma has been documented previously in mice (Inderdeo *et al.* 1996) and rats (Curry *et al.* 2001). Immunoreactive TIMP-4 was mainly restricted to granulosa and thecal cells of bovine preovulatory follicles, which is consistent with the reported localization of TIMP-4 protein to both granulosa and thecal cells of equine follicles (Riley *et al.* 2001). TIMP-4 mRNA has been localized primarily to the thecal layer surrounding follicles on diestrus and proestrus in the rat (Simpson *et al.* 2003). Localization of TIMP-4 mRNA to cells of the vascular wall of large

Figure 3 Effect of the preovulatory gonadotropin surge on abundance of TIMP-3 protein in extracts of the apex and base of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection ($n = 4$ or 5 per timepoint). (a) Representative Western blot for immunoreactive TIMP-3 demonstrating increase in signal intensity with increasing amounts of sample protein analyzed. (b, c) Western blots demonstrating that two forms of TIMP-3 (46 and 30 $M_r \times 10^{-3}$) were present in the apex (b) and base (c) of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection to induce the gonadotropin surge (representative samples). (d, e) Relative abundance of TIMP-3 proteins (LS mean $\pm$ average S.E.M.; as determined by densitometry) in the apex (d) and base (e) of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection to induce the gonadotropin surge (individual samples; $n = 4$ or 5 per timepoint). Solid bars represent data for 46 000 (higher) M_r form of TIMP-3 and open bars represent data for 30 000 (lower) M_r form of TIMP-3. Timepoints without a common letter (solid bars) or symbol (open bars) are different at $P < 0.05$.

blood vessels in the rat ovary has also been observed (Simpson *et al.* 2003). The overlapping intraovarian distribution of TIMP-3 and TIMP-4 in both granulosa and thecal cells and the similar observed temporal regulation of TIMP-3 and TIMP-4 mRNAs suggest that common regulatory mechanisms may potentially control TIMP-3 and TIMP-4 expression in bovine preovulatory follicles.

Elucidation of the intrafollicular signaling pathways induced in response to the gonadotropin surge that mediate the observed transient increase in TIMP-3 and TIMP-4 mRNA and proteins is of extreme interest. Evidence in rodents supports an obligatory role of intrafollicular progesterone receptor signaling pathways in the ovulatory process (Lydon *et al.* 1995). Progesterone receptor mRNA is transiently increased in bovine preovulatory follicles within 6 h after GnRH injection (Cassar *et al.* 2002, Jo *et al.* 2002). Future studies will be required to determine if

the transient increase in TIMP-3 and TIMP-4 in bovine preovulatory follicles is mediated by intrafollicular progesterone receptor signaling.

Western blot analysis revealed new information about the molecular masses of TIMP-3 and TIMP-4 proteins expressed in bovine preovulatory follicles. Two immunoreactive proteins of 46 and 30 $M_r \times 10^{-3}$ were detected for TIMP-3, while a single immunoreactive protein of approximately 28 $M_r \times 10^{-3}$ was detected for TIMP-4 in bovine preovulatory follicle homogenates. Two immunoreactive forms of TIMP-3 were also detected in the ovine CL, including a prominent larger form of 48 000 M_r (Ricke *et al.* 2002). The nature of the larger M_r form of TIMP-3 was not determined in the present studies, but information supplied with antibodies utilized in Western blot analysis indicates it may represent a dimeric form of TIMP-3 (www.chemicon.com). In contrast, a single 28 $M_r \times 10^{-3}$

Figure 4 Effect of the preovulatory gonadotropin surge on abundance of TIMP-4 protein in extracts of the apex and base of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection ($n = 4$ or 5 per timepoint). (a) Representative Western blot for immunoreactive TIMP-4 demonstrating increase in signal intensity with increasing amounts of sample protein analyzed. (b, c) Western blots demonstrating that the TIMP-4 antibody recognizes a 28 kDa form of TIMP-4 present in the apex (b) and base (c) of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h following GnRH injection to induce the gonadotropin surge (representative samples). (d, e) Relative abundance of TIMP-4 protein (LS mean $\pm$ average S.E.M.; as determined by densitometry) in the apex (d) and base (e) of bovine preovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection to induce the gonadotropin surge (individual samples; $n = 4$ or 5 per timepoint). Timepoints without a common letter are different at $P < 0.05$.

form of TIMP-4 was detected in bovine preovulatory follicles. Immunoreactive TIMP-4 of 28 kDa was detected in the rat ovary (Simpson *et al.* 2003). A similar M_r form of TIMP-4 was observed in extracts of rat carotid arteries (Dollery *et al.* 1999). A 29 kDa of TIMP-4, which co-migrated with a purified human TIMP-4 standard, was detected (using the same antibody as used in the present studies) in human cartilage (Huang *et al.* 2002). Therefore, the observed M_r of TIMP-3 and TIMP-4 in the present studies are consistent with those reported previously for other species and tissue types.

