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Prolactin delays gonadotrophin-induced ovulation and down-regulates expression of plasminogen-activator system in ovary

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This study was conducted to determine whether prolactin (PRL) suppresses gonadotrophin-induced ovulation and disturbs the co-ordinated gene expression of tissue type plasminogen activator (tPA) and plasminogen activator inhibitor type-1 (PAI-1) in rat ovary. Immature female rats were injected with 10 IU pregnant mare's serum gonadotrophin to stimulate follicle growth, and 48 h received different doses of prolactin followed by 7 IU human chorionic gonadotrophin (HCG). The oviducts were examined for the presence of ova, and the amounts of tPA and PAI-1 mRNA present in the ovary were measured at various times after the hormone treatment. PRL had no significant effect on ovarian weight but caused a dose-dependent decrease in ovulation number. In the control animals receiving HCG alone, 13.3 ± 1.3 (mean ± SEM) ova/oviduct were found; while in animals receiving HCG plus 50, 100 or 200 μg PRL, the ovulation number was dose-dependently suppressed by 53.6, 66.9 and 76% respectively at 18 h after treatment. PRL suppression of HCG-induced ovulation was time-dependent. By 24 h after treatment, the number of ova in the oviducts in HCG- and HCG plus PRL-treated groups was not significantly different. PRL also suppressed HCG-induced tPA gene expression in a dose- and time-dependent manner. At all time points examined, tPA mRNA content of whole ovaries and granulosa cells (GC) in PRL-treated groups was lower than in the HCG-treated controls. The activities of PAI-1 in ovarian extracellular fluid (OEF) and PAI-1 mRNA in the theca–interstitial cells (TI) in the PRL-treated groups were higher than in the HCG-treated controls. The highest stimulation by PRL of PAI-1 activity in OEF and of PAI-1 mRNA in TI was observed at 9 h and 6 h after HCG treatment respectively. The localization of tPA and PAI-1 antigens in the ovaries was consistent with changes in the mRNA and activity levels. These data suggest that PRL temporarily delays, but does not completely inhibit, HCG-induced ovulation, which may be caused by a suppression of PA-mediated proteolysis.

Key words: ovulation/plasminogen activator inhibitor type-1/prolactin/tissue type plasminogen activator

Introduction

Hyperprolactinaemia, or elevation of prolactin (PRL) secretion during lactation, is associated with anovulation (McNeilly *et al.*, 1982). Clinical studies have indicated that PRL acts directly on developing human follicles to inhibit ovarian steroidogenesis, follicular maturation and ovulation (McNatty *et al.*, 1974; McNatty, 1979; Jacobs *et al.*, 1976; McNeilly, 1987). Specific PRL receptors have been found in the ovary of several mammalian species (Saito and Saxena, 1975). PRL treatment *in vitro* has been shown to cause a decrease in ovarian aromatase activity (Tsai-Morris *et al.*, 1983; Hu and Liu, 1995). Using an in-vitro perfused rabbit ovarian system, PRL was found to inhibit human chorionic gonadotrophin (HCG)-induced ovulation (Hamada *et al.*, 1980), indicating that PRL acts directly on the ovary by interfering with the mechanisms causing rupture of the follicle. Results from many studies indicate that the plasminogen activator system is instrumental for the induction of ovulation: (1) there is a transient increase in tissue type plasminogen activator (tPA) activity in the rat ovary just prior to ovulation (Liu *et al.*, 1987); (2) plasminogen is present in follicular fluid, and plasmin has been shown to weaken the follicle wall *in vitro* (Beers, 1975); (3) intrabursal injection of protease inhibitors and antibodies against tPA partially blocks gonadotrophin-induced ovulation in rats (Reich *et al.*, 1985; Tsafriri *et al.*, 1989; Guerre *et al.*, 1991); (4) not only PAs, but also the specific PA inhibitor PAI-1, are regulated during the periovulatory period (Liu, 1988; Liu *et al.*, 1991); (5) in-situ localization studies revealed that tPA and PAI-1 are expressed in a cell specific and time-co-ordinated fashion (Chun *et al.*, 1992; Peng *et al.*, 1993). These data suggest that proteolytic activity provided by tPA and modulated by PAI-1 may cause a directed proteolysis leading to rupture of the selected follicles during ovulation.

Using in-vitro perfused viable rabbit ovaries, it has been shown that PRL causes a decrease in the PA activity of mature follicles (Yoshimura *et al.*, 1990, 1992). The objective of this study was to examine the effect of PRL on ovulation, and the expression of tPA and PAI-1 in rat ovaries.

