Expression of protease nexin-1 and plasminogen activators during follicular growth and the periovulatory period in cattle

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Abstract

Extracellular matrix remodeling occurs during ovarian follicular development, mediated by plasminogen activators (PAs) and PA inhibitors including protease nexin-1 (PN-1). In the present study we measured expression/activity of the PA system in bovine follicles at different stages of development by timed collection of ovaries during the first follicular wave and during the periovulatory period, and in follicles collected from an abattoir. The abundance of mRNA encoding PN-1, tissue-type PA (tPA), urokinase (uPA) and PA inhibitor-1 (PAI-1) were initially upregulated by human chorionic gonadotropin (hCG) in bovine preovulatory follicular wall homogenates. PN-1, PAI-1 and tPA mRNA expression then decreased near the expected time of ovulation, whereas uPA mRNA levels remained high. PN-1 concentration in follicular fluid (FF) decreased and reached the lowest level at the time of ovulation, whereas plasmin activity in FF increased significantly after hCG. Follicles collected from the abattoir were classified as non-atretic, early-atretic or atretic based on FF estradiol and progesterone content: PN-1 protein levels in FF were significantly higher in non-atretic than in atretic follicles, and plasmin activity was correspondingly higher in the atretic follicles. No changes in PN-1 levels in FF were observed during the growth of pre-deviation follicles early in a follicular wave. These results indicate that PN-1 may be involved in the process of atresia in non-ovulatory dominant follicles and the prevention of precocious proteolysis in periovulatory follicles.

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Introduction

The growth of bovine follicles from the primordial to the preovulatory stage is characterized by the proliferation of cells and considerable increase in size of the follicle (Lussier *et al.* 1987). The granulosa and theca cell layers are separated by the basal lamina, and the theca cells are enclosed in a dense extracellular matrix (ECM). Remodeling of the basal lamina and ECM occurs as follicles expand, and changes in the chemical composition of the basal lamina have been described during follicular development (Rodgers *et al.* 2003). Extensive breakdown and remodeling of the basal lamina and connective tissue of the follicular wall is required for ovulation (Curry *et al.* 2001, Richards *et al.* 2002).

The plasminogen activator (PA) system has been implicated as one of the important mediators of ECM remodeling and follicular rupture at ovulation (Ny *et al.* 2002, Liu 2004). The PA system consists of the ubiquitous proenzyme, plasminogen, that is converted to an active enzyme, plasmin, by the tissue-type (tPA) and urokinase

(uPA) PAs. The activity of PA is regulated in part by inhibitors, including PA inhibitor-1 (PAI-1) and protease nexin-1 (PN-1, also known as serine protease inhibitor E2). PN-1 is a secreted glycoprotein, and is a broad and rapid inhibitor of a number of serine and cysteine proteases including tPA, uPA and plasmin (Silverman *et al.* 2001). The expression and regulation of PN-1 has been examined in ovarian follicles. In contrast to PAI-1, which is predominantly expressed in thecal–interstitial cell layers, PN-1 was exclusively expressed in granulosa cells in mice (Hägglund *et al.* 1996), rats (Hasan *et al.* 2002) and cattle (Bédard *et al.* 2003).

Previous studies indicate that ovulation requires coordinated expression of the PAs and their inhibitors. In rats and monkeys, there is an upregulation of tPA and PAI-1 expression by human chorionic gonadotropin (hCG) during the periovulatory period, followed by a marked decrease in PAI-1 expression just before ovulation. This may allow a narrow window of increased tPA activity that results in follicular rupture and ovulation (Liu *et al.* 1987,

Shen *et al.* 1997, Liu 2004). PN-1 is also expressed in preovulatory follicles, although its role is not clear. PN-1 expression decreased following an ovulatory dose of hCG in rats (Hasan *et al.* 2002) but not in mice (Hägglund *et al.* 1996). How the proteolytic cascade is controlled in cattle is not well understood, as tPA activity increases prior to ovulation, whereas PAI-1 activity does not change (Dow *et al.* 2002a, 2002b). The potential role of PN-1 during the periovulatory period in cattle remains to be determined.

The PA system may also be involved in tissue remodeling at earlier stages of folliculogenesis. In rats, there is a switch from uPA to tPA activity during follicular growth (Karakji & Tsang 1995). In cattle, follicular growth occurs in waves, during which the dominant, potential ovulatory follicle undergoes rapid growth and the subordinate follicles become atretic (Fortune *et al.* 2001, Ginther *et al.* 2001b). The PA system may be involved in the growth of the dominant follicle, as cellular uPA activity was higher in granulosa cells from small antral follicles compared with those from large follicles, and PN-1 secretion and expression was lower in granulosa cells from small follicles compared with those of large follicles of rodents and cattle (Hägglund *et al.* 1996, Bédard *et al.* 2003, Cao *et al.* 2004). Follicular regression may also involve PA activity, as plasminogen activation markedly decreased attachment of Chinese hamster ovary fibroblasts to ECM components *in vitro*, resulting in detachment-induced cell death (anoikis) (Rossignol *et al.* 2004). Interestingly, PN-1 inhibited PA-induced anoikis in these cells (Rossignol *et al.* 2004).

