BIOLOGY OF REPRODUCTION **59,** 409–416 (1998)

Prolactin Regulation of Tissue Type Plasminogen Activator and Plasminogen Activator Inhibitor Type-I Gene Expression in eCG-Primed Rat Granulosa Cells in Culture[1]

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ABSTRACT

The present study was designed to investigate the effect of prolactin (PRL) on plasminogen activator inhibitor-I (PAI-I) and tissue type plasminogen activator (tPA) gene expression in eCG-primed granulosa cells in vitro. At 46 h after the hormone treatment, ovaries were removed, and granulosa cells were prepared for culture. Cells were incubated for various times in serum-free medium in the presence or absence of LH and PRL alone or in combination. tPA and PAI-I activities in the media were assayed by fibrin overlay and reverse fibrin autograph, respectively. Cytoplasmic RNA from granulosa cells was prepared using the NP-40 method and was assayed for PAI-I and tPA mRNA levels. We demonstrated the following. 1) PRL increased PAI-I mRNA production in cultured granulosa cells. Inclusion of LH with PRL had a synergistic effect on increasing PAI-I mRNA levels. After 48-h culture, 3-fold increases in PAI-I mRNA levels were seen with LH in combination with PRL as compared with PRL alone. The synergistic increase in PAI-I mRNA levels occurred in a dose- and time-dependent manner. 2) The increase in PAI-I mRNA synthesis by PRL alone, or by PRL in combination with LH, was well correlated with the changes in PAI-I activity and antigen levels in the conditioned media. 3) PRL in the culture also dramatically decreased LH-induced tPA mRNA and activity in a dose- and time-dependent fashion. The decrease in the tPA activity by PRL was also correlated with an increase in the amount of PA-PAI-I complexes in the cell-conditioned media. 4) In situ hybridization of tPA and PAI-I mRNAs in the cultured granulosa cells also showed that PRL was capable of enhancing PAI-I mRNA while diminishing tPA mRNA production induced by LH. This suggests that the dose- and time-dependent decrease in the gonadotropin-induced tPA activity in the culture by the presence of PRL may be due to decreasing tPA mRNA synthesis on one hand and to neutralization of the tPA activity by the increased PAI-I activity on the other.

INTRODUCTION

Prolactin (PRL), a hormone with partial structural homology to growth hormone, is secreted by the lactotroph cells of the anterior pituitary and is primarily involved in the stimulation of milk production. However, PRL is also necessary for ovarian function. Specific PRL receptors have been found in the ovaries of several mammalian species [1, 2]. An antigonadal or antigonadotropic action of this hormone has been demonstrated in several species [3]. Elevation of PRL in plasma is often associated with reduction of follicular maturation and steroidogenesis [4, 5]. In vitro studies have documented that excess PRL can act directly on the ovary to inhibit gonadotropin-induced follicular growth and secretion of estrogen [2, 5–7] and progestin [8]. However, McNatty et al. [9] found that human granulosa cells in culture in the absence of PRL failed to secrete progesterone, even in the presence of an adequate amount of gonadotropin. Studies in mice suggest that PRL may enhance LH-induced progesterone production while inhibiting the gonadotropin-induced preovulatory estrogen surge in luteinized granulosa cells [10]. Progesterone production in cultured porcine granulosa cells from immature follicles was dose-dependently inhibited by the addition of PRL. In contrast, granulosa cells isolated from large mature follicles produced more progesterone in the presence of PRL [11], indicating that PRL may exert an action on the ovary, depending on the stage of ovarian cell differentiation and the number of PRL receptors in the ovary.

Many studies have indicated that gonadotropin-induced ovulation is associated with an increase in ovarian plasminogen activator (PA) levels [12–17]. Using an in vitro perfused rabbit ovarian system, Hamada et al. [18] showed that the presence of PRL in the perfusion medium inhibited hCG-induced ovulation. Yoshimura et al. [19, 20] further demonstrated that PA activity induced by hCG in the perfused rabbit ovary was significantly inhibited by high doses of PRL in the perfusate. However, the PA system is complex and consists of two PAs, tissue-type PA (tPA) and urokinase-type PA (uPA), as well as a specific PA inhibitor PAI-I, which may have an important role in the regulation of PA activity in the ovary. Ovarian cells have the capacity to synthesize tPA, uPA, and PAI-I [21, 22]. Since the interaction among PAs and PAI-I leads to the formation of inactive complexes and neutralization of PA activity, the absolute level of PA activity is determined by the relative level of PAs and the inhibitor. Our studies suggest that the time-coordinated and cell-specific gene expression of tPA and PAI-I in the ovary and the interaction of these molecules allow a narrow ''window'' of preovulatory increase in tPA activity that may play an essential role in the process of follicular rupture [23–26]. Just recently we have further demonstrated that treatment of eCG-primed immature rats with PRL significantly delays, but does not completely inhibit, ovulation in a time- and dose-dependent manner [27].

This study was designed to investigate whether PRL affects the coordinate regulation of tPA and PAI-I gene expression in eCG-primed rat granulosa cells in vitro.

