Gonadotrophin surge-induced upregulation of mRNA for plasminogen activator inhibitors 1 and 2 within bovine periovulatory follicular and luteal tissue

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The serine proteinases, tissue-type (tPA) and urokinase (uPA) plasminogen activator, are implicated in the ovulatory processes via their ability to convert plasminogen to its active form, plasmin. One mechanism for regulation of plasmin-directed ovarian extracellular matrix remodelling during follicle rupture and corpus luteum formation is through inhibition of plasminogen activation by the plasminogen activator inhibitors (PAI-1 and PAI-2). The effect of the preovulatory gonadotrophin surge on the temporal and spatial regulation of expression of PAI-1 and PAI-2 mRNA and PAI activity in preovulatory bovine follicles and new corpora lutea collected at 0, 6, 12, 18, 24 and 48 h after a GnRH-induced gonadotrophin surge was examined. Both PAI-1 and PAI-2 mRNAs were upregulated markedly after

the gonadotrophin surge, with the highest expression observed in follicles collected at about the time of ovulation (24 h) and in corpora lutea (48 h). PAI-1 mRNA was localized primarily to the thecal layer of preovulatory follicles. In contrast, PAI-2 mRNA was localized specifically to the granulosa cell layer. Significant PAI activity was detected in follicle extracts, but temporal or spatial differences in PAI activity were not detected in response to the gonadotrophin surge. These results indicate that PAI-1 and PAI-2 mRNAs are upregulated in preovulatory bovine follicles after the gonadotrophin surge in a cell-specific way. Regulation of PAI-1 and PAI-2 may help to control plasminogen activator activity associated with ovulation and early corpus luteum formation.

Introduction

The oocyte must be liberated from the preovulatory follicle for fertilization and subsequent pregnancy to occur. The gonadotrophin surge initiates the production of proteinases that mediate the degradation of the extracellular matrix and cellular remodelling required for follicular rupture and corpus luteum formation. One such family of proteinases implicated in the above processes is the plasminogen activators. The plasminogen activators consist of two enzymes, tissue-type (tPA) and urokinase (uPA) plasminogen activator, that convert ubiquitous plasminogen into the broad-spectrum serine proteinase, plasmin. One mechanism for the temporal and spatial regulation of plasminogen activator activity is through the action of two members of the serine protease inhibitor (serpin) gene family, plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). Both inhibitors bind to uPA or tPA in a 1:1 stoichiometry *in vitro*. Structurally, both serpins have an exposed reactive centre loop (RCL) of approximately 20 amino acids, which

inhibits the activity of plasminogen activators when inserted into the active site of the enzymes (Loskutoff *et al.*, 1988). *In vivo*, PAI-1 is an efficient inhibitor of both tPA and uPA, whereas PAI-2 is thought to inhibit uPA primarily (Holmberg *et al.*, 1978; Lecander *et al.*, 1984). PAI-2 exists as both an intracellular non-glycosylated form and a secreted glycosylated form with different biological activities (Kruithof *et al.*, 1987). Furthermore, PAI-2 is 20–100-fold less efficient in inhibiting plasminogen activator activity than PAI-1 (Kruithof *et al.*, 1986).

Evidence indicates that PAI-1 may play a key role in the temporal and spatial regulation of plasminogen activator activity in rat preovulatory follicles during the periovulatory period. mRNA for tPA and PAI-1 shows parallel increases until just before the time of ovulation, when PAI-1 mRNA abundance decreases markedly but tPA mRNA remains high. The spatial localization of tPA and PAI-1 is also distinct in rat preovulatory follicles; activity for tPA is associated primarily with granulosa cells, whereas PAI-1 immunoreactivity is concentrated predominantly in the thecal cells of preovulatory follicles collected near the time of ovulation (Peng *et al.*, 1993; Liu, 1999). This differential regulation of tPA and PAI-1 may provide a temporal and spatial window of increased tPA activity that may be

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important for mediating the ovulatory process in rats. Even less information is available about the intrafollicular localization and regulation of PAI-2 mRNA during the periovulatory period.

To date, the periovulatory regulation and potential roles of PAI-1 and PAI-2 in the regulation of remodelling of the ovarian extracellular matrix, which are characteristic of follicle rupture and corpus luteum formation, are not completely understood. Therefore, the aim of the present study was to determine the effect of the preovulatory gonadotrophin surge on localization and regulation of PAI-1 and PAI-2 mRNAs and PAI activity in bovine periovulatory follicular and luteal tissue.

