

**Expression of Luteinizing Hormone (LH) subunit genes in the rat ovary**

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**Luteinizing Hormone (LH) subunit**

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## INTRODUCTION

The pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are synthesized and secreted by gonadotropes in anterior pituitary, and they play a pivotal role in both male and female reproduction. LH and FSH belong together with other pituitary hormone, thyroid stimulating hormone (TSH), and placental chorionic gonadotropin (CG) to the family of glycoprotein hormones (Pierce & Parsons, 1981; Albanese *et al.*, 1996). Each hormone consists of non-covalently linked  $\alpha$ - and  $\beta$ -subunits, which are encoded by separated genes (Ryan *et al.*, 1988). In mammals, the  $\alpha$ -subunit ( $C_\alpha$ ) is encoded by a single gene and common to all of the glycoprotein hormones while the unique  $\beta$ -subunits encoding by different genes provide the biological specificity of each hormone (Fiddes & Goodman, 1981; Ryan *et al.*, 1988).

Though the anterior pituitary had been thought to be the only source of LH for a long time, recent studies from the Finnish research group clearly demonstrated the novel expression of LH  $\beta$ -subunit ( $LH_\beta$ ) genes in the rat testis (Zhang *et al.*, 1995a). Three  $LH_\beta$  clones with length of 3.2, 2.4 and 0.86 Kb were identified in a rat testicular cDNA library, and the nucleotide sequence analysis of the clones indicated that the size differences are due to the existence of testis-specific exons and alternative RNA splicing (Zhang *et al.*, 1995b). Regarding the role(s) of the LH-like peptides in rat testis, any direct evidence has not been reported yet. This locally produced

hormone seems to be functional and be involved in the modulation of spermiogenesis since the transcripts for LH $\beta$  and immunoreactive LH molecules are predominantly detected in differentiating spermatids (Zhang *et al.*, 1995a).

The present study was performed to analyze the expression of LH gene in the rat ovary, a female counterpart of testis. Expression of LH $\beta$  and C $\alpha$  gene in the rat ovary was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR). To assess the production of immunoreactive LH-like molecules in rat ovary, radioimmunoassay (RIA) was performed using antibody which can recognize the rat LH $\beta$  polypeptide.

## MATERIALS AND METHODS

### 1. Animals and collection of tissue samples

Immature Sprague-Dawley rats (day 25) were ip injected with 15 IU pregnant mare's serum gonadotropin (PMSG; Sigma) or 0.2 ml saline. Rats were sacrificed at different time intervals, such as 24, 48 and 72 h after injection, respectively. After decapitation, the morphologies of ovaries and uteri were investigated briefly to ensure the administration of PMSG induced sexual maturation properly, then ovaries and pituitaries were removed and stored at  $-70^{\circ}\text{C}$  until use. Granulosa cells were obtained from ovaries by needle puncture (Lee *et al.*, 1994b). Serum was collected from trunk blood and stored at  $-20^{\circ}\text{C}$  prior to LH radioimmunoassay.

### 2. LH RIA

Tissues and cell extracts were homogenized in ice-cold 1 M acetic acid. The homogenates were centrifuged at 10,000 g for 10 minutes, and the supernatants were neutralized with 2 N NaOH and used for assay. Tissue contents and serum level of LH were measured by a double antibody RIA method (Lee *et al.*, 1994a). The reference standard (rLH-RD-2) and anti-LH antiserum (anti-rLH-S-6; 1:1,000,000 final dilution) were donated from NIDDK pituitary hormone program (NIH, USA) and the anti-rabbit gamma globulin (ARGG) was purchased from Sigma. The detection limit for the LH assay was 20 pg/tube and the intra-, inter-assay precisions were 7.2 and 9.4%, respectively.

### **3. RNA Extraction**

The total RNA was extracted from the tissues by using Trizol solution (Gibco-BRL, USA), a mixture based on the acid guanidium thiocyanate-phenol-chloroform RNA extraction method (Chomczynski & Sacchi, 1987). Tissues (100 mg/tube) were homogenized with 1 ml of Trizol solution, subsequently 0.2 ml of chloroform-isoamyl alcohol (49:1) was added to the homogenates with mixing vigorously by hand. Samples were centrifuged at 10,000 g for 20 min at 4°C. After spin, the upper part (aqueous phase) was collected, mixed with 1 ml of isopropanol, and placed at -20°C for 1 h. Sedimentation (at 10,000 g) for 20 min at 4°C was performed and the resulting pellets were washed in 75% ethanol and dissolved in DEPC-treated water. RNA samples were quantitated by UV spectrophotometry at 260 nm and stored at -70°C until further reaction.

