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Temporal Changes in Ovarian Gonadotropin-Releasing Hormone mRNA Levels by Gonadotropins in the Rat

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The present study examines whether gonadotropins are involved in the regulation of ovarian GnRH gene expression and how ovarian GnRH gene expression temporally correlates with alterations in hypothalamic GnRH, pituitary LH_β gene expression in response to gonadotropins. Hypothalamic and ovarian GnRH mRNA and pituitary LH_β mRNA levels were determined by respective RNA-blot hybridizations, and ovarian GnRH and estradiol contents and serum LH levels were measured by respective radioimmunoassays. Three animal models such as 1) PMSG-treated, 2) PMSG and hCG-treated immature rats and 3) proestrous stage of adult rats were used. Immature rats (25-days old) were administered with PMSG (10 iu) at 10:00 h and 48 h later with hCG (10 iu) to induce ovulation. In the PMSG-injected model, hypothalamic GnRH mRNA levels were markedly augmented about 9-fold at 50 h, and pituitary LH mRNA 3-fold at 52 h after PMSG administration. Serum LH levels were increased to the preovulatory surge levels at 56 h, and ovarian GnRH mRNA levels were augmented 4-fold at 60 h after PMSG injection. Administration of hCG also induced a marked enhancement in ovarian GnRH mRNA levels in comparison to the values shown in both intact and PMSG-treated rats at 52 h and 54 h, respectively. In the proestrous stage of normal adult rats, pituitary LH_β mRNA levels were peaked at 16:00 h. The preovulatory LH surge was evident at 4 h before increment in ovarian GnRH mRNA levels as shown in PMSG-treated rats. The present study clearly showed the sequential increase in hypothalamic GnRH mRNA, pituitary LH_β mRNA and ovarian GnRH mRNA levels, indicating that ovarian GnRH may play a possible role in the control of follicular maturation and the ovulation process.

The mammalian ovary is a highly heterogenous organ composed of many functionally different cells including granulosa, theca, interstitial and luteal cells, and oocyte. There is an unique spatial and temporal expression of a specific set of genes that orchestrates cell growth and differentiation of these cell types which are regulated by hormones in the hypothalamic-pituitary-ovarian axis (Hall *et al.*, 1991). Numerous neuropeptides and growth factors are known to act as intraovarian mediators. For instance, GnRH exerts inhibitory and/or stimulatory effects on intraovarian function (Sharper, 1982; Hsueh *et al.*, 1983). GnRH regulates the formation of gonadotropin receptor (Hsueh *et al.*, 1983), and increases the biosynthesis of progesterone in the ovary (Davis *et al.*, 1988). GnRH also stimulates maturation of oocytes (Hillensjo and LeMaire, 1980; Dekel *et al.*, 1985), and ovulation (Ekholm *et al.*, 1981).

GnRH or GnRH-like peptide is evidently present in the ovary of various species including cows, sheep, humans and rats (Ying *et al.*, 1981; Aten *et al.*, 1986; Ireland *et al.*, 1988). Existence of the amplified GnRH transcript in the rat ovary was recently demontrated by a reverse transcription-polymerase chain reaction method (Oikawa *et al.*, 1990; Goubach et al., 1992; Bauer-Dantoin *et al.*, 1993). Our laboratory also demonstrated the existence and localization of GnRH transcript and its gene products in the ovary using in situ hybridization histochemistry and immunohistochemistry (Park *et al.*, 1988; Choi *et al.*, 1990). It is, however, yet unknown whether the ovarian GnRH gene expression is physiologically regulated by gonadotropins, although it can be hypothesized that the regulation of ovarian GnRH may be under the influence of hypothalamic-pituitary-ovarian hormonal axis.

The present study examines whether gonadotropins

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The abbreviations used are: PMSG, pregnant mare serum gonadotropins; hCG, human chorionic gonadotropins; MOPS, 3-(n-morpholino)propanesulfonic acid; CV, coefficients of variation; LSD, least significance difference; tPA, tissue plasminogen activator.

are involved in the regulation of ovarian GnRH gene expression and how ovarian GnRH gene expression temporally correlates with alterations in hypothalamic GnRH and pituitary LH_β gene expression in response to gonadotropins. Since administration of pregnant mare serum gonadotropins (PMSG) to immature female rats resulted in the stimulation of follicular maturation and endogenous preovulatory-like LH surge (Bahr and Ben-Jonathan, 1981), the immature female rats administered with PMSG and/or human chorionic gonadotropins (hCG) were used as a model system in the present study.

