Production of matrix metalloproteinases by cultured bovine theca and granulosa cells

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Abstract

Matrix metalloproteinases (MMPs) degrade the proteinaceous components of the extracellular matrix and are presumably essential for follicular growth culminating in ovulation or atresia. The objectives of this study were to characterize the gelatinolytic and caseinolytic MMPs secreted by cultured bovine thecal and granulosa cells and to determine the effect of luteinizing hormone (LH) on MMP secretion. Thecal and granulosa cells were collected from small bovine follicles (<5 mm) on day 2 or 5 of the estrous cycle (day 0 = estrus). A serum-free culture system was utilized in which bovine thecal and granulosa cells do not spontaneously luteinize, but produce androstenedione and estradiol in response to physiological concentrations of LH and follicle-stimulating hormone (FSH) respectively. The effect of LH (0, 1 or 100 ng/ml) on MMP production was determined in conditioned media collected every 48 h for 144 h. MMPs were detected by gelatin and casein zymography and MMP activity was quantified by image analysis. Thecal and granulosa cell conditioned media contained MMPs that had a relative molecular size (M_r) ranging from 53 000 to 200 000 and addition of 1,10 phenanthroline (MMP inhibitor) blocked gelatinolytic and caseinolytic activity. Patterns of gelatinolytic activity in thecal and granulosa cell conditioned media differed over time with the M_r 62 000 and 83 000 MMPs being increased ($P < 0.05$) and the M_r 53 000 MMP being decreased ($P < 0.05$) at 96 h of culture. LH (1 or 100 ng/ml) increased ($P < 0.05$) gelatinolytic activity of the M_r 53 000 and 62 000 gelatinases within thecal cell conditioned media but not granulosa cell conditioned media. The M_r 62 000 and 83 000 gelatinolytic activities corresponded to the active forms of gelatinase A (M_r 62 000) and B (M_r 83 000) and gelatinase A was detected in thecal cell conditioned media by Western blot analysis. Caseinolytic activity (M_r 83 000) was detected in both thecal and granulosa cell conditioned media and increased from 48 to 96 h. In summary, thecal and granulosa cells secrete gelatinolytic and caseinolytic MMPs and thecal cell production of gelatinase A was stimulated by LH.

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Introduction

Follicular growth culminating in ovulation or atresia is a cyclical process that requires extensive extracellular matrix (ECM) remodeling. In addition, components of the ovarian ECM undergo cyclic changes during the murine estrous cycle based on changes in mRNA expression of type III, IV, and VI collagens (Oksjoki *et al.* 1999). The ECM consists of proteinaceous and nonproteinaceous molecules that provide the tissue-specific, extracellular architecture to which cells attach (Luck 1994, Rodgers *et al.* 1999). Binding of ECM proteins to cellular receptors (i.e. integrins) can affect cellular structure, second

messenger generation, and gene expression (Tremble *et al.* 1995, Giancotti *et al.* 1997).

Although numerous studies report the effects of various hormones and growth factors on follicular growth and steroidogenesis, there is very little information on the mechanisms by which follicular expansion occurs within the confines of the ovarian stroma. Development of a bovine primordial follicle into a preovulatory follicle requires an increase in follicular surface area of >317 000-fold (van Wezel & Rodgers 1996). Follicular expansion within the confines of the ovarian stroma necessitates ECM remodeling of the basement membrane and the surrounding stromal tissue in addition to angiogenesis within the thecal

layer. Two families of enzymes that regulate remodeling of ECM include the matrix metalloproteinase (MMP) and plasminogen activator/plasmin families. MMPs are metal-dependent enzymes capable of degrading each of the proteinaceous components of the ECM, and are reported to have an important role in ovulation, tissue growth and angiogenesis (Egeblad & Werb 2002, Smith *et al.* 2002, Curry & Osteen 2003). The objectives of this study were to characterize the gelatinolytic and caseinolytic MMPs secreted by cultured bovine thecal and granulosa cells and to determine the effect of luteinizing hormone (LH) and time on MMP secretion.

Materials and Methods

Materials

The following items were obtained from Gibco (Gibco BRL, Life Technologies Ltd, Paisley, Renfrewshire, UK): Medium 199, Hepes, glutamine, Dulbecco's PBS without calcium and magnesium (DPBS⁻), and amphotericin. The following items were obtained from Sigma Chemical Ltd (Poole, Dorset, UK): McCoy's 5a medium with sodium bicarbonate, Penstrep (containing 10 000 IU penicillin and 10 mg/ml streptomycin), BSA, transferrin, selenium, bovine insulin, androstenedione, estradiol, neutral red, donor calf serum, trypan blue, Dulbecco's minimum essential medium: Hams F-12 medium (DMEM: F-12; 1:1 mixture), type II and type IV collagenase, hyaluronidase (from bovine testes), pronase, and deoxyribonuclease I. An IGF-1 analog (Long R3 IGF-1, which does not bind to IGF binding proteins) was obtained from Groprep (Groprep Pty Ltd, Adelaide, SA, Australia). Bovine follicle stimulating hormone (FSH) (USDA-bFSH-I-2; bioactivity potency 854 IU/mg) was provided by the United States Department of Agriculture and ovine LH (NIADDK-oLH-26) was provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Torrance, CA, USA). The following items were obtained from Nunclon (Life Technologies, Paisley, Renfrewshire, UK): culture bottles, plates and dishes.