### **4. RT-PCR**

First strand cDNA was synthesized from the total RNA (1 µg) using SuperScript RNase H reverse transcriptase (GIBCO-BRL) and oligo d(T)<sub>25</sub> primer. PCR reactions were performed in a thermal cycler (PTC-150, MJ Research). Generally, the reactions were performed in a total volume of 50 µl containing 5 µl of dNTP-premixed 10 x buffer, 100 pM of each primer, 20-50 ng of template DNA, and 0.25 unit of Taq DNA polymerase (Takara). The final reaction mixture was overlaid with 100 µl of mineral oil (Sigma) to prevent evaporation. Primers and their sequences used in this study are

shown in Table 1. Thirty-five to forty cycles were usually run, and each cycle consists of 1 min at 94°C (denaturation), 1 min at 52°C (annealing) and 1 min at 72°C (extension). In each PCR reaction, a negative control sample (RT mixture without RNA) was used. The amplified cDNA fragments were subjected to 2.0% agarose gel electrophoresis, and stained with ethidium bromide (EtBr) for visualizing. To confirm that the cDNAs for LH subunits were specifically amplified, the nucleotide sequence of the PCR products was determined partially by using PCR sequencing kit (Korea Bioneer).

## **5. Statistics**

Student's *t*-test was used for analysis of the differences in tissue contents and serum levels of LH between control and PMSG-injection groups.

## RESULTS

### 1. RT-PCR of the transcripts for rat LH subunits

RT-PCR analysis using the primers for amplification of rat LH $\beta$  cDNA yielded a fragment of 306 bp in ovary samples derived from immature rats (Fig. 1A, lane IV). A cDNA fragment of identical size was obtained in pituitary and testis samples which were used as positive controls (Fig. 1A, lanes II and III), while no PCR product was produced in negative control (Fig. 1A, lane V). To examine whether the rat ovarian LH $\beta$  transcripts contain the recently identified testis-specific exons (Zhang *et al.*, 1995b), the second set of primers corresponding to the major testicular LH $\beta$  exon 1 of 2.4 kb transcript was used in the next PCR reaction. A 428 bp DNA fragment was obtained from ovarian tissue (Fig. 1B, lane IV), and from testis used as positive control tissue (Fig. 1B, lane III). Negative control reactions, the pituitary samples and RNA-free RT mixture, did not yield any PCR product (Fig. 1B, lanes II and V).

The amplification of cDNA fragments from the C $\alpha$  transcripts in pituitary, testis and ovary were shown in Fig. 1C. Partial sequence analysis of the PCR products confirmed that all the PCR reactions were specifically occurred.

### 2. Competition curves with increasing amounts of LH standard and tissue extracts

Significant amount of LH-like molecules were detected in crude ovarian

extracts, and the competition curves with increasing amount of tissue extracts were parallel with those of standard peptide (rLH-RD-2), indicating that the ovarian immunoreactive LH-like material is similar to authentic pituitary LH molecule (Fig. 2). The LH contents in ovarian granulosa cells from both immature and PMSG-injected rats were only traceable, the majority of LH molecule might be present in theca-interstitial cells (unpublished data).

### **3. Effect of PMSG on changes in serum LH levels and ovarian LH contents**

The administration of PMSG to immature rats resulted in the stimulation of follicular maturation and endogenous preovulatory LH surge (Bahr & Ben-Jonathan, 1981). As expected, serum LH level after 48 h of PMSG injection significantly increased 5-fold when compared to those of control and PMSG 24 h group. Following then, the LH level declined at PMSG 72 h group, but the level was still higher than that of control and PMSG 24 h group (Fig. 3A). In contrast, single injection of PMSG resulted in a sharp decrease of the ovarian LH content at 24 h, and the lowered level was not recovered at 48 h and 72 h post-injection (Fig. 3B).

## DISCUSSION

The present results are first to report that genes for the LH subunits are expressed in the rat ovary. This demonstration is based on two evidence; (1) the generation of identical PCR products from two known sources of LH, pituitary and testis, as well as from ovary, and (2) detection of immunoreactive LH-like molecules from ovary by specific LH. The present study also provide evidence that the level of ovarian LH can be regulated physiologically by FSH.

From early 80's, cDNAs and genomic genes of the gonadotropin subunits have been isolated and characterized in several species (Albanese *et al.*, 1996). In each species, a single gene encodes the  $C_\alpha$  and shows considerable size variation (8-16.5 kb). The  $C_\alpha$  mRNAs range in size from 0.73-0.8 kb in mammalian species so far analyzed, and the translated product has a signal peptide 24 amino acids in length and the mature peptide has 92-96 amino acids in all species (Ryan *et al.*, 1988). Gene encoding the rat pituitary LH $\beta$  is relatively small, with total length of 1.5 kb, composed of three exons and two introns, and the transcript is 0.8 kb in size (Jameson *et al.*, 1984).