Materials and methods

Materials

McCoy's 5a medium (modified, without serum), penicillin–streptomycin solution, L-glutamine and fetal calf serum were purchased from Gibco (Paisley, UK); pregnant mare's serum gonadotrophin (PMSG), was obtained from Sigma Chemical Company (St Louis, MO, USA); acrylamide *N*,*N*-methylene-bis-acrylamide, sodium dodecyl sulphate, tetramethylene diamine (TEMED), ammonium persulphate, Coomas-

sie brilliant blue were from Bio-Rad Laboratories (Richmond, CA, USA); nylon filters were obtained from Amersham (UK); urokinase type plasminogen activator was obtained from Serono S.A. (Aubonne, Switzerland); plasminogen was obtained from BioPool (Umeå, Sweden); human fibrinogen was purchased from KabiVitrum (Stockholm, Sweden) and further purified by ethanol precipitation as described by Blombäck and Blombäck (1956). T7 RNA polymerase was obtained from Promega (Madison, WI, US) and [α-^{32}P] UTP was from Amersham (UK). Restriction enzymes were purchased from Boehringer Mannheim (Bromma, Sweden) and collagenase was obtained from Worthington Biochemical Corporation (New Jersey, USA). Ovine PRL (NIDDK-oPRL-15, 31 IU/mg) was obtained from National Institute of Diabetes, Digestive and Kidney Diseases; Human chorionic gonadotrophin (HCG CR-127, 14 900 IU/mg) was obtained from National Institute of Child Health and Human Development (NICHHD, USA).

Animal treatment

Immature female rats of the Sprague–Dawley strain (21–22 days old) were obtained from Alab Labortorietjänst AB (Stockholm, Sweden) or Institute of Zoology, Chinese Academy of Sciences (Beijing, China), and were fed with chow and water *ad libitum*. A 14:10 h light-dark cycle was maintained with the light cycle initiated at 0600 h. The animals were injected s.c. with a superovulating dose (10 IU) of pregnant mare's serum gonadotrophin (PMSG) to stimulate follicle growth. Forty-eight hours later, 7 IU HCG was given to induce ovulation. In the indicated groups 50–200 μg PRL in 100 μl of saline was also given 15 min before HCG injection. The animals were killed at 6, 9, 12, 16, 18 and 24 h after injection of HCG or HCG plus various doses of PRL. The oviducts were examined for the presence of ova. The ovaries were isolated, and the ovarian extracellular fluid (OEF), granulosa cells and theca–interstitial tissue were prepared for tPA, PAI-1 mRNA and activity measurement (Liu *et al.*, 1991).

Sodium dodecyl sulphate gel electrophoresis (SDS–PAGE)

PA and PAI-1 activities in the samples were fractionated by SDS–PAGE according to Laemmli (1970). Before electrophoresis, the samples were adjusted to 2.5% SDS (for PA-activity assay) and 2.5% SDS and 12.5 mM DTT (for PAI-1 activity assay). Electrophoresis was performed at 50 V until the dye front reached the bottom of the gel (~16 h). After electrophoresis, gels were incubated 2×45 min in 2.5% (v/v) Triton X-100 to remove SDS in the gel, rinsed with distilled water and applied onto the surface of a fibrin-agar indicator gel.

Fibrin overlay and reverse fibrin autography assay

The fibrin-agar indicator gel was prepared as previously described (Granelli-Piperno *et al.*, 1978). The fibrin-agar gel contained 50 μg/ml plasminogen, 2.4 mg/ml fibrinogen, and 0.5 U/ml thrombin for generation of fibrin as the substrate for plasmin. After electrophoresis, the gel was laid onto the fibrin-agar indicator gel and incubated at 37°C in a humid chamber until the lysis zones became visible, indicating the presence of PAs. For detection of the PAI-1 activity, the samples were analysed by a reverse fibrin autograph (Erickson *et al.*, 1984). In addition to plasminogen, fibrinogen and thrombin, RFA indicator gel contained 0.01 U/ml urokinase to allow autolysis. The development of an opaque, lysis-resistant zone indicated the presence of PAI-1 activity.

Synthesis of RNA and DNA probes

A rat PAI-1 cDNA was cloned into the pBluescript vector (Zeheb and Geehrter, 1988) and a 400 bp of EcoRI fragment from the rat tPA cDNA clone lambda 15 (Ny *et al.*, 1988) was subcloned into pGEM-1 vector. Both vectors were linearized by Hind III and used as templates for probe synthesis using an in-vitro transcription system (Promega, Madison, WI, USA). A 400 bp tPA probe complementary to the 5′ region of rat tPA mRNA, and a 376 bp PAI-1 probe, complementary to 3′-untranslated region of PAI-1 mRNA were obtained using T7 and T3 promoters respectively. The ^{32}P-labelled antisense RNA probes were tested for specificity by hybridization to ovarian total RNA fractionated by formaldehyde agarose gel electrophoresis and blotted to nylon filters (Thomas, 1980; Maniatis *et al.*, 1982). The probes were found to be specific for tPA and PAI-1 mRNA, since they hybridized only to specific mRNA species with the size corresponding to tPA (Ny *et al.*, 1988), and PAI-1 mRNA (Zeheb and Geehrter, 1988). A 250 nucleotide long single stranded β-actin DNA probe was prepared by primer extension as described (Ohlsson *et al.*, 1988).

