Regulation of granulosa cell-derived ovarian metalloproteinase inhibitor(s) by prolactin

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Increased prolactin concentrations are known to inhibit the ovarian proteolytic enzyme cascade associated with follicular rupture. It is not known whether there is also an effect of prolactin on endogenous proteinase inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs). We sought to study the effect of prolactin on ovarian metalloproteinase inhibitors in cultured rat granulosa cells. Granulosa cells were cultured for 24 h with prolactin (0–1000 ng ml⁻¹) in the absence or presence of LH. Metalloproteinase inhibitor activity in the conditioned culture media was measured by a colorimetric assay. Prolactin at 1000 ng ml⁻¹ increased inhibitor activity by 2.86 ± 0.63 times. Expression of mRNA encoding TIMP-1 measured by Northern analysis increased by 2.34 ± 0.34 times with 100 ng prolactin ml⁻¹ and by 2.43 ± 0.42 times with 1000 ng prolactin ml⁻¹ compared with control cultures (no LH, no prolactin). In the presence of LH, expression of mRNA encoding TIMP-1 and inhibitor activity increased by 2.60 ± 0.6 and 4.60 ± 0.54 times, respectively. However, no further change in mRNA expression or inhibitor activity was apparent with the addition of prolactin to LH-treated cultures. Prolactin had no effect on expression of mRNA encoding TIMP-3 in the absence or presence of LH, although LH stimulated a 1.7-fold increase in mRNA encoding TIMP-3 compared with controls. Addition of prolactin had no effect on media concentrations of oestradiol or progesterone. These data demonstrate that metalloproteinase inhibitor activity increases with increasing doses of prolactin; however, when LH was added, this effect was no longer seen. With an increase in metalloproteinase inhibitor activity, tissue metalloproteinase action could be decreased, providing a possible explanation for the local inhibition on pre- and peri-ovulatory pathways by hyperprolactinaemia.

Introduction

Prolactin plays a critical role in various aspects of reproduction in many species. In addition to being crucial for lactogenesis, prolactin directly affects the menstrual cycle. Increased concentrations of prolactin disrupt normal ovulatory function, potentially leading to amenorrhoea and infertility (Bohnet *et al.*, 1976; Kaupila *et al.*, 1982). While there is a recognized central effect on the hypothalamic–pituitary axis, there is also evidence for a direct ovarian effect of prolactin (Demura *et al.*, 1982; McNeilly *et al.*, 1982). The effect on ovarian physiology is at several stages, including follicular maturation, steroidogenesis, ovulation, luteinization and corpus luteum function. Prolactin is part of the luteotropic complex in many species including rats, mice, pigs and sheep (Denamur *et al.*, 1973), and may also be luteotrophic during the early stages of luteal formation in humans (McNatty *et al.*, 1974).

Hyperprolactinaemia has been shown to interfere with ovulation (Lin *et al.*, 1980; McNeilly, 1993; Yoshimura *et al.*, 1994) and this effect cannot be overridden by increasing

gonadotrophin concentrations (Hamada *et al.*, 1980; Yoshimura *et al.*, 1991). Rabbit ovaries perfused with increasing concentrations of prolactin show a dose-dependent decrease in the percentage of follicles ovulating. On morphological examination of the ovarian surface, increased prolactin is shown to directly interfere with connective tissue breakdown of the follicular apex (Yoshimura *et al.*, 1991, 1994). Thus, prolactin interferes with ovulation, in part, by preventing the mechanical events leading to rupture of the mature follicle.

