Secretion of a putative metalloproteinase inhibitor by ovine granulosa cells and luteal tissue*

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Summary. Ovine granulosa cells respond, through transcriptional mechanisms, to preovulatory concentrations of gonadotrophins by secreting an M_r 30 000 polypeptide. To determine the stages of the oestrous cycle at which this polypeptide is secreted, corpora lutea were collected on Days 3, 7, 10, 13 and 16 (Day 0 = oestrus; $n = 4$ per group), cut into 1-mm slices, and incubated for 6–7 h with [35 S]methionine. Radio-labelled polypeptides of intra- and extra-cellular origin were separated by polyacrylamide gel electrophoresis and quantitated by densitometry. The M_r 30 000 polypeptide was secreted at all stages of the luteal phase tested (Days 3–16), and represented approximately 24% of the total labelled polypeptide present in the medium; polypeptides of approximate M_r 14 000, 25 000 and 46 000 accounted for most of the other secreted proteins. Neither pituitary hormones (LH, FSH, prolactin) nor cholera toxin (chosen to activate adenylate cyclase) affected the rate of production of M_r 30 000 polypeptide, indicating that, once secretion has been initiated in the granulosa cells, it is not readily modulated by hormonal intervention after luteinization.

Incubation of luteinized granulosa cells with tunicamycin (inhibits *N*-linked glycosylation reactions) showed that the secreted polypeptide consists of a heavily glycosylated amino acid backbone of approximately M_r 20 000. Western blot analysis established further that the polypeptide was not an inhibin subunit. However, NH_2 -terminal amino acid sequencing of the first 25 amino acids revealed a 68% sequence identity between the secreted polypeptide (M_r 30 000) and a human tissue inhibitor of metalloproteinases.

Keywords: granulosa cell; corpus luteum; polypeptide secretion; metalloproteinase inhibitor; sheep

Introduction

Ovarian cells secrete a variety of proteins which have an important endocrine, paracrine, or autocrine role in regulating reproductive function. Numerous studies have reported ovarian proteins which are involved in the regulation of mitosis, gonadotrophin binding, angiogenesis, luteinization and many other ovarian processes (Tsafriri, 1988).

The preovulatory gonadotrophin surge altered the pattern of follicular protein secretion in sheep (Moor & Crosby, 1987). More specifically, when intact follicles or granulosa cells were exposed to gonadotrophins (*in vivo* or *in vitro*) the secretion of M_r 46 000–60 000 polypeptides decreased over time, while that of an M_r 30 000 polypeptide was begun. Furthermore, inhibition of transcription in follicles after gonadotrophin stimulation inhibited synthesis of the M_r 30 000 polypeptide which is phosphorylated and glycosylated but not sulphated.

The results of Moor & Crosby (1987) raise a number of important questions concerning the functions of these follicular polypeptides. Therefore, the objectives of the present experiments were

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to (1) determine whether the secretion of the M_r 30 000 polypeptide continues after ovulation and luteinization, (2) determine whether luteal secretion of the M_r 30 000 polypeptide could be regulated by pituitary hormones or 3',5'-cyclic AMP, (3) determine whether the M_r 30 000 polypeptide is an inhibin subunit, and (4) obtain an NH_2 -terminal amino acid sequence for the identification of the protein by sequence identity.

Materials and Methods

Collection and culture of granulosa cells. Ovaries were collected from Welsh Mountain ewes at slaughter 36 h after injection (i.m.) of 900 i.u. pregnant mares' serum gonadotrophin (Folligon: Intervet Laboratories Ltd, Cambridge, UK) during the luteal phase. Non-atretic follicles (> 3 mm in diameter) were dissected from ovaries at room temperature in Dulbecco's phosphate buffered saline. Granulosa cells were collected, explanted into 4-well plates (Nunc, Inter Med., Roskilde, Denmark) and cultured in a CO_2 incubator (5% CO_2 gas phase) at 38°C in Medium 199 (enriched M199) containing Earles salts (Gibco Ltd, Paisley, UK), supplemented with 5% fetal calf serum and 5% sheep serum (Sera Lab Ltd, Crawley, Sussex, UK). Once confluence was attained the granulosa cells were washed three times with serum-free Medium 199 in preparation for protein radiolabelling. Luteinization of granulosa cells was characterized by the ability of these cells to secrete predominantly progesterone.