Materials and Methods

Animal care

Mature Holstein cows (*Bos taurus*) aged ≥ 2 years were fed a balanced corn silage diet and housed at the Michigan State University Beef Cattle Research Center during the course of the experiments. All experiments were approved by the All University Committee on Animal Use and Care at Michigan State University (Approval 04/98-056-00).

Experimental model

Follicle development and timing of the preovulatory gonadotrophin surge were synchronized in Holstein cows using the Ovsynch procedure (Pursley *et al.*, 1997). In brief, 100 mg GnRH was injected to start a new wave of follicle growth and, thus, a new dominant follicle. After 7 days, 25 mg PGF_{2 α} was administered to regress corpora lutea. A second GnRH injection was administered 36 h later to induce an LH surge, resulting in ovulation of the dominant follicle. Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection to verify follicle synchrony and to exclude animals that turned over a new follicular wave before the second GnRH injection. Average time of ovulation is approximately 29 h after the second GnRH injection (Pursley *et al.*, 1995). Ovaries containing ovulatory follicles or new corpora lutea were collected by colpotomy (under epidural anaesthesia) at 0, 6, 12, 18, 24 and 48 h (corpora lutea) after the second GnRH injection. Blood samples were collected at the time of PGF_{2 α} injection and at the time of the second GnRH injection. Serum progesterone concentrations in these samples were measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) to ensure that all animals included in the study responded to the PGF_{2 α} injection with a decrease in serum progesterone concentration to < 1 ng ml⁻¹, indicating corpus luteum regression. Intra- and interassay coefficients of variation were 5.6 and 9.1%, respectively. Three blood samples at 15 min intervals were collected every 8 h beginning 16 h after the PGF_{2 α} injection until the time of ovariectomy or GnRH injection,

to verify that none of the animals included in the study underwent a preovulatory gonadotrophin surge before the second GnRH injection. A premature LH surge was not detected in any of the animals included in the 0 h (pre-gonadotrophin surge group). Blood samples were also collected every hour for 4 h after the second GnRH injection to confirm that a gonadotrophin surge was elicited by the second GnRH injection. In the other animals, the LH surge occurred after GnRH injection only, verifying control of timing of the gonadotrophin surge in our model system. Concentrations of serum LH were measured by radioimmunoassay (Matteri *et al.*, 1987; Jimenez Krassel *et al.*, 1999). Intra- and interassay coefficients of variation were 5.8 and 15.6%, respectively.

Tissue collection

For mRNA quantification and proteinase inhibitor activity assay, ovaries containing the ovulatory follicle or new corpora lutea were collected at 0, 6, 12, 18, 24 and 48 h ($n = 5-6$ in each group) after the second GnRH injection. After ovariectomy, the ovulatory follicle or new corpus luteum was isolated by cutting away all remaining ovarian stroma and small follicles such that the ultra-structure at the apex of the follicle remained intact. The follicles were cut in half transversely. One half was used for total RNA isolation. For protein analysis, the other half was cut sagittally into two equal pieces, one containing the follicle apex and one the base. New corpora lutea collected at 48 h after GnRH injection were used for mRNA analyses only. Samples were frozen at -80°C within 15 min of ovariectomy. For *in situ* hybridization, ovaries containing the ovulatory follicles were collected at 0, 6 and 24 h ($n = 3$ in each group) after GnRH injection. Ovulatory follicles were dissected from the ovary, immersed immediately in embedding medium, frozen over liquid nitrogen vapour and stored at -80°C until cut into sections.

Preparation of cDNA probes for PAI-1 and PAI-2

The nucleotide sequence of bovine PAI-1 has been reported (GenBank accession no. X16383). The reported sequence was used to prepare oligonucleotide primers, which were used in combination with RNA isolated from bovine corpora lutea in the RT-PCR to amplify a 491 bp cDNA that encoded bovine PAI-1. A set of degenerate primers (CTCCTCRTTCACATCCAC and GCTTTATCCTTTCCGTGTMAA; R = G + A; M = A + C) was designed based on the nucleotide sequence of PAI-2 from humans, rats and mice to obtain a bovine PAI-2 cDNA. These primers were used to amplify a 442 bp PAI-2 cDNA by RT-PCR from bovine luteal RNA. PCR products (PAI-1 and PAI-2) were subcloned into pBluescript SK(+) vectors (Stratagene, La Jolla, CA) and their identities and orientations were confirmed by fluorescent dye terminator sequencing. The partial bovine PAI-2 cDNA (GenBank accession no. AF416234) shared 84% identity with human PAI-2

(Schleuning *et al.*, 1987). In addition, a shorter PAI-2 cDNA (247 bp) was amplified from the PAI-2 cDNA using an internal primer and one original PAI-2 primer, and was subcloned and sequenced as described above.