Preparation of total RNA

Cytoplasmic RNA from granulosa cells was prepared using the NP-40 method (Maniatis *et al.*, 1982). Total RNA was prepared from whole ovaries and from theca–interstitial tissue using CsCl gradient density separation method (Peng *et al.*, 1993). For hybridization analysis, total RNA was fractionated by agarose gel electrophoresis in the presence of formaldehyde (Holmes *et al.*, 1986) and transferred to nylon filters (Thomas, 1980) or immobilized directly by using a slot blot filtration apparatus (Schleicher & Schull, Dassal, Germany). Serial dilution of RNA from each sample was applied to nylon filter for hybridization with PAI-1, tPA and β-actin probes. Nylon filters were cross-linked using a Stratalinker (Stratagene, Sweden), and prehybridized in 50% formamide, 5×saline sodium citrate (SSC), 8×Denhardt's solution (1.6 mg/ml Ficoll, 1.6 mg/ml polyvinylpyrrolidone, 1.6 mg/ml BSA), 0.1% SDS, 10 mM EDTA, 25 mM Tris-HCl, pH 7.0, 250 μg/ml heat denatured herring sperm DNA, 250 μg/ml yeast tRNA at 62°C for 2 h. The hybridization was carried out in the same solution containing 2.5×10^6 c.p.m./ml of each probe for 16 h at 64 °C. The filters were washed in 2×SSC, 0.1% SDS twice for 15 min at room temperature and followed by two washes in 0.1×SSC, 0.1% SDS for 40 min at 66 °C. Hybridization using the β-actin probe was performed at 42°C as described (Ohlsson *et al.*, 1988). After hybridization, the filters were analysed by phosphorimager (Molecular Dynamics, Sweden), or were exposed to autoradiographic films.

In-situ hybridization

The ovaries left for in-situ hybridization were fixed in Bouin's fluid and embedded in paraffin prior to sectioning (4 μm), according to standard procedures. The deparaffinized sections were treated with 8 mg/ml proteinase K (E. Merck, Darmstadt, Germany) for 10 min and washed in PBS for 5 min. Sections were then fixed in 4% paraformaldehyde in PBS for 5 min and washed in PBS for 10 min. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air-dry. The sections were prehybridized with 50% formamide and 2×SSC for 2 h at room temperature, then hybridized overnight with digitoxigenin (DIG)-labelled tPA RNA probe in hybridization buffer (10 mM Tris–HCl, pH 7.5, 2×SSC, 50% deionized formamide, 1×Denhardt's, 2.5 mM DTT, 5% dextran sulphate, 250 μg/ml yeast tRNA, and 0.5% SDS) at 48°C. After hybridization, the sections were thoroughly washed in 2×, 1×, and 0.1×SSC, each for 2×15 min at 40°C. The sections were then rinsed in DIG buffer 1 (0.1 M maleic acid, 150 mM NaCl, pH 7.5) for 5 min, and blocked with 1% blocking reagent in buffer 1. The sections were incubated with alkaline phosphatase conjugated anti-DIG IgG diluted 1:200 in buffer 1 containing 1% blocking reagent for 2 h, washed in PBS for 3×5 min. The bound antibody was detected by a standard immuno-alkaline phosphatase reaction, using 5-bromo-4-

Table I. Dose-dependent inhibition of HCG-induced ovulation by prolactin in PMSG-primed immature rats

μg PRL	No. animals	Ovarian wt	No. ovulating rats	No. ova/ovary	% inhibition
0	13	39.2 ± 1.7	13	13 ± 1.3	0
50	8	39.2 ± 2.6	6	6.3 ± 1.0*	47.4
100	10	40.6 ± 2.9	7	4.4 ± 0.8*	66.9
200	17	34.5 ± 2.3	8	3.2 ± 0.8*	76.0

*$P < 0.01$ compared with control (0 PRL). Results are expressed as mean ± SEM.
HCG = human chorionic gonadotrophin; PMSG = pregnant mare's serum gonadotrophin.

Table II. Time-dependent inhibition of HCG-induced ovulation by prolactin in PMSG-primed immature rats

Hours after HCG injection	HCG treatment	No. rats	No. ovulating rats	No. ova/ovary	% inhibition
12	HCG	10	2	2.0 ± 1.2	80
	HCG + PRL	10	1	0.4 ± 1.3*	
16	HCG	6	4	9.3 ± 3.1	100
	HCG + PRL	6	0	0**	
18	HCG	9	8	11.0 ± 2.7	90
	HCG + PRL	9	2	1.1 ± 0.9**	
20	HCG	7	7	12.6 ± 2.1	79
	HCG + PRL	7	3	2.7 ±1.3**	
24	HCG	14	13	13.9 ± 1.3	7.1
	HCG + PRL	14	12	12.9 ± 1.8	

*$P < 0.05$, **$P < 0.01$ compared with control group (HCG alone).
HCG = human chorionic gonadotrophin; PMSG = pregnant mare's serum gonadotrophin.