Collection of ovaries

Research was conducted under a Home Office project licence and was approved by an institutional committee. Fifteen normally cycling beef (*Bos taurus*; Friesian cross) heifers received a progestational-releasing intravaginal device (PRID; Sanofi Animal Health, Watford, UK) for 10 days and an intramuscular injection of prostaglandin F_{2α} (25 mg Lutalyse) in the neck at PRID removal. Heifers were detected for estrus and ovaries were collected on day 2 or 5 of the estrus cycle (day 0 = estrus) during three separate trips to a local abattoir (five heifers per trip). Ovaries were immediately placed in collection media (Medium 199, containing 20 mM Hepes, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 1 µg/ml amphotericin) at 37°C and transported to the laboratory. Ovaries were

trimmed of excess tissue, placed in 70% ethanol for 30 s, and rinsed and maintained in collection media (37°C) until follicle dissection. Follicles (<5 mm) were manually dissected and visibly atretic follicles were discarded. Atretic follicles were identified as having reduced vascularity, evidence of degenerated granulosa cells, and a detached cumulus-oocyte-complex. Gutierrez *et al.* (1997) reported that granulosa cells from small bovine follicles differentiate *in vitro* and secretion of estradiol increased with time such that it was similar to large follicles. Therefore, in the present experiment, granulosa and thecal cells were collected from small follicles (<5 mm) and placed in culture as described below. The experiment was replicated three times (five heifers per replicate) with four wells per treatment (see below).

In order to characterize gelatinolytic and caseinolytic activity within bovine serum, a blood sample was collected from each of the five heifers on day 5 of the cycle. Blood was collected by venipuncture and allowed to clot for 1 hour before being placed overnight at 4°C. Serum was harvested from each animal and frozen at -20°C until zymographic analyses (see below). Immediately following collection of ovaries, follicles were dissected from each pair of ovaries from each of the five heifers. Follicles were manually dissected and sorted by follicular diameter into the following groups by animal: small (<5 mm), medium (5 to 8 mm), and large (>8 mm). Follicular fluid was aspirated from each follicular size class and stored at 4°C until zymographic analyses (see below). Within an animal, follicular fluid from follicles was pooled within a size class.

Granulosa cell culture

A serum-free culture system was utilized in which bovine granulosa cells do not spontaneously luteinize and estradiol secretion can be stimulated with physiological concentrations of FSH (Gutierrez *et al.* 1997). Following the removal of follicular fluid, follicles (<5 mm) were hemisected and placed in a petri dish containing DPBS⁻. Granulosa cells were removed from the walls of the follicles by flushing the hemisected follicles up and down through a 3 ml syringe without a hypodermic needle. The resulting cell suspension (5 ml) was placed into a tube and supplemented with 10 ml culture media (McCoy's medium containing sodium bicarbonate, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin). The cells were centrifuged at 800 g for 10 min and washed twice in culture media. Cell number and viability were determined in a hemacytometer using Trypan blue exclusion.

Granulosa cells were seeded into 96-well plates (between 43 000 and 75 000 viable cells/well) in McCoy's 5a medium containing sodium bicarbonate and supplemented with 20 mM Hepes, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 3 mM L-glutamine, 0.1% BSA, 10⁻⁷ M androstenedione, 5 µg/ml transferrin, 4 ng/ml selenium, 10 ng/ml insulin, 1 ng/ml IGF analog (long R3

IGF-I), and 1 ng/ml bovine FSH. No serum was added to the culture at any time. Granulosa cells (50 µl aliquot) were added to each well containing 200 µl of pre-equilibrated media. The cell density that was utilized has been reported to optimize estradiol secretion (Gutierrez *et al.* 1997). The cells were incubated at 37 °C in a humidified atmosphere of 4% CO₂ for 6 days.

The effects of 0, 1 and 100 ng/ml of LH on gelatinolytic and caseinolytic activity were tested. LH was diluted in culture media and added at 0, 48 and 96 h of culture (5 µl of the corresponding treatment to each well containing 0, 5 or 500 ng/ml LH). Media (175 µl) was collected at 48, 96 and 144 h of culture and frozen at –20 °C until determination of gelatinolytic or caseinolytic activity. After 6 days of culture, media were collected and the number of viable granulosa cells per well determined by the uptake of a vital red dye, neutral red (Campbell *et al.* 1998).

Thecal cell culture

Bovine thecal cells were cultured in a serum-free system in which the cells do not spontaneously luteinize and androstendione secretion can be stimulated with physiological concentrations of LH (Campbell *et al.* 1998). Following collection of granulosa cells, thecal cells were enzymatically dispersed in 20 ml DPBS[–] (containing 100 mg collagenase type II, 20 mg hyaluronidase, and 20 mg pronase) at 37 °C with gentle agitation. After 20 min, 0.4 mg deoxyribonuclease I was added and the incubation continued until the thecal cells were dispersed (approximately 30–45 min). The enzymatic reaction was terminated following addition of 200 µl donor calf serum. The enzyme mix (10 ml) was added to a 15 ml centrifuge tube containing 5 ml culture media (DMEM/F12 with 3 mM L-glutamine, 5 µg/ml transferrin, 4 ng/ml selenium, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 20 ng/ml estradiol, and 0.1% BSA) and centrifuged at 800 g for 10 min. The thecal cell pellets were washed twice and cell number and viability determined by Trypan blue exclusion. Seventy-five thousand thecal cells were seeded in a volume of 50 µl into pre-equilibrated 96-well plates containing 200 µl of culture media. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 6 days.

The effects of 0, 1, and 100 ng/ml LH on MMP activity were tested. LH was diluted in culture media and added at 0, 48, and 96 h of culture (50 µl of the corresponding treatment to each well containing 0, 5, or 500 ng/ml LH). Media (175 µl) were collected at 48, 96, and 144 h of culture and frozen at –20 °C until determination of gelatinolytic or caseinolytic activity (see below). After 6 days of culture, media were collected and the number of thecal cells per well determined by the uptake of a vital red dye, neutral red (Campbell *et al.* 1998).