Apical connective tissue remodelling during follicular rupture occurs through the action of proteolytic enzymes such as the matrix metalloproteinases (reviewed in Espey and Lipner, 1994). Matrix metalloproteinases are a family of enzymes involved in remodelling of the extracellular matrix, and include collagenases, gelatinases, and stromelysins. However, proteolytic activity in the extracellular space must be highly controlled for precise, localized connective tissue degradation. Proteolytic control in the extracellular space involves the metalloproteinase inhibitors, two major classes of which are distinguished: tissue derived and serum borne inhibitors (Birkedal-Hansen *et al.*, 1993). The serum borne inhibitors include the macroglobulins, such as α_2 -macroglobulin, which is

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a 720 kDa inhibitor that has the ability to inhibit a variety of proteinases as well as several growth factors (Barrett and Starkey, 1973). The tissue-derived inhibitors are referred to as tissue inhibitors of metalloproteinases (TIMPs) and comprise three distinct proteins (Matrisian, 1990; Kleiner and Stetler-Stevenson, 1993). TIMP-1, a 29 kDa protein, binds to the active form of matrix metalloproteinases on a 1:1 basis. TIMP-2 (21 kDa) and TIMP-3 (23 kDa) have been shown to be differentially regulated from TIMP-1 and may act selectively on specific matrix metalloproteinases (Pavloff *et al.*, 1992; Roswit *et al.*, 1992; Leco *et al.*, 1994). In addition, TIMP-1 and TIMP-2 are secreted and remain available to bind to free enzyme, whereas TIMP-3 is secreted and then predominantly bound to the extracellular matrix, further contributing to the selectivity and specificity of TIMP action (Pavloff *et al.*, 1992; Roswit *et al.*, 1992).

In the ovary, TIMPs are believed to act in part to control the site and extent of follicular connective tissue remodelling associated with ovulation. The effect of prolactin on expression of mRNA encoding TIMPs and inhibitor activity has not been investigated. In the present study we examined the effect of prolactin on ovarian metalloproteinase inhibitor production and activity to understand further the mechanism by which prolactin may act to disrupt follicular rupture. We specifically examined expression of mRNA encoding TIMP-1 because mRNA encoding TIMP-1 is abundant in rat and human granulosa cells, is stimulated by the periovulatory LH surge, is secreted in response to hormonal stimuli, and TIMP-1-like activity is found in granulosa cell conditioned media (Rapp *et al.*, 1990; Mann *et al.*, 1991). We were interested in TIMP-3 because this metalloproteinase inhibitor provides an additional regulatory stop-point for metalloproteinase activity, as it is largely bound to the extracellular matrix (Pavloff *et al.*, 1992). Differential regulation of TIMP-1 and TIMP-3 has been observed (Leco *et al.*, 1994). Furthermore, expression of mRNA encoding TIMP-3 has not been characterized in the ovary. TIMP-2 is located in the thecal cells and so was not studied (Smith *et al.*, 1995).

Materials and Methods

Immature female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) were housed in controlled environmental conditions under the care and supervision of a licensed veterinarian. Rats were maintained on a 14 h light:10 h dark cycle and provided with water and rat chow *ad libitum*. All animal procedures for these experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee. All reagents were obtained from Sigma Chemical Company (St Louis, MO) except where noted. Between 9:00 h and 10:00 h on day 23–24 of age, rats were injected with 20 iu of pregnant mares' serum gonadotrophin (PMSG) to induce follicular development and were killed 48 h later. Granulosa cells were obtained from preovulatory follicles as described by Mann *et al.* (1991). Briefly, aseptic techniques were used to remove ovaries which were cleaned of any remaining adnexa, and placed in Ham's F-12/Dulbecco's modified Eagles medium (DMEM) (GIBCO, Gaithersburg, MD) containing Hepes buffer (25 mmol l⁻¹), gentamicin (50 µg ml⁻¹), and 0.1% (w/v) BSA.

