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Progesterone Inhibits Luteinizing Hormone β Subunit (LHβ) Gene Expression in the Rat Pituitary in a Synergic Manner with Estrogen

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The present study examines the inhibitory effect of progesterone (P) on luteinizing hormone (LH)β subunit gene expression in anterior pituitary of ovariectomized, estradiol-treated adult rats. A single injection of P (1mg) further decreased the estradiol-induced decrease in LHβ mRNA levels in ovariectomized rats in a time-dependent manner. P suppressed LHβ mRNA levels at lower doses (0.1 and 1mg), but increased LHβ mRNA levels at a high dose (10mg). The inhibitory action of P on LHβ mRNA was restored when RU486, a P receptor antagonist, was administered 1h before P treatment. These data clearly indicate that P inhibits gene expression of LHβ in the rat pituitary in a synergic manner with estrogen.

KEY WORDS: LHβ, Gene expression, Progesterone, Estrogen, Pituitary

Luteinizing hormone (LH) plays a crucial role in development and reproduction including steroidogenesis and gametogenesis (Pierce and Parsons, 1981). Gonadal steroids affect at the level of the hypothalamus leading to change in the pattern of GnRH secretion as well as at the level of gonadotroph regulating LH secretion and the responsiveness to GnRH (Marshall and Kelch, 1986). Gonadectomy affects to rise plasma LH, pituitary LH content, pituitary GnRH receptors as well as GnRH pulse frequency (Marchetti *et al.*, 1982; Steiner *et al.*, 1982). Gonadectomy and replacement of gonadal steroids also regulate α and LHβ mRNA levels (Abbot *et al.*, 1985; Gharib *et al.*, 1986; Papavasiliou *et al.*, 1986b).

Progesterone (P) is known to exert an important biphasic effect on LH secretion, depending on the dose and mode of P administration, the time in ovariectomized animals primed with estrogen (E) or the estrous cycle (Freeman *et al.*, 1976; Goodman, 1978). P can inhibit the frequency of GnRH pulses which are accompanied by a decline in pituitary GnRH receptor, and the pulse frequency and amplitude of LH (Marchetti *et al.*, 1982; Soules *et al.*, 1984; Marshall and Kelch, 1986). In the cultured rat anterior pituitary cells primed with E, a short-term exposure to P can lead to an acute potentiation of the sensitizing effect of E followed by a decrease in LH release over longer time intervals (Lagace *et al.*, 1980; Drouin and Labrie, 1981). In spite of extensive studies on the antagonistic effect of P on LH release (Caligaris *et al.*, 1971; Freeman *et al.*, 1976; Goodman, 1978), little is known about the role of P in change in LHβ gene expression in ovariectomized, E-treated adult rats. In this study we delineate an inhibitory effect of E and/or P on LHβ gene expression using Northern blot analysis.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (weighing 150-200 g; Seoul National University Laboratory Animal Center, Seoul) were maintained under an adjusted photocycle (14 h of light, 10 h of darkness; light on at 06:00 h) and temperature-controlled condition with food and water available *ad libitum*. Animals were bilaterally ovariectomized (OVX) under light ether anesthesia and used two weeks later. They were assigned to experiments as described below.

Experimental design

Exp. 1. To establish the dose effect of E on LHβ mRNA levels, OVX rats were implanted with Silastic capsules (10 mm in length, inner diameter 1.575 mm, outer diameter 3.175 mm; Silastic Medical Grade Tubing, Dow Corning) containing different doses of E (78, 235 and 705 μg/ml in sesame oil, Sigma) designated as 1/3 X E, 1 X E and 3 X E, respectively (Yoon *et al.*, 1994). Animals were sacrificed 48 h after E implants.

Exp. 2. To examine the time course changes of LHβ mRNA levels in response to E implants, OVX and OVX+E-treated rats were sacrificed 1 h and 1, 2, and 3 days after E implants.

Exp. 3. To investigate the effect of P and/or E on LHβ mRNA levels, a single injection of P (P, 1 mg; Sigma) or vehicle (V) was administered s.c. to either OVX+V- or OVX+E-treated rats 42 h following E or V treatments. Four experimental groups were employed: 1) OVX+V+V, 2) OVX+V+P, 3) OVX+E+V, 4) OVX+E+P.

Exp. 4. The time course and dose effect of P on LHβ mRNA levels were also examined. Animals in OVX+E+V and OVX+E+P groups were sacrificed at different time points such as 2, 4, and 6 h after administration of P. Different doses of P (0.01-10 mg) were administered s.c. to OVX+E-treated rats and animals were sacrificed 6 h after P injection.