Luteal cell culture

Bovine ovaries containing corpora lutea were collected at a local abattoir during early luteal development (~day 4

of the estrous cycle, day 0 = estrus) according to criteria published by Fields and Fields (1996). Corpora lutea were dissected from ovaries under sterile conditions, and sliced into 0.5 mm sections using a hand-held microtome. Luteal sections (0.5 mm) were dissociated in Ca⁺⁺- and Mg⁺⁺-free Hank's Balanced Salt Solution containing 100 U ml^{–1} penicillin, 100 µg ml^{–1} streptomycin sulphate, 5 µg ml^{–1} insulin, 5 µg ml^{–1} transferrin, and 5 ng ml^{–1} selenium in the presence of 16 units type IV collagenase per gram of tissue and 17.5 mg deoxyribonuclease I per corpus luteum. Luteal tissue was dissociated for 45 to 60 min at 37 °C with moderate agitation. After dissociation, cells were washed twice with DMEM/Ham's F-12 media containing 100 U ml^{–1} penicillin, 100 µg ml^{–1} streptomycin sulphate, 5 µg ml^{–1} insulin, 5 µg ml^{–1} transferrin, and 5 ng ml^{–1} selenium (DMEM/HAM's F-12 complete media). Cells were cultured overnight in 24-well plates (~400 000 cells/well) containing DMEM/HAM's F-12 complete media supplemented with 5% fetal bovine serum in an atmosphere of humidified air with 5% CO₂. Culture wells containing luteal cells were washed three times with serum free DMEM/HAM's F-12 media and subsequently cultured for 24 h in serum free DMEM/HAM's F-12 complete media. Following collection, luteal cell conditioned media was concentrated 17-fold and stored at –20 °C until utilized for Western blot analysis.

Gelatin and casein zymography

To identify the types and relative abundance of gelatinolytic (gelatinases) and caseinolytic (stromelysins) MMPs within granulosa and thecal conditioned media, gelatin and casein substrate zymography were performed respectively. Granulosa or thecal conditioned media (50 µl per well) were collected from each of four wells per treatment, pooled within treatment, and mixed with Laemmli sample buffer lacking β-mercaptoethanol. Samples were not boiled prior to electrophoresis. Granulosa secreted proteins (15 µl per lane), thecal secreted proteins (15 µl per lane), blood serum, or bovine follicular fluid (1 µl per lane) were electrophoresed in 0.75 mm thick, 10% one-dimensional SDS-polyacrylamide gels containing 1 mg/ml porcine skin gelatin (175 bloom) or 1 mg/ml casein. Some of the gelatin gels included gelatinase A (MMP-2) and gelatinase B (MMP-9) standards (kindly provided by Hideaki Nagase, Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College, London). No caseinolytic (stromelysins) MMP standards were available for this study.

Following electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 for 1 h to remove SDS. Gels were rinsed three times (20 min per rinse) in water and incubated for 15 to 18 h at 37 °C in incubation buffer (50 mM Tris–HCl (pH 7.5), 10 mM CaCl₂, and 5 µM ZnCl₂) in the presence or absence of 12 mM 1,10 phenanthroline (inhibitor of MMP activity). Gels were subsequently stained with 0.5% (w/v) Coomassie Brilliant Blue R250 and destained. Gelatin- and casein-degrading enzymes were identified as

clear zones of digested gelatin or casein respectively. Prestained molecular weight markers were used for molecular weight estimation of the bands resolved by gel zymography.

MMP activity was quantified by densitometry (Bio Rad Model GS 700 Imaging Densitometry, Molecular Analyst, Molecular Bioscience Group, Hercules, CA, USA). Gelatinolytic and caseinolytic activities for both granulosa and thecal conditioned media were expressed as arbitrary units per 50 000 cells. The relative molecular mass of each gelatinolytic or caseinolytic band was determined from the migration of prestained molecular weight markers.

Western blot analysis for MMP-2

Bovine granulosa, thecal, or luteal (positive control) cell secreted proteins (15 µl per lane) were electrophoresed in 0.75 mm thick, 10% one-dimensional SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. Nonspecific binding sites were blocked by incubating nitrocellulose strips (6 mm) in Tris-buffered saline containing 0.05% Tween (TBST; pH 7.5) and 3% non fat dry milk for 0.5 h. Strips were washed three times in TBST. Nitrocellulose strips were incubated (2 h at 4 °C) in TBST containing 1% non fat dry milk with a polyclonal rabbit anti-human gelatinase A (MMP-2) serum (gift from WG Stetler-Stevenson, NIH, Bethesda, MD, USA) or normal rabbit serum at a 1:300 dilution. Strips were subsequently washed three times in TBST and incubated with goat anti-rabbit immunoglobulin conjugated to biotin at a 1:10 000 dilution in TBST. After rinsing three times in TBST, strips were incubated for 0.5 h at 20 °C in an avidin-biotin-peroxidase complex solution. Strips were rinsed three times in TBST, incubated in a peroxidase substrate, and photographed. The relative molecular mass of each band was determined from the migration of prestained molecular weight markers.

Statistics

Repeated measurements of gelatinolytic and caseinolytic activity from three replicate experiments were analysed by the mixed procedure for mixed model analysis of variance of SAS (SAS Institute Inc., Cary, NC, USA) and differences among means determined by a protected Least Significant Difference test.