Characterization of PAI-1 and PAI-2 mRNA abundance

Total RNA was isolated according to the manufacturer's instructions using the Trizol reagent (Invitrogen, Carlsbad, CA). Approximately 15 µg pooled RNA from each sample per time point was subjected to northern blot analysis to determine transcript size and number, and to optimize the specificity of hybridization conditions (Sambrook *et al.*, 1989). For quantitation of PAI-1 and PAI-2 mRNA abundance, 5 µg total RNA from each sample was applied in duplicate to a Zeta probe nylon membrane (Bio-Rad, Hercules, CA) using a dotblot apparatus (Bio-Rad; Sambrook *et al.*, 1989). Northern and dotblot analysis were performed using specific bovine PAI-1, PAI-2 or ribosomal protein L-19 (RPL19) [³²P]-labelled cDNA probes generated by PCR. RPL19 was used for normalization purposes. Each 20 µl PCR reaction included 1 × PCR buffer, 2.5 mmol MgCl₂ l⁻¹, 1.6 µmol l⁻¹ each of dATP, dGTP, dTTP, 0.25 µmol of each primer l⁻¹, 100 pg DNA template, 1.5 U Taq polymerase and 0.825 µmol [³²P]dCTP l⁻¹ (3000 Ci mmol⁻¹; NEN® Life Science Products, Boston, MA). The amplification conditions were: 95°C for 5 min; 94°C for 0.5 min, 52°C for 1 min, 72°C for 1.5 min for 40 cycles; 72°C for 10 min; hold at 4°C. After amplification, the PCR samples were made up to 100 µl with NETS (150 mmol NaCl l⁻¹, 10 mmol EDTA l⁻¹, 50 mmol Tris l⁻¹, 0.1% (w/v) SDS) and the unincorporated ³²P was removed by spun column chromatography through G-50 Sephadex minicolumns (Sambrook *et al.*, 1989). The membranes were incubated overnight at 42°C in 25 ml prehybridization buffer (50% (v/v) formamide, 5 × SSC (saline-sodium citrate buffer: 1 × SSC is 0.15 mol NaCl l⁻¹ and 0.015 mol sodium citrate l⁻¹, pH 7.0), 5 × Denhardt's (single strength is 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) BSA), 0.05 mol sodium phosphate l⁻¹ (pH 6.9), 0.1% (w/v) SDS and 250 µg denatured herring sperm DNA ml⁻¹). The prehybridization buffer was discarded and 25 ml fresh hybridization buffer (50% (v/v) formamide, 5 × SSC, 1 × Denhardt's, 0.02 mol sodium phosphate l⁻¹, 0.1% (w/v) SDS, 10% (w/v) dextran sulphate, 100 µg denatured herring sperm DNA ml⁻¹ and 1 × 10⁶ c.p.m. labelled probe) was added and the membranes were incubated overnight at 42°C. The membranes were washed in 1 × SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate at 42°C for 15 min, followed by consecutive washes in 0.1 × SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate at 42°C and 47°C for 15 min each. After washing, the filters were exposed to a phosphorimager cassette. After exposure (2–24 h), the cassette was scanned using a phosphorimager (Bio-Rad). After northern blot analyses, the size of the RNA transcripts was determined based on relative migration of RNA molecular mass markers (Roche, Indianapolis, IN). After

hybridization for PAI-1 or PAI-2, the membranes were stripped and reprobed with the [³²P]-labelled RPL19 cDNA. Preliminary experiments demonstrated that RPL19 mRNA abundance in preovulatory bovine follicles and new corpora lutea is not regulated by the gonadotrophin surge (data not shown). Relative densitometric units for PAI-1 and PAI-2 were quantified and adjusted relative to RPL19 mRNA expression using Molecular Analyst Version 1.5 software (Bio-Rad). Preliminary northern blot experiments demonstrated that the hybridization and washing conditions used in subsequent dotblot analyses were specific and yielded hybridization to single transcripts of the expected size for each mRNA of interest. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected after hybridization of each cDNA to increasing amounts of sample RNA (1–10 µg).