Materials and Methods

Animals

Immature (25 days old) and adult female (2-3 months old) Sprague-Dawley rats (Seoul National University Animal Breeding Center, Seoul, Korea) were allowed *ad libitum* to water and food and maintained under 14 h light, 10 h dark conditions (light on 06:00). Twenty five-day-old rats were ip administered with either 10 iu PMSG (Sigma) alone or 10 iu PMSG+10 iu hCG (Sigma). hCG was administrated 48 h after PMSG. In the adult rats, the estrous cycle was monitored by a daily vaginal smear procedure and those rats showing at least two consecutive 4-day cycles were used in the present study. After decapitation, ovaries, pituitaries and hypothalamic tissues were removed and stored at −70 ℃ until use. Serum was also collected from trunk blood and kept at −20 ℃ prior to assay.

Preparation of the rat GnRH and LH_β gene probes

GnRH antisense RNA transcript or GnRH oligomer were used as a hybridization probe. GnRH cDNA clone inserted into plasmid pGEM4 (a gift from Dr. Kelly Mayo, Northwestern University, Evanston, Ill.) was linearized and transcribed using SP6 RNA polymerase to specific activity (1.0×10^9 cpm) (Seong *et al.*, 1993). GnRH oligomer (3′-CG/GTC/GTG/ACC/AGG/ATA/CCC/AAC/GCG/GGA-5′; 29 mer) complementary to the sequence of the rat GnRH mRNA coding for amino acids −1 to 9 of decapeptide (Adelman *et al.*, 1986) was 5′ end-labeled with γ-32P-ATP (S.A.; 3,000 Ci/mmole, NEN) to specific activity (2.1×10^8 cpm/50 pmole) (Lee *et al.*, 1990). Rat LH cDNA probe (a gift from Dr. J. L. Roberts, Mt. Sinai, New York) was labeled by a random primer labelling method (Feinberg and Vogelstein, 1984).

Total RNA preparation and RNA analysis

Total RNAs from hypothalami, pituitaries and ovaries were extracted by an acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987). GnRH mRNA levels in hypothalami and ovarian tissues were determined by Northern blot analysis or slot blot hybridization as described previously (Kim *et al.*, 1989; Lee *et al.*, 1990; Seong *et al.*, 1993). For Northern blot analysis, total RNAs (20 μg of each group) were dissolved and denatured in 50% formamide, 7.4% formaldehyde, 20 mM 3-(n-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA at 60 ℃. RNA was then fractionated by size using electrophoresis on 1.2% agarose gel containing 6.4% formaldehyde and 20 mM MOPS (Sambrook *et al.*, 1989) and transferred to Nytran membrane (Schleicher & Schuell; 0.22 μm pore size). For slot blot hybridization, RNAs (10 μg of each group) were solubilized in buffer consisting of 7.4% formaldehyde and 6× SSC. Nytran filters were prehybridized with 10 ml hybridization buffer for 3 h at room temperature in a heat sealable plastic bag (Kapak) followed by hybridization at 62 ℃ overnight with a gentle shaking. Hybridization buffer for GnRH RNA blot hybridization consists of 2× SSC, 2× Denhardt's solution, 150 μg/ml of denatured salmon sperm DNA and 200 μg/ml of yeast tRNA (Lee *et al.*, 1990). Following hybridization, Nytran membranes were washed with 2× SSC at 62 ℃ for 5 min and then three times with 2× SSC at 52 ℃ for 30 min each. LH mRNA levels in pituitary were determined by RNA blot hybridization at 42 ℃ for 16 h. The details for hybridization procedure are described elsewhere (Tepper and Roberts, 1984). The filters were autoradiographed with X-ray film (Kodak, X-Omat RP film) and the density of bands was quantitated by densitometric scanning (Transdyne General Corp.). Nytran membrane was rehybridized with an 18S rDNA control probe. GnRH and LH_β mRNA levels were normalized by 18S RNA control values and expressed as an arbitrary unit.

