Pattern of protein production by bovine corpora lutea during luteolysis and characterization of expression of two major secretory products of regressing corpora lutea

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Although the decrease of progesterone in serum and in luteal tissue during luteal regression is well characterized, relatively little is known about changes in proteins produced by the corpus luteum during this time. The first objective was to examine changes in patterns of protein secretion that might be associated with functional and structural luteal regression. The second objective was to characterize the expression of two major secretory products of regressing corpora lutea. Thirty normally cyclic heifers were randomly assigned at day 15–16 of the oestrous cycle (oestrus = day 0) to be ovariectomized at 0 h (no PGF_{2α}; *n* = 5) or at 4, 8, 12, 24 or 48 h after PGF_{2α}-induced luteal regression (*n* = 5 per time point). Total cellular RNA was isolated from tissue frozen at the time of ovariectomy. Thin slices (< 1 mm) of tissue were placed in methionine-deficient minimum essential media with [³⁵S]methionine and placed in a humidified CO₂ incubator at 38°C. Media and tissues were collected 6 h later. Changes in profiles of secreted proteins were analysed by one-dimensional SDS-PAGE. A number of proteins (relative molecular mass ranging from 14 300 to 200 000) were produced by luteal tissue at each time point (0–48 h). The major secretory proteins had relative molecular masses of approximately 21 500, 28 200, 43 700 and 46 000. Secretion of the relative molecular mass 46 000 protein(s) increased (*P* < 0.05) between 4 and 24 h after PGF_{2α} injection compared with the 0 h group. Western blot analyses with either tissue inhibitor of metalloproteinase-1 or tissue inhibitor of metalloproteinase-2 antisera detected immunoreactive proteins of relative molecular mass 28 200 and 21 500, respectively. Concentrations of mRNA encoding tissue inhibitor of metalloproteinases-1 increased (*P* < 0.01) by 8 h after PGF_{2α} injection, remained stable (*P* > 0.20) through 24 h and decreased (*P* < 0.05) by 48 h after PGF_{2α}. Concentrations of tissue inhibitor of metalloproteinases-2 mRNA were highest (*P* < 0.05) at 8 h after PGF_{2α} injection and lowest (*P* < 0.05) 48 h following induction of luteolysis. In summary, the profile of luteal protein production changed during luteolysis and two secretory products (tissue inhibitor of metalloproteinases-1 and -2) were identified. Metalloproteinase inhibitors may have an important role in tissue remodelling during structural luteolysis.

Introduction

Prostaglandin F_{2α} (PGF_{2α}) of uterine origin causes luteal regression in several species including cattle (for review see Knickerbocker *et al.*, 1988). PGF_{2α}-induced luteolysis may involve any of the following mechanisms: (1) decrease in blood flow (Niswender, 1981), (2) direct action on luteal cells (Hansel *et al.*, 1991; Wiltbank *et al.*, 1991); (3) production of oxygen radicals (Riley and Behrman, 1991); or (4) recruitment of immune cells (Murdoch *et al.*, 1988). The precise mechanisms

associated with luteolysis are not clearly understood; however, complex communication between different luteal cell populations seems likely. Hansel *et al.* (1991) proposed that luteal cell types cooperate to obtain peak progesterone secretion during the luteal phase and that loss of this positive interaction could play a role in luteolysis. In addition, large luteal cells that contain large numbers of PGF_{2α} receptors may regulate small luteal cell function during luteolysis in sheep, since the number of small cells, which contain few PGF_{2α} receptors, decreased before that of the large cells (Braden *et al.*, 1988). The agents responsible for communication between luteal cells are not known, but may include steroids, prostaglandins, and proteins. Morphological (O'Shea *et al.*, 1977; Sawyer *et al.*, 1990) and

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biochemical (Juengel *et al.*, 1993) evidence indicates that apoptosis is associated with luteal cell death. Apoptosis is a process of cell death that eliminates a distinct population of cells by activation of metabolic events within the cells themselves. In some systems apoptosis requires synthesis of specific proteins (Arends and Wyllie, 1991); changes in protein synthesis may therefore occur during luteolysis. One morphological characteristic of cells undergoing apoptosis is detachment from the extracellular matrix and subsequent loss of contact with neighbouring cells (Kerr *et al.*, 1972). Metalloproteinases and their inhibitors are extensively involved in regulation of extracellular matrix remodelling (Woessner, 1991) and thus may be involved in control of cell death and tissue reorganization during luteolysis.

One aspect of luteal regression that has not been examined is the changes in protein production that occur during luteolysis. The specific objectives of this study were: (1) to determine changes in protein production during luteolysis and (2) to characterize the expression of two major secretory products of regressing corpora lutea (tissue inhibitor of metalloproteinases-1 (TIMP-1) and -2 (TIMPs-2)).