MATERIALS AND METHODS

Materials

McCoy's 5a medium (modified, without serum), penicillin-streptomycin solution, L-glutamine, and fetal calf se-

Accepted April 1, 1998.
Received December 16, 1997.
[1]Project supported by the ''Pang-Dun'' Program, WHO, Chinese Academy of Sciences, National Natural Science Foundation of China, and Swedish Medical Research Council.
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rum were purchased from Gibco (Paisley, Scotland); eCG, LH, poly-D-lysine (M_r 70 000–150 000), Triton X-100, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), trypan blue dye, and thrombin were obtained from Sigma Chemical Company (St. Louis, MO); acrylamide *N,N*-methylene-*bis*-acrylamide, SDS, tetramethylene diamine (TEMED), ammonium persulfate, and Coomassie brilliant blue dye were from Bio-Rad Laboratories (Richmond, CA); nitrocellulose filters were obtained from Schleicher and Schuell GmBH (Dassal, Germany); Hybond nylon filters were obtained from Amersham (Buckinghamshire, England); prestained protein molecular weight standard was obtained from Bethesda Research Laboratories (Gaithersburg, MD); rabbit antiserum against rat PAI-I was purchased from American Diagnostics Inc. (New York, NY); anti-rabbit alkaline phosphate-conjugated IgG (Fc) fraction and a riboprobe in vitro transcription system were purchased from Promega-Biotech (Madison, WI); uPA was obtained from Serono S.A. (Aubonne, Switzerland); plasminogen was obtained from BioPool (Umeå, Sweden); human fibrinogen was purchased from Kabi-Vitrum (Stockholm, Sweden) and further purified by ethanol precipitation as described by Blombäck et al. [28]. T_7 RNA polymerase was obtained from Promega-Biotech, and [α-^{32}P]uridine triphosphate (UTP) was from New England Nuclear (Boston, MA). Restriction enzymes were purchased from Boehringer Mannheim (Bromma, Sweden), and collagenase was obtained from Worthington Biochemical Corporation (Freehold, NJ). Ovine FSH (NIH-FSH-S15, FSH activity 20 × NIH FSH-S1 U/mg, LH activity 0.04 × NIH-LH-SI U/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIADDK; ovine PRL (NIAMDD-OPRL-15, 30.5 IU/mg) was obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases); and hCG CR-121 (13 550 IU/mg) was obtained from the National Institute of Child Health and Human Development (NICHHD).

Granulosa Cell Culture

Immature rats (21–22 days old) received injections of 10 IU eCG to stimulate follicle growth. At 46 h after the hormone treatment, the animals were killed, and the ovaries were removed for preparation of granulosa cells. The procedure for this preparation has been described in detail previously [29]. To determine PA and PAI-I levels, 5×10^5 viable cells per well were preincubated for 6 h in 16-mm 24-well culture plates in 0.5 ml McCoy's 5a medium supplemented with 2 mmol L-glutamine, 100 U/ml penicillin, and 100 mg streptomycin sulfate. At the end of the incubation, the cells were washed once with 0.5 ml fresh McCoy's 5a medium and further incubated in fresh serum-free medium for 48 h in the presence or absence of LH (100 ng/ml) and PRL (10–1000 ng/ml) alone or in combination. For extraction of tPA and PAI-I mRNA, $2–3 \times 10^6$ viable granulosa cells were precultured for 6 h in poly-D-lysine-coated glass slides (22 × 22 mm) in 1 ml of McCoy's 5a medium containing 5% calf serum supplemented with the same concentrations of L-glutamine penicillin and streptomycin. After being washed, the cells were further incubated for the designated times in 1 ml fresh serum-free McCoy's 5a medium in the presence or absence of the hormones. All the cell cultures were performed in a humidified 95% air: 5% CO_2 incubator at 37°C. Media were collected at the end of incubation, adjusted to 0.01% Tween 80, and kept at −20°C until assayed for PAI-I activity.

SDS-PAGE

PA and PAI-I activities in samples were fractionated by SDS-PAGE [30]. Before electrophoresis, the samples were adjusted to 2.5% SDS (for PA activity assay), and 2.5% SDS and 12.5 mM dithiothreitol (DTT; for PAI-I activity assay). Electrophoresis was performed at 50 volts until the dye front reached the bottom of the gel (approximately 16 h). After electrophoresis, the gels were incubated twice for 45 min each in 2.5% (v:v) Triton X-100 to remove SDS in the gel, rinsed with distilled water, and applied to the surface of a fibrin-agar indicator gel.

Fibrin Overlay and Reverse Fibrin Autography (RFA) Assay

The fibrin-agar indicator gels were prepared as previously described [31]. The fibrin-agar gel contained 50 mg/ml plasminogen as zymogen for PA. Fibrinogen (2.4 mg/ml) and thrombin (0.5 U/ml) were also added to allow the formation of fibrin as the substrate for plasmin. After electrophoresis, gels were laid carefully onto the fibrin-agar gel and were incubated at 37°C in a humid chamber until the lysis zones became visible, indicating the presence of PAs. For detection of PAI-I activity, the samples were analyzed by RFA [32]. In addition to containing plasminogen, fibrinogen, and thrombin, RFA indicator gels contained 0.01 U/ml urokinase to allow autolysis. The development of an opaque, lysis-resistant zone indicated the presence of PAI-I activity.