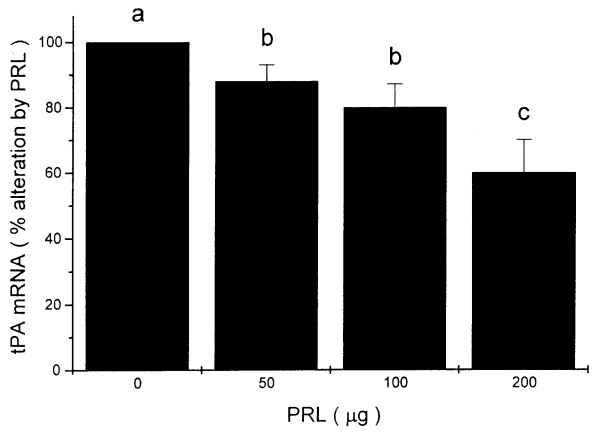

Figure 1. Dose-dependent effect of prolactin on ovarian tissue plasminogen activator (tPA) mRNA levels. Ovarian samples were obtained following treatment with human chorionic gonadotrophin (HCG) as described in Materials and methods. The total RNA was analysed for tPA and β-actin mRNA. The relative amount of tPA mRNA was estimated by phosphorimager and normalized against the corresponding relative amounts of β-actin mRNA. Results are expressed as mean ± SEM. Different letters indicate significant difference between points ($P < 0.05$).

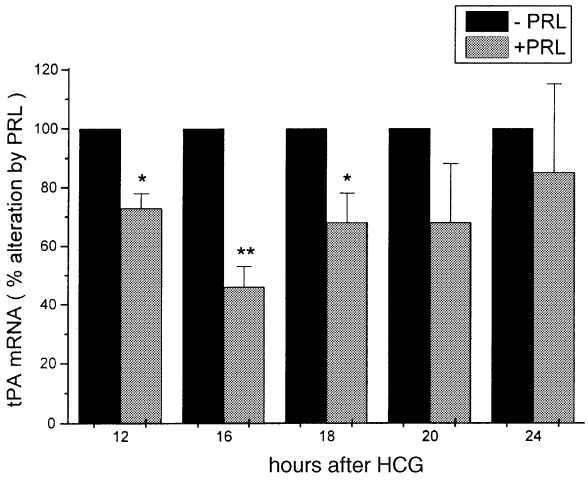

Figure 2. Time-dependent effect of prolactin (PRL) on ovarian tissue plasminogen activator (tPA) mRNA content. Ovarian samples were obtained following treatment with human chorionic gonadotrophin (HCG) and PRL for different time periods as described in Materials and methods. The total RNA was analysed for tPA and β-actin mRNA. The relative amount of tPA mRNA was estimated by phosphorimager and normalized against the corresponding relative amount of β-actin mRNA. Results are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with corresponding control groups (Student's *t*-test).

chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) as substrate. The DIG RNA labelling kit and all the reagents used for DIG detection were from Boehringer Mannheim GmbH Biochemica (Mannheim, Germany).

Data analysis

All experiments measuring PA and PAI-1 activity were repeated at least three times. A photographic record using dark-field illumination of one representative experiment is shown.

The relative amount of specific mRNA was determined by quantitation by densitometric scanning of autoradiographic films, and was normalized against the corresponding relative amount of β-actin in the samples. The data are expressed as fold increase relative to the control group or as mean ± SEM of at least three separate experiments. Data were analysed by one-way analysis of variance. Differences among groups were detected by Tukey's Multiple Comparison test (Sokal and Roulf, 1981). Paired comparisons, when required, were