Materials and Methods

Animal care

All procedures described where animals were used were approved by the University of Missouri Animal Care and Use Committee (Protocol no. 1903).

Experimental design

Beef heifers (15–24 months of age) exhibiting normal oestrous cycles (18–24 days) were housed in dry pens and fed corn silage with supplemental grain to meet National Research Council growth requirements. Heifers were randomly assigned to a treatment group after being observed in standing oestrus (day = 0). On day 15–16, heifers were either ovariectomized ($n = 5$; 0 h) or given 25 mg PGF_{2α} (Lutalyse; Upjohn Co., Kalamazoo, MI) to induce luteolysis. Heifers that received PGF_{2α} were ovariectomized at 4, 8, 12, 24 or 48 h ($n = 5$ per time point) after injection of PGF_{2α}. Blood was collected daily for 4 days preceding ovariectomy or PGF_{2α} injection, at the time of PGF_{2α} injection, and at ovariectomy. Concentration of progesterone in all sera was determined by radioimmunoassay (Copelin *et al.*, 1989) in two assays; intra-assay coefficients of variation averaged 15.8% and the inter-assay coefficient of variation was 2.9%.

Ovary removal and tissue culture

Lidocaine (2%) was used locally to anaesthetize the surgical site. Ovaries containing corpora lutea were removed by aseptic technique with an ecraseur inserted through a flank incision, rinsed in sterile saline and transported to the laboratory. Corpora lutea were blunt dissected from ovaries and weighed. Thin slices (approximately 1 mm) were obtained from the central portion of the gland with a hand microtome and tissue

explants (one slice, 10–40 mg per slice) were cultured in 0.5 ml of methionine deficient media (MEM; Sigma, St Louis, MO) with 50 µCi L-[³⁵S]methionine (cell labelling grade, Amersham, Arlington, Heights, IL) for 6 h in a humidified 5% CO₂ 38°C incubator on an orbital shaker. The remaining tissue was frozen in liquid N₂ and stored at –70°C until DNA and RNA analysis. Tissues were frozen or cultures were initiated within 30 min of removal of ovaries.

Analysis of secreted proteins

Media were collected at the end of the 6 h culture, centrifuged at low speed to remove any tissue debris, and stored at –70°C. Samples were lyophilized, resuspended in reducing gel loading buffer, and trichloroacetic acid precipitable radioactivity determined. Equivalent amounts of radioactivity per lane were separated by PAGE (12.5% gel; Moor *et al.*, 1981). Radiolabelled proteins were visualized by fluorography using Kodak X-Omat AR-5 film (Rochester, NY). The percentage of total radiolabelled protein represented in each major protein band was determined by densitometry.

Western blot analysis

Secreted proteins were collected from corpora lutea removed 12 h after PGF_{2α}-induced luteolysis. Conditions for culture were the same as those outlined above except that L-[³⁵S]methionine was replaced with non-labelled methionine. Reduced proteins (100 µg) were separated by PAGE (10% gel), and transferred to nitrocellulose (Fisher Scientific, St Louis, MO) by electroblotting. Lanes were blocked with 5% non-fat dry milk (NFDm) in Tris-buffered saline with 0.05% Tween (TBS-T, Fisher Scientific) at room temperature for 4 h. Nitrocellulose strips were washed (three times, 5 min each) in TBS-T and incubated with normal rabbit serum or antisera (described below) in TBS-T with 1% NFDm at room temperature overnight. Immunoreactive TIMP-1 was detected by incubating nitrocellulose strips in IgG purified normal rabbit serum (control; 1:1000 dilution) or in rabbit anti-human TIMP-1 serum (1:3600 dilution, donated by I. M. Clarke, Cambridge, UK and funded by Smith Kline Beecham Pharmaceuticals, Philadelphia, PA). Immunoreactive TIMP-2 was detected by incubating nitrocellulose strips in normal rabbit serum (control; 1:750 dilution) or rabbit anti-human TIMP-2 antisera (1:750 dilution; donated by W. G. Stetler-Stevenson, Bethesda, MD). Strips were washed (three times, 5 min each) in TBS-T and incubated with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) at 1:3000 dilution in TBS-T with 1% NFDm for 4 h at room temperature. Strips were washed (once in TBS-T, 5 min; twice in TBS, 5 min) and transferred to TBS containing 4-chloro-1-naphthol (Sigma) and H₂O₂ (Fisher Scientific).