Results

Gelatinolytic activity in thecal cell conditioned media

Thecal cell conditioned media contained gelatinases having a relative molecular size (M_r) ranging from 53 000 to 200 000 (Fig. 1; panel A). The primary gelatinolytic bands had an M_r of 53 000, 62 000, 83 000, and 200 000 and addition of 12 mM 1,10 phenanthroline (inhibitor of MMP activity) strongly inhibited all gelatinolytic activity

(Fig. 1; panel B). Additional gelatinolytic bands were detected that had an M_r of 92 000, 86 000, 72 000, and 66 000. MMPs having an M_r of 62 000 and 83 000 comigrated with the active forms of MMP 2 (gelatinase A; M_r 62 000) and MMP 9 (gelatinase B; M_r 82 000) standards respectively (data not shown). Gelatinolytic activity differed over time with the M_r 62 000 and 83 000 MMPs increasing ($P < 0.05$) from 48 to 96 h and decreasing from 96 to 144 h (Fig. 2; panels B and C). The M_r 200 000 gelatinase also increased ($P < 0.05$) from 48 to 96 h in the presence of 1 or 100 ng/ml LH (Fig. 2; panel D). The M_r 53 000 MMP decreased ($P < 0.05$) from 48 to 96 to 144 h of culture in the presence of 1 or 100 ng/ml LH (Fig. 2; panel A).

Mono-dispersed thecal cells organized into cellular aggregates and addition of LH (1 or 100 ng/ml) increased the amount of aggregate formation (data not shown). LH (1 or 100 ng/ml) increased ($P < 0.05$) the gelatinolytic activity of the M_r 53 000 (48 h), 62 000 (48, 96, and 144 h), and 200 000 (48 and 96 h) MMPs relative to the control group (0 ng/ml; Fig. 3; panels A–C) but had no effect on the M_r 83 000 MMP.

Gelatinolytic activity in granulosa cell conditioned media

Granulosa cell conditioned media contained gelatinolytic activity having a relative molecular size of 62 000, 83 000, and 200 000 (Fig. 4; panel A) and MMPs (in granulosa cell conditioned media) having an M_r of 62 000 and 83 000 comigrated with the active forms of the MMP 2 (gelatinase A) and MMP 9 (gelatinase B) standards respectively (data not shown). Similar to the thecal cell conditioned media, addition of 1,10 phenanthroline strongly inhibited gelatinolytic activity within granulosa cell conditioned media. There was an LH x time interaction for the M_r 83 000 gelatinolytic activity but not the M_r 62 000 or 200 000 activity in granulosa cell conditioned media (Fig. 5). Gelatinolytic activity of granulosa cell conditioned media differed over time with the M_r 62 000 and 200 000 gelatinases increasing ($P < 0.05$) from 48 to 96 h and remaining the same at 144 h (Fig. 5; panels A and C). The M_r 83 000 gelatinolytic activity increased ($P < 0.05$) from 48 to 96 h (0 ng/ml) but decreased ($P < 0.05$) from 96 to 144 h (100 ng/ml; Fig. 5; panel B). There was no effect ($P > 0.05$) of either dose of LH on M_r 62 000 or 200 000 gelatinolytic activity at any time point in granulosa conditioned media. However, LH (1 or 100 ng/ml) increased M_r 83 000 gelatinolytic activity at 48 h and decreased (100 ng/ml LH) M_r 83 000 activity at 144 h relative to the control group (0 ng/ml LH) in granulosa cell conditioned media (Fig. 6).

Caseinolytic activity in thecal and granulosa cell conditioned media

Caseinolytic activity (M_r 45 000 and 57 000) was detected in thecal and granulosa cell conditioned media (Fig. 1;

Figure 1 Representative gelatin (panels A & B) and casein (panels C & D) zymograms of MMP activities in bovine thecal cell conditioned media (15 μ l media per lane) collected at 48, 96, and 144 h of culture. Thecal cells were obtained from small follicles (<5 mm) and cells were cultured with 0, 1, or 100 ng/ml of LH. Zymography was performed in the presence (B & D) or absence (A & C) of 12 mM 1,10 phenanthroline (inhibitor of MMP activity). Areas of gelatinolytic (A) and caseinolytic (C) activity are visualized as clear zones of digested gelatin or casein respectively.

panel C and Fig. 4; panel C) respectively. The M_r 45 000 and 57 000 caseinolytic bands may represent the active and latent forms of stromelysin-1 (MMP-3), -2 (MMP-10) or -3 (MMP-11), based on the relative molecular mass (active forms M_r 44 000 to 45 000; latent forms, 51 000 to 58 000) and the disappearance of the activities in the presence of 1,10 phenanthroline (Fig. 1; panel D). The preceding activities were below the limits of detection by densitometry.

Caseinolytic activity (M_r 83 000) was detected in both thecal (Fig. 1; panel C) and granulosa cell (Fig. 4; panel C) conditioned media and the activity was inhibited by 1,10 phenanthroline (Fig. 1; panel D). There was an increase ($P < 0.05$) in M_r 83 000 caseinolytic activity within both thecal and granulosa cell conditioned media from 48 to 96 h (Fig. 7; panels A and B). At 144 h, M_r 83 000 caseinolytic activity decreased ($P < 0.05$) in thecal but not granulosa cell conditioned media (Fig. 7; panels A and B). There was no effect ($P > .05$) of LH on M_r 83 000 caseinolytic activity in thecal cell conditioned

media. However, LH (100 ng/ml) decreased ($P < 0.05$) M_r 83 000 caseinolytic activity in granulosa cell conditioned media (densitometric units = 4647 ± 388 , 3663 ± 388 , and 2798 ± 388 for 0, 1, and 100 ng/ml LH respectively).