Granulosa cells were also explanted into culture flasks (Nunc) containing Medium 199 and allowed to grow to confluence. The cells were washed with serum-free medium as previously described and incubated with serum-free Medium 199 which was collected daily for 7 days. After collection, the medium (containing secreted proteins) was dialysed and lyophilized.

Collection and incubation of luteal tissue. Ovaries were collected at slaughter from 20 Welsh Mountain and Clun Forest ewes on Days 3, 7, 10, 13 and 16 ($N = 4$ per group) after oestrus (Day 0). Corpora lutea were dissected from ovaries at room temperature, cut into 1-mm slices, and washed in serum-free Medium 199 pending radiolabelling.

Action of hormones and drugs on protein secretion. Luteinized granulosa cells and luteal slices were incubated with pituitary hormones (single treatment including NIH-LH-S18, $5 \mu\text{g/ml}$; NIH-FSH-S12, $2.5 \mu\text{g/ml}$; and NIH-prolactin-S9, 20 ng/ml) or cholera toxin (250 ng/ml) for 7 h (see Methods below) to determine whether either the pattern or rate of protein secretion could be modulated. The cells or luteal slices were radiolabelled as described below. In separate experiments the relative molecular mass of the peptide backbone of the glycosylated M_r 30 000 molecule was determined by preincubating luteinized granulosa cells in serum-free medium containing tunicamycin (0, 0.125, 0.25, 0.5, 1.0 or $2.0 \mu\text{g/ml}$) for 6 h. Thereafter, the preincubation medium was discarded and replaced with medium containing an equivalent amount of tunicamycin in preparation for radiolabelling the cells as described below. Tunicamycin is an antibiotic which inhibits synthesis of N -glycosidically linked oligosaccharides (White *et al.*, 1978).

Radiolabelling of cells. Granulosa cells or luteal slices were incubated for 6–7 h with $0.1 \text{ mCi L-[}^3\text{S]methionine/ml}$ (sp. act. $> 1000 \text{ Ci/mmol}$) in Eagles Minimum Essential Medium which did not contain methionine (MEM Eagle Modified; Flow Laboratories, Irvine, UK). After radiolabelling of cells, the medium was collected, centrifuged ($500 g$ for 5 min) to pellet any suspended cells and the supernatant was collected for analysis of secreted polypeptides. Granulosa cells and luteal tissue were also collected, washed with saline, and stored until analysis of intracellular polypeptide synthesis.

Electrophoretic analysis of polypeptides. One-dimensional SDS polyacrylamide gel electrophoresis was used for separation of the labelled polypeptides (secreted and intracellular; Moor *et al.*, 1981). Medium and cells were lyophilized and dissolved in reducing SDS buffer. Incorporation of $[^3\text{S}]$ methionine into TCA-precipitable protein was determined (Moor *et al.*, 1981) and an equal number of counts was placed into each lane of 8 to 15% linear gradient SDS polyacrylamide gels. Labelled polypeptides were visualized by fluorography by using preflashed Kodak X-Omat film. Densitometric analysis of fluorograms was conducted on a Chromascan 3 (Joyce Lobel, Gateshead, UK).

Production of monoclonal antibodies. Polypeptide (M_r 30 000) was electroeluted from a one-dimensional polyacrylamide gel. Female rats were injected intramuscularly with approximately $10 \mu\text{g}$ polypeptide emulsified in complete Freund's adjuvant. A second subcutaneous injection, containing an equivalent amount of polypeptide emulsified in incomplete Freund's adjuvant, was injected 34 days later. Antibody production was determined 13 days after the booster injection by enzyme-linked immunoabsorbent assay (ELISA) and the immunoblotting technique of Burnette (1981). After a booster injection of $30 \mu\text{g}$ M_r 30 000 polypeptide, spleen cells from the most reactive rat (72 h after boosting) were fused with the rat myeloma line Y3 Ag1.2.3 (Galfre *et al.*, 1979).

Positive cell lines were identified by ELISA and immunoblot and those were single cell cloned to produce a variety of hybridoma cell lines against the M_r 30 000 polypeptide. One of the monoclonal antibodies was selected for use in this study.