Preparation of probes

Complementary DNA probes for TIMP-1 (Smith *et al.*, 1994) and TIMP-2 (Smith *et al.*, 1992) isolated from an ovine corpus luteum cDNA library were used for hybridization. The 900 bp TIMP-1 cDNA and 428 bp TIMP-2 cDNA were

random prime labelled with the Klenow fragment of DNA polymerase I (Multi Prime Labelling System, Amersham) to a specific activity of approximately 1.8×10^8 cpm μg^{-1} DNA with $\alpha[^{32}\text{P}]\text{dCTP}$ (3000 Ci mmol^{-1} , Amersham).

Northern and dot blot analysis

Total cellular RNA was isolated by the method of Chomczynski and Sacchi (1987). The amount of DNA was determined by the method of Labarca and Paigen (1980). For northern analysis, equal concentrations of total cellular RNA from each sample were combined by treatment (0, 4, 8, 12, 24 and 48 h). Five (TIMP-2) or 7.5 (TIMP-1) μg total cellular RNA from combined samples, total cellular RNA isolated from bovine corpora lutea (positive control) and bovine spleen (negative control) were subjected to electrophoresis in a 1.5% agarose formaldehyde gel and transferred to nylon filters (Biotrans[®]; ICN Biomedicals, Inc. Irvine, CA) by capillary action (Maniatis *et al.*, 1982). The filters were air dried for 20 min and subsequently baked for 2 h at 70–80°C.

Complementary RNA for TIMP-1 and TIMP-2 was transcribed *in vitro* according to the protocol provided by the manufacturer (Stratagene, La Jolla, CA). For dot blot analysis, total cellular RNA ($1 \mu\text{g} \mu\text{l}^{-1}$) isolated from individual corpora lutea were spotted as 2 μl spots onto 0.2 μm nylon filters [Biotrans[®] (ICN)]. In addition, RNA transcripts (cRNA; 2 μl spots; 1000–7.8 pg in bovine spleen $1 \mu\text{g} \mu\text{l}^{-1}$ total cellular RNA) and total cellular RNA isolated from bovine corpora lutea (1–3 μl spots; $1 \mu\text{g}$ total cellular RNA μl^{-1}) were included as positive controls, and total cellular RNA isolated from bovine spleen (2 μl spots; $1 \mu\text{g}$ total cellular RNA μl^{-1}) was included as a negative control. Filters were allowed to air dry and then baked at 70–80°C for 2 h.

Filters were prehybridized in $5 \times \text{SSC}$ (Fisher Scientific), 0.05% (w/v) Ficoll 400 (Sigma), 0.05% (w/v) polyvinylpyrrolidone (Sigma), 0.05% (w/v) BSA (Sigma), 0.1% (w/v) SDS (Fisher Scientific), 50% (v/v) deionized formamide (Fisher Scientific) and 50 mmol $\text{NaPO}_4 \text{ l}^{-1}$ (Fisher Scientific) with heat denatured herring sperm DNA (Fisher Scientific; 300 $\mu\text{g} \text{ml}^{-1}$) overnight at 42°C. The filters were hybridized with 2×10^6 c.p.m. ml^{-1} of appropriate $[^{32}\text{P}]\text{cDNA}$ in prehybridization buffer (without herring sperm DNA) for 24–36 h at 42°C. All filters were washed in $2 \times \text{SSC}$, 0.1% SDS, 42°C for 20 min, and $0.1 \times \text{SSC}$, 0.1% SDS, 65°C for 30 min. Filters were exposed to Kodak X-Omat AR-5 film for 1–5 days and subjected to densitometry.

Determination of concentration of mRNA for TIMP-1 and TIMP-2

All mRNA concentrations were determined by comparing dot blot samples to a standard curve of *in vitro* synthesized cRNA on the same filter. Hybridization signals were measured by a video densitometer (Bio-Rad Laboratories). Coefficients of variation were 14.7% and 14.5% for TIMP-1 and TIMP-2, respectively.

Statistical analysis

Data were analysed by using Statistical Analysis Systems (SAS, 1985). Standard GLM procedures (analysis of variance)

Fig. 1. Autoradiograph of $[^{35}\text{S}]$ methionine-labelled proteins secreted from bovine corpora lutea during luteolysis. Two of five animals are shown for each time point. Time of removal of corpora lutea is indicated across the top. Migration of standard proteins are shown on the left.

were used and mean separation test (protected least significant difference) was used where applicable. Before analysis, percentages of density represented in protein bands were arcsin transformed; however, data are presented as means $\pm$ SEM of percentages.