Radioimmunoassays for GnRH, LH and 17β-estradiol

GnRH concentrations in ovarian extracts were measured in duplicate by a RIA procedure using Chen-Ramirez anti-GnRH antiserum (CRR-11-B-72) at a final dilution of 1 : 20,000 (Kim *et al.*, 1989). Ovarian tissues were homogenized in 600 μl of 0.1 N HCl, neutralized with 10 N NaOH and centrifuged at 15,000 × *g* for 20 min at 4 ℃. Synthetic GnRH (Sigma) was used as radioiodination and a reference standards. The sensitivity at 80% binding was about 0.5 pg/tube. The intra- and interassay coefficients of variation (CV) were about 6 and 7%, respectively. Serum LH levels were measured by a double antibody RIA method using reagents supplied by the NIADDK. rLH-I-5 and rLH-RD-2 were used as radioiodination and a reference standard, respectively. Anti-LH antiserum (anti-rLH-S-6) was used at a final dilution of 1 : 1,000,000. The detection limit for the LH assay was 20 pg/tube and the intra- and interassay CV were 5.9 and 8.9%, respectively. The ovarian concentrations of 17β-estradiol were determined using reagents supplied by the WHO Matched Reagent Program. Labeled ligand was [2, 4, 6, 7, 16, 17-3H]-estradiol (3.73×10^{11} dpm/ mole, NEN). The intra- and interassay CV were 9.6 and 4.1 %, respectively.

Statistical analysis

Changes in GnRH and LH_β mRNA and serum LH levels were analyzed by a one way analysis of variance (ANOVA). Fisher's least significance difference (LSD) test was used for post-hoc comparison with $P<0.05$ required for statistical significance. Student's t-test was also employed for analysis of the differences in hypothalamic and ovarian GnRH contents between the control and PMSG-treated groups.

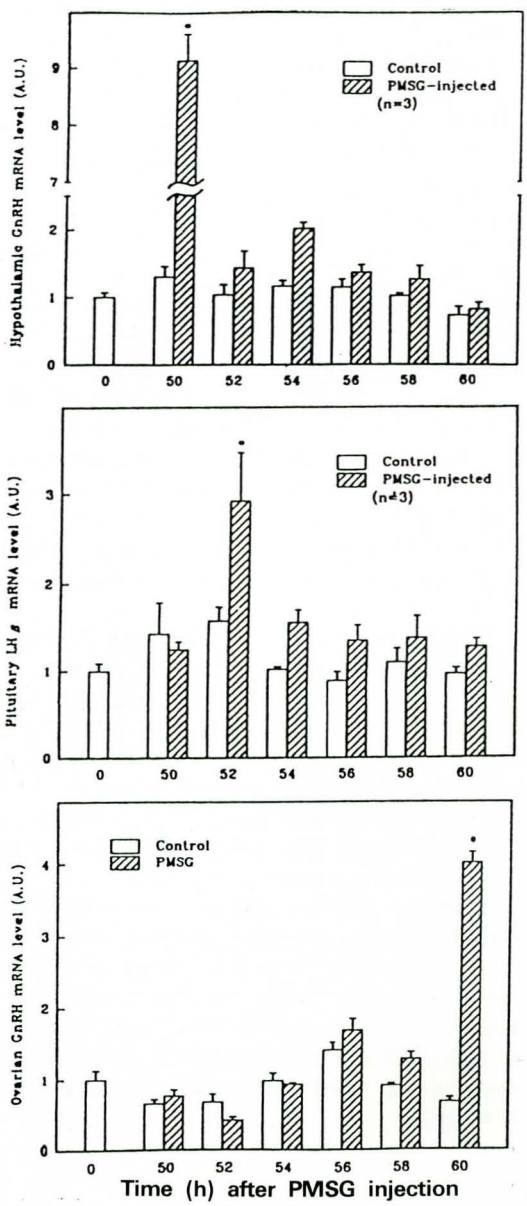

Figure 1. Effect of PMSG on hypothalamic GnRH mRNA (upper panel), pituitary LH_β mRNA (middle panel) and ovarian GnRH mRNA (lower panel) levels in the control and PMSG-treated rats. mRNA levels are expressed as an arbitrary unit (A.U.) and bar represents the mean (± S.E.) of repeated experiments (n=3). *, vs the control and other time points ($P<0.01$).