In situ hybridization

Follicles were cut on a Leica cryostat (W. Nuhsbaum, McHenry, IL) into 12 µm sagittal sections and mounted onto positively charged slides (Fisher Scientific, Chicago, IL). A sagittal section allows a view of the types of cell contained at both the apex and the base of the follicle. Before hybridization, the sections were prewarmed to room temperature for 10 min, fixed in 3.7% (v/v) formaldehyde in PBS for 5 min, rinsed twice in 2 × SSC for 2 min each, incubated in 0.25% (v/v) acetic anhydride in 0.1 mol triethanolamine-HCl l⁻¹ (pH 8.0) for 10 min, dehydrated in increasing concentrations of ethanol (70, 80, 95 and 100%; all v/v) for 2 min each, delipidated in absolute chloroform for 5 min, rinsed in 100% and 95% ethanol for 2 min each, and air dried for 1 h. Hybridizations for each mRNA were performed on serial sections in triplicate using antisense and sense (negative controls) [³⁵S]- or [³³P]-labelled cRNA probes generated from previously described PAI-1 and PAI-2 cDNAs. Both antisense and sense [³⁵S]UTP (1250 Ci mmol⁻¹; NEN® Life Science Product; PAI-1) or [³³P]UTP (3000 Ci mmol⁻¹; PAI-2) cRNA probes were generated using linearized cDNA templates and an *in vitro* transcription kit (Stratagene) according to the manufacturer's directions. Plasmid DNA containing the shorter (247 bp) PAI-2 cDNA was used as a template for cRNA synthesis and hybridized to follicle sections. Both PAI-2 cDNAs yielded identical results in northern blot analyses (data not shown). The transcription reaction was incubated at 37°C for 1 h and template DNA was removed by incubation with 20 U RNase-free DNase (Stratagene) at 37°C for 15 min. After DNase treatment, the reaction was diluted to 100 µl with NETS and unincorporated radionucleotides were removed as described above. Before hybridization, labelled probes were diluted in hybridization buffer to a concentration of 1.0 × 10⁶ c.p.m. ml⁻¹. Hybridization buffer included 50% (v/v) formamide, 0.3 mol NaCl l⁻¹, 10 mmol Tris l⁻¹ (pH 8), 1 mmol EDTA l⁻¹, 1 × Denhardt's, 50 mmol dithiothreitol l⁻¹ (DTT), 0.5 mg yeast tRNA ml⁻¹ and 10% (w/v) dextran sulphate. Hybridizations were performed by adding 60 µl diluted probe

per slide and incubating the slide in a humidified oven at 55°C for 16 h. After hybridization, the slides were washed twice by shaking in $2 \times \text{SSC}$ for 15 min at room temperature and were treated with RNase-A ($50 \mu\text{g ml}^{-1}$ in $2 \times \text{SSC}$) for 1 h at 37°C. The slides were washed at 55°C in $2 \times \text{SSC}$ containing 0.1% (v/v) β -mercaptoethanol (BME) for 15 min, $1 \times \text{SSC}/0.1\%$ (v/v) BME for 15 min, $1 \times \text{SSC}/50\%$ (v/v) formamide/ 0.1% (v/v) BME for 30 min, and twice in $0.1 \times \text{SSC}/0.1\%$ (v/v) BME for 15 min. The slides were dehydrated in increasing ethanol concentrations (60, 80, 95 and 100%), air dried for 1 h and dipped in 50% (v/v) NTB-2 emulsion (Eastman Kodak, Rochester, NY). Slides were exposed to autoradiographic emulsion for either 10 days (PAI-1) or 50 days (PAI-2) at 4°C and developed, followed by counterstaining with haematoxylin and eosin. Exposure time for detection of a given mRNA was the same for all time points. Digital bright-field and dark-field images were acquired on a Leica research microscope equipped with SPOT Model 1.1.0 camera and Version 3.2.4 software (W. Nuhsbaum, McHenry, IL).

Follicle homogenization procedure and chromogenic PAI assay

Follicles were homogenized using procedures previously described by Murdoch and McCormick (1992). In brief, the apical or basal sections of follicles were homogenized using a polytron homogenizer (Fisher Scientific) in $800 \mu\text{l}$ of 10 mmol calcium chloride I^{-1} and 0.25% (v/v) Triton-X100. The homogenates were centrifuged at 9000 g for 30 min at 4°C and the supernatants were collected and frozen at -20°C until assayed.