Immunoblot Analysis

For immunoblot analysis, culture media were fractionated by SDS-PAGE [30]. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper and immunoblotted as described [33] using an antiserum against rat PAI-I (1:1000 dilution). Antibody binding was visualized using anti-IgG antibodies conjugated with alkaline phosphatase.

Synthesis of RNA and DNA Probes

A rat PAI-I cDNA cloned into a pBluescript vector [34] and a 400-basepair (bp) *Eco*RI fragment from rat tPA cDNA clone lambda 15 [35] was subcloned into a pGEM-1 vector. Both vectors were linearized by *Hin*dIII and used as templates for probe synthesis using an in vitro transcription system (Promega-Biotech). A 400-bp tPA probe complementary to the 5′ region of rat tPA mRNA and a 376-bp PAI-I probe complementary to the 3′-untranslated region of PAI-I mRNA were obtained using the T7 and T3 promoters [24], respectively. The ^{32}P-labeled antisense RNA probes were tested for specificity by hybridization to ovarian total RNA fractionated by formaldehyde agarose gel electrophoresis and blotted to nylon filters [36, 37]. The probes were found to be specific for tPA and PAI-I mRNA since they hybridized only to specific mRNA species with the size corresponding to tPA [35] and PAI-I mRNA [34]. A 250-nucleotide single-stranded β-actin DNA probe was prepared by primer extension as described [38].

Preparation of Total RNA

Total RNA from granulosa cells was prepared using the NP-40 method [24]. For hybridization analysis, total RNA was fractionated by agarose gel electrophoresis in the presence of formaldehyde [36] and transferred to nitrocellulose

filters [37] or immobilized directly by using a slot blot filtration apparatus (Schleicher & Schuell). Dilutions of RNA from each sample were applied to nitrocellulose filters in triplicate for hybridization with PAI-I, tPA, and β-actin probes.

Nylon filters were cross-linked by UV light in a Stratalinker (Stratagene, La Jolla, CA) and prehybridized at 62°C for 2 h in 50% formamide, 5-strength saline sodium citrate (SSC; single-strength SSC IS 0.15 M sodium chloride, 0.015 M sodium citrate), 8-strength 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 mg/ml heat-denatured herring sperm DNA, and 250 mg/ml yeast tRNA. The hybridization was carried out in the same solution containing 2.5×10^6 cpm/ml of each probe for 16 h at 64°C. The filters were washed in double-strength SSC, 0.1% SDS twice for 15 min at room temperature and then washed twice in 0.01-strength SSC, 0.1% SDS for 40 min at 66°C. Hybridizations using the β-actin probe was performed at 42°C as described [38]. After hybridization, the filters were exposed to autoradiographic films or analyzed by Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

In Situ Hybridization of tPA and PAI-I mRNA in Cultured Granulosa Cells

To examine the expression and localization of tPA and PAI-I mRNA in cultured granulosa cells, 2×10^6 viable cells obtained from the eCG-primed ovaries were precultured for 6 h on poly-D-lysine-coated glass slides (22 × 22 mm) in a dish, in 1 ml of McCoy's 5a medium containing 5% calf serum. After being washed, the cells were further incubated for 6 h in 1 ml fresh serum-free medium in the presence of LH (100 ng/ml) with or without PRL (1000 ng/ml).

The glass slides were air-dried and fixed in 4% paraformaldehyde in PBS. After fixation, the slides were digested with proteinase K for 10 min at 37°C, rinsed in PBS, and fixed again in 4% paraformaldehyde for 5 min. After being washed in PBS for 5 min, the slides were dehydrated in a series of ethanol and air-dried. They were then prehybridized in 50% formamide/double-strength SSC for 2 h at room temperature. Then the slides were hybridized with dig-labeled antisense cRNA probes in hybridization solution. Hybridization was performed in a humidified box for 20 h at 48°C. The slides were washed in double-strength, then single-strength, and then 0.1-strength SSC, each twice for 15 min at 40°C. The hybridized probes were detected using an alkaline phosphatase-coupled anti-dig *Fab* fragment. The color reaction was developed by incubation with NBT and BCIP in color development buffer. The reactions were terminated by immersing the slides in buffer I [27].

Data Analysis

All experiments measuring PA and PAI-I mRNA and their activities were repeated at least three to five times. Each experiment was designed to obtain enough granulosa cells from about 40–46 ovaries of eCG-primed immature rats for the measurements of both tPA and PAI-I mRNAs as well as their activities at the same time for the comparison. A photographic record of tPA or PAI-I activities on the agarose indicator gel was accomplished by using dark-field illumination.