The objective of this study was to determine if cell-specific and temporal regulation of PN-1 and PA expression and secretion contribute to follicular development and ovulation in cattle. We assessed the role of PN-1 as a candidate for regulation of PA activity in bovine follicles at three stages of folliculogenesis: (i) the periovulatory period; (ii) in healthy, early-atretic and atretic dominant follicles classified on biochemical criteria; and (iii) during the first follicular wave before and during follicular deviation.

Materials and Methods

Experiments 1 and 3 were performed with cross-bred heifers aged between 1.5 and 3 years. The animals were housed indoors on the University of Montreal farm for the duration of the experiment and were fed concentrate and hay twice daily. Water was freely available. All animal experimentation was approved by the Animal Care Committee of the Faculty of Veterinary Medicine, University of Montreal, and performed in accordance with Canadian Council of Animal Care Guidelines.

Experiment 1: hCG-induced periovulatory follicles

Experimental design

Ten heifers were induced to ovulate as described (Bédard *et al.* 2003). Animals with a corpus luteum were

synchronized with one injection of prostaglandin F_{2α} (PGF_{2α}) (25 mg, i.m.) (Lutalyse; Upjohn, Kalamazoo, MI, USA), and behavioral estrus was monitored at 12 h intervals, from 48 to 96 h following PGF_{2α} injection. Ovarian follicular development was monitored by daily transrectal ultrasonography performed with a real-time linear scanning ultrasound system (LS-300; Tokyo Keiki Co., Ltd, Tokyo, Japan) equipped with a 7.5 MHz transducer (Lussier *et al.* 1994). Preovulatory follicles were obtained following a second injection of 25 mg PGF_{2α} 7 days after estrus to induce luteolysis, thereby allowing the development of the dominant follicle of the first follicular wave into a preovulatory follicle (Sirois 1994). An ovulatory dose of hCG (3000 IU) (APL; Ayerst Lab., Montreal, QC, Canada) was injected 36 h after the induction of luteolysis, and ovaries bearing the preovulatory follicle were collected by ovariectomy at 0, 6, 12, 18 and 24 h after hCG injection.

Follicular fluid (FF) was aspirated from the follicles with a 21 G needle, centrifuged (3000 *g* for 2 min at 4 °C) and stored at −20 °C for PN-1 and PA assay. The follicle walls were then homogenized in lysis buffer (4 M guanidium isothiocyanate, 0.5% sodium *N*-laurylsarcosine, 25 mM sodium citrate, pH 7) (Chomczynski & Sacchi 1987), and total RNA was sedimented on a cesium chloride cushion by centrifugation (Ndiaye *et al.* 2005). The concentration of total RNA was evaluated by optical density at 260 nm, and quality was estimated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde–agarose gel in the presence of ethidium bromide.

Granulosa cells were collected from individual follicles at 0, 12 and 24 h following hCG injection for protein extraction. Cells were homogenized in M-PER Reagent (Pierce, Rockford, IL, USA) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Laval, QC, Canada). Lysis of cells was achieved by repeated passage through a 25 G needle attached to a 3 ml syringe. Cell lysates were centrifuged (16 000 *g* for 15 min at 4 °C) and supernatant was stored at −80 °C until analysis of PN-1 and PA activity.

mRNA reverse transcriptase and semi-quantitative RT-PCR

One microgram of total RNA was reverse transcribed and amplified for 15 cycles with the SMART PCR cDNA synthesis kit (BD Biosciences Clontech, Mississauga, ON, Canada) as described (Ndiaye *et al.* 2005). The resulting cDNA pool was diluted to 50 µl in TE buffer (10 mM Tris pH 8, 1 mM EDTA), and 1 µl of the aliquot was used in a secondary 100 µl PCR reaction for 18 cycles using the Advantage 2 DNA Polymerase Mix (BD Biosciences Clontech) and the PCR primer AAGCAGTGGTAACAACGCAGAGT.

cDNA from the secondary PCR reactions were diluted 10-fold in TE buffer, and used as a template in subsequent

semi-quantitative RT-PCR for the target genes PN-1, PAI-1, tPA and uPA. Table 1 summarizes the gene-specific PCR primers used and PCR conditions. GAPDH was used as the housekeeping control (Ndiaye *et al.* 2005). Briefly, an aliquot of 2 µl of the diluted cDNA was amplified using Advantage 2 DNA polymerase (0.6 µl) in a 25-µl PCR reaction containing 0.4 mM dNTP mix, and 0.8 µM specific primers (except for GAPDH, 0.4 µM). Target cDNA was amplified in a PCR thermal cycler (Gene AMP PCR System 9700; Applied Biosystems, Foster City, CA, USA) under the following conditions: (i) an initial denaturation step for 1 min at 95 °C; and (ii) amplification cycles with denaturation at 95 °C for 30 s, annealing for 45 s at the temperatures indicated in Table 1 for each gene, and elongation at 68 °C for 1.5 min. The number of PCR cycles was optimized for each gene to be analyzed (see Table 1). The amplicons were separated on 1% agarose gel containing ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was normalized to GAPDH mRNA abundance.