MMP activity in bovine serum and follicular fluid

Gelatinolytic (M_r 62 000) but not caseinolytic activity was detected in serum and follicular fluid collected on day 5 postestrus (Fig. 4; panels B and D) and gelatinolytic activity in both serum and follicular fluid was inhibited by 1, 10 phenanthroline. A faint gelatinolytic band at M_r 72 000 was detected in the serum but not follicular fluid of each animal. There was no difference among animals in M_r 62 000 gelatinolytic activity of serum; however, there were differences in gelatinolytic activity of follicular fluid collected from different sized follicles. Gelatinolytic activity within large (>8 mm) follicles (475 ± 43.9 arbitrary units) was decreased ($P < 0.01$) compared with small

Figure 2 Effect of time (48, □; 96, ▨; or 144 h, ■) on mean ($\pm$ S.E.M.) gelatinolytic (M_r 53 000 (53K), 62 000 (62K), 83 000 (83K), and 200 000 (200K); panels A, B, C and D respectively) activity of bovine thecal cell conditioned media following treatment with LH at 0, 1, or 100 ng/ml. There was no time $\times$ LH interaction ($P > 0.05$) for the 83K gelatinolytic activity; therefore, the data from wells treated with LH were pooled. Thecal cells were obtained from small follicles (< 5 mm). Gelatinolytic activities were determined by image analysis of zymograms and expressed as arbitrary units of MMP activity per 50 000 cells. Conditioned media was collected at 48, 96, and 144 h (h) of incubation. Means ($\pm$ S.E.M.) for specific gelatinolytic activities (53K, 62K, and 200K) having different superscripts are different ($^{abc}P < 0.05$). In panel C the three doses of LH are combined into a single value where there was no interaction.

(< 5 mm; 910 ± 43.9 arbitrary units) or medium (5 to 8 mm; 962 ± 43.9 arbitrary units) follicles.

Immunodetection of MMP-2 (M_r 62 000)

An anti-human MMP-2 antibody detected a band (M_r 62 000) corresponding to the active form of MMP-2 (gelatinase A) in both thecal and granulosa cell

conditioned media (Fig. 8). This band was similar in size to a band that was detected in the luteal cell conditioned media (positive control).

Discussion

MMPs are zinc- and calcium-dependent enzymes that collectively degrade the proteinaceous components of

Figure 3 Effect of LH (0 (□), 1 (▨), or 100 (■) ng/ml) at 48, 96, or 144 h on changes in gelatinolytic activity (M_r 53 000 (53K), 62 000 (62K), and M_r 200 000 (200K)) of bovine thecal cell conditioned media (panels A, B, and C respectively). Thecal cells were obtained from small follicles (<5 mm). Gelatinolytic activity was determined by image analysis of gelatin zymograms and expressed as arbitrary units of MMP activity per 50 000 cells. Prior to gelatin zymography, conditioned media was collected at 48, 96, and 144 h of incubation and from cells that were treated with LH (0, 1, or 100 ng/ml). Means ($\pm$ S.E.M.) for specific gelatinolytic activities having different superscripts differ ($^{abc}P < 0.05$).

the ECM and cleave signaling proteins (Egeblad & Werb 2002, Smith *et al.* 2002). The MMPs are a growing gene family of greater than 26 enzymes that can generally be subdivided into the collagenases, gelatinases, stromelysins, and membrane type (mt) MMPs (Nagase & Okada 1997, Smith *et al.* 1999, Egeblad & Werb 2002). Numerous studies have focused on the role of the MMP family in

ovulation; however, recent studies indicate that this enzyme system may have an essential role during follicular growth and differentiation (Curry & Osteen 2003).

Results from this study indicate that bovine granulosa and thecal cells produce MMPs that have gelatinolytic and caseinolytic activities *in vitro*. Evidence that the enzymes are MMPs produced by follicular cells include the following: (1) gelatinolytic and caseinolytic activities in thecal and granulosa cell conditioned media were inhibited by an MMP inhibitor (1, 10 phenanthroline; chelates zinc); (2) the relative molecular mass (M_r) of the 62 000 and 83 000 gelatinases corresponded to the relative molecular mass of the active form of gelatinase A (M_r 62 000) and B (M_r 82 000) respectively, and comigrated with gelatinase A and B standards; (3) the M_r 72 000 and 92 000 gelatinases corresponded to the latent forms of gelatinase A (M_r 72 000) and B (M_r 92 000) respectively; (4) the M_r 62 000 protein crossreacted with antisera directed against gelatinase A (MMP-2); (5) although gelatinase A was detected in both serum (latent and active forms), and follicular fluid (active form), granulosa and thecal cells appear to be a source of this gelatinase since both cell types were cultured in serum free-media; and (6) the relative molecular mass (M_r) of the 45 000 and 57 000 caseinolytic activities corresponded to the relative molecular mass of the active (M_r 44 000 to 45 000) and latent (M_r 51 000 to 58 000) forms of stromelysin-1, -2, or -3, respectively.

MMPs contain several conserved domains including a propeptide that maintains the latency of the enzyme (Nagase & Woessner 1999). Upon secretion of these zymogens, cleavage of the propeptide is necessary for enzyme activation. Gelatinase activity at M_r 72 000 and 92 000 in thecal cell conditioned media corresponded to the latent forms of gelatinase A (MMP-2; M_r 72 000) and gelatinase B (MMP-9; M_r 92 000). Although the latent forms of gelatinase A and B are enzymatically inactive *in vivo*, they are active in the presence of SDS during zymography. Gelatinase activity at M_r 66 000 and 86 000 likely represent intermediate forms of gelatinase A and B respectively. Intermediate forms of gelatinase A are known to exist, and have been previously reported for ovine luteal tissue (Ricke *et al.* 2002). At the present time it is not clear how MMP-9 is activated; however, intermediate forms are likely.