Results

Effect of PMSG on hypothalamic GnRH, pituitary LH_β and ovarian GnRH mRNA levels

The validation of GnRH RNA blot hybridization was previously well documented (Kim *et al.*, 1989; Lee *et al.*, 1990; Seong *et al.*, 1993). The sizes of GnRH and LH_β mRNA were approximately 0.6 kb (Kim *et al.*, 1989) and 0.72 kb (Tepper and Roberts, 1984) as shown previously. The time course changes in hypothalamic GnRH, pituitary LH_β and ovarian GnRH mRNA levels were examined at 2 h intervals from 50 h to 60 h after PMSG treatment. Fifty hours following PMSG administration, hypothalamic GnRH mRNA levels were markedly augmented. Hypothalamic GnRH mRNA levels were then dramatically declined to the control value and remained unchanged until 60 h (Fig. 1, upper panel). Pituitary LH_β mRNA levels were significantly increased 2-fold at 52 h after PMSG injection and then returned to the basal levels (Fig. 1, middle panel). Note that the peak of hypothalamic GnRH mRNA levels was 2 h earlier than that of pituitary LH_β mRNA levels. Ovarian GnRH mRNA levels did not increase until 58 h after PMSG

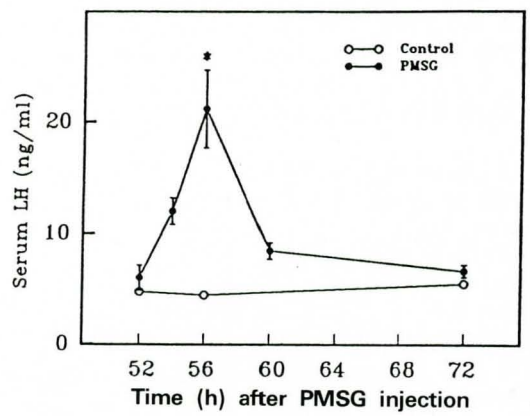

Figure 2. Serum LH levels of control and PMSG-injected rats. Each point represents the mean S.E. (n=6−11). *, vs other time points ($P<0.05$).

Table 1. Effects of PMSG on ovarian GnRH and estradiol contents

Time(h) after injection	GnRH content		Estradiol content	
	control	PMSG	control	PMSG
24	ND	29.8± 11	ND	136.6± 19.0
48	20.8± 3.1	64.9± 1.7[a]	19.5± 4.9	181.2± 38.3[a]
56	8.5± 1.7	67.6± 7.5[a]	18.0± 3.9	287.8± 41.9[a]
72	2.6± 0.6	4.2± 0.9	20.0± 3.2	111.7± 28.5[a]

GnRH and estradiol contents represent the mean ± S.E. (pg/pair of ovaries and pmol/pair of ovaries, respectively). Experiments were repeated six to eight times. ND; not determined. [a]PMSG vs control: significantly ($P<0.05$) different.

injection. Ovarian GnRH mRNA levels were significantly higher by 6-fold than the control values at 60 h following PMSG administration (Fig. 1, lower panel). Serum LH levels started to increase and reached a peak at 56 h and then declined to the control levels. Serum LH levels in the controls remained unchanged throughout the period examined (Fig. 2).

Effect of PMSG on ovarian GnRH and estradiol contents

Table 1 shows the stimulatory effects of PMSG on ovarian GnRH and estradiol contents. A similar pattern of both GnRH and estradiol contents was observed with maximal values at 56 h after PMSG injection. While ovarian GnRH contents in the control group were endogenously fluctuated with a high level at 24 h, ovarian GnRH contents began to increase at 24 h, reached a peak at 56 h, and then returned to nadir levels at 72 h. Estradiol contents rose 15-fold and then rapidly decreased.

Effect of PMSG and hCG on hypothalamic GnRH, pituitary LH_β and ovarian GnRH mRNA levels

As shown in Figure 1, hypothalamic GnRH mRNA levels were markedly increased at 50 h after PMSG injection when compared to the control values. However, this increment was not observed at 50 h and even 52 h after administration of hCG to PMSG-primed rats (Fig. 3, upper panel). Similarly, an increase in pituitary LH_β mRNA levels at 52 h post-PMSG injection was not observed in PMSG and hCG-injected rats (Fig. 3, middle panel). These data indicated that the animals had not been affected by the endogenous LH surge and negative effects of hCG on hypothalamic-pituitary neural circuitry. On the other hand, administration of hCG to PMSG-pretreated immature rats significantly enhanced ovarian GnRH mRNA levels 4-fold at 2 h and about 7-fold at 4 h after hCG-administration (Fig. 3, lower panel).

Temporal changes in pituitary LH_β mRNA, serum LH and ovarian GnRH mRNA levels during the proestrous stage in normal cycling rats

The time course changes in LH_β mRNA levels during the proestrous stage were then examined. The pituitary LH_β mRNA levels were increased about 2-fold at 16:00 h and then returned to the basal levels (Fig. 4, upper panel). At the same time, serum LH levels were significantly elevated by 16:00 h in the proestrous stage and then gradually decreased to the basal levels (Fig. 4, middle panel). Ovarian GnRH mRNA levels, however, were markedly increased at 14:00 h as compared to those at 10:00 h, and remained at similar values until 18:00 h with a peak at 20:00 h (Fig. 4, lower panel). Likewise, in the PMSG-injected immature rats, serum LH levels were elevated at 16:00 h, 4 h before the peak of ovarian GnRH gene expression.