Experiment 2: healthy and atretic large follicles

Tissue collection

Ovaries were obtained from an abattoir local to the São Paulo State University campus in Botucatu, and transported to the laboratory in saline on ice. Follicles were dissected from the ovaries and measured with calipers. Ovaries with a follicle ≥ 9 mm in diameter (and therefore post-deviation, dominant follicles) were selected, and FF from that follicle was aspirated, centrifuged and frozen for steroid, PN-1 and PA assay. The antral cavity was flushed repeatedly with cold saline and granulosa cells recovered by centrifugation at 1200 g for 1 min, and pooled with the

FF cell pellet. The remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette, and pooled with the flushed cells. The theca layer was then removed with forceps and washed in saline by passing repeatedly through a 1 ml syringe. Granulosa and theca cell were collected into Trizol (Invitrogen; São Paulo, Brazil) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol.

Follicles containing >100 ng estradiol/ml and <100 ng progesterone/ml were classified as non-atretic ($n = 7$), those containing <40 ng estradiol/ml and <100 ng progesterone/ml were classified as early atretic ($n = 7$), and those containing <40 ng estradiol/ml and >100 ng progesterone/ml were classified as atretic ($n = 4$). These represent mature dominant growing, static and regressing follicles respectively (Price *et al.* 1995). Cross-contamination of theca and granulosa cells was tested by detection of mRNA encoding cytochromes P450 aromatase (Cyp19) and 17 α -hydroxylase (Cyp17) in each sample by PCR (Buratini *et al.* 2005). Only granulosa cell samples negative for Cyp17, and only thecal samples negative for Cyp19 amplicons were included in the analysis (number of follicles used given above).

Semi-quantitative RT-PCR

PN-1, tPA and uPA mRNA expression in granulosa cells, and PAI-1, tPA and uPA mRNA expression in theca cells were measured by semi-quantitative RT-PCR. Briefly, for both theca and granulosa cells, total RNA (1 µg) was incubated with DNase I (Invitrogen) and reverse transcribed with SuperScript II (Invitrogen) and oligo-d(T) primer (Buratini *et al.* 2005). An aliquot (0.4 µl) of the cDNA template was amplified by PCR using 0.2 µl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech, Inc., Oakville, ON, Canada) in a 20 µl PCR buffer (Amersham Pharmacia

Table 1 Summary of RT-PCR protocols in Experiments 1 and 2.

Gene	Primers	Size (bp)	Annealing temperature (°C)		Cycles		Reference
			Expt 1	Expt 2	Expt 1	Expt 2	
PN-1	S: 5'-TCCGTGACGTTGCCCTCTGTG-3'	555	64	62	17	24	Bédard <i>et al.</i> (2003)
	AS: 5'-CCGTGATCTCCACAAACCCTT-3'						
PAI-1	S: 5'-GAACAAGGATGAGATCAGCACAGC-3'	696	64	62	22	30	NM_174137
	AS: 5'-GACACGTACAGAAACTCTTGATCTG-3'						
tPA	S: 5'-AAGGTTGCAGAAAGATGG-3'	479	56	55	20	26	Macchione <i>et al.</i> (2000)
	AS: 5'-GTGAGGCGGGTACCTCTCCTGGAA-3'						
uPA	S: 5'-GTCTGGTGAATCGAACTGTGGC-3'	511	58	65	25	30	Balcerzak <i>et al.</i> (2001)
	AS: 5'-GGCTGCAACCAAGGCTG-3'						
GAPDH	S: 5'-TGTTCCAGTATGATCCACCCACG-3'	600	64	–	21	–	Fayad <i>et al.</i> (2004)
	AS: 5'-CTGTTGAAGTCGCAGGAGACAACC-3'						
H2a	S: 5'-GTCGTGGCAAGCAAGGAG-3'	182	–	55	–	30	Robert <i>et al.</i> (2002)
	AS: 5'-GATCTCGGCCGTTAGGTACTC-3'						
GAPDH	S: 5'-TGTTCCAGTATGATCCACC-3'	860	–	55	–	26	Tsai <i>et al.</i> (1996)
	AS: 5'-TCCACCACCCTGTTGCTG-3'						

Biotech) containing 0.1 mM dNTP mix, and 0.2 μ M specific primers (Cao *et al.* 2004). Target cDNA was amplified under the following conditions: (i) an initial denaturation step for 3 min at 94 °C, except uPA, which was for 5 min at 95 °C; (ii) amplification cycles with denaturation at 94 °C for 15 s (PN-1), 30 s (uPA, tPA, PAI-1 and GAPDH) or 45 s for histone H2a (H2a), annealing for 30 s for H2a and 45 s for all other genes, at the temperatures indicated in Table 1, and elongation at 72 °C for 1 min; and (iii) final elongation at 72 °C for 5 min. The number of cycles is given in Table 1.

Semi-quantitative RT-PCR was validated for each gene product (Cao *et al.* 2004). The PCR products (10 μ l) were separated on 1% agarose gels containing ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to H2a mRNA abundance in granulosa cells, and to GAPDH mRNA abundance in theca cells.