Exp. 5. To determine whether blockade of P action may affect P-inhibited LHβ mRNA levels in OVX+E-treated rats, a well-known P receptor antagonist, RU486, was used. A single injection of RU486 (100 μg in oil, a gift from Roussel, UCLAF, France) was adminstered to OVX+E-treated rats 1 hr before P treatment, and animals were sacrificed 6 h following P administration.

The pituitaries were removed for determination of LHβ mRNA levels by Northern analysis as described below. Trunk blood was collected and sera were kept frozen until assayed for serum levels of E and P.

Total RNA extraction

Total RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Briefly, the rat pituitaries (2 per each group) were homogenized in 600 μl denaturing solution (solution D) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosine, 0.1 M 2-mercaptoethanol. Sixty μl of 2 M sodium acetate (pH 4), 600 μl of water-saturated phenol, and 120 μl of chloroform-isoamyl alcohol mixture (49:1) were added to the samples. After cooling on ice for 15 min, samples were centrifuged at 10,000 $\times$g at 4°C for 20 min. The upper phase was transferred to a fresh tube and precipitated with ethanol. RNA was finally dissolved in 20 μl of distilled water, and RNA was quantified at A_{260}. Ratios of A_{260}/A_{280} ranged from 1.8 to 2.0.

Electrophoresis

Cytoplasmic total RNA was dissolved in distilled water and denatured in 50% formamide, 6.2% formaldehyde, 20 mM MOPS [3-(N-morpholino) propanesulfonic acid], 5 mM sodium acetate and 1 mM EDTA at 60°C for 5 min. RNA was then fractionated by size using electrophoresis on 1.2% agarose gel containing 6.2% formaldehyde and 20 mM MOPS. Electrophoresis was performed at 70 volts for 1.5 h in submarine gel. RNA was then transferred to Nytran filters (pore size: 0.45 μm, Schleicher & Schuell) according to the diffusion blotting. After at least 6 h transfer, the filters were dried in air and baked at 80°C for 2 h.

Probe preparation

The rat LHβ cDNA (kindly provided by Dr. J. Roberts, Mt. Sinai, New York, USA) was labeled

with ^{32}P-dCTP (New England Nuclear) by a random primer labeling method (Feinberg and Vogelstein, 1983). ^{32}P-labeled LHβ probe was separated from unincorporated free ^{32}P-dCTP by Sephadex G-50 (Pharmacia Fine Chemical) column chromatography. The specific activity of labeled probe was approximately 1-2 × 10^9 cpm/μg DNA.

Northern blot hybridization

The hybridization procedure previously described by Papavasiliou *et al.* (1986a) was used with a slight modification. RNA samples blotted onto the filter were prehybridized and then hybridized with radio-labeled probe in a heat-sealable plastic bag (Kapak). Prehybridization was performed with 10 ml hybridization buffer at 42°C for 3 h. Hybridization buffer consists of 50% deionized formamide, 5 X Denhardt's solution (1 X : 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% ficoll), 5 X SSPE (1 X : 0.18 M NaCl, 10 mM Na_2PO_4, pH 7.7, 1 mM EDTA), 0.1% SDS, and 200 μg/ml of sonicated and heat-denatured salmon sperm DNA. The labeled probe was added and then incubated at 42°C for 24 h. After hybridization, the filters were washed three times with 2 X SSC and 0.1% SDS at room temperature for 5 min and then twice with 0.1 X SSC and 0.1% SDS at 42°C for 30 min each. Nytran filters were dried and autoradiographed with X-ray film (X-Omat RP film, Eastman Kodak) at −70°C for 1 day. Density of each autoradiographed band on film was scanned with densitrometric scanner (Transidyne General Corp.).

Results

Dose effect of E on LHβ mRNA levels.

Validation of hybridizational signals of LHβ mRNA, such as the tissue-specificity and the dose dependence of LHβ mRNA using different amounts of total RNA was studied. LHβ gene was expressed in anterior pituitary, but not in kidney and liver (Fig. 1). The size of LHβ mRNA was approximately 0.72 kb, which was in a good agreement with the previous report (Tepper and Roberts, 1984).

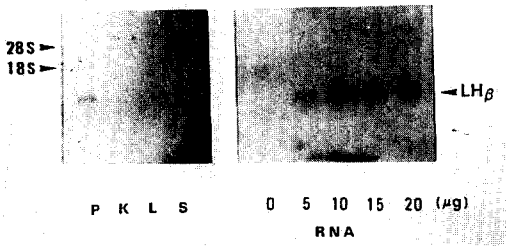

Fig. 1. Validation of LHβ mRNA. Total RNA (10 μg) from rat pituitary (P), kidney (K), and liver (L) were hybridized with ^{32}P-labeled rat LHβ cDNA (left). Increasing amounts of total RNA ranging from 0 to 20 μg resulted in a dose-dependent increase in LHβ hybridization signal (right).