Two chromogenic assays were required to determine quantitatively the plasminogen activator inhibitor (PAI-1 and PAI-2) activity present in the above samples. In the first assay, endogenous plasminogen activator activity (tPA plus uPA) was measured. Dow *et al.* (2002) reported changes in endogenous plasminogen activator activity. In the second assay, a fixed amount of exogenous uPA was added to the samples and allowed to react with the PAIs. The residual plasminogen activator activity was determined and the PAI activity was expressed as a percentage of plasminogen activator activity (endogenous and exogenous) quenched in each sample. In both assays, the endogenous or residual plasminogen activator activity was quantified using a procedure described by Coleman and Green (1981). A sample in which plasminogen was omitted as a substrate was used as a negative control. Standard curves using a plasminogen activator standard (uPA; Sigma Chemical Co., St Louis, MO) were used to interpolate plasminogen activator activity (endogenous or residual) in samples. Preliminary experiments established that an increase in plasminogen activator and PAI activity was detected with increasing amounts of sample protein (25–200 μg). Each sample was run in duplicate for both assays and all samples were run in a single assay. The intra-assay coefficient of variation was 8.6%. Validation of the PAI activity assay has been reported previously (Politis *et al.*, 1990; Pandolfi *et al.*, 2001).

Statistical analysis

Differences in mRNA abundance or PAI activity were determined by one-way ANOVA using the General Linear Models procedure of SAS (Version 8.0). For PAI activity, percentages were arcsine-transformed before statistical analysis. Individual comparisons of mean RNA concentrations or PAI activity were performed using Fisher's protected least significant differences test. When heterogeneity of variance was detected, data were log-transformed before statistical analysis.

Results

Regulation of PAI-1 and PAI-2 mRNA abundance during the periovulatory period

mRNA for PAI-1 was detected as a single transcript of 2.9 kb (Fig. 1a). The abundance of mRNA for PAI-1 increased after the gonadotrophin surge (Fig. 1b; $P < 0.05$), with the highest mRNA abundance detected in follicles collected at about the time of ovulation (24 h) and in early corpora lutea (48 h). There was also a transient significant increase in PAI-1 mRNA in follicles collected at 6 h ($P < 0.05$), but expression then decreased to pre-surge concentrations by 12 h (Fig. 1b).

The gonadotrophin surge also upregulated PAI-2 mRNA expression in bovine periovulatory follicular and luteal tissue. A predominant transcript of 1.9 kb for PAI-2 was detected by northern blot analysis (Fig. 2a). Relative abundance of PAI-2 mRNA was increased transiently at 6 h ($P < 0.05$), but expression had decreased to pre-surge values in follicles collected at 12 h. Subsequently, PAI-2 mRNA abundance also increased in 24 h follicles and was increased further in new corpora lutea (48 h; $P < 0.05$; Fig. 2b) collected after GnRH injection.

Localization of PAI-1 and PAI-2 mRNAs in preovulatory bovine follicles

The cell-specific regulation of expression of PAI-1 and PAI-2 mRNA in response to the gonadotrophin surge was distinct. mRNA for PAI-1 was detected in the thecal layer at all times examined (Fig. 3d–f; 0, 6 and 24 h depicted). However, a low expression was also observed in the granulosa cell layer in follicles collected at about the time of ovulation (Fig. 3f; 24 h). In contrast, expression of PAI-2 mRNA was localized specifically to the granulosa cell layer of preovulatory follicles collected at 6 and 24 h (Fig. 4e,f). Significant expression in the thecal layer and adjacent ovarian stroma was not detected.

Effect of the gonadotrophin surge on PAI activity in preovulatory bovine follicles

Exogenous uPA activity added to preovulatory follicle homogenates (apex and base) was inhibited by 60–80%, indicating the presence of significant endogenous PAI activity in follicles collected during the periovulatory period

Fig. 1. Effect of a GnRH-induced gonadotrophin surge on abundance of plasminogen activator inhibitor 1 (PAI-1) mRNA in periovulatory bovine follicular and luteal tissue. (a) Northern blot analysis of expression of PAI-1 mRNA: note hybridization to a single 2.9 kb transcript. (b) Effect of the preovulatory gonadotrophin surge on relative abundance of PAI-1 mRNA in preovulatory bovine follicles and new corpora lutea. Data in (b) are expressed as relative units (PAI-1 mRNA per unit ribosomal protein L-19 (RPL19) mRNA) $\times 100$. Data are mean $\pm$ SE. ^{a-e}Times without a common superscript are significantly different ($P < 0.05$).