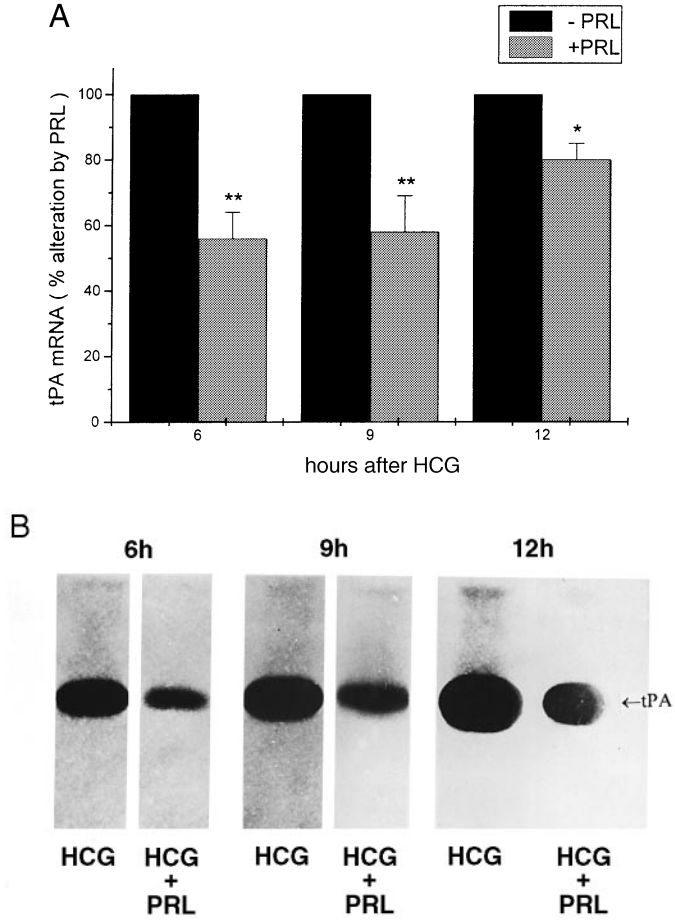

Figure 3. Prolactin inhibition of human chorionic gonadotrophin (HCG)-induced tissue plasminogen activator (tPA) mRNA (**A**) and activity (**B**) in granulosa cells. Immature female rats (21–22 days old) were injected s.c. with 10 IU PMSG and 48 h later with 7 IU HCG, or HCG plus 200 μg of prolactin (PRL). At the indicated times the animals were killed. (**A**) Cytoplasmic RNA was obtained from the granulosa cells, was fractionated by agarose gel electrophoresis and hybridized with tPA and β-actin probes. The relative amount of specific mRNA was determined by densitometric scanning of autoradiographic films and normalized against corresponding relative amount of β-actin in the samples. The data are expressed as % of inhibition to the control (100%). $*P < 0.05$, $**P < 0.01$ compared with corresponding control groups (–PRL) (Student's *t*-test). (**B**) tPA was extracted from 0.5×10^6 granulosa cells, separated by SDS–PAGE, and analysed by fibrin overlay technique.

made by using Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Dose- and time-dependent inhibition of HCG-induced ovulation by prolactin in PMSG-primed immature rats

To study the effect of PRL on ovulation, immature rats were injected s.c. with 10 IU PMSG in saline, and subsequently with 7.0 IU HCG alone or HCG plus the indicated doses of PRL. Eighteen hours after the hormone treatment the ovaries were removed and weighed and the oviducts were examined for presence of ova. As shown in Table I, PRL at all doses used had no significant effect on ovarian weight, but dose-dependently decreased the number of ova present in the oviducts, by 47.4, 66.9 and 76%, at doses of 50, 100 and 200 μg PRL respectively.

To study whether the PRL inhibition of ovulation was time-dependent, ovulation was induced with 10 IU PMSG, followed by HCG in the presence and absence of PRL (200 μg). At the indicated times, the animals were killed, and the oviducts were examined for the presence of ova. As shown in Table II, PRL inhibition of ovulation was time-dependent. The average number of ova in the PRL-treated group was significantly decreased at 12, 16, 18 and 20 h after hormone treatment, but by 24 h the difference was not significant. This indicates that PRL did not completely inhibit, but temporarily delayed, HCG-induced ovulation.

Prolactin inhibition of HCG-induced tPA mRNA content and activity in the ovary

To examine whether PRL inhibited the synthesis of tPA in the ovary, ovarian samples were analysed for tPA mRNA and activity. As shown in Figure 1, the ovarian tPA mRNA content induced by injection of HCG at 18 h was significantly decreased in a dose-dependent manner by treatment with PRL. At all time points during ovulation, the tPA mRNA levels were lower in the groups treated with PRL (200 μg) as shown in Figure 2. The PRL inhibition of HCG-induced tPA mRNA production in the ovary was most pronounced at 16 h after HCG and PRL injection and thereafter the suppression gradually decreased. The profile of tPA activity in the ovary was consistent with the changes in tPA mRNA during the hormone treatment (data not shown).

Granulosa cells produce the most tPA activity in the ovary, the tPA being secreted into the extracellular compartment (OEF). We therefore examined the changes in both tPA mRNA and tPA activity levels in granulosa cells and OEF at times preceding ovulation, 6, 9, and 12 h after hormone treatment. As shown in Figure 3A, the tPA mRNA content of granulosa cells in PRL-treated groups were 56, 58 and 80% of those of the corresponding HCG control groups at 6, 9, and 12 h. The PRL-induced decrease in tPA mRNA in granulosa cells was well correlated with the decrease in the cellular content of tPA activity (Figure 3B), and the tPA activity in OEF (data not shown).

In-situ localization of HCG-induced tPA mRNA in the ovary

To study the effect of PRL on HCG-induced tPA mRNA localization in the ovary, non-radioactive in-situ hybridization was performed. As shown in Figure 4, tPA mRNA levels in granulosa cells induced by injection of HCG after 6 (A), 9 (C), and 12 h (E) were considerably inhibited by the co-injection of PRL after 6 (B), 9 (D), and 12 h (F).

Effect of prolactin on PAI-1 expression in the ovary

PAI-1 is a rapidly secreted protein, barely detectable in the cell lysates. To estimate the PAI-1 activity in the extracellular environment, OEF was prepared from ovaries at 6, 9, and 12 h after HCG treatment, and the samples were analysed for PAI-1 activity by SDS–PAGE. As shown in Figure 5, at 6, 9, and 12 h after the hormone injection, PAI-1 activity in the