Experiment 3: early in the follicular wave

Seven heifers were first synchronized with two injections of PGF $_{2\alpha}$ given 11 days apart. Ovulation and follicular development was monitored daily or twice daily by transvaginal ultrasonography, and follicles were punctured as described (Ouellette *et al.* 2005) when the largest follicle of the first wave had reached 6.5, 7.5, 8.5 or 9.5 mm internal diameter (referred to as 'follicular stage'), corresponding to approximately 1, 1.5, 2 and 2.5 days after wave emergence (Ginther *et al.* 2001b). Follicular deviation is expected to occur when the largest follicle reaches 8.5–9 mm diameter (Beg *et al.* 2001). FF from the largest three follicles (F1, F2 and F3) was collected separately for each follicle ('follicular rank'). Each animal was used once during a follicular wave, and 5–6 days after follicular puncture each animal received a single injection of PGF $_{2\alpha}$ to initiate ovulation and a new first follicular wave. Each animal was in this manner sampled on four consecutive estrous cycles. Only clear FF samples without blood contamination were used. The FF was centrifuged for 15 min at 2000 g and the supernatant frozen at –20 °C until assayed for steroid concentrations, PN-1 content and PA activity.

Casein zymography

Casein zymography was used to measure plasmin, tPA and uPA activity in FF and cell extracts as described (Cao *et al.* 2004). Briefly, 2 μ l FF or 30 μ g cell protein were subjected to electrophoresis in 10% non-denaturing polyacrylamide gels containing 0.2% casein (Sigma), 0.1% SDS and 3.75 mU/ml bovine plasminogen (Sigma). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and placed in incubation buffer (50 mM Tris, 0.1 M NaCl, pH 7.6) at 37 °C for 16 h with gentle shaking. The gels were then stained using

0.05% Coomassie blue in 10% acetic acid, 40% methanol for 2 h, destained in 10% acetic acid, 40% methanol, and then fixed in 10% glycerol. The identity of the enzymatic activities was investigated by comparing molecular size with human tPA (Calbiochem, Darmstadt, Germany) and uPA (NIBSC, Herts, UK) standards. Plasminogen-free gels were used to confirm that the activity detected was plasminogen dependent. Bands of plasmin activity were visualized as clear zones where casein degradation occurred, against a dark (blue) background. Images of gels were captured with an Alphamager (Alpha Innotech Corp., San Leandro CA, USA), digitally inverted to give black proteolytic bands against a white background, and quantified with NIH Image software. To correct for gel-to-gel variation, all samples were expressed relative to a control sample (conditioned medium) that was included in every gel.

Western blot

PN-1 protein abundance in FF and cell lysates was analyzed by Western blot as described (Cao *et al.* 2004). Samples were subjected to electrophoresis in 10% denaturing polyacrylamide gels. Proteins were then electrotransferred onto nitrocellulose membrane (0.45 μ m) (Bio-Rad, Hercules, CA, USA) at 22 V overnight at 4 °C in transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol, pH 8.3). After blocking for 1 h in TTBS (0.2% Tween 20, 10 mM Tris-HCl, 150 mM NaCl), blots were incubated with 1:5000 rabbit anti-bovine PN-1 (Bédard *et al.* 2003) for 4 h with agitation, followed by three washes (10 min each) with 0.2% TTBS. The blots were then incubated with 1:5000 alkaline phosphatase-linked anti-rabbit IgG (Sigma) for 1.5 h with agitation, followed by three washes (10 min each) with TTBS. Finally, the blots were incubated with NBT/BCIP solution (Roche Diagnostics, Indianapolis, IN, USA). Images of blots were captured with an Alphamager and quantified with NIH Image software. Rainbow-colored protein molecular mass markers (Pharmacia, Piscataway, NJ, USA) were used to estimate molecular size of the target protein, and a bovine FF sample (2 μ l) was used as positive control in all blots.

Steroid assays

Estradiol and progesterone in FF from Experiment 2 were assayed by RIA using iodinated tracers and antibodies furnished in the 3rd Generation Estradiol RIA (DSL-39100) and the DSL-3400 Progesterone RIA kits (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The standard curves were prepared from crystalline steroids (Sigma) in PBS–gelatin (0.02 M sodium phosphate, 0.15 M sodium chloride, 0.1% gelatin, 0.01% sodium azide, pH 7.5). The assay protocols were as described in the kits, except that the estradiol antibody and tracer were each diluted 1:1 with PBS–gelatin before use, and the progesterone antibody and tracer were diluted 3:2 and 7:3 respectively. FF samples were diluted in PBS–gelatin before assay.

Intra- and inter-assay coefficients of variation were 7.4 and 13.5% respectively for estradiol, and 6.8 and 7% respectively for progesterone. The sensitivities of the assays were 0.05 ng/ml for estradiol (at 1:25 dilution of FF) and 0.2 ng/ml for progesterone (at 1:10 dilution).

Concentrations of estradiol and progesterone in FF from Experiment 3 were analyzed by RIA using double antibody precipitation and charcoal absorption methods, as validated for use with bovine FF (Carrière & Lee 1994, Price *et al.* 1995). Spiking FF samples with 2 pg/tube and 5 pg/tube of estradiol gave 81 and 107% recovery respectively. Spiking FF samples with 100 pg/tube and 500 pg/tube of progesterone gave 115 and 97% recovery respectively. Intra- and inter-assay coefficients of variation were 5 and 10% for estradiol, and 7 and 8% for progesterone respectively.