Follicles were punctured with a 26 gauge needle and granulosa cells were gently expressed and pooled. Cells were centrifuged at 600 *g* for 5 min and resuspended in fresh media at a concentration of 1 × 10⁶ cells ml⁻¹. Number of cells and their viability were determined by Trypan blue exclusion. With this method, the average yield of viable cells per ovary was 2.5 × 10⁶. The cells were cultured in the absence or presence of 100 ng LH ml⁻¹ with or without different concentrations of rat prolactin (1, 10, 100, 1000 ng ml⁻¹, supplied by NIDDK, National Hormone and Pituitary Program, Baltimore, MD) for 24 h. These concentrations of prolactin were chosen on the basis of previous published work evaluating the effect of prolactin on ovulation (Chedrese *et al.*, 1988; Gitay Goren *et al.*, 1989; Kransnow *et al.*, 1990; Yoshimura *et al.*, 1991). Ovine prolactin was also used at a dose of 1000 ng ml⁻¹ to confirm the effect of rat prolactin.

After culture for 24 h, the granulosa cells were separated from the conditioned media by centrifugation (5 min at 600 *g*). Total RNA was extracted from the granulosa cell pellets and the concentrations of mRNA encoding TIMP-1 and TIMP-3 were determined by northern analysis. Inhibitor activity, progesterone, and oestradiol concentrations were determined in the conditioned media.

Northern blot analysis: detection of mRNA encoding TIMP

Total RNA was isolated from the control and prolactin-treated granulosa cells (3–6 × 10⁶ cells) by extraction in guanidinium thiocyanate and centrifugation at 100 000 *g* for 18 h over a caesium chloride gradient (Chirgwin *et al.*, 1979; Mann *et al.*, 1991). RNA samples (3–20 µg per lane) underwent electrophoresis through a 1% agarose gel containing formaldehyde and were transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH). cDNA probes for mouse TIMP-1, mouse TIMP-3 and rat 18S were prepared by isolation of excised fragments and random primer labelling to a specific activity of 1.0–2.5 × 10⁸ c.p.m. µg⁻¹ using [α -³²P]dCTP (New England Nuclear, Boston, MA). cDNA probes were 825 bp in length for TIMP-1, 1800 bp for TIMP-3 and 1100 bp for the 18S (supplied by D. Edwards, Calgary, Alberta). Membranes were hybridized concomitantly with the radiolabelled cDNAs for 18 h at 45°C. Membranes were washed at 42°C in 2 × standard saline citrate (SSC) (0.3 mol NaCl l⁻¹, 0.03 mol citric acid l⁻¹) + 0.1% (w/v) SDS, and then washed at 65°C in 0.2 × SSC + 0.1% SDS. The reaction product was visualized by autoradiography on Kodak X-AR film (Eastman Kodak, Rochester, NY). The resulting blots were analysed with an LKB Ultrascan XL laser (LKB instruments, Rockville, MD). Equality of sample loading and capillary transfer to the membrane were assessed by quantitating the blots against the 18S ribosomal band to normalize the expression of mRNA encoding TIMP-1 and TIMP-3. Relative mRNA content encoding each TIMP was then expressed as arbitrary units that were calculated by setting the control 0 h transcript amount equal to 1.0 and expressing each other transcript amount as a fold change from that value. Control tissues included lung, liver, kidney and ovary collected from the PMSG-primed animals at the time the animals were killed for granulosa cell collection. Corpus luteum tissue was obtained from rats primed with PMSG and then by hCG 48 h later, and collected 24 h after hCG.

Metalloproteinase inhibitor assay

Ovarian metalloproteinase inhibitor activity was measured by a colorimetric assay for metalloproteinase inhibitors as described by Mann *et al.* (1991). Briefly, the assay is based on the inhibition of a uterine neutral metalloproteinase by metalloproteinase inhibitors in the granulosa cell conditioned culture media. Central to this assay is the degradation of a colorimetric substrate, Azocoll (Calbiochem, La Jolla, CA) by the uterine metalloproteinase. The released red azodye is quantitated spectrophotometrically at 520 nm. In the presence of metalloproteinase inhibitors, the activity of the uterine metalloproteinase is reduced and there is a decrease in the digestion of the Azocoll substrate and a concomitant decrease in absorbance.