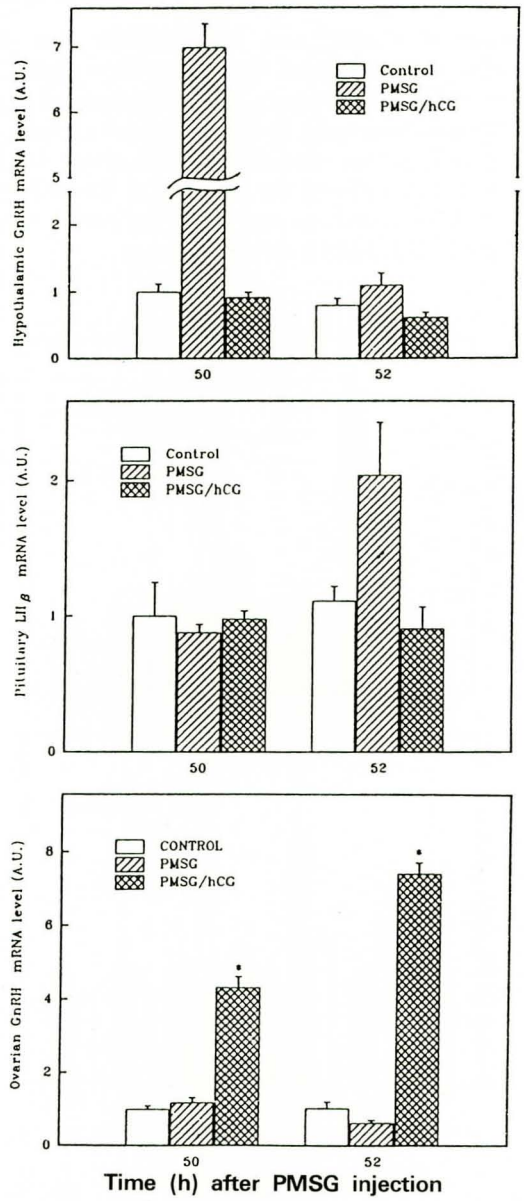

Figure 3. Effects of PMSG and hCG on hypothalamic GnRH mRNA (upper panel), pituitary LH_β mRNA (middle panel) and ovarian GnRH mRNA (lower panel) levels. Fourth-eight hours after PMSG injection, animals were administered with saline and 10 iu hCG, then sacrificed at indicated times. Bar represents the mean (± S.E.) of repeated experiments (n=3). *, vs the control and PMSG-injected, group ($P<0.01$).

Discussion

The present study provides evidence that there are temporal relationships between hypothalamic GnRH, pituitary LH_β and ovarian GnRH gene expression in PMSG-treated rats. These data indicate that ovarian GnRH gene expression appears to be under the inf-