The identity of the M_r 53 000 gelatinolytic activity in thecal cell conditioned media is not clear. It seems unlikely that the M_r 53 000 gelatinase represents stromelysin-1 (MMP-3; M_r 56 000), since the substrate affinity of stromelysin-1 for casein is much greater than for gelatin. No prominent bands were detected at M_r 53 000 in thecal cell conditioned media with casein zymography. Although a caseinolytic band was detected in thecal cell conditioned media at approximately M_r 45 000, this activity is more likely to be due to the active form of stromelysin-1, -2, or -3 (MMP-3, -10, or -11 respectively). The gelatinolytic activity (M_r 53 000) may represent a cleavage product of gelatinase A since LH (1 and 100 ng/ml) increased the

Figure 4 Representative gelatin (panel A) and casein (panel C) zymograms of matrix metalloproteinases in bovine granulosa cell conditioned media (15 μ l media per lane) collected at 48, 96, and 144 h of culture. Granulosa cells were obtained from small follicles (<5 mm) and cells were cultured with 0, 1, or 100 ng/ml of LH (panels A and C). Gelatin zymograms of enzymes in bovine serum (panel B; 1 μ l per lane) and follicular fluid (panel D; 1 μ l per lane). Serum samples from five heifers (1 to 5) are shown in panel B. Follicular fluid from small (S; < 5 mm), medium (M; 5–8 mm), and large (L; > 8 mm) follicles (Foll.) collected from three heifers (1 to 3; Animals) are shown in panel D. Areas of gelatinolytic (panels A, B, and D) and caseinolytic (panel C) activity are visualized as clear zones of digested gelatin or casein respectively.

gelatinolytic activity of both the M_r 53 000 and 62 000 gelatinases relative to the control group at 48 h. Alternatively, the activity at M_r 53 000 might represent the ectodomain shedding of membrane type 1 MMP (MT1-MMP; M_r 56 000) (Toth *et al.* 2002) or a novel MMP as described by Kubler *et al.* (1998). Non-autocatalytic shedding of MT1-MMP from the cellular surface produced an active enzyme (M_r 50 000) that was inhibited by TIMP-2 (Toth *et al.* 2002). Mt1-MMP is expressed in bovine follicles and corpora lutea (G W Smith, unpublished observations, Zhang *et al.* 2002).

The gelatinolytic band at approximately M_r 200 000 probably represents gelatinase A and B bound to α -2 macroglobulin. MMPs are inhibited by two primary endogenous inhibitors: tissue inhibitors of metalloproteinases and α -2 macroglobulin. Alpha-2 macroglobulin is a large protein (M_r 720 000) that is made up of four

identical subunits (M_r 185 000). This proteinase is present in serum and has also been detected in human follicular fluid (Curry *et al.* 1990), rat ovarian follicles (Gaddy-Kurten *et al.* 1989), and rat corpora lutea (Gaddy-Kurten *et al.* 1989). Furthermore, mRNA and protein expression for α -2 macroglobulin and its receptor were detected in bovine granulosa cells from dominant and subordinate follicles (Jimenez-Krassel *et al.* 2002). Alpha-2 macroglobulin increased estradiol production *in vitro*, by granulosa cells collected from dominant or subordinate follicles by 30- or 50-fold respectively (Jimenez-Krassel *et al.* 2002).

Binding of α -2 macroglobulin to a proteinase is the result of a proteolytic attack on the 'bait region' of α -2 macroglobulin. Following proteolytic cleavage of the bait region there is a conformational change in α -2 macroglobulin that entraps the proteinase without blocking the active site of the enzyme (Barrett & Starkey 1973). Both

Figure 5 Effect of time (48, 96, or 144 h) on changes in gelatinolytic (62 000 (62K), 83 000 (83K), and 200 000 (200K)) activities of bovine granulosa cell conditioned media following treatment with LH at 0, 1, or 100 ng/ml (panels A, B, and C respectively). There was no time $\times$ LH interaction ($P > 0.05$) for the M_r 62 000 and 200 000 gelatinolytic activities; therefore, the data from wells treated with LH were pooled. Granulosa cells were obtained from small follicles (< 5 mm). Gelatinolytic activity was determined by image analysis of zymograms and expressed as arbitrary units of MMP activity per 50 000 cells. Conditioned media (15 μ l per group) was collected at 48, 96, and 144 h of incubation from cells that were treated with LH (0, 1, or 100 ng/ml). Means ($\pm$ S.E.M.) for specific gelatinolytic activities having different superscripts differ ($^{ab}P < 0.05$). In panels A and C the three doses of LH are combined into a single value where there is no interactions.

gelatinase A and B have been reported to be proteolytically active, as determined by gelatin zymography, while bound to α -2 macroglobulin (Nagase *et al.* 1994).

Stromelysins (MMP-3, -10 and -11) have a broad substrate specificity and are capable of degrading the major constituents of basement membranes (Egeblad & Werb 2002); however, there is very little published data on stromelysin activity of follicular cells (Curry & Osteen 2003). Casein zymography is frequently utilized to detect stromelysins (MMP-3, -10, and -11) and in the present study caseinolytic activity was detected in thecal (M_r 45 000)

Figure 6 Effect of LH (0 (open bars), 1 (hatched bars), or 100 (black bars) ng/ml) at 48, 96, or 144 h on changes in M_r 83 000 (83K) gelatinolytic activity of bovine granulosa cell conditioned media. Granulosa cells were obtained from small follicles (< 5 mm). Gelatinolytic activity was determined by image analysis of gelatin zymograms and expressed as arbitrary units of MMP activity per 50 000 cells. Prior to gelatin zymography, conditioned media was collected at 48, 96, and 144 h of incubation and from cells that were treated with LH (0, 1, or 100 ng/ml). Means ($\pm$ S.E.M.) for specific gelatinolytic activities having different superscripts differ ($^{ab}P < 0.05$).