The relative amount of specific mRNA was determined by densitometric scanning of autoradiographic films or by quantitation of counts per minute by Phosphorimager. Each value was normalized against the corresponding relative amount of β-actin in the sample. In each experiment, the data are expressed in terms of fold increase in relation to the control group.

Data were analyzed by ANOVA. Differences among groups were detected by Tukey's multiple-comparison test [39]. Differences were considered significant when $p < 0.05$.

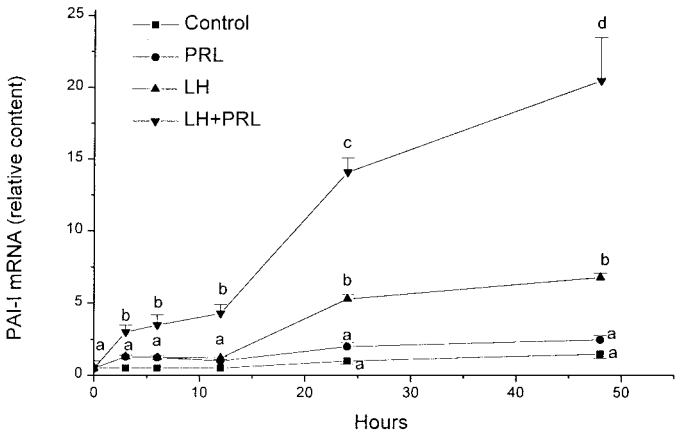

FIG. 1. Time-dependent induction of PAI-I mRNA levels by PRL and LH in cultured eCG-primed granulosa cells. Immature female rats (21–22 days old) received injections of 10 IU eCG to stimulate follicular growth; 46 h later, the animals were killed, and the ovaries were removed for preparation of granulosa cells. Granulosa cells (3×10^6) were preincubated for 6 h in poly-D-lysine-coated polypropylene dishes (10 × 35 mm, 5 μg/cm²) in 1 ml of McCoy's 5a medium containing 2% calf serum. After being washed, the cells were further incubated for the designated times in 1 ml fresh serum-free McCoy's 5a medium in the presence or absence of the indicated hormones. The cells in the dishes were quickly frozen in liquid nitrogen and processed for PAI-I mRNA measurement as described in *Materials and Methods*. Mean ± SEM of three experiments with duplicate incubations per experiment; analyzed by ANOVA followed by Tukey's multiple comparison test. Groups with at least one identical letter not significantly different.

RESULTS

Time-Dependent Effect of PRL on PAI-I mRNA and Activity in eCG-Primed Granulosa Cells

To examine whether PRL stimulates PAI-I expression in cultured granulosa cells, 2×10^6 cells obtained from eCG-treated ovaries were preincubated for 6 h in the presence of 2% calf serum, and then incubated in fresh serum-free medium for the indicated times with or without LH (100 ng/ml) and PRL (1000 ng/ml) alone or in combination. The conditioned media were collected for PAI-I assay, and the cells were frozen in liquid nitrogen for mRNA preparation. As shown in Figure 1, LH and PRL alone slightly induced PAI-I mRNA production in a time-dependent manner. After 48-h incubation, 1.6- and 4.5-fold increases in the mRNA production were observed in the presence of LH and PRL, respectively, as compared with the control. Cotreatment of LH with PRL had a synergistic effect on PAI-I mRNA production, with an 8.2-fold induction in relation to LH alone after 48-h incubation. The increase in the cell PAI-I mRNA levels in the culture was well correlated with the increase in PAI-I activity in the conditioned media. After 12-h culture, a small amount of PAI-I activity could be detected in the groups incubated with PRL alone and with LH plus PRL. After 24-h incubation, an obvious synergistic increase

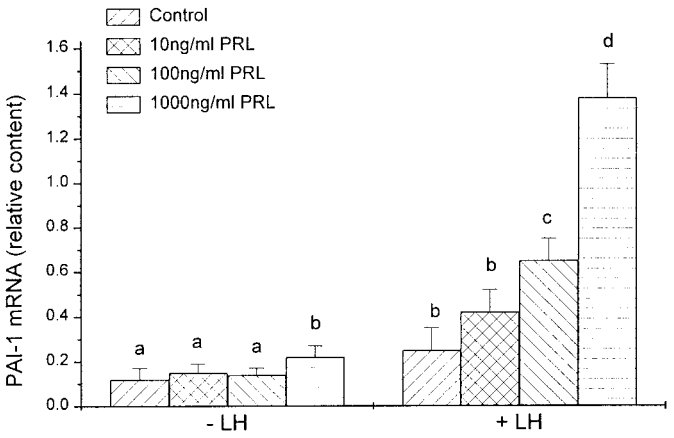

FIG. 2. Dose-dependent effect of PRL on PAI-I mRNA levels in the cultured granulosa cells. Granulosa cells (3×10^6 cells) were prepared and cultured as indicated in Figure 1 in the presence or absence of LH (100 ng/ml) and various concentrations of PRL (10–1000 ng/ml) alone or in combination. The cells in the dishes after removal of media were quickly frozen in liquid nitrogen, and processed for the PAI-I mRNA measurement as described in *Materials and Methods*. Mean ± SEM of three experiments with duplicate incubations per experiment; analyzed by ANOVA followed by Tukey's multiple comparison test. A different letter denotes a significant difference ($p < 0.05$).