The potential physiological significance of the transient increase in TIMP-3 and TIMP-4 proteins in the apex of bovine follicles following the gonadotropin surge is not clear. In the apex of bovine preovulatory follicles, abundance of the 46 kDa form of immunoreactive TIMP-3, but not the 30 kDa form of TIMP-3, was

increased at 12 h after GnRH injection, but returned to pre-gonadotropin surge levels prior to ovulation (24 h post GnRH injection). Similar temporal changes in abundance of TIMP-4 protein were observed in the apex of bovine preovulatory follicles. Temporal changes in abundance of TIMP-3 and TIMP-4 proteins in the apex of bovine preovulatory follicles correspond well to observed changes in TIMP-3 and TIMP-4 mRNAs in the present studies. However, abundance of TIMP-3 and TIMP-4 proteins was not significantly up-regulated in the base of preovulatory follicles after GnRH injection. The intrafollicular cell types for which TIMP-3 and TIMP-4 immunoreactivity were localized were consistent at both timepoints analyzed (0 and 20 h post GnRH injection) and in both the follicular apex and base. Unfortunately, samples for immunohistochemical analyses were not collected at 12 h post GnRH injection and thus we cannot infer the cellular source of

the transient increase in TIMP-3 and TIMP-4 proteins detected specifically within the apex of preovulatory follicles at the 12 h timepoint.

The increase in TIMP-3 and TIMP-4 proteins in the apex of preovulatory follicles was transient and thus suggestive of a potential role for the inhibitors in temporal regulation of preovulatory MMP activity. The observed differential regulation of TIMP-3 and TIMP-4 proteins in the apex vs the base of preovulatory follicles was not expected and seems counterintuitive. We recently reported that abundance of protein for collagenase-3 (MMP-13) is increased in both the apex and the base of bovine preovulatory follicles at 24 h after GnRH injection (Bakke *et al.* 2004). The observed regulation of MMP-13, TIMP-3 and TIMP-4 in the apex vs the base of preovulatory follicles in itself does not present a viable explanation for the selective ECM degradation that occurs at the apex of preovulatory follicles (Espey 1967, Murdoch & McCormick 1992). Results would on the surface appear to favor a potential shift in the MMP:TIMP ratio in the follicular apex toward ECM degradation as ovulation approaches (24 h timepoint post GnRH injection). However, we previously reported that inhibitory activities for both TIMP-1 and TIMP-2 (as determined by reverse zymography) are increased in bovine preovulatory follicular fluid collected 24 h after GnRH injection (Bakke *et al.* 2002). Therefore, changes in other MMPs and TIMPs also have to be considered. Ultimately, direct measurements of MMP and MMP inhibitory activity will be necessary to confirm biochemically the assumed shift in MMP:TIMP ratio in favor of ECM degradation in bovine follicles near the time of ovulation.

Although the ability of TIMPs to control preovulatory ECM degradation may help regulate the ovulatory process, additional functions for the TIMPs have been described. In particular, TIMP-3 and TIMP-4 have been shown to promote apoptosis in other systems (Smith *et al.* 1997, Tumulapalli *et al.* 2001). Interestingly, the proapoptotic effects of TIMP-3 on apoptosis of colon carcinoma cells are mediated through stabilization of cell surface tumor necrosis factor (TNF)- α receptors (Smith *et al.* 1997). Apoptosis of cells in the follicular apex, particularly the ovarian surface epithelial cells, is characteristic of the ovulatory process in ewes (Murdoch 1995) and evidence indicates this phenomenon is mediated by TNF- α (Murdoch *et al.* 1997). Establishment of a role for TIMP-3 and TIMP-4 in the ovulatory process of cattle that is independent of regulation of ECM remodeling will require further investigation.

Collectively, results of the present studies indicate that TIMP-3 and TIMP-4 mRNA and protein in bovine preovulatory follicles are transiently increased following the preovulatory gonadotropin surge. Furthermore, increased abundance of TIMP-3 and TIMP-4 proteins was found within the apex, but not the base of preovulatory follicles. Increased TIMP-3 and TIMP-4 expression in preovulatory follicles may potentially help temporally and spatially

regulate the extent of MMP activity during the interval preceding follicle rupture.

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