Western blot analysis. Polypeptides from luteinized sheep granulosa cells were reduced (2-mercaptoethanol), separated by one-dimensional SDS polyacrylamide electrophoresis and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper (transferred proteins) was incubated overnight in 10% fetal calf serum in

phosphate-buffered saline (0.05% (v/v) Tween) at 4°C to block non-specific binding sites. The nitrocellulose was cut into strips (3–4 mm) which were incubated with PBS–Tween (Control), rabbit anti porcine 1–26 α chain inhibin antiserum (1/1000 dilution in PBS–Tween; gift from Dr A. S. McNeilly, MRC Reproductive Biology Unit, Edinburgh EG3 9EW, UK, to Dr P. Wooding, AFRC, Babraham, Cambridge), or monoclonal antibody against the M_r 30 000 polypeptide (undiluted culture medium) for 18 h at room temperature. The strips were washed 5 times (10 min each) in PBS–Tween and incubated with goat anti-rabbit or anti-rat immunoglobulin conjugated to alkaline phosphatase at 1/1000 dilution in PBS containing 10% fetal calf serum. Strips were washed 5 times (5 min each) in veronal acetate buffer (0.15 M-sodium barbitone; pH 9.6) and transferred to veronal acetate buffer containing 5 bromo-4 chloro-indoyl phosphate (5 mg/ml in dimethyl formamide) and nitroblue tetrazolium (1 mg/ml in veronal acetate buffer).

NH_2 -terminal amino acid sequence. Protein secreted by luteinized granulosa cells was separated by one-dimensional SDS polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Matsudaira, 1987) and the M_r 28 000–30 000 band was subjected to microsequencing. Gas-phase Edman degradation (Applied Biosystems 470A) was utilized to obtain an NH_2 -terminal amino acid sequence.

Results

Polypeptide secretion during the oestrous cycle

The first experiment was conducted to determine whether the M_r 30 000 polypeptide was synthesized and secreted by luteal tissue. Although there were slight differences, the overall pattern of polypeptides, and in particular the M_r 30 000 polypeptides secreted by luteal slices were similar to those secreted by luteinized ovine granulosa cells (Fig. 1). The amount of M_r 30 000 polypeptide which was released did not appear to vary during the oestrous cycle and its absence from intracellular polypeptide profiles indicated that it was rapidly released by luteal tissue.

Fig. 1. Fluorograph of a one-dimensional SDS-polyacrylamide gel showing changes in patterns of ovine luteal polypeptide secretion into medium on Days 3–16 after oestrus (Day 0 = oestrus; each lane represents a different animal). Labelled polypeptides secreted by luteinized ovine granulosa cells are also presented.

Other major polypeptides secreted throughout the luteal phase included molecules with an M_r of 14 000, 25 000 and 46 000. The mean percentage of polypeptide as measured by densitometry, represented by the M_r 14 000, 25 000, 30 000 and 46 000 polypeptides was 3, 9, 24 and 23%, respectively.

Fig. 2. Fluorograph of a one-dimensional SDS-polyacrylamide gel showing the effect of pituitary hormones (LH, FSH, prolactin) and cholera toxin (activates adenylate cyclase) on pattern of polypeptide secretion by (a) luteinized ovine granulosa cells and (b) ovine luteal tissue (adjacent lanes within treatment are replicates).

Effect of pituitary hormones and cholera toxin

Addition of hormones (LH, FSH and prolactin) or cholera toxin did not alter the pattern of polypeptide secretion by luteinized granulosa cells (Fig. 2a) or luteal tissue (Fig. 2b; Day 10 after

Fig. 3. Fluorograph of a one-dimensional SDS-polyacrylamide gel showing the effect of different concentrations of tunicamycin on the pattern of polypeptide secretion by luteinized ovine granulosa cells (adjacent lanes within treatment are replicates).

oestrus). The only exception was a polypeptide (M_r 120 000) which was released by luteinized granulosa cells after stimulation with cholera toxin.

Effect of tunicamycin

The M_r 30 000 polypeptide was shown to be glycosylated (Moor & Crosby, 1987); however, the degree of glycosylation was not determined. Therefore a third experiment was conducted to determine the effects of tunicamycin on the relative molecular mass of the M_r 30 000 polypeptide. Incubation of luteinized granulosa cells with tunicamycin decreased the relative molecular mass of the polypeptide to 20 000 (Fig. 3), but this reduction was not affected by the concentration of tunicamycin (0.125–2.0 $\mu\text{g/ml}$). A polypeptide product (M_r 32 000) remained after tunicamycin treatment and there may be more than one polypeptide present at this molecular weight. The relative molecular mass of several other secreted proteins was also reduced but not as dramatically as that of the M_r 30 000 polypeptide.