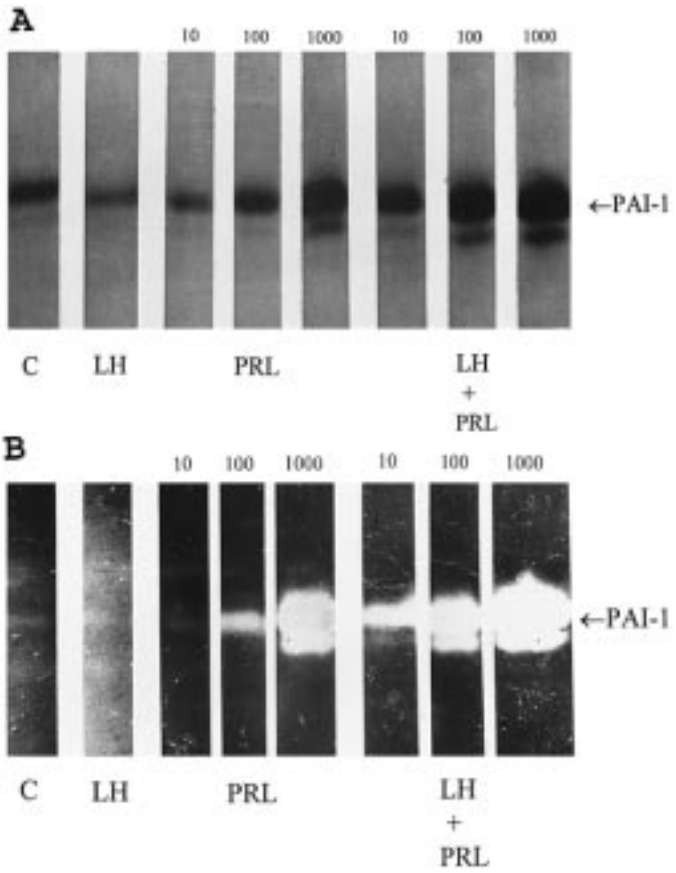

FIG. 3. Dose-dependent stimulation PAI-I antigen (**A**) and activity (**B**) in cultured granulosa cells. For determination of PAI-I activity and antigen in the conditioned media, granulosa cells (5×10^5 cells/well) obtained from the ovaries at 46 h after eCG injection were preincubated for 6 h in 0.5 ml McCoy's 5a medium containing 2% serum and further incubated for 48 h in fresh medium in the presence or absence of LH (100 ng/ml) and various concentrations of PRL (10–1000 ng/ml) alone or in combination; PAI-I protein in the medium was fractionated by SDS-PAGE, and activity and antigen were determined by RFA and Western blotting, respectively. Representative experiment from three similar experiments is shown.

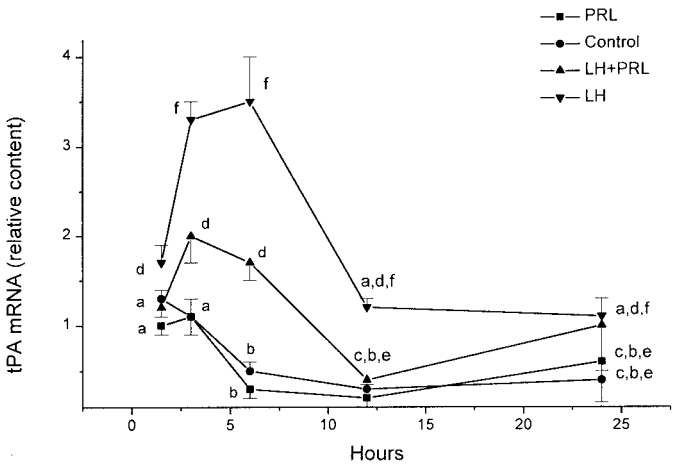

FIG. 4. Time-dependent inhibitory effect of PRL on tPA mRNA levels in the cultured granulosa cells. Granulosa cells (2×10^6 cells) were prepared and precultured as indicated in Figure 1. After being washed, the cells were further incubated for the indicated times in serum-free medium in the presence or absence of LH (100 ng/ml), PRL 1000 ng/ml), or LH plus PRL. The cells in the dishes after removal of media were quickly frozen in liquid nitrogen and processed for tPA mRNA measurement as described in *Materials and Methods*. Mean ± SEM of three experiments with duplicate incubations per experiment; analyzed by ANOVA, followed by Tukey's multiple comparison test. Groups with at least one identical letter were not significantly different. CPX, PA-PAI-I complexes.

in PAI-I activity could be observed in the culture of LH with PRL (data not shown).