Results

Serum progesterone concentrations

Mean concentration of progesterone (SEM; ng ml^{-1}) was not different among groups for any of the 4 days preceding or at the time of $\text{PGF}_{2\alpha}$ injection (range 2.6 ± 0.3 to 4.2 ± 0.4). At the time of surgery (tissue removal), serum concentrations of progesterone of the 0 and 4 h group were not different (3.0 ± 0.5 versus 2.6 ± 0.4), decreased ($P < 0.01$) in the 8 h group (1.3 ± 0.3), and remained low thereafter (1.2 ± 0.2 , 0.8 ± 0.1 , and 0.6 ± 0.2 in the 12, 24 and 48 h groups, respectively). These results were published by Juengel *et al.* (1993).

Secreted proteins

Tissue explants collected from all time points secreted a wide array of proteins (relative molecular mass (M_r) approximately 14 300 to $> 200\,000$; Fig. 1). Four major secretory products (M_r 21 500, 28 200, 43 700 and 46 000) were chosen for one-dimensional scanning. Secretion of the M_r 21 500 protein(s) was greatest ($P < 0.05$) by the 0 h tissue explants compared with explants removed at later time points (12–48 h; Table 1). Incorporation of L- $[^{35}\text{S}]$ methionine into the protein(s) at M_r 28 200 was lower ($P < 0.02$) at 48 h than at any other time point. Secretion of the 43 700 M_r protein(s) did not change over time. The protein(s) at M_r of 46 000 increased ($P < 0.05$) during luteal regression.

Table 1. Percentage of total density (one-dimensional analysis) represented in selected secreted protein bands

Molecular mass of protein ($\times 10^{-3}$)	Time after injection of PGF _{2α} (h)					
	0	4	8	12	24	48
21.5	16.2 $\pm$ 2.8 ^a	13.2 $\pm$ 1.0 ^{ab}	12.1 $\pm$ 1.7 ^{ab}	11.1 $\pm$ 0.9 ^{bc}	11.6 $\pm$ 1.2 ^{ac}	7.0 $\pm$ 1.4 ^c
28.2	12.2 $\pm$ 1.3 ^a	14.6 $\pm$ 1.0 ^a	14.5 $\pm$ 1.1 ^a	15.8 $\pm$ 0.9 ^a	12.9 $\pm$ 2.3 ^a	7.1 $\pm$ 1.1 ^b
43.7	13.3 $\pm$ 1.0 ^a	11.9 $\pm$ 1.6 ^a	10.4 $\pm$ 1.4 ^a	11.5 $\pm$ 0.9 ^a	8.0 $\pm$ 0.8 ^a	11.0 $\pm$ 2.1 ^a
46.0	7.1 $\pm$ 1.6 ^a	13.2 $\pm$ 2.4 ^b	15.9 $\pm$ 1.3 ^b	13.7 $\pm$ 2.0 ^b	14.5 $\pm$ 1.8 ^b	ND

ND: not determined

^{a,b,c}Percentages with different superscripts within a row are significantly different ($P < 0.05$). **Fig. 2.** Western blot analysis of *in vitro* secreted proteins (100 μ g per lane) from corpora lutea collected 12 h after luteolysis was initiated. Nitrocellulose strips were incubated with normal rabbit serum (N), tissue inhibitor of metalloproteinase (TIMP-1) antiserum (T-1), or TIMP-2 antiserum (T-2). Migration of standard molecular mass markers are indicated on the left.

Western blot analysis of secreted luteal proteins for TIMP-1 and TIMP-2

Since the ovine corpus luteum was found to produce TIMP-1 and -2 (Smith and Moor, 1991; Smith *et al.*, 1992b, 1993), antibodies specific for TIMP-1 and -2 were used to provide evidence that the major secreted radiolabelled bands at M_r 28 200 and 21 500, respectively, were composed, at least in part, of these proteins. The TIMP-1 and TIMP-2 antisera specifically recognized proteins of M_r 28 200 and 21 500, respectively (Fig. 2).

Expression of TIMP-1 and TIMP-2 mRNA during luteolysis

The ovine TIMP-1 cDNA hybridized specifically to a band of approximately 900 bases in all sample lanes and in the positive control (Fig. 3). This transcript was similar in size to that previously reported in bovine corpora lutea (Freudenstein *et al.*, 1990). There is a lack of hybridization in the negative

Fig. 3. Autoradiograph of bovine regressing corpus luteum total cellular RNA (7.5 μ g per lane) hybridized to ovine tissue inhibitor of metalloproteinase-1 (TIMP-1) cDNA. Time of removal of corpora lutea is indicated across the top. Migration of 28S and 18S ribosomal bands is indicated on the right. Film was exposed for 3 days.

control lane, bovine spleen (Fig. 3). Concentrations of TIMP-1 mRNA (ng mg^{-1} DNA) increased ($P < 0.01$) by 8 h after initiation of luteolysis, remained stable through 24 h, then decreased ($P < 0.05$) at 48 h to concentrations similar to 0 h (Fig. 4a).