The conditioned media (100 μ l) was mixed with the uterine neutral metalloproteinase, brought to a total volume of 400 μ l with assay buffer (50 mmol Tris-HCl l^{-1} , 10 mmol $CaCl_2$ l^{-1} , 0.2 mol NaCl l^{-1} , 0.05% (w/v) sodium azide, and 0.167% (v/v) Brij 35, pH 7.5) and preincubated for 1 h at 37°C to allow inhibitors present in the conditioned media to interact with the uterine metalloproteinase. Next, 1 mg of Azocoll suspended in 850 μ l of assay buffer was added to each sample and incubated for 18 h at 37°C. Inhibition of the uterine metalloproteinase activity was then quantitated on a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA).

Progesterone and oestradiol radioimmunoassay

Steroid content in conditioned media from cultured granulosa cells was determined by radioimmunoassay using Coat-A-Count tubes (Diagnostic Products Corp., Los Angeles, CA) as described by Jones *et al.* (1987). These are direct, solid phase ^{125}I -labelled radioimmunoassay kits. A 100 μ l aliquot of the granulosa cell conditioned culture media (diluted 1:10) and 1.0 ml of ^{125}I -labelled steroid were added to the precoated primary antibody tubes and incubated at room temperature for 3 h. Samples were decanted, counted in a gamma counter, and steroid concentrations determined. Progesterone and oestradiol assay sensitivities are 30 pg ml^{-1} and 8 pg ml^{-1} , respectively. Intra-assay and interassay coefficients of variation are 4.7% and 9.0% for progesterone and 5.4% and 8.4% for oestradiol, respectively.

Experimental design

Each experiment was designed to test the effects of prolactin on granulosa cell derived inhibitor activity, mRNA concentrations, and steroidogenesis. The variability between different experimental replicates was normalized by expressing data as the mean fold change $\pm$ SEM compared with the control (no LH, no prolactin). Each experimental replicate represents tissue generated from a separate group of rats collected at different time points. Differences in metalloproteinase inhibitor activity, steroid production, and mRNA abundance between prolactin treatments were tested with a one-way analysis of variance (ANOVA). Dunnett's test was used for posthoc group comparisons; $P < 0.05$ was considered significant.

Results

Effect of prolactin on expression of mRNA encoding TIMP-1 and TIMP-3

Northern analysis of granulosa cell expression of mRNA encoding TIMP-1 and TIMP-3 displayed single transcripts of approximately 900 bp and 4500 bp, respectively (Fig. 1), similar to the reported sizes of TIMP-1 and TIMP-3 (Leco *et al.*, 1994). The expression of TIMP-1 and TIMP-3 in the positive and negative control tissues was as reported by Leco *et al.* (1994). The digitized values for mRNA encoding TIMP-1 ($n = 3$) and TIMP-3 ($n = 2$) were normalized against the 18S ribosomal band and expressed as a fold increase in mRNA expression over the control value. The addition of 1 or 10 ng ml^{-1} of prolactin to granulosa cells cultured in the absence of LH (100 ng ml^{-1}) had no significant effect on expression of mRNA encoding TIMP-1. However, the addition of prolactin to the granulosa cells resulted in a significant increase in expression of mRNA encoding TIMP-1 at 100 ng prolactin ml^{-1} (2.34 ± 0.34 -fold) and 1000 ng prolactin ml^{-1} (2.43 ± 0.42 -fold; Fig. 2). The addition of LH significantly increased expression of mRNA encoding TIMP-1 by 2.6 ± 0.57 times ($P < 0.05$) above cells cultured without LH. The addition of prolactin in the presence of LH had a variable but nonsignificant effect on expression of mRNA encoding TIMP-1 compared with cells treated with LH alone (Fig. 2).