Western blot analysis for inhibin

The fourth experiment was conducted to determine whether the M_r 30 000 polypeptide was an inhibin subunit. Protein collected from luteinized granulosa cells was treated with 2-mercaptoethanol to reduce disulphide bonds and separated on a one-dimensional polyacrylamide gel. A polyclonal antibody to the α subunit of inhibin (rabbit anti-porcine 1–26 α chain inhibin) bound specifically to M_r 22 000 and 67 000 polypeptides but not to the M_r 30 000 polypeptides; the monoclonal antibody directed against the M_r 30 000 polypeptide specifically bound M_r 30 000 polypeptides (Fig. 4). Detection of higher molecular weight forms of ovine inhibin has been reported (Findlay & Clarke, 1987). Two separate bands were present in the lanes probed with monoclonal antibody directed against M_r 30 000 polypeptide.

NH₂-terminal amino acid sequence

The NH₂-terminal sequence of the first 25 amino acids is shown in Fig. 5. The M_r 30 000 polypeptide had 68% sequence identity with a human tissue inhibitor of metalloproteinase.

Fig. 4. Western blot analysis of protein secreted (*in vitro*) by luteinized ovine granulosa cells. Equal amounts of protein were added to each lane and adjacent lanes within treatment are replicates. Nitrocellulose strips were incubated with normal rabbit serum (Control), rabbit anti-porcine 1-26 α chain inhibin antiserum (Inhibin antibody) or a monoclonal antibody directed against the M_r 30 000 polypeptide. Two separate bands are present (arrow) in the lanes probed with monoclonal antibody directed against the M_r 30 000 molecules.

M_r 30 000	T	P	V	P	P	A	P	Q	T	A	F	C	N	S	E	V	V	I
TIMP	T	C	V	P	P	H	P	Q	T	A	F	C	N	S	D	L	V	I
M_r 30 000	I	C	A	F	C	G	T											
TIMP	R	A	K	F	V	G	T											

Fig. 5. Sequence identity between the NH_2 -terminal amino acid sequences of the M_r 30 000 polypeptide and a human tissue inhibitor of metalloproteinase (TIMP). Amino acids are expressed as single letters.

Discussion

The emphasis in studies on the corpus luteum has shifted recently from the regulation of progesterone synthesis and secretion (Niswender & Nett, 1988) to the characterization, regulation and physiological role of luteal peptides and proteins (Schams, 1987, 1989). However, with the exception of oxytocin, relaxin and inhibin, very little information has so far been published on the secretion of other luteal proteins. This is addressed in our experiments, the results of which show that sheep luteal cells secrete a variety of polypeptides throughout the luteal phase and during luteolysis. Two interesting general points emerge from these studies. First, it is clear that the secretory polypeptides from luteal tissue are largely similar to those secreted by luteinized sheep granulosa cells. Secondly, although the steroidogenic capacity of the corpus luteum is markedly reduced by Day 16 (Diekmann *et al.*, 1978), this is not paralleled by changes in the pattern of polypeptides which remain remarkably constant throughout the cycle.

It is well known that cultured granulosa cells secrete numerous proteins (Savion & Gospodarowicz, 1980; Knecht *et al.*, 1986; Skinner & Osteen, 1988). That gonadotrophins acting via transcriptional mechanisms dramatically alter the secretion of polypeptides has been clearly demonstrated for sheep granulosa cells (Moor & Crosby, 1987). This alteration is characterized by the switch from the secretion of M_r 46 000–60 000 polypeptides to the M_r 30 000 secretory product after the LH surge. Our present results show that these lower molecular weight polypeptides are secreted not only by the membrana granulosa but also by luteal cells throughout the ensuing oestrous cycle. Although LH initiates the production of the M_r 30 000 polypeptides, neither pituitary hormones nor cyclic AMP are able thereafter to modulate further its pattern or rate of secretion by luteal cells. This is in marked contrast to the luteotrophic action of LH and its cyclic nucleotide intracellular mediator on progesterone synthesis and secretion (Rothchild, 1981; Niswender, 1981; Niswender & Nett, 1988).