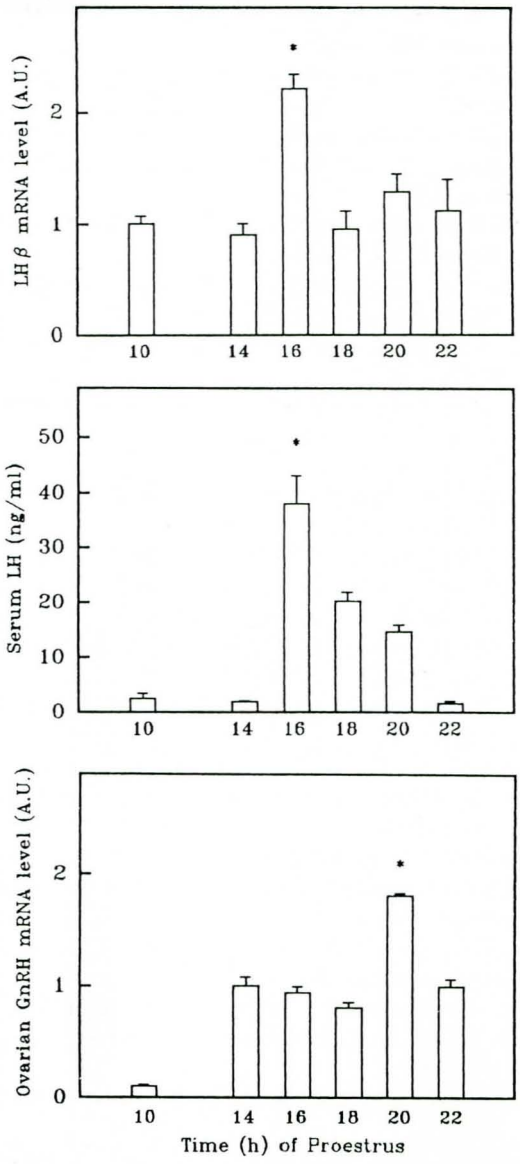

Figure 4. Temporal changes in pituitary LH_β mRNA (upper panel), serum LH (middle panel) and ovarian GnRH mRNA levels (lower panel) during proestrous stage in normal cycling rats. Pituitary LH_β and ovarian GnRH mRNA levels are expressed as an arbitrary units (pituitary LH_β mRNA level at 10:00 h and ovarian GnRH mRNA level at 14:00 h were set to 1.0 A.U.). Bar indicates the mean S.E. of repeated experiments (n=4). *, vs other time points ($P \pm 0.01$).

luence of classical hypothalamic-pituitary-ovarian hormonal axis in the rat. In the endocrine axis, activation of hormonal genes, such as hypothalamic GnRH, pituitary LH and ovarian GnRH was sequentially occurred in a well-ordered manner. The changes in ovarian GnRH and estradiol contents by PMSG also showed a similar pattern, indicating that ovarian GnRH is involved in ovarian steroidogenesis. It can be hypothesized that activation of ovarian GnRH gene expression in preovulatory follicles may require the endogenous LH surge. Administration of hCG to PMSG-treated rats suppressed hypothalamic GnRH and pituitary LH_β mRNA levels which were thought to be responsible for inducing the LH surge. It appears that there is a negative short-loop feedback of LH, which inhibited hypothalamic GnRH activity. During the proestrous stage of normal rats, ovarian GnRH production was significantly enhanced at 4 h after the preovulatory LH surge (Fig. 4). These results support the above notion.

Hypothalamic GnRH is transported to pituitary gonadotropes through a specialized portal vessel and stimulates the release of gonadotropins (FSH and LH). Gonadotropins then regulate the production of steroids which modulate ovarian functions, such as production of intracellular proteins, cell proliferation and activation of steroidogenic enzymes. Several intraovarian regulators including GnRH can affect local cellular response and directly respond to gonadotropins (Tonetta and diZerega, 1989). Indeed, PMSG and/or hCG can stimulate ovarian inhibin (Davis *et al.*, 1988), thymosin (Hall *et al.*, 1991) and tissue plasminogen activator (tPA) (Ny *et al.*, 1987; O'Connell *et al.*, 1987). It has been known that PMSG is capable of inducing LH/hCG receptors in granulosa cells of preovulatory follicles (Richards et al., 1989), and hCG exerts the LH-like effects on enhancing GnRH production in granulosa cells. The present study suggests that FSH may act as a primer and LH as a stimulator in ovarian GnRH gene expression. However, the possibility that FSH may have a direct stimulatory effect on GnRH production can not be excluded.

Special notice has also taken of the regulatory mechanism(s) of oocyte maturation and ovulation. Since the preovulatory LH surge-like response is elicited by GnRH and its agonist (Hillensjo and LeMaire, 1980; Dekel *et al.*, 1983), and, more importantly, the GnRH receptor is present in rat oocyte membrane (Dekel *et al.*, 1988), it is possible to presume that there may be some common mechanism shared by GnRH and LH in the rat ovary (Dekel *et al.*, 1985). Several lines of evidence suggest that tPA plays an important role in gonadotropins-induced ovulation process (Beers, 1975; Reich *et al.*, 1985). Gonadotropins stimulate tPA activity and the activity of this protease is temporally correlated with ovulation. GnRH is able to increase tPA activity and mRNA levels in cultured rat granulosa cells (Ny *et al.*, 1987). It appears then that an increase in tPA may represent a common pathway in the mechanism of ovulation induced by LH/hCG or GnRH. Indeed, in the present study, hCG directly enhanced ovarian GnRH synthesis before ovulation. It is possible to presume that LH/hCG may increase ovarian GnRH which, in turn, may induce tPA and eventually ovulation. Further studies are necessary for elucidation of the precise molecular mechanism of ovulation.

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