Statistics

Data are presented as least-squares means $\pm$ S.E.M. Data were transformed to logarithms when they were not normally distributed (Shapiro–Wilk test). All analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The data from Experiments 1 and 2 were analyzed by ANOVA for effect of time (Experiment 1) or follicular class (Experiment 2), with gel or blot included as random effect terms where samples were analyzed in several gels/blots. Where main effects were found, means

comparisons were performed by the Tukey–Kramer HSD test. In Experiment 3, data were analyzed by two-way ANOVA with follicular stage and rank as main effects. Owing to the lack of normal distribution of the estradiol data, log estradiol values were analyzed for effect of follicular rank within follicular stage. The time of follicular deviation was defined as the earliest change in diameter between the largest follicle (F1) and the second-largest follicle (F2). Correlations between PA activity or PN-1 secretion and FF steroid concentration or follicular diameter were assessed with Pearson's product-moment correlation coefficient (*r*).

Results

Experiment 1: hCG-induced periovulatory follicles

PN-1, PAI-1, tPA and uPA mRNA expression were determined in follicular wall homogenates by RT-PCR. Abundance of mRNA encoding PN-1, PAI-1 and tPA all increased transiently after hCG injection ($P < 0.05$; Fig. 1), reaching maximal values at 6 h after hCG, and returning to pretreatment levels by 24 h after hCG injection. The pattern of uPA mRNA abundance differed from the other genes examined, as mRNA levels increased following hCG administration and remained elevated at 24 h after hCG injection ($P < 0.05$; Fig. 1C).

Figure 1 Analysis of (A) PN-1, (B) PAI-1, (C) uPA and (D) tPA mRNA expression in follicular wall lysates of preovulatory follicles by RT-PCR (Experiment 1). Total RNA was extracted from bovine preovulatory follicular walls collected at 0, 6, 12, 18 and 24 h after hCG injection, and was employed in mRNA expression analyses as described in Materials and Methods. GAPDH was used as a control gene, and showed no significant difference in expression levels between samples (A). Data are least-squares means (relative units) $\pm$ S.E.M. of two animals. Different letters denote means that are significantly different ($P < 0.05$).

Figure 2 Analysis of (A) PN-1 protein content and (B) proteolytic enzyme activities in granulosa cell lysates from periovulatory bovine follicles at 0, 12 and 24 h after hCG (Experiment 1). For PN-1, samples (50 µg cell protein) were subjected to SDS-PAGE followed by blotting with an antibody raised against bovine PN-1. PA activity was measured in aliquots of 30 µg cell protein by casein zymography. The zymograph presented in (B) shows lytic zones produced by human recombinant uPA standard (uPA) and samples collected at 0, 12 and 24 h after hCG injection. Location of plasmin, tPA and uPA activities are indicated to the right of the zymograph. Different contrast and brightness settings were used for uPA and plasmin image capture, and although the data are plotted on the same axis, uPA activity was significantly weaker than plasmin activity. Data are least-squares mean densitometry units $\pm$ S.E.M. of two animals. For each enzyme, different letters denote means that are significantly different ($P < 0.05$).

To support the gene expression data, PN-1 protein and PA activities in granulosa cell lysates were measured. A major immunoreactive band corresponding to PN-1 was detected by Western blot in granulosa cell lysates, and abundance of this protein band did not differ between 0, 12 or 24 h after hCG injection (Fig. 2A). Zymography demonstrated an increase in proteolytic activity corresponding to plasmin (approximately 82 kDa) and uPA (approximately 45 kDa) in preovulatory granulosa cell lysates after hCG injection ($P < 0.05$; Fig. 2B), whereas tPA activity was weak to undetected (Fig. 2B).

Figure 3 Analysis of (A) PN-1 protein content and (B) proteolytic enzyme activity in FF collected from periovulatory follicles at 0, 6, 12, 18 and 24 h after hCG (Experiment 1). The representative Western blot (for PN-1) and composite zymograph (for uPA and plasmin activity) presented show data for one animal at each time point, plus the control (Ctrl) sample (FF) used in Western blotting. Image-capture parameters for plasmin and uPA activity were different owing to the significantly weaker uPA activity. Results shown represent least-squares means $\pm$ S.E.M. densitometry units of two animals. For each enzyme, bars with different letters are significantly different ($P < 0.05$); *mean different from time 0 ($P < 0.05$).

Table 2 Estradiol and progesterone concentrations, and mean diameters of follicles in non-atretic, early-atretic and atretic follicles in Experiment 2.

Follicle class	Diameter (mm (range))	Estradiol (ng/ml (range))	Progesterone (ng/ml (range))
Non-atretic (<i>n</i> = 7)	11.6 ± 0.8 ^a (10–14)	1392 ± 687 ^a (396–4084)	33 ± 2 ^a (28–38)
Early-atretic (<i>n</i> = 7)	9.7 ± 0.6 ^a (9–13)	1.3 ± 0.6 ^b (0.6–4.6)	34 ± 6 ^a (18–63)
Atretic (<i>n</i> = 4)	10.0 ± 0.6 ^a (9–11)	13 ± 8 ^b (0.4–31.0)	207 ± 86 ^b (106–465)

For each variable, means with different superscripts are significantly different ($P < 0.05$).