Implantation of E-containing capsules to OVX rats for two days decreased LHβ mRNA levels. Note that a high dose of E (705 μg/ml) implants resulted in a clear suppression of LHβ mRNA levels (Fig. 2).

Time course changes in LHβ mRNA levels following E implants to OVX rats

To determine the time course changes in LHβ mRNA levels by E implants to OVX rats, OVX rats were implanted with Silastic capsules containing E (235 μg/ml) and then sacrificed at 0, 1, 2, and 3 days after E implantation. Following E implantation, LHβ mRNA levels were decreased in a time-dependent manner (Fig. 3). OVX+E (for 2 days)-treated rats were used for the following experiments.

Synergism of the E-decreased LHβ mRNA levels by P

To determine the synergistic effect of P on E-induced suppression of LHβ mRNA levels, P (1 mg) was administered to OVX+V- or OVX+E-treated rats and animals were sacrificed at 6 h following administration of P. When P was administered to OVX+V rats, there was no change in LHβ mRNA levels between OVX+V+V- and OVX+V+P-treated rats. However, P further suppressed E-induced decrease in LHβ mRNA levels in OVX+E+P rats, while E alone slightly decreased LHβ mRNA levels (Fig. 4).

To investigate the time course change of P

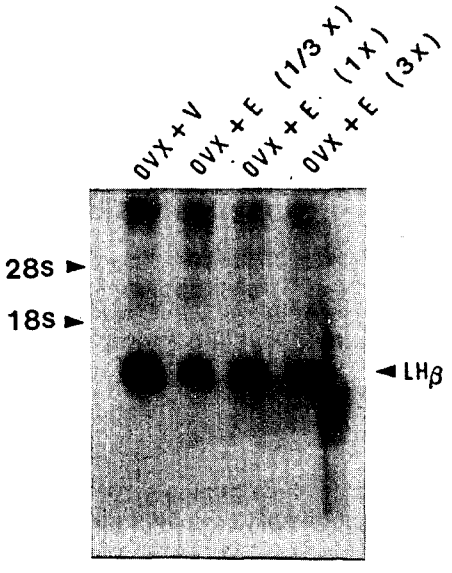

Fig. 2. Dose effect of E on LHβ mRNA levels in OVX rats. Three different size of Silastic capsules containing E (235 μg/ml) were implanted into the neck region of OVX rats. Size markers (18S and 28S) are shown at left

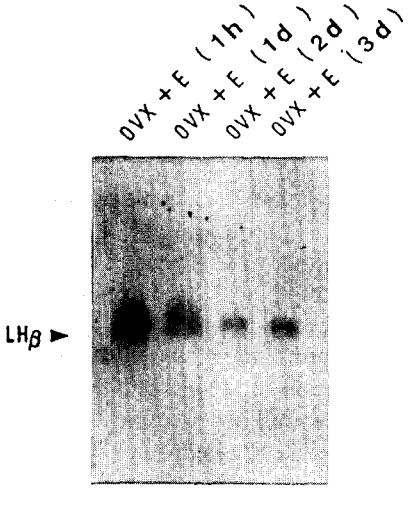

Fig. 3. Time course changes in LHβ levels by E. Animals were sacrificed 1 h, 1 d, 2 d, and 3 d after implants of E (235 μg/ml) to OVX rats.

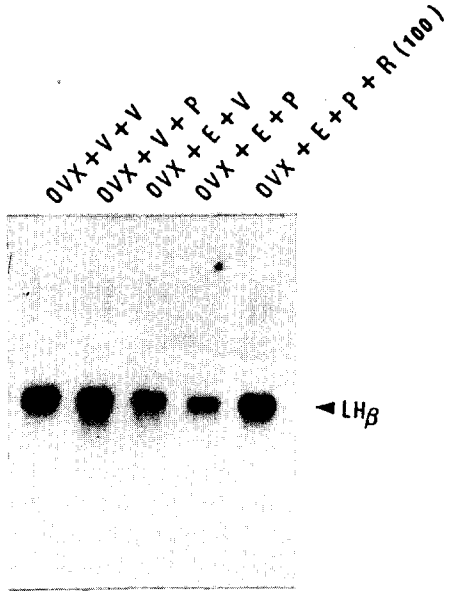

Fig. 4. Effect of RU486 on LHβ mRNA levels in OVX+E+P-treated rats. RU486 (100 μg) was administered 1 h before P injection (1 mg). Animals were sacrificed 6 h after P injection (at 16:00).

action, P was administered to OVX+E-treated rats and sacrificed at different time points, 2, 4, and 6 h after administration of P or V. Comparing with control group at each time point, the inhibitory effect of P was seen from 2 h to 6 h after administration of P to OVX+E-treated rats. Six h after P injection, LHβ mRNA evidently decreased in comparison with its control group (Fig. 5).