PRL Dose-Dependent Stimulation of the Synthesis of PAI-I mRNA and Activity Levels in Cultured Granulosa Cells

To estimate the dose-dependent effect of PRL on PAI-I mRNA levels in the culture, granulosa cells (2×10^6 cells/dish) were preincubated for 6 h in medium containing 2% calf serum, and then incubated for 24 h in serum-free medium. As shown in Figure 2, addition of various doses of PRL in the presence or absence of LH stimulated PAI-I mRNA induction in a dose-dependent manner. The presence of LH resulted in a synergistic induction of PAI-I mRNA levels.

To analyze PAI-I antigen and activity levels in the conditioned medium, granulosa cells (5×10^5 cells/well) were preincubated for 6 h in medium containing 2% serum and further incubated for 48 h in fresh serum-free medium in the presence or absence of LH (100 ng/ml) with various doses of PRL alone or in combination with LH. As shown in Figure 3A, PAI-I antigen in the control group was hardly detected. Addition of LH (100 ng/ml) to the culture did not increase the PAI-I antigen level. Addition of PRL induced PAI-I antigen production in a dose-dependent manner. PRL induction of PAI-I antigen was further enhanced by the addition of LH to the culture. PAI-I activity in the sample was also assayed by RFA, as shown in Figure 3B. LH alone did not significantly increase the PAI-I activity. Addition of PRL to the culture increased PAI-I activity levels in a dose-dependent manner, and this increase was greatly enhanced by cotreatment with LH.

Time- and Dose-Dependent Inhibition of LH-Induced tPA mRNA and Activity Levels by PRL in Cultured Granulosa Cells

To examine whether PRL inhibits LH-induced tPA gene expression in cultured granulosa cells, 2×10^6 cells ob-

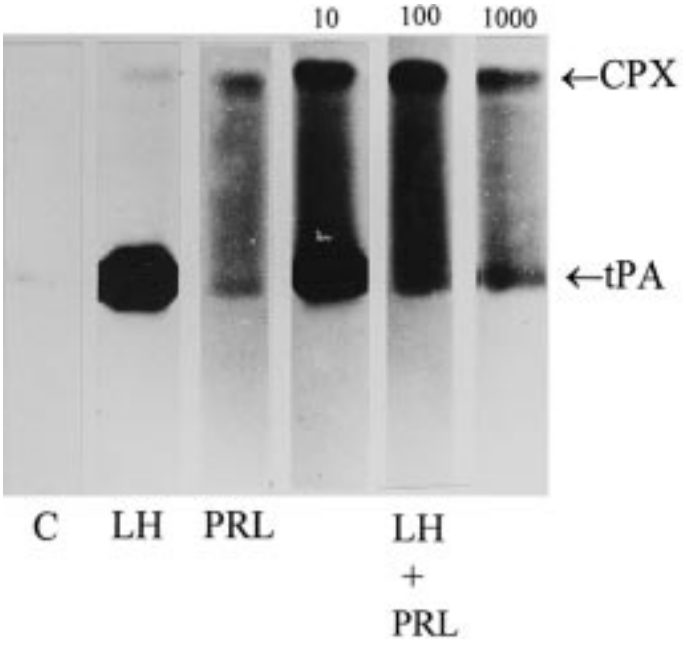

FIG. 5. Dose-dependent inhibitory effect of PRL on LH-induced tPA activity in the cultured granulosa cells. 5×10^5 viable granulosa cells per well were preincubated for 6 h in 0.5 ml of McCoy's 5a medium containing 2% serum. At the end of incubation, the cells were washed and further incubated for 48 h in the presence or absence of LH (100 ng/ml), and various doses of PRL. Media were collected at the end of incubation. Activity of tPA in the media was determined by fibrin-overlay technique as described in *Materials and Methods*. Representative experiment from three similar experiments is shown.

tained from eCG-primed ovaries were preincubated for 6 h in the presence of 2% calf serum, and then further incubated in serum-free medium for the indicated times. As shown in Figure 4, LH alone dramatically stimulated tPA mRNA levels in a time-dependent manner. Cotreatment with PRL time-dependently decreased the LH-induced tPA mRNA levels. Significant inhibition could be observed at 3-, 6-, and 12-h culture, with 36.4%, 51.4%, and 66.7% decreases, respectively, of the LH-induced tPA mRNA levels.

To analyze the dose-dependent effect of PRL on LH-induced tPA activity levels, eCG-primed granulosa cells (5×10^5 cells) were preincubated in 0.5 ml of McCoy's 5a medium containing 2% serum for 6 h, and further incubated in serum-free medium with the indicated hormones for 48 h. The tPA activity in the medium is shown in Figure 5. LH stimulated synthesis of tPA in the culture, and the increase of tPA activity was diminished markedly by cotreatment with PRL in a dose-dependent fashion. The presence of high-molecular weight PA-PAI-I complexes indicated the neutralization of LH-induced tPA activity by the increased PAI-I activity.