Figure 7 Effect of time (48, 96, or 144 h) on mean ($\pm$ S.E.M.) caseinolytic (M_r 83 000 (83K)) activity of bovine thecal (Theca; panel A) and granulosa (Gran; panel B) cell conditioned media following treatment with LH at 0, 1, or 100 ng/ml. There was no time $\times$ LH interaction ($P > 0.05$) for the 83K caseinolytic activity for theca or granulosa cell conditioned media; therefore, the data from wells treated with LH were pooled. Thecal and granulosa cells were obtained from small follicles (< 5 mm). Caseinolytic activities were determined by image analysis of zymograms and expressed as arbitrary units of MMP activity per 50 000 cells. Conditioned media was collected at 48, 96, and 144 h of incubation. Means ($\pm$ S.E.M.) for specific caseinolytic activities having different superscripts differ ($^{ab}P < 0.05$).

Figure 8 Western blot analysis of polypeptides secreted *in vitro* by bovine luteal, granulosa, or thecal cells between 48 and 96 h of culture. Nitrocellulose strips were incubated with a polyclonal rabbit anti human MMP-2 or normal rabbit serum (NRS) at a 1:300 dilution. MMP-2 (M_r 62 000) was detected in luteal, granulosa, and thecal cell conditioned media.

and granulosa (M_r 57 000) cell conditioned media that corresponded to the active (M_r 44 000 to 45 000) and latent (M_r 51 000 to 57 000) forms of stromelysins respectively. However, definitive evidence that stromelysins are produced by bovine thecal and/or granulosa cells awaits further investigation. Unfortunately, the caseinolytic bands were below the limit of detection to conduct densitometry; therefore, the effect of time and/or LH on caseinolytic activity could not be examined.

The source of the M_r 83 000 caseinolytic activity in bovine thecal and granulosa cell conditioned media is not known. The relative molecular weight of the caseinolytic band corresponds to plasmin (M_r 82 000). Plasminogen activators (urokinase and tissue plasminogen activator) convert plasminogen to plasmin and have been localized to both granulosa and thecal cells in several species (Li *et al.* 1997). Furthermore, ovarian plasminogen activator activity is reported to be upregulated by LH/hCG. Plasmin is known to have substrate specificity for components of the extracellular matrix (collagens, gelatin, fibronectin, laminin, and proteoglycans) and the latent form of several MMPs (interstitial collagenase, gelatinase A, gelatinase B, and stromelysin 1). Plasmin is a serine protease that is not inhibited by 1,10 phenanthroline; however, in the present study the caseinolytic activity was strongly inhibited by this specific inhibitor of MMP activity. The preceding observation suggests that the caseinolytic activity is due to an MMP rather than a serine protease. In addition, detection of plasmin with casein zymography normally requires the incorporation of plasminogen into the gel, which was not the case in the present study. Furthermore, no caseinolytic activity was detected at M_r 82 000 in follicular fluid

even though bovine follicular fluid is known to contain significant amounts of plasmin (Beers 1975, Dow *et al.* 2002). It also seems unlikely that the caseinolytic activity is due to gelatinase B (MMP-9) since the substrate affinity of gelatinase B is much greater for gelatin compared with casein (Egeblad & Werb 2002). If the caseinolytic activity was due to gelatinase B then one would expect much greater gelatinolytic activity compared with caseinolytic activity at M_r 83 000.

A distinct advantage of the granulosa and thecal cell culture systems utilized in the present study, is that both cell types retain their follicular phenotype in serum-free media (Gutierrez *et al.* 1997, Campbell *et al.* 1998). Physiological concentrations of FSH, insulin, and IGF-1 (identical to the concentrations utilized in the present study) stimulated granulosa cell proliferation and estradiol secretion (Gutierrez *et al.* 1997) and physiological concentrations of LH stimulated androstenedione secretion by thecal cells (Campbell *et al.* 1998). More specifically, the amount of estradiol secreted by granulosa cells during the first 48 h of culture was reported to be directly proportional to follicular size and the granulosa cells from small follicles (<4 mm) differentiated, *in vitro*, such that secretion of estradiol was similar to that of large follicles (>8 mm) (Gutierrez *et al.* 1997). Consequently, the production and regulation of specific MMPs was investigated in a physiologically relevant culture system without complications due to contamination of follicular fluid and/or serum.

Although a direct effect of MMPs has not been demonstrated, there is circumstantial evidence that this enzyme system is involved in follicular growth (Curry & Osteen 2003). The physiological role of follicular MMPs is likely associated with mechanisms of follicular expansion, angiogenesis, and bioavailability of growth factors. Gelatinases (gelatinase A and B) are known to cleave gelatin (denatured helix of collagen) and type IV collagen, a major component of the basement membrane separating the thecal and granulosa cell compartments. Gelatinases have been postulated to have a key role during ovulation by breaking down the basement membrane and further hydrolyzing the denatured collagen fibrils following their initial cleavage by collagenases (Smith *et al.* 2002, Curry & Osteen 2003). In the present study, gelatinase A (M_r 62 000), B (M_r 83 000), and caseinolytic activity (M_r 83 000) in thecal and granulosa cell conditioned media increased from 48 to 96 h (thecal and granulosa cells) and gelatinolytic activity decreased from 96 to 144 h (thecal cells). Expression of mRNA (MMP-2 and -9), protein (MMP-2 and 9), and gelatinolytic activity have been associated with follicular growth in rodent ovaries (Bagavandoss 1998, Cooke *et al.* 1999, Curry *et al.* 2001). Increases in gelatinolytic and caseinolytic activities *in vivo* may be associated with follicular remodeling and differentiation.