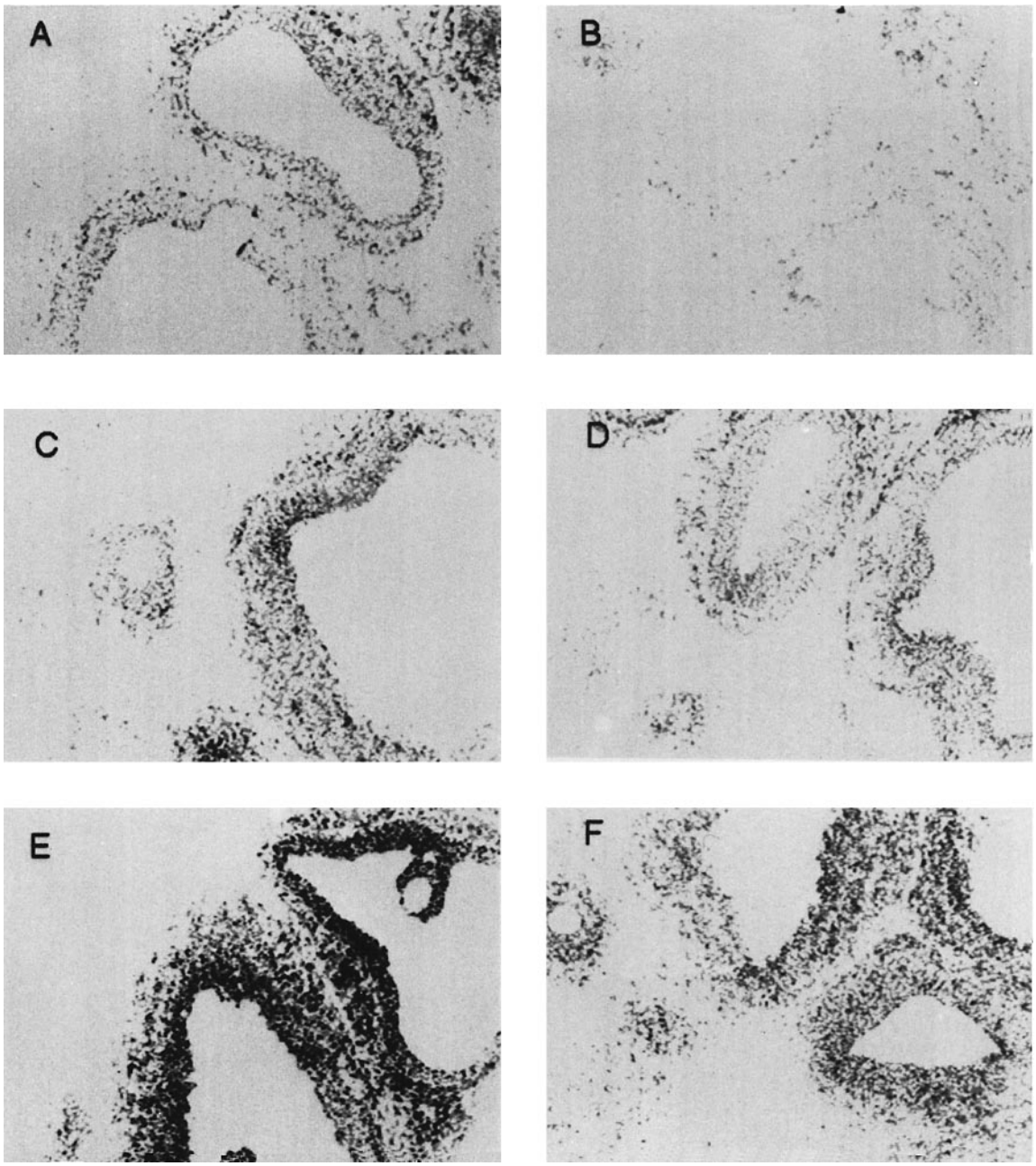

Figure 4. In-situ localization of tissue plasminogen activator (tPA) mRNA in ovarian cells. Immature female rats (21–22 days old) were injected s.c. with 10 IU pregnant mare's serum gonadotrophin (PMSG) and 48 h later with 7 IU human chorionic gonadotrophin (HCG), or HCG plus 200 μg of prolactin (PRL). After 6, 9 or 12 h, the animals were killed, the ovaries were removed and fixed in Bouin's fluid. Non-radioactive in-situ hybridization was performed as described in materials and methods. (**A**), (**C**) and (**E**): Localization of tPA mRNA in the ovaries 6, 9, and 12 h after HCG treatment respectively. (**B**), (**D**) and (**F**): Localization of tPA mRNA 6, 9 and 12 h respectively after treatment with HCG plus PRL.

OEF collected from the PRL-treated group was considerably higher than that in the HCG-treated group. The absolute PAI-1 activity in both groups was remarkably decreased by 12 h after the hormone treatment, perhaps due to a pre-ovulatory burst of tPA activity at this time point. The presence of multiple molecular weight forms is analogous to that found for PAI-1 secreted by rat HTC hepatoma cells (Zeheb *et al.*, 1987), and is mainly due to differences in glycosylation (Zeheb *et al.*, 1987; Liu *et al.*, 1991).

Theca–interstitial cells have been shown to secrete the most PAI-1 activity in the ovary. To estimate the effect of PRL on PAI-1 gene expression in theca tissue, ovaries were punctured, granulosa cells were removed, and the residue tissue was examined for PAI-1 mRNA levels. As shown in Figure 6, the levels of PAI-1 mRNA were significantly higher in the groups receiving treatment with both HCG and PRL than in HCG-treated groups. The PAI-1 mRNA contents of the tissues of PRL-treated groups were 154, 120, and 135% of that in the corresponding HCG groups at 6, 9, and 12 h respectively after the hormone treatment.