In Situ Hybridization of tPA and PAI-I mRNA in the Cultured Granulosa Cells

To examine whether PRL in culture in combination with LH stimulates PAI-I and inhibits tPA mRNA expression, $1–2 \times 10^6$ eCG-primed granulosa cells were cultured for 6 h in the presence of LH with or without PRL. The tPA and PAI-I mRNAs in the cells were localized by in situ hybridization. As shown in Figure 6, PRL was capable of decreasing LH-induced tPA mRNA expression (Fig. 6, A and B), while increasing PAI-I mRNA level, in the cells (Fig. 6, C and D).

DISCUSSION

In the present study, we have demonstrated that PRL stimulates PAI-I gene expression in a time- and dose-dependent manner in eCG-primed rat granulosa cells in vitro. This stimulation of PAI-I expression is dramatically enhanced by the presence of LH. PRL is also capable of inhibiting LH-induced tPA expression. The mechanism by which PRL with the gonadotropin synergistically super-induces PAI-I, while inhibiting tPA expression, in the eCG-primed granulosa cells is unknown. It has been demonstrated that a low dose of FSH through activation of adenylate cyclase induces PAI-I production in granulosa cells [21]. The present study further demonstrated that PRL alone in higher doses is also capable of inducing PAI-I gene expression by a mechanism that does not appear to involve activation of adenylate cyclase in the cell membrane because cAMP does not mimic the action of PRL (data not shown). GnRH or phorbol 12-myristate 13-acetate (PMA) has been shown to markedly stimulate PAI-I mRNA and activity levels in cultured granulosa cells [40]. The increase in PAI-I expression in the cells by GnRH and PMA was completely inhibited by the coaddition of FSH to the culture [41]. During GnRH-induced ovulation in hypophysectomized rats, both PAI-I mRNA and activity were also increased in a time-dependent fashion [25]. These data suggest that the action of PRL on PAI-I gene expression in granulosa cells might be through a protein kinase C-dependent pathway.

PRL is a pituitary hormone mainly involved in stimulating milk production. However, there is abundant evidence to show that the ovary is also one of the primary target tissues for PRL. Specific PRL receptors have been found in the granulosa cells and other compartments of the ovary [1, 2, 6, 42]. Although a large body of evidence has shown that an increase in PRL dose inhibits steroidogenesis in cultured granulosa cells [9, 11, 42], McNatty et al. [9] reported that steroid production by cultured human granulosa cells is stimulated by a low physiological dose of PRL, whereas high doses are inhibitory, suggesting that PRL may exert an effect on ovarian function depending on the stage of ovarian cell differentiation. Studies in rats and mice have indicated that PRL binding sites on granulosa cells are low before ovulation but increase in response to the preovulatory LH surge [43, 44]. Therefore, progesterone production induced by LH is further enhanced by PRL [43]. A similar sequence of events appears to occur in the pig. Increases in the number of PRL receptors on granulosa cells are associated with increases in the capacity of progesterone secretion by PRL [11]. These results suggest that the effects of PRL on ovarian cells may largely depend on the differentiation of PRL receptors. Using a eCG-primed mouse granulosa cell culture model, we have demonstrated that PRL significantly enhances FSH- and LH-induced progesterone production, while it inhibits aromatase activity [45]. In vivo experiments also showed that PRL inhibits hCG-induced ovulation in mice by decreasing ovarian PA activity and serum estrogen concentrations [10]. Both FSH and LH have been demonstrated to induce tPA mRNA and activity in cultured granulosa cells [21, 22, 46, 47]. This study further provided evidence to show that tPA activity induced by LH is dramatically inhibited by the presence of PRL in a dose-dependent manner. This result is consistent with the previous reports that PRL significantly decreased hCG-in-