The suppression of LHβ mRNA levels by P was not in a dose-dependent manner: lower doses of P (0.1 and 1 mg) decreased LHβ mRNA levels but a high dose of P (10 mg) rather increased LHβ mRNA levels (Fig. 6).

Blockade of the P-inhibited LHβ mRNA by RU486.

To determine whether blockade of P action may affect P-inhibited LHβ mRNA levels, RU486 was administered 1 h before administration of P in OVX+E-treated rats. Treatment with RU486 (100 μg) clearly restored LHβ mRNA levels (Fig. 4).

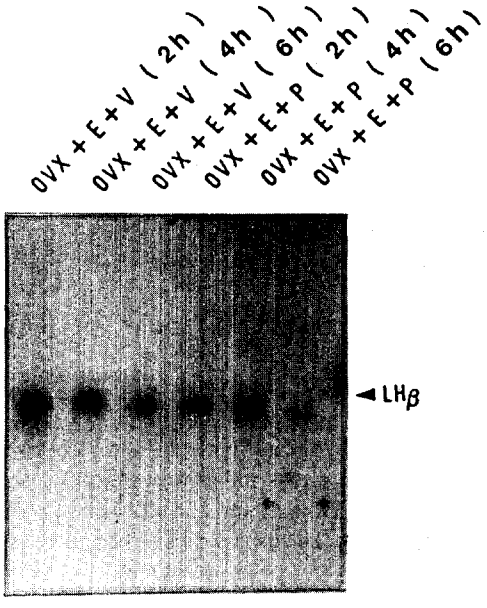

Fig. 5. Time course change of LHβ mRNA by P. Animals were sacrificed 2 h, 4 h, and 6 h after a single injection of P (1 mg) or vehicle (V) to OVX+E-treated rats.

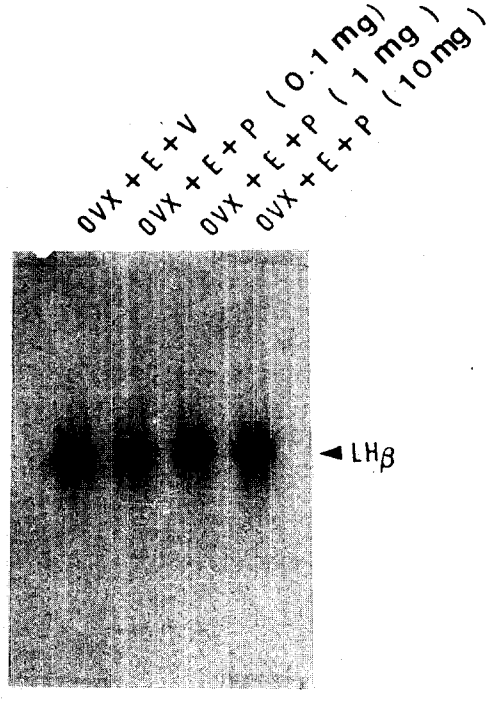

Fig. 6. Dose response of P on LHβ mRNA in OVX+E rats. Different dose of P (0.1, 1, and 10 mg) were injected s.c. to OVX+E rats.

Discussion

Ovariectomized (about 2 weeks) rats offer an excellent model system for study of steroids (E and/or P) on the regulation of pituitary LH gene expression. After ovariectomy, serum LH levels, pituitary content of LH and both α and LHβ subunit mRNA level were markedly elevated (Corbani *et al.*, 1984; Papavasiliou, 1986a). Treatment with E after OVX decreased LHβ mRNA levels *in vivo* (Gharib *et al.*, 1986; Shupnik *et al.*, 1988) and *in vitro* (Shupnik *et al.*, 1989). The dose and time course changes in LHβ mRNA levels by E implants to OVX rats shown in this study are in accordance with the previous findings (Gharib *et al.*, 1986; Shupnik *et al.*, 1988).