(Fig. 5). However, PAI activity in the preovulatory follicle apex and base was not regulated temporally by the gonadotrophin surge (Fig. 5).

Discussion

Controlling and focusing extracellular proteolytic activity is essential for many reproductive processes, including ovulation. There is a marked preovulatory increase in both mRNA abundance and activity for tPA and uPA in preovulatory bovine follicles (Dow *et al.*, 2002). The induction of PAIs is thought to be one of the key means for regulation of plasmin-mediated proteolysis initiated by the plasminogen activators. However, the regulation of the PAIs in preovulatory bovine follicles during the periovulatory period has not been reported. The results of the present study show clearly that PAI-1 and PAI-2 mRNAs were upregulated in a cell-specific way in response to the gonadotrophin surge, but temporal changes in PAI activity

Fig. 2. Effect of a GnRH-induced gonadotrophin surge on abundance of plasminogen activator inhibitor 2 (PAI-2) mRNA in periovulatory bovine follicular and luteal tissue. (a) Northern blot analysis of expression of PAI-2 mRNA: note hybridization predominantly to a single 1.9 kb transcript. (b) Effect of the preovulatory gonadotrophin surge on relative abundance of PAI-2 mRNA in preovulatory bovine follicles and new corpora lutea. Data in (b) are expressed as relative units (PAI-2 mRNA per unit ribosomal protein L-19 (RPL19) mRNA) $\times 100$. As a result of heterogeneity of variance, values for PAI-2 mRNA were log-transformed before analysis. Data shown as mean $\pm$ average SE. ^{a-e}Times without a common superscript are significantly different ($P < 0.05$).

were not detected. Furthermore, the regulation of PAI-1 and PAI-2 mRNAs and PAI activity observed are distinct from reports in other species to date.

A transient increase in the abundance of PAI-1 mRNA was observed within 6 h after the gonadotrophin surge, with increased expression detected at about the time of ovulation (24 h follicles) and in new corpora lutea. This finding is in contrast to reported changes in PAI-1 mRNA abundance in preovulatory rat and monkey follicles in response to hCG administration. In the above species, PAI-1 mRNA is upregulated transiently but subsequently decreases in preovulatory follicles at about the time of ovulation (Liu *et al.*, 1991; Peng *et al.*, 1993). Furthermore, high expression of tPA mRNA continues in preovulatory rat and monkey follicles at about the time of ovulation, several hours after the decrease in PAI-1 mRNA. At about the time of

A significant increase in PAI-2 mRNA was also observed in preovulatory bovine follicles at about the time of follicular rupture (24 h) and PAI-2 mRNA was localized specifically to the granulosa cell layer. Very limited information is available regarding the temporal and cell-specific regulation of PAI-2 mRNA during the periovulatory period in other species. mRNA for PAI-2 has been detected in both human and mouse ovarian tissues. Both human cumulus cells and granulosa-luteal cells collected from patients (36 h after hCG administration; a few hours before ovulation) undergoing IVF express PAI-2 mRNA (Piquette *et al.*, 1993). A small increase in PAI-2 mRNA abundance was also observed in mouse ovaries at 4 h after hCG injection. The localization of PAI-2 mRNA in the mouse ovary was distinct from that

Fig. 4. *In situ* localization of plasminogen activator inhibitor 2 (PAI-2) mRNA within preovulatory bovine follicles collected at 0, 6 and 24 h after GnRH injection. Representative bright-field micrographs of preovulatory follicles collected at (a) 0 h, (b) 6 h and (c) 24 h, and stained with haematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at (d) 0 h, (e) 6 h and (f) 24 h, and hybridized with [³³P]-labelled antisense PAI-2 cRNAs. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at (g) 0 h, (h) 6 h and (i) 24 h, and hybridized with [³³P]-labelled sense PAI-2 cRNAs ($n = 3$ per time; total = 9). Note localization of PAI-2 mRNA to the granulosa cell layer (G) of follicles collected at 6 and 24 h. T: thecal layer. Scale bar represents 250 μ m.

observed in cattle and was restricted primarily to a few individual cells within the thecal layer that were thought to be macrophages (Leonardsson *et al.*, 1995).