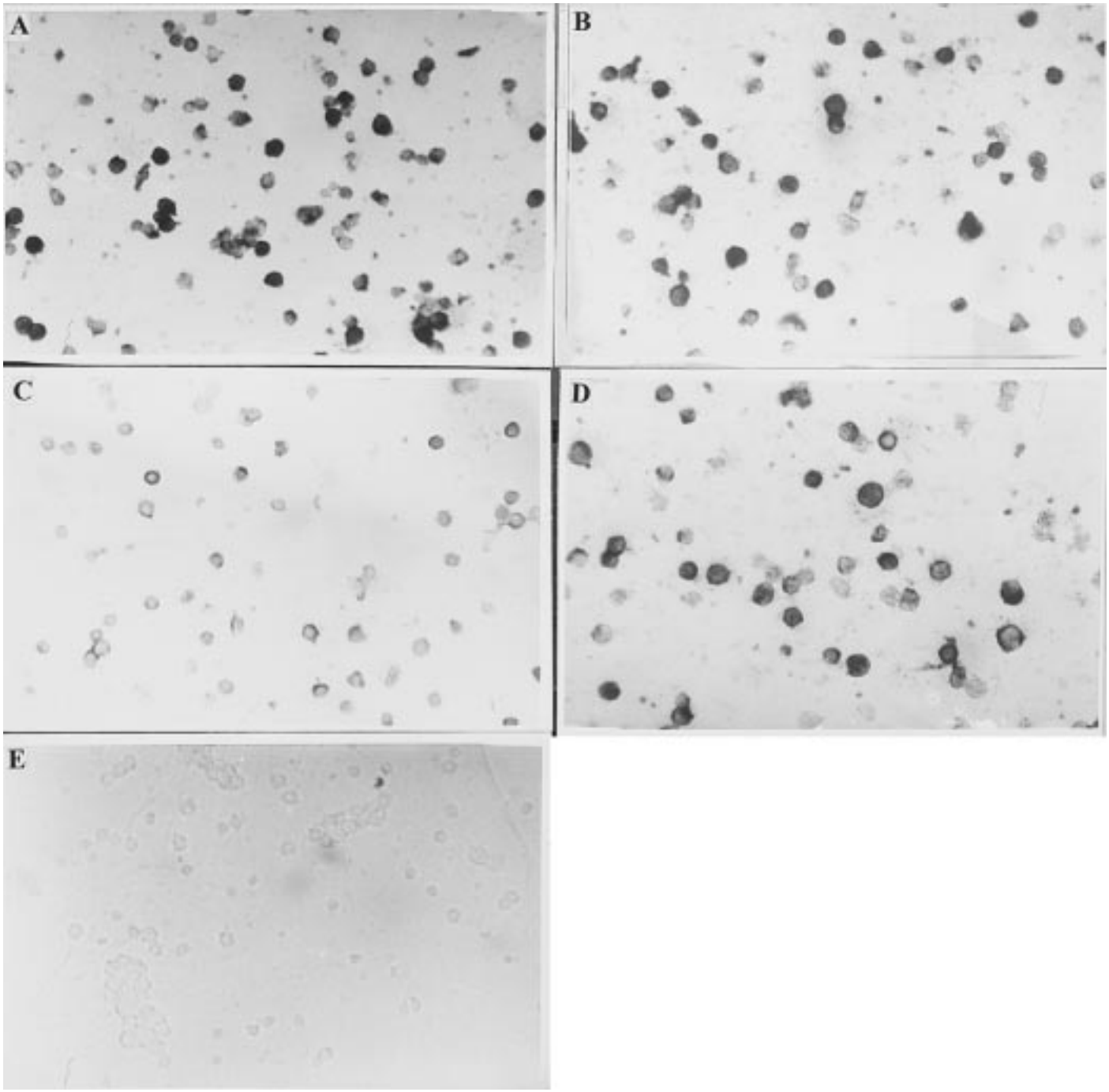

FIG. 6. Effects of PRL on LH-induced tPA and PAI-I mRNA levels in cultured granulosa cells. Granulosa cells (2×10^6 cells) were prepared and precultured as indicated in Figure 1. After being wash, the cells were further incubated for the indicated times in serum-free medium in the presence or absence of LH (100 ng/ml), PRL (1000 ng/ml), or LH plus PRL. The cells were fixed and processed for tPA and PAI-I mRNA localization by in situ hybridization as described in *Materials and Methods*. **A, C**) Granulosa cells cultured with LH alone. **B, D**) Granulosa cells cultured with LH plus PRL. **E**) Sense probe of hybridization of the cultured granulosa cells. **A, B**) tPA mRNA level. **C, D**) PAI-I mRNA level. Incubation time: tPA based on time course, PAI-I based on time course. ×400.

duced ovulation by inhibiting ovarian PA activity in vitro [18, 19] and in vivo [27]. PRL was also demonstrated to inhibit hCG-induced ovulation in mice [10]. Both in vivo and in vitro experiments showed that PRL inhibition of gonadotropin-induced tPA expression was regulated by decreasing tPA mRNA synthesis on the one hand, and by neutralizing PA activity by the increased PAI-I molecules on the other [27]. This suggestion is further supported by the fact that the decrease in tPA activity by PRL is correlated with a formation of PA-PAI-I complexes. The PA and PAI complex formation subsequently leads to the cleavage and inactivation of both tPA and PAI-I activity. Any change in tPA and PAI-I activity in the cells or in the cell-conditioned media is therefore determined by the relative level of both of these molecules.

PAI-I is a primary regulator of plasminogen activation [48] and is synthesized in a highly regulatory manner by different cell types [48]. In vitro studies have shown that PAI-I biosynthesis is regulated by diverse agents such as growth factors, hormones, and cytokines [48]. PAI-I activ-

ity has been detected in rat [21], human [49], monkey [50], pig [51], and hen [46] granulosa cells, which have been known to produce the most tPA activity in the ovary. PAI-I gene expression in the cells may be modulated in a coordinated way by different regulatory signals. Pretreatment with a low dose of FSH of granulosa cells obtained from the ovaries of diethylstilbestrol-implanted hypophysectomized rats induced PAI-I activity in the culture; subsequent treatment with a high concentration of FSH down-regulated the PAI-I production but induced a high level of tPA activity [21]. These data plus the present finding indicate that the expression of tPA and PAI-I genes regulated by the gonadotropin can occur independently and coordinately.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Philip F. Smith for providing NIDDK hormone, oFSH, and PRL, and Ron Zeheb and Thomas Gelehrter for the rat PAI-I cDNA plasmid.

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