FF PN-1 protein content decreased with time after hCG injection, reaching the lowest levels at 24 h after hCG ($P < 0.05$; Fig. 3A). Plasmin activity in FF increased after hCG ($P < 0.05$; Fig. 3B) whereas uPA activity decreased (Fig. 3B). Proteolytic activity corresponding to tPA was not detected in FF of preovulatory follicles.

Experiment 2: healthy and atretic large follicles

Estradiol and progesterone concentrations and diameters of the follicles in each class are given in Table 2. Non-atretic and early-atretic differed by estradiol content but not by progesterone content or diameter; non-atretic and atretic follicles differed by estradiol and progesterone content, but not by follicular diameter. Plasmin activity in FF collected from non-atretic follicles was significantly lower than that in early-atretic and atretic follicles ($P < 0.05$; Fig. 4A). uPA activity in FF did not differ between groups (Fig. 4A). tPA activity was not detected in any the follicles examined. PN-1 protein abundance in FF of non-atretic follicles was significantly higher than that of early-atretic and atretic follicles ($P < 0.05$; Fig. 4B). Plasmin activity in FF was negatively correlated with PN-1 content ($r = -0.6$, $P < 0.05$) but not with uPA activity ($P > 0.05$).

mRNA for tPA, uPA, and PN-1 was detected in granulosa cells. PN-1 mRNA levels were lower in granulosa cells of early-atretic follicles than in those of non-atretic and atretic follicles ($P < 0.05$; Fig. 5A), but there were no differences in tPA or uPA mRNA levels. mRNA for tPA, uPA and PAI-1 was detected in theca cells, and message levels did not differ significantly between groups (Fig. 5B).

Overall, granulosa cell PN-1 mRNA abundance and FF PN-1 protein abundance were positively correlated with FF estradiol concentrations ($r = 0.73$ and 0.62 respectively, $P < 0.01$). Plasmin activity in FF was negatively correlated with FF estradiol concentration ($r = -0.65$, $P < 0.01$). Follicular diameter was correlated with granulosa cell tPA mRNA abundance ($r = 0.57$, $P < 0.05$) but with no other variable.

Experiment 3: early in the follicular wave

Mean diameters and estradiol and progesterone concentrations in the F1, F2 and F3 follicles are summarized in Table 3. A significant difference in diameter between the largest (F1) and second-largest (F2) follicle occurred when

the F1 had reached 9.5 mm. Estradiol concentrations did not differ between the three largest follicles of a wave when the F1 was 6.5 mm diameter, but was significantly lower in the F3 compared with F1 at all subsequent stages. Differences in estradiol between F1 and F2 occurred only after follicular deviation. Progesterone concentrations did not differ between follicles at any stage of the wave. Based on the follicular health criteria used in Experiment 2, all F3 and three F2 follicles in the 7.5 mm group, all F3 and two F2 follicles in the 8.5 mm group, and all F3 and all F2 follicles in the 9.5 mm group were early-atretic.

A single band corresponding to PN-1 was detected in FF by immunoblotting, and PN-1 abundance did not significantly change with stage of the follicular wave or between F1, F2 or F3 follicles (Fig. 6A). Plasmin and uPA (but not tPA) activities were detected in FF, and no differences were observed between follicular stages or rank (Fig. 6B). However, uPA activity was correlated with FF estradiol concentration (not shown) and the estradiol:progesterone ratio (Fig. 7) in subordinate but not in dominant (F1) follicles.

Discussion

The pattern of expression and potential role of PN-1 during follicular growth is not well known. In the present study, we measured PN-1 and PA activity in bovine follicles at three stages of follicular development. We demonstrate that during the periovulatory period the expression of PN-1 is initially upregulated by hCG, and then declines in a pattern similar to the expression of PAI-1 and tPA. This profile of PN-1 expression appears to differ from that observed in rodents, where no change in expression was observed until the onset of ovulation (Hägglund *et al.* 1996, Hasan *et al.* 2002), although these are *in situ* studies that do not lend themselves well to quantification. We also demonstrate a potential role for PN-1 in dominant follicular growth, as follicular protein levels are higher in non-atretic than in early-atretic and atretic follicles.

We first measured the pattern of expression of PA system members in the follicle wall in response to an ovulatory dose of hCG, as the ovarian PA system is best known for its role during ovulation. Follicular tPA, uPA, PAI-1 and PN-1 mRNA abundance increased sharply by 6 h after hCG, and mRNA levels declined thereafter, except for uPA mRNA, which remained high until the expected time of ovulation. Overall, these results support previous

Figure 4 Plasmin and uPA enzyme activities (A) and PN-1 protein (B) in FF from non-atretic ($n = 7$), early-atretic ($n = 7$) and atretic ($n = 4$) follicles (Experiment 2). Follicular classification is described in Materials and Methods. Inserts are zymographs (A) and Western blots (B) showing raw data for all follicles. Lanes marked 'C' are control samples used to correct for variation between gels. Data are least-squares means (relative units) $\pm$ S.E.M. Bars with different letters are significantly different ($P < 0.05$).