The ovine TIMP-2 cDNA hybridized predominantly to a band of approximately 1100 bases in total cellular RNA isolated from tissue collected at all time points; however, a minor band of approximately 3500 bases was also detected. The size of the transcripts was similar to the reported size of TIMP-2 in other species; however, their relative intensities were reversed (Stetler-Stevenson *et al.*, 1990). The negative

Fig. 4. (a) Mean concentrations of mRNA for tissue inhibitor for metalloproteinase-1 (TIMP-1) in bovine corpora lutea during luteolysis. (b) Mean concentrations of mRNA for TIMP-2 in bovine corpora lutea during luteolysis. Bars having different superscripts are significantly different ($P < 0.05$; $n = 5$ per time point).

control, bovine spleen, did not show any specific hybridization (Fig. 5). Concentrations of TIMP-2 mRNA (ng mg^{-1} DNA) were greatest ($P < 0.05$) at 8 h and lowest ($P < 0.05$) at 48 h (Fig. 4b).

Discussion

A number of points have emerged from these experiments. First, luteal tissue secretes a variety of proteins and the pattern of secretion changes during luteolysis. Changes in the pattern of protein production suggest a potential endocrine, paracrine, or autocrine role for these proteins during luteolysis. Fitz *et al.* (1993) found that treatment of ovine luteal cell cultures with the transcription inhibitor, actinomycin D blocks the ability of PGF_{2α} to inhibit progesterone secretion. Consequently, protein synthesis may be necessary for PGF_{2α}-induced luteolysis. Second, although progesterone secretion was dramatically reduced, luteal tissue continued to secrete a number of proteins as late as 48 h after injection of PGF_{2α}.

Secretion of a protein(s) of M_r 46 000 was observed, the pattern of secretion of which changed early during luteolysis. The potential role of the 46 000 M_r protein(s) in luteal regression may be determined upon its identification and characterization.

Two additional proteins that were shown to be produced by bovine corpora lutea during luteolysis included TIMP-1 and -2. The relative molecular masses of TIMP-1 (M_r 28 000–30 000; Docherty *et al.*, 1990) and -2 (21 000; Stetler-Stevenson *et al.*, 1990) in other species correspond closely

Fig. 5. Autoradiograph of bovine regressing corpus luteum total cellular RNA (5 µg per lane) hybridized to ovine tissue inhibitor of metalloproteinase-2 (TIMP-2) cDNA. Time of removal of corpora lutea is indicated across the top. Migration of 28S and 18S ribosomal RNA is indicated at the right. Film was exposed for 5 days.

to the relative molecular masses of two proteins (28 200 and 21 500, respectively) secreted by luteal tissue in this study.

Changes in expression of TIMP-1 and -2 mRNA were detected during luteolysis. Metalloproteinases (collagenase, gelatinase, stromelysin) are metal-dependent enzymes that degrade the proteinaceous components of the extracellular matrix. These enzymes have an important role in tissue remodelling and TIMP-1 and -2 are important regulators of metalloproteinase activity. It has been proposed that metalloproteinases and their inhibitors have an important physiological role in various processes including ovulation, implantation (reviewed in Sakamoto and Sakamoto, 1988), angiogenesis, cell migration and tumour cell metastasis (reviewed in Liotta *et al.*, 1991; Liotta, 1992). In ovarian function, TIMP-1 and -2 may have an important role in regulating metalloproteinase activity preceding ovulation and in the regulation of tissue remodelling during the luteal phase. During luteolysis, corpora lutea undergo extensive tissue remodelling; it is therefore, not surprising that expression of TIMP-1 and -2 mRNA increased during this period.

As previously mentioned, there is evidence of apoptosis in bovine luteal tissue during luteolysis (Juengel *et al.*, 1993). Cells undergoing apoptosis become detached from the extracellular matrix and begin to shrink (Kerr *et al.*, 1972). Metalloproteinases and their inhibitors are considered to be associated with detachment of cells from the extracellular matrix. The transient increase in TIMP-1 mRNA precedes the onset of oligonucleosome formation, reported by Juengel *et al.* (1993). Proteins

involved with regulation of tissue remodelling may, therefore, have an important role in apoptotic cell death of luteal cells.

In summary, protein production by corpora lutea changes during luteolysis. Two major secretory proteins of regressing corpora lutea have been identified as TIMP-1 and -2. Changes in protein expression may play an important role in luteal regression.

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