PAI-2 is a major product of macrophages and monocytes in response to inflammatory conditions (Schwartz *et al.*, 1988; Gyetko *et al.*, 1992). Furthermore, the two different forms of PAI-2 (extracellular and intracellular) in monocytes probably have distinct functions. The extracellular form inhibits uPA activity, whereas the predominant intracellular form inhibits tumour necrosis factor α (TNF- α)-directed apoptosis (Dickinson *et al.*, 1995, 1998). Plasmin production at the site of follicle rupture in preovulatory sheep follicles

facilitates liberation of membrane-anchored TNF- α and subsequent apoptosis of ovarian surface epithelial cells (Murdoch, 1995a,b, 1998, 1999; Murdoch *et al.*, 1997). Although the presence of the two forms of PAI-2 in bovine granulosa cells has not been established, it is possible that intracellular PAI-2 may have a protective role against TNF- α -directed apoptosis in the granulosa cell layer of preovulatory bovine follicles.

Formation of the corpus luteum involves marked biochemical and morphological processes including luteinization, angiogenesis and cellular proliferation and migration. In the present study, the highest relative abun-

Fig. 5. Detection of total plasminogen activator inhibitor activity (PAI-1 and PAI-2) in homogenates of the apex (■) and base (□) of periovulatory bovine follicles using a chromogenic assay. Data are expressed as percentage PAI activity per 100 µg protein. As a result of heterogeneity of variance, the data were log-transformed. Percentages were arcsine-transformed before statistical analysis. Data are mean $\pm$ average SE ($n = 5$ – 6 per time). PAI activity in the preovulatory follicle apex and base were not regulated in response to the gonadotrophin surge.

dance of PAI-1 and PAI-2 mRNA was observed in tissue collected during corpus luteum formation. Furthermore, it is possible that the increased abundance of PAI-1 and PAI-2 mRNA detected at about the time of ovulation may in fact be more relevant to regulation of luteal development. In rats, PAI-1 mRNA is localized adjacent to uPA-expressing capillaries of the early corpus luteum and is thought to play an important role in the regulation of angiogenesis (Bacharach *et al.*, 1992). Studies using PAI-1 deficient mice have shown the absolute requirement for PAI-1 during tumour-induced angiogenesis (Bajou *et al.*, 1998; Gutierrez *et al.*, 2000). The role of PAI-2 (intracellular or extracellular) during corpus luteum formation is unclear. More investigation will be critical to understanding the exact roles of PAI-1 and PAI-2 during the periovulatory period in cattle.

Significant PAI activity (PAI-1 and PAI-2) was detected in the apex and base of preovulatory bovine follicles. However, no temporally or spatially significant changes in PAI activity were detected in response to the gonadotrophin surge. Increased tPA mRNA abundance and activity have been observed during the periovulatory period in preovulatory bovine follicles (Dow *et al.*, 2002). Furthermore, tPA activity is spatially regulated in preovulatory bovine follicles after the gonadotrophin surge. In the follicle apex, tPA activity is increased in response to the gonadotrophin surge and remains high until 24 h later. In contrast, tPA activity in the follicle base peaks within 12 h after the gonadotrophin surge and decreases to pre-surge values by 24 h (Dow *et al.*, 2002). A differential increase in PAI activity was not detected in the base of preovulatory bovine

follicles at about the time of follicular rupture. Therefore, the differential upregulation of tPA activity in the follicular apex versus the base is not likely to be a result of regional differences in upregulation of PAI activity in response to the gonadotrophin surge. This difference in the regulation of plasminogen activator (tPA) and PAI abundance may provide a temporal and spatial window of increased plasminogen activator activity that may help to facilitate follicular rupture.

In conclusion, we have demonstrated that PAI-1 and PAI-2 mRNAs are upregulated in preovulatory bovine follicles in response to the preovulatory gonadotrophin surge with maximal expression during corpus luteum formation. Furthermore, the temporal and spatial regulation of PAI-1 and PAI-2 mRNAs in preovulatory bovine follicles in response to the gonadotrophin surge was distinct from reports to date in other species. Further investigation will be required to elucidate the precise physiological roles of PAI-1 and PAI-2 during the periovulatory period in cattle.

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