Recent data indicate that the negative feedback effect of E on LH secretion may reside primarily at the level of the anterior pituitary, although the participation of the central nervous system cannot be ruled out. GnRH release was not increased 4 or 8 days following ovariectomy (Levine *et al.*, 1986) when LHβ mRNA levels were high (Gharib *et al.*, 1986; Shupnik *et al.*, 1988). E produced a robust inhibition of LH release in hypophysectomized rats in which a pituitary was grafted to kidney capsule (Strobl and Levine, 1988). This clean-cut demonstration indicates that an inhibitory action of E on LH release is exerted by a direct action of pituitary gonadotroph. Indeed, E is able to directly affect the transcriptional changes in LHβ gene expression since there is an estrogen response element (ERE) in the 5'-flanking region of LHβ gene (Shupnik *et al.*, 1989).

P is known to exert an important biphasic effect on LH secretion, depending upon the dose and

mode of administration in ovariectomized animals primed with E or during the estrous cycle (Freeman *et al.*, 1976; Goodman 1978; Karsch *et al.*, 1987). P can increase GnRH release from the hypothalamus of OVX+E-treated rats (Leadem and Kalra, 1984; Kim and Ramirez, 1985; Levine *et al.*, 1985). P also augments the GnRH-induced LH release from cultured pituitary cells derived from OVX+E rats (Lagace *et al.*, 1980; Drouin and Labrie, 1981). However, P can inhibit the frequency of GnRH pulses which is accompanied by a decline in pituitary GnRH receptors, and the pulse frequency and amplitude of LH (Marchetti *et al.*, 1982; Soules *et al.*, 1984). GnRH agonist did not influence on LHβ mRNA levels (Hubert *et al.*, 1988; Simard *et al.*, 1988). Recent study by Weiss *et al.* (1990) shows that continuous or pulsatile administraton of GnRH did not produce any effect on LHβ mRNA levels in OVX+E-treated rats. Moreover, it is of importance to note that LH release caused by GnRH (Salton *et al.*, 1988) or testosterone (Yoon *et al.*, 1994) is not always coupled to LHβ gene expression indicating a differential regulation of GnRH and LH gene expression.

The present findings show that the inhibitory effect of P on LHβ mRNA levels began 2 h after administration of P and was restored by RU486 (100 μg), indicating that P may exert its action through its receptor-mediated mechanism. Recently, we found that P was able to inhibit the E-induced prolactin gene expression in the rat pituitary and a blockade of P receptor by RU486 relieved the inhibitory action of P on prolactin mRNA levels (Cho *et al.*, 1993). There appears a similar mechanism underlying the inhibitory action of P on gene expression of pituitary hormones (LH and prolactin). The P receptor is known to be exclusively localized at the gonadotrophs (Fox *et al.*, 1990). It is also possible that the inhibitory action of P may be mediated through the change of E receptor. The E receptor is abundant in the pituitary rather than in the hypothalamus (Smanik *et al.*, 1983). P can decrease the nuclear E receptor in the anterior pituitary, but not in the hypothalamus within 2 h after P treatment (Smanik *et al.*, 1983: Calderon *et al.*, 1987). Thus, we cannot rule out the possibility that P can exert its inhibitory action through the interaction between P and E receptors.

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프로제스테론은 흰쥐 뇌하수체에서 LHβ 유전자 발현을 에스트로젠과 상승작용으로 억제한다.

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본 연구는 난소절제후 에스트론젠을 처리한 성숙한 흰쥐를 사용하여 프로제스테론이 뇌하수체의 LHβ 유전자 발현에 억제적 역할을 함을 조사하였다. 프로제스테론 (1 mg)을 난소절제후 에스트로젠을 처리한 흰쥐에 주사하고 2, 4, 6 시간뒤에 쥐를 희생시켜 LHβ 유전자 발현 양상을 Northern blot hybridizaton 방법으로 조사하였다. 에스트로젠에 의해 감소된 LHβ mRNA 수준은 프로제스테론 처리에 의해 시간에 의존적으로 더욱 감소하였다. 프로제스테론을 낮은 농도 (0.1 과 1 mg)로 처리하였을 때에는 LHβ 유전자 수준이 감소되었으나, 높은 농도 (10 mg)으로 처리하였을 때에는 반대로 증가하는 양상을 관찰할 수 있었다. 프로제스테론 수용체의 길항제인 RU486을 프로제스테론 처리 한시간 전에 처리하였을 때 프로제스테론에 의한 LHβ의 억제적 효과는 사라졌다. 본 연구 결과는 프로제스테론이 흰쥐 뇌하수체에서 LHβ 유전자 발현을 에스트로젠과 상승작용으로 억제함을 시사한다.