studies in cattle (Dow *et al.* 2002a, 2002b), in which tPA, uPA and PAI-1 mRNA levels were upregulated by an induced gonadotropin surge. This is in contrast to that observed in other species, in which only one PA was increased in response to the gonadotropin surge. For instance, only tPA in rats (Li *et al.* 1997) and monkeys (Liu *et al.* 2004) or uPA in mice (Macchione *et al.* 2000) and sheep (Colgin & Murdoch 1997) was upregulated during ovulation. The specific time points when mRNA abundance increased and then decreased is slightly different between the present study and the previous studies (Dow *et al.* 2002a,b), possibly caused by the different methods of inducing ovulation (hCG vs gonadotropin-releasing hormone (GnRH)).

Changes in periovulatory PN-1 expression have not previously been described in ruminants. In mice, granulosa cell PN-1 expression generally did not vary throughout the periovulatory period (Hägglund *et al.* 1996), although a decrease in immunostaining was observed 12 h after hCG injection in PMSG-stimulated rats (Hasan *et al.* 2002). The present data clearly show a transient upregulation of PN-1 expression within 6 h of hCG treatment, indicating that PN-1 regulation in cattle is different from that of rodents. The only comparable work in ruminants is a gene-profiling study that described lower PN-1 expression in periovulatory follicles 24 h after hCG injections compared with dominant non-ovulatory follicles on day 5 of the estrous cycle

A Granulosa cells

B Theca cells

Figure 5 RT-PCR analysis of mRNA expression of PA and PA inhibitors in (A) granulosa and (B) theca cells from non-atretic ($n = 7$), early-atretic ($n = 7$) and atretic ($n = 4$) follicles (Experiment 2). Follicular classification is described in Materials and Methods. Inserts are composite images of agarose gels showing all samples. Data are least-squares means (relative units) $\pm$ S.E.M. Group means that differed are indicated by an asterisk ($P < 0.05$).

Table 3 Mean $\pm$ S.E.M. diameter and FF steroid content of the dominant (F1) and the two largest subordinate follicles (F2, F3) collected early in a follicular wave, when the dominant follicle reached approximately 6.5, 7.5, 8.5 or 9.5 mm diameter Experiment.

Follicle stage (mm)	Follicle rank	Diameter (mm)	Estradiol (ng/ml) [†]	Progesterone (ng/ml)
6.5	F1	6.5 $\pm$ 0.1	114 $\pm$ 33	29 $\pm$ 6
	F2	6.1 $\pm$ 0.3	68 $\pm$ 17	30 $\pm$ 8
	F3	5.6 $\pm$ 0.3	40 $\pm$ 18	61 $\pm$ 41
7.5	F1	7.4 $\pm$ 0.1	226 $\pm$ 42 ^a	37 $\pm$ 8
	F2	6.5 $\pm$ 0.1	127 $\pm$ 51 ^{ab}	29 $\pm$ 6
	F3	5.7 $\pm$ 0.2	32 $\pm$ 6 ^b	23 $\pm$ 5
8.5	F1	8.2 $\pm$ 0.5	423 $\pm$ 124 ^a	30 $\pm$ 6
	F2	6.8 $\pm$ 0.6	168 $\pm$ 68 ^a	26 $\pm$ 4
	F3	6.4 $\pm$ 0.6	14 $\pm$ 10 ^b	60 $\pm$ 28
9.5	F1	9.7 $\pm$ 0.1*	682 $\pm$ 45 ^a	46 $\pm$ 6
	F2	6.8 $\pm$ 0.3	3 $\pm$ 1 ^b	74 $\pm$ 43
	F3	6.1 $\pm$ 0.1	11 $\pm$ 8 ^b	19 $\pm$ 5

[†] Within follicle stage group, different superscripts denote differences between F1, F2 and F3 follicles ($P < 0.05$).

* F1 follicle was significantly different from F2 follicle. $n = 7$ follicles/group.

(Bédard *et al.* 2003). In rats, a model was proposed for the tight regulation of proteolytic activity in periovulatory follicles, in which both tPA and PAI-1 expression increase initially to generate high levels of inhibited enzyme within the follicular wall (Liu *et al.* 1987, Shen *et al.* 1997). According to this model, PAI-1 but not tPA expression then decreases, thus triggering activation of accumulated tPA and degradation of the follicular wall. In cattle, a modified version of this model can be proposed, in which there is an initial upregulation of tPA, uPA, PAI-1 and PN-1, followed by a decrease in tPA, PAI-1 and PN-1 expression while maintaining uPA expression. The activation of accumulated PA in theca (owing to reduced PAI-1 expression) and granulosa (owing to reduced PN-1 expression) cells would contribute to the proteolytic cascade at ovulation in this species. The cell-specific expression of PA inhibitors is physiologically relevant, as tPA expression is localized predominantly to the granulosa layer in bovine follicles (Dow *et al.* 2002a), thus regulation of PN-1 expression is probably important to prevent precocious proteolytic activity on the antral side of the basal lamina.