In contrast to expression of mRNA encoding TIMP-1, addition of prolactin to the granulosa cells had no effect on concentrations of mRNA encoding TIMP-3 in either the absence or presence of LH. However, the addition of LH stimulated an increase of 1.7 ± 0.04 ($P < 0.05$) times in mRNA expression compared with control cultures (data not shown).

Effect of prolactin on metalloproteinase inhibitor activity

Conditioned media from 24 h control cultures (no LH, no prolactin) demonstrated 9.5% inhibition of the uterine metalloproteinase. The addition of 1, 10 or 100 ng prolactin ml^{-1} to granulosa cells cultured in the absence of LH had no significant effect on inhibitor activity (Fig. 3). In contrast, the addition of 1000 ng ml^{-1} of either rat or ovine prolactin resulted in a 2.86-fold increase ($P < 0.05$) in metalloproteinase inhibitor activity in the absence of LH compared with control cultures (Fig. 3). The addition of LH alone (without prolactin) to the granulosa cells resulted in a significant increase in the inhibitor activity (4.59 times the control; $P < 0.05$), as reported by Mann *et al.* (1991). In the presence of LH, however, no further increase in inhibitor activity was seen with the addition of prolactin (Fig. 3).

Effect of prolactin on steroidogenesis

After 24 h of culture, media from control cultures contained 1.82 ng progesterone ml^{-1} . The addition of LH to the granulosa cell culture resulted in an increase of 18.5 ± 6.9 times ($P < 0.05$) in progesterone production over control, confirming cell viability and hormonal responsiveness. The addition of

Fig. 1. Representative northern analysis of granulosa cell mRNA encoding tissue inhibitor of metalloproteinase 1 (TIMP-1) and TIMP-3. Rat granulosa cells were cultured for 24 h in the presence of increasing concentrations of prolactin under basal (no LH) or LH-stimulated conditions (100 ng LH ml⁻¹). RNA samples were prepared from the cell pellets and hybridized with a full length mouse cDNA probe for TIMP-1 ($n = 3$) and TIMP-3 ($n = 2$). Samples were normalized to the 18S ribosomal RNA. CL: corpus luteum.

Fig. 2. Effect of prolactin on granulosa cell mRNA encoding tissue inhibitor of metalloproteinase 1 (TIMP-1). Changes in mRNA encoding TIMP-1 were normalized to the 18S ribosomal RNA and are expressed as the fold change versus the 24 h control (0 ng prolactin, no LH). *Significantly different ($P < 0.05$) from the 24 h control. Although the cultures with LH (100 ng ml⁻¹) were all significantly different from the 24 h control without LH ($P < 0.05$), there were no significant differences within this group (LH added) after prolactin treatment ($n = 3$).

prolactin in either the presence or absence of LH did not effect progesterone production by granulosa cells after culture for 24 h.

Fig. 3. Effect of prolactin on granulosa cell derived inhibitor activity. Rat granulosa cells were cultured for 24 h in the presence of increasing concentrations of prolactin under basal (no LH) or LH-stimulated conditions (100 ng LH ml⁻¹). Changes in inhibitor activity are expressed as the fold change $\pm$ SEM versus the 24 h control (no prolactin, no LH). *Significantly different ($P < 0.05$) from the 24 h control. Although the cultures with LH were all significantly different from the 24 h control without LH ($P < 0.05$), there were no significant differences within this group (LH added) after prolactin treatment ($n = 6$ in duplicate).

There was 902 pg oestradiol ml⁻¹ in the conditioned media from control cultures. The addition of LH induced an increase of 3.02 times in oestradiol production compared with the control. Similar to the findings with progesterone, the addition

of prolactin in either the presence or absence of LH did not affect oestradiol production by granulosa cells after culture for 24 h.

Discussion

These studies are the first demonstration that expression and activity of mRNA encoding metalloproteinase inhibitor are enhanced by prolactin. Furthermore, these findings are the first report of mRNA encoding TIMP-3 in the ovary, specifically in granulosa cells, and that LH stimulates ovarian expression of mRNA encoding TIMP-3.