Inhibin suggested itself as an obvious candidate molecule in our first identification experiments because of its similar relative molecular mass and its known cellular source of synthesis. Ovine inhibin is a glycoprotein (M_r 32 000) which is secreted by granulosa cells, consists of two subunits ($\alpha = M_r$ 21 000, $\beta = M_r$ 16 000) and selectively suppresses secretion of FSH (Leversha *et al.*, 1987; Findlay & Clarke, 1987). Inhibin is known to be secreted (*in vitro*) by bovine and ovine luteinized granulosa cells (Luck *et al.*, 1989; Campbell, 1989), but inhibin secretion by luteal tissue has been controversial. Although granulosa cells are considered the primary source of inhibin, luteal tissue of humans (Davis *et al.*, 1987), rats (Davis *et al.*, 1986), sheep (Tsonis *et al.*, 1988) and cattle (Juengel *et al.*, 1990) also contains inhibin. Inhibin α subunit mRNA has been detected in human (Davis *et al.*, 1987), rat (Davis *et al.*, 1986) and bovine (Juengel *et al.*, 1990) luteal tissue, but other investigators have failed to detect inhibin α subunit mRNA in the luteal tissue of rats (Woodruff *et al.*, 1988), sheep (Rodgers *et al.*, 1989) or cattle (Rodgers *et al.*, 1989). Clearly the M_r 30 000 polypeptide is not inhibin since the molecular weight of the polypeptide did not decrease under reducing conditions (2-mercaptoethanol) and the inhibin antiserum did not detect the M_r 30 000 polypeptide by Western blot analysis.

Monoclonal antibody production and microsequencing were used in the second series of experiments on the identification of the M_r 30 000 secretory polypeptide. In two separate laboratories (Babraham and University of Missouri) NH_2 -terminal amino acid sequences revealed that a close sequence identity exists between the M_r 30 000 polypeptides and a human tissue inhibitor of metalloproteinase (TIMP; Docherty *et al.*, 1985). In addition to similarities in NH_2 -amino acid sequence identity, the secreted polypeptide and metalloproteinase inhibitor (TIMP) share similar physicochemical properties. TIMP normally has a molecular weight ranging from 28 000 to 30 000 and the molecular weight of TIMP secreted into medium by cells cultured with tunicamycin was 20 000 (Murphy & Werb, 1985). The unglycosylated form of the M_r 30 000 glycoprotein was also M_r 20 000. Metalloproteinase inhibitor activity has been detected in human ovarian follicular fluid (Curry *et al.*, 1988) and rat granulosa cell-conditioned medium (Slackman *et al.*, 1986; Mann *et al.*, 1990). Furthermore, stimulation of rat granulosa cells with LH or a phorbol ester increased the secretion of metalloproteinase inhibitor activity (Mann *et al.*, 1990). The highest level of expression of TIMP mRNA was detected in the corpora lutea of mice (Nomura *et al.*, 1989).

Metalloproteinases are metal-dependent enzymes which are capable of degrading proteinaceous components of the extracellular matrix. The regulation of extracellular matrix turnover is a precisely controlled process which is designed to maintain normal cellular organization (Mullins & Rohrich, 1983). Krane (1985) proposed the following three mechanisms by which turnover of the extracellular matrix may be regulated: (1) rate of latent enzyme synthesis, (2) proteolytic activation of latent enzymes, and (3) synthesis of specific protease inhibitors. Tissue inhibitors of metalloproteinases (TIMP) form irreversible inactive complexes with metalloproteinases and thereby regulate the extracellular activity of these proteolytic enzymes. Metalloproteinase inhibitors are reported to have an important role in the regulation of collagenase activity within the extracellular

spaces of bone (Cawston *et al.*, 1981), skin (Stricklin & Welgus, 1983) and cartilage (Dean & Woessner, 1984).

The preovulatory gonadotrophin surge increases the synthesis of proteolytic enzymes (i.e. plasminogen activator and collagenase) which are reported to be involved in proteolytic degradation of the preovulatory follicular wall (Lipner, 1988). Although collagenase activity is known to increase following LH stimulation there is very little information on the regulation of collagenolysis within the follicular wall. In fibroblasts (Murphy *et al.*, 1985) the secretion of collagenase and inhibitors of collagenase increased simultaneously. Therefore, metalloproteinase inhibitors may have an important role in the regulation of collagenolysis after the gonadotrophin surge and may continue to be involved in cellular remodelling during the luteal phase.

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