This model for the control of the proteolytic cascade at ovulation in cattle is supported by the increased uPA and plasmin activity in granulosa cell lysates observed 12–24 h after hCG, at a time when granulosa cell PN-1 protein levels were not different from pretreatment controls (see Fig. 2). The increase in plasmin activity in granulosa cells was reflected by an increase in FF plasmin activity. This is consistent with the plasmin activity detected in sheep follicular walls (Murdoch 1998), but in contrast to a previous study in cattle (Dow *et al.* 2002a) in which FF plasmin activity increased in

GnRH-treated animals, but was not detected in the lysates of whole follicular wall. The reason for this discrepancy is unknown but may be related to the methods used to prepare lysate samples and/or the use of whole follicular wall compared with granulosa cells. We also observed an increase in uPA activity in granulosa cell lysates after hCG but a decrease in activity in FF. This discrepancy may be caused by a redistribution of uPA to the cell surface or the ECM (Macchione *et al.* 2000) or specific regulation of protein secretion, or be a consequence of changes in thecal contribution to total FF levels of uPA.

The expression of PN-1 has been detected in small antral follicles (Hägglund *et al.* 1996, Hasan *et al.* 2002, Bédard *et al.* 2003) suggesting that it might play a role in follicular growth prior to the preovulatory period. We explored this by measuring PN-1 and PA activity/expression in non-atretic and atretic dominant follicles, and during follicular deviation early in the follicular wave. Changes in PN-1 or PA activity in FF were not detected during the growth of the follicular cohort before deviation, even though FF estradiol content increased in the largest follicle and decreased in smaller follicles of the cohort. Interestingly, uPA activity in FF was positively correlated with follicular estradiol content in subordinate follicles early in a follicular wave but not in the largest, presumably dominant follicle of the cohort. This suggests that the estrogenic subordinate follicles may be growing or have the potential to grow, and may have different requirements for uPA activity than the less-estrogenic subordinate follicles whose growth may have ceased. In support of this, it has been demonstrated that larger subordinate follicles have the capacity to develop into dominant follicles if the existing dominant follicle is ablated (Ginther *et al.* 2001a). The dominant follicle is actively growing and therefore would be expected to undergo constant ECM remodeling irrespectively of estradiol content, which might explain the absence of a correlation between estradiol and uPA in the dominant follicle. The absence of changes in PN-1 protein or expression in pre-deviation follicles suggests that PN-1 may not be tightly regulated prior to deviation, or may not play a role at this stage of development.

In dominant follicles, however, PN-1 may be regulated and/or play a more important role. FF PN-1 content was lower in early-atretic and atretic follicles compared with non-atretic follicles, and FF plasmin activity was correspondingly higher in the early-atretic and atretic follicles. Plasmin activity in FF appears to be regulated by PN-1, as plasmin activity was correlated with PN-1 but not uPA, and uPA activity did not change significantly with follicular health. PN-1 has been suggested to be an anti-apoptotic factor in adherent cells, as it inhibited plasminogen activation-induced anoikis in these cells (Rossignol *et al.* 2004). Atresia in dominant bovine follicles is characterized by apoptosis

Figure 6 PN-1 protein (A) and uPA enzyme activity (B) in FF samples collected from growing dominant (F1) and subordinate (F2, F3) follicles during the first follicular wave (Experiment 3). FF was collected by ultrasound-guided aspiration when the dominant follicle reached approximately 6.5, 7.5, 8.5 and 9.5 mm diameter (follicle stage). Inserts are representative Western blots (A) and zymographs (B). Data are least-squares means (relative units) $\pm$ S.E.M. of seven animals at each follicular stage.

and detachment of granulosa cells near the antrum (Irving-Rodgers *et al.* 2001), and in sheep there are changes in collagen and fibronectin content of early-atretic follicles (Huet *et al.* 1998). Therefore it is possible that certain endocrine or paracrine signals may inhibit PN-1 synthesis and/or secretion from dominant follicles at the end of their growth phase, and these lowered PN-1 levels may be involved in the process of atresia in the membrana granulosa.

In summary, PN-1 expression/secretion changes in a manner dependent on stage of follicular development. Specifically, no changes in PN-1 or PA activity were observed during the growth of follicles early in the follicular wave

before follicular deviation occurred, suggesting a minor role, if any, for these proteins before follicular deviation. In dominant follicles, however, PN-1 levels in FF were lower in atretic compared with non-atretic dominant follicles, and were inversely correlated with follicular plasmin activity. We suggest that PN-1 may be involved in atresia in non-ovulatory dominant follicles. In periovulatory follicles, PN-1, PAI-1, tPA and uPA mRNA levels were transiently upregulated by hCG, and all decreased at the expected time of ovulation except for uPA, which remains elevated. These data suggest a role for PN-1 in preventing precocious proteolysis in the granulosa cell layer before ovulation.

Figure 7 Correlation between estradiol:progesterone ratio and uPA activity in FF from growing dominant (F1) and subordinate (F2, F3) follicles during the first follicular wave (Experiment 3). FF was collected by ultrasound-guided aspiration when the dominant follicle reached approximately 6.5, 7.5, 8.5 and 9.5 mm diameter (follicular stage). Each data point represents an individual follicle, and correlation coefficients are given for each follicular rank

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