The decrease in gelatinase A activity in follicular fluid of large compared with medium or small bovine follicles is consistent with an effect of time on gelatinase A

(M_r 62 000) activity of thecal and granulosa cells (Fig. 2; panel B and Fig. 5; panel A). Gelatinase A activity increased from 0 to 96 h in both thecal and granulosa cell conditioned media but decreased by 144 h (thecal cells) or remained the same (granulosa cells). Therefore, the effect of time (0 to 96 h) was similar to the effect of follicular diameter (small, medium, and large) on pattern of secretion of gelatinase A activity *in vitro*. Gutierrez *et al.* (1997) reported that 96 h were required for follicular cells to proliferate and differentiate in the culture system utilized in the present study. Interestingly, this is similar to the time it takes a recruited follicle (5–6 mm) to develop into an ovulatory size follicle (>10 mm) in cattle.

In the present study, LH had a demonstrable effect on thecal cells, since there was increased formation of cellular aggregates in the presence of LH, as previously described for ovine thecal cells (Campbell *et al.* 1998). Furthermore, LH (1 or 100 ng/ml) increased gelatinase A (M_r 62 000) activity in thecal cell conditioned media (48, 96, and 144 h) and M_r 83 000 gelatinolytic activity in granulosa cell conditioned media (48 h). *In vivo*, bovine granulosa cells do not acquire LH receptors until around the time of follicular selection (follicle diameter = >8 mm; Bao *et al.* 1997). In the present study, granulosa cells were obtained from small follicles (<5 mm) that presumably did not express receptors for LH. However, the increase in M_r 83 000 gelatinolytic activity implies that LH receptors are present on the granulosa cells by 48 h of culture.

Stimulation of follicular growth with eCG resulted in increased gelatinase A and B immunoreactivity in rat pre-ovulatory follicles (Bagavandoss 1998). Furthermore, expression of mRNA for gelatinase A, gelatinase B, collagenase 3 (Cooke *et al.* 1999) and TIMP-1 (Kennedy *et al.* 1996) and gelatinolytic and collagenolytic activity (Cooke *et al.* 1999) were also increased in rat ovaries in response to eCG treatment. An LH-stimulated increase in gelatinase A may have a role in the regulation of follicular angiogenesis. LH-stimulated production of an endothelial cell migration factor by bovine luteal tissue *in vitro* (Redmer *et al.* 1988). MMPs and their inhibitors promote angiogenesis through regulating the degradation of the basal lamina, allowing separation of existing endothelial cells, and facilitating the migration of endothelial cells from existing vessels towards the angiogenic stimulus. Expression of the integrin $\alpha_v\beta_3$ on angiogenic blood vessels was associated with angiogenesis and antagonists of $\alpha_v\beta_3$ inhibited new blood vessel formation without affecting existing blood vessels (Brooks *et al.* 1994). Within melanomas, active gelatinase A was associated with the integrin $\alpha_v\beta_3$ on the surface of endothelial cells (Brooks *et al.* 1996). A naturally occurring, noncatalytic fragment of gelatinase A competed with gelatinase A for binding to the $\alpha_v\beta_3$ integrin and may function to limit the extent of migration and invasion of endothelial cells during angiogenesis (Brooks *et al.* 1998). The activities of membrane-bound or associated MMPs may provide a mechanism for the migration of endothelial cells during follicular development.

Follicular MMPs may have a role in regulating the bio-availability of growth factors. Although the role of growth factors in control of ovarian cell proliferation and differentiation has been extensively examined, the mechanisms controlling the availability and activity of growth factors is less well understood. Growth factors are frequently secreted constitutively and sequestered in the extracellular matrix in an inactive form or in association with specific binding proteins where they can subsequently be liberated by proteolysis of the ECM (Flaumenhaft & Rifkin 1992, Logan & Hill 1992). For example, MMPs can liberate fibroblast growth factor molecules bound to heparan sulfate proteoglycans in the extracellular matrix and hence increase growth factor availability to nearby cells (Tamura *et al.* 1996). Furthermore, MMPs have been demonstrated to release bioactive vascular endothelial growth factor from extracellular stores to promote angiogenesis (Bergers *et al.* 2000, Coussens *et al.* 2000). Other growth factors and growth factor binding proteins that are capable of binding to components of the ECM include transforming growth factor- β , hepatocyte growth factor, transforming growth factor α , and insulin like growth factor binding proteins (Armstrong & Webb 1997, Taipale & Keski-Oja 1997). Therefore, MMPs likely play a key role in regulating the availability of growth factors and their activities within developing follicles.

In summary, follicular growth, differentiation, and ovulation or atresia are dependent upon cyclical remodeling of the extracellular matrix. The results of this study indicate that gelatinolytic and caseinolytic MMPs are temporally and spatially regulated within the thecal and granulosa compartments of bovine follicles. Furthermore, gelatinase A (MMP-2) activity was increased in response to physiological concentrations of LH. The controlled degradation of ECM proteins by MMPs and their inhibitors (TIMPs) may be essential for preserving a microenvironment conducive to follicular function.

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