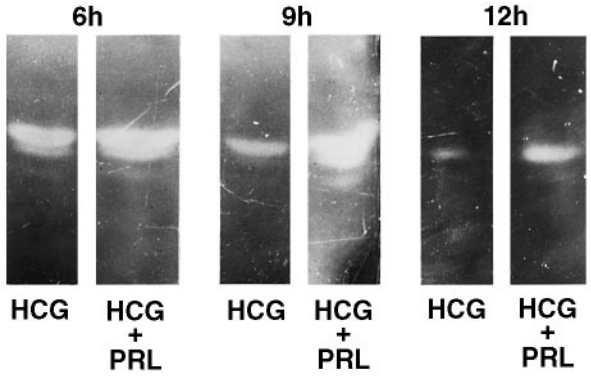

Figure 5. Prolactin stimulation of PAI-1 activity in ovarian extracellular fluid. Extracellular fluid (OEF) was obtained from the ovaries and fractionated by SDS–PAGE. PAI-1 activity in the gel was analysed by reverse fibrin autography as described in Materials and methods.

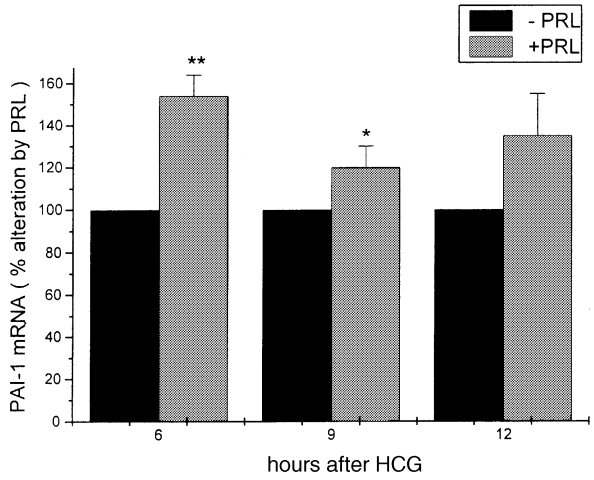

Figure 6. Prolactin stimulation of PAI-1 mRNA in theca-interstitial tissue. Theca–interstitial tissue was obtained from ovaries, following treatment with human chorionic gonadotrophin (HCG) and prolactin (PRL) for various time periods, and after removal of granulosa cells, the tissues were analysed for PAI-1 mRNA. Results are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$ compared with corresponding control group (–PRL) (Student's *t*-test).

Discussion

We have demonstrated that treatment of PMSG-primed immature rats with PRL significantly inhibits and delays HCG-induced ovulation in a dose- and time-dependent manner. Further studies have suggested that PRL inhibition and delay of ovulation may be due to temporal suppression of gonadotrophin-induced tPA gene expression in granulosa cells on one hand, and stimulation of PAI-1 production on the other. These studies provide further evidence to show that PRL acts on the ovarian PA-PAI-1 system to interfere with mechanisms leading to ovulation. These findings are consistent with previous reports in mouse (Hu and Liu, 1993, 1995) and rabbit (Hamada *et al.*, 1980). Using a well designed in-vitro perfused rabbit ovary preparation, Hamada *et al.* (1980) demonstrated that addition of PRL to the perfusate significantly decreased HCG-induced ovulation. Yoshimura *et al.* (1992) further demonstrated that PA activity induced by HCG in the perfused rabbit ovary was significantly inhibited by the high doses of PRL in the perfusate. That PRL, a compound that is known to inhibit ovulation, decreases both mRNA and activity levels of tPA activity in the ovary, further highlights the importance of the PA system in the ovary. These results strongly support the hypothesis that the PA system is the primary proteolytic enzyme responsible for follicle rupture (Beers, 1975; Strickland and Beers, 1976; Canipari and Strickland, 1985; Reich *et al.*, 1985; Liu *et al.*, 1987; Hsueh *et al.*, 1988). However, the precise mechanism by which PRL affects tPA mRNA and tPA activity is unknown.

Differences between the mouse and rat have been demonstrated. Mouse ovary mainly expresses uPA (Canipari *et al.*, 1985; Liu *et al.*, 1992; Leonardsson *et al.*, 1995), a lesser amount of tPA (Liu *et al.*, 1992; Leonardsson *et al.*, 1995) and various types of matrix metalloproteinases (K. Liu *et al.*, unpublished data). No PAI-1, but α_2-antiplasmin was detected in mouse granulosa cells (Liu *et al.*, 1992). Both uPA and tPA in the mouse ovary were stimulated by the injection of PMSG/HCG and reached maximum levels just prior to ovulation (Liu *et al.*, 1992), implying that both tPA and uPA may play a role in the processes of follicular rupture in the mouse. Recent studies on autologous plasminogen activator gene replacement in mice have shown that in mice with a deficiency of tPA, uPA or PAI-1, the litter sizes and the life-span were normal (Carmeliet *et al.*, 1994). However, in mice with combined deficiencies of tPA and uPA, fertilization was significantly reduced (Carmeliet *et al.*, 1994; Leonardsson *et al.*, 1995). In-vivo and in-vitro studies also showed that in gonadotrophin-treated mice, PRL inhibited both tPA and uPA, and also ovulation (Hu *et al.*, 1993, 1995). These data suggest that other proteases may provide proteolytic activity sufficient for follicular rupture in the absence of PA in the mouse. A functionally redundant mechanism for plasmin formation may operate in mouse ovary during gonadotrophin-induced ovulation. The PA, together with other proteases, generate the proteolytic activity required for follicular wall degradation.