The present study demonstrates that metalloproteinase inhibitor activity secreted in the conditioned culture media is enhanced by increased concentrations of prolactin. The increased activity is associated with a stimulation of cellular mRNA encoding TIMP-1, suggesting that the enhanced activity is a result of increased TIMP-1 gene expression. It is unlikely that the prolactin-induced increase in inhibitor activity reflects changes in TIMP-3 because prolactin does not alter the abundance of mRNA encoding TIMP-3. Furthermore, since TIMP-3 is predominately bound to the extracellular matrix, the contribution of TIMP-3 to inhibitor activity found in the conditioned medium would be expected to be minimal. In the presence of LH, there is a marked overall enhancement of secreted inhibitor activity in the conditioned media with no further prolactin stimulation of activity. The lack of an additive prolactin effect on metalloproteinase inhibitors in the presence of LH may be explained by LH induction of multiple intracellular pathways leading to metalloproteinase inhibitor production. Thus, the specific effect of prolactin is not evident in the overall LH-enhanced effect on granulosa cell metalloproteinase inhibitor activity. Our findings can be interpreted that increasing concentrations of prolactin stimulate TIMP-1 which may regulate tissue remodelling prior to and during the gonadotrophin surge.

Increased mRNA encoding TIMP-1 is apparent at 100 ng prolactin ml^{-1} and 1000 ng prolactin ml^{-1} , while increased inhibitor activity is not apparent until 1000 ng prolactin ml^{-1} . Follicular concentrations of prolactin have been found to be closely correlated with serum concentrations of prolactin, such that a follicular concentration of 100 ng ml^{-1} is consistent with moderate hyperprolactinaemia, while a concentration of 1000 ng ml^{-1} is consistent with marked hyperprolactinaemia (Ohwaki *et al.*, 1992).

Physiological concentrations of prolactin do not appear to alter the role of the TIMPs in follicular remodelling. However, a moderate increase in the prolactin concentration (100 ng ml^{-1}) caused an increase in mRNA encoding TIMP-1, while marked hyperprolactinaemia (1000 ng ml^{-1}) resulted in an increase in inhibitor activity as well as an increase in mRNA encoding TIMP-1. The findings reported here are consistent with the observation that ovulation rates are reduced in perfused rabbit ovaries at a dose of 1000 ng ml^{-1} , but not at 10 or 100 ng ml^{-1} (Yoshimura *et al.*, 1991).

Northern analysis demonstrated that the ovary contains mRNA encoding TIMP-3, that granulosa cells are a source of TIMP-3 and that LH stimulates expression of mRNA encoding TIMP-3. The relative abundance of this inhibitor is markedly

reduced compared with TIMP-1 based upon equivalent exposure times of concomitantly hybridized cDNA probes of equivalent specific activity. The role that TIMP-3 plays in ovarian function remains to be determined; however, it may play an important role in maintaining the integrity of the granulosa cell compartment during follicular rupture and as the follicle remodels to form the corpus luteum.

In summary, our results show that in a short term granulosa cell culture *in vitro*, increased concentrations of prolactin stimulate TIMP-1 production and inhibitor activity. This effect is independent of a central effect on steroidogenesis, as progesterone and oestradiol production were unaffected. Our findings are supported by the observation that prolactin inhibits hCG-induced ovulation in a dose-dependent fashion without affecting progesterone production (Yoshimura *et al.*, 1991). However, the present report examines only mRNA expression and activity of the metalloproteinase inhibitors and does not address the possible prolactin-induced changes in metalloproteinase action. As a working model, we would hypothesize that metalloproteinases and their associated inhibitors are precisely coordinated to regulate follicular connective tissue remodelling during the ovulatory process and that pathologically increased concentrations of prolactin could act to disrupt this coordinated balance and thus interfere with ovulation.

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