PRL is the primary pituitary hormone known to be involved in stimulating milk production. Marshall *et al.* (1986) observed high levels of tPA activity present in the colostrum and transitional milk, which was inversely related to the duration of lactation. However, it is most likely that the tPA activity found in the milk is synthesized in the breast tissue and is not derived from other sources. Both normal and malignant breast tissue have been shown to carry various steroid receptors (Isotalo *et al.*, 1983; Reiner *et al.*, 1984), and the increased cortisol, oestrogen and progesterone secretion during early lactation may be responsible for the increase in tPA production (Isotalo *et al.*, 1983; Bulter *et al.*, 1983; Huft and Lippman, 1984; Reiner *et al.*, 1994). No evidence has been found that prolactin is responsible for the increase in tPA activity in the breast tissue.

There is abundant evidence to show that the ovary is also one of the primary target tissue for PRL. Specific receptors for PRL have been found in granulosa cells and other compartments of the ovary (Saito *et al.*, 1975). There have been several reports that increased levels of PRL in plasma are associated with reduced follicular maturation and steroid production (Jacobs *et al.*, 1976; McNeilly *et al.*, 1982). However,

McNatty *et al.* (1974) reported that steroid production by cultured human granulosa cells was stimulated by low physiological doses of PRL whereas high doses are inhibitory (Jacobs *et al.*, 1976; McNeilly *et al.*, 1982). This suggests that PRL may exert a complex effect on ovarian function. The role of PRL in the ovary may also depend on the state of cell differentiation, and may operate by more than one mechanism in the ovulatory cycle.

Hyperprolactinaemia during lactation has been reported to be associated with infertility (McNeilly, 1982). However, the precise link between the release of prolactin during lactation and the duration of lactational infertility has been not resolved (Tay *et al.*, 1996). However, Harlow *et al.* (1996) have recently provided data to show an increase in serum prolactin concentrations during stimulated in-vitro fertilization (IVF) treatment. There was no such increase in a control group having laparoscopic surgery unrelated to infertility or in a group having unstimulated IVF. This evidence suggests that high serum prolactin levels in some infertile women may be causally related to the suppression of fertility at the ovarian level.

Although a large body of evidence has shown that PRL directly suppresses ovarian steroidogenesis and ovulation *in vivo* (McNatty *et al.*, 1974; Jacobs *et al.*, 1976; McNatty, 1976; McNeilly *et al.*, 1982; McNeilly, 1987), the evidence accumulated to date cannot explain the complete role of PRL in the regulation of physiological events during the cycle. Two peaks of secretion of PRL from the pituitary have been detected in each oestrus cycle in rats (Butcher *et al.*, 1974; Haisenleder *et al.*, 1989) and cows (Dusza, 1989). The first peak coincided with the luteinizing hormone (LH) and follicle stimulating hormone (FSH) surge in association with the increasing level of oestrogen; and the second peak usually occurred late at oestrus, just before or after ovulation. The increases in PRL secretion during pro-oestrus and oestrus in rats appear to be correlated with an increase in PRL mRNA levels from the pituitary observed on each of these days (Haisenleder *et al.*, 1989). Two forms of PRL receptors have been described in granulosa cells of the rodent species (Shirota *et al.*, 1990). The role of these isoforms co-existing in the cells is unknown. In our previous studies we have demonstrated one peak level of tPA and two peak levels of PAI-1 mRNA and activity in the rat ovary during the periovulatory period (Liu *et al.*, 1991). The tPA peak appeared at 12 h after HCG injection, just prior to ovulation. The first peak of PAI-1 occurred 6 h after HCG injection, mainly produced by theca–interstitial cells, while the second peak appeared several hours after ovulation, produced by both granulosa cells and theca–interstitial cells. The two peaks of PAI-1 secretions allow a narrow 'window' for a pre-ovulatory increase in tPA activity which may be important for the regulation of the ovulatory process. Although we do not know the changes of circulating PRL levels or the mode of operation of its two forms of receptors in the ovary during PMSG/HCG-induced ovulation, the time-correlated increases in PRL secretion from the pituitary (Butcher *et al.*, 1974; Haisenleder *et al.*, 1989; Dusza, 1989) and the changes in the two forms of PRL receptors (Shirota *et al.*, 1990) as well as the co-ordinated expression of tPA and PAI-1 molecules in the ovary during the periovulatory period (Liu *et al.*, 1991) may provide important information for further study of the physiological role of PRL secretion by the pituitary.

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