Until fairly recently, the anterior pituitary gland was the only known source of LH. In fact, the finding of testicular LH expression was quite accidental, since the testis was originally considered for the negative control (Zhang *et al.*, 1995a). The rat  $C_\alpha$  mRNA had a size of 0.8 kb in the

pituitary and testis, while the major LH $\beta$  transcript was 0.8 and 2.7 kb in the rat pituitary and testis, respectively. Subsequently, three transcriptional variants for the rat testicular LH $\beta$  with length of 3.2, 2.4, and 0.86 kb were identified and their sequence analysis indicated that the size difference of these variants and the pituitary LH $\beta$  was due to alternative splicing and differences at the 5' ends of transcripts (Zhang *et al.*, 1995b). RT-PCR amplification in the present study demonstrated that the ovarian LH $\beta$  transcripts contain at least two parts of the published cDNA structure; the pituitary exons 1, 2 and 3 including the translated region which were detected in the pituitary and testis, as well as the part of testicular exons 1 and 2 found in the major LH $\beta$  transcripts (2.7 kb) in the rat testis. RT-PCR for amplification of the C $\alpha$  transcripts shown the identical cDNA product from the positive control tissues (pituitary and testis) and ovary, indicating that the LH subunit peptides might be produced in the rat ovary. Actually, immunoreactive LH-like molecules were detected in ovary using RIA, and the molecules seem to be physiological substance because the ovarian contents of LH-like were significantly altered by single injection of PMSG.

Mammalian ovary and testis show extreme morphological differences due to the sexual dimorphism. In many aspects, however, these organs share common functional natures, such as germ cells production, synthesis and secretion of gonadal factors, and regulation of their functions by

pituitary gonadotropins (Griffin, 1996; Ojeda, 1996). In the few years studies provided clear evidence for the production of LH or LH-like molecule in rat testis, so it was plausible to hypothesize that LH might be expressed in rat ovary. In fact, the present study demonstrated that genes for LH subunits are expressed in the rat ovary, and the presence of "gonadal LH" and indicated that the LH<sub>β</sub> in both ovary and testis might be expressed in gonad-specific manner with alternative splicing mechanism(s) and unique transcription start sites. A similar phenomenon has also been reported with some hormone genes, classically assorted as "non-gonadal", are expressed in rat gonads, e.g. growth hormone releasing hormone (GHRH) and pituitary adenylate cyclase activating polypeptide (Bagnato *et al.*, 1992; Srivastava *et al.*, 1995; Hurley *et al.*, 1995; Gräs *et al.*, 1996).

Most of hormones are acting on gonad through an endocrine control mechanism. The major ovarian function is primarily regulated by pituitary gonadotropins that transverse the general circulation to reach gonad. However, there is ample evidence that intraovarian factors are indispensable for fine tuning in the regulation of highly complex but harmonized events in ovary (Richards, 1994). Together, gonadotropins, steroids and locally produced peptidergic factors consist a biological network that modulates the follicular maturation, ovulation, corpus luteum formation and luteolysis (Adashi & Rohan, 1992). In this context, it is intriguing that LH may exert its role on the control of both male and female reproduction

through dual pathways. One is an endocrine mechanism for primary action of pituitary LH, the other is an auto- and/or paracrine mechanism for fine tuning action of the locally produced LH. However, the physiological significances such as intraovarian roles, interactions between other trophic factors, and tissue-specific regulation mechanism of the rat ovarian LH remain to be elucidated.

## SUMMARY

The present study was performed to analyze the expression of LH genes in the rat ovary. Expression of LH subunit genes in the rat ovary was demonstrated by amplification of ovarian RNA by RT-PCR. The ovarian LH $\beta$  transcripts contained at least two parts of the published cDNA structure, the pituitary exons 1, 2 and 3 and the part of testicular exon 1 in the major transcripts form in rat testis. Using RIA, significant amount of LH-like molecules were detected in crude ovarian extracts, and the competition curves with increasing amount of tissue extracts were parallel with those of standard peptide, indicating that the ovarian immunoreactive LH-like material is similar to authentic pituitary LH molecule. The administration of PMSG to immature rats resulted in a sharp decrease of the ovarian LH contents after 24 h post-injection. In conclusion, these findings demonstrate that genes for LH subunits are expressed in the rat ovary, and suggest that LH can play a central role in regulation of female reproduction with both endocrine (by pituitary LH) and auto-, para-crine (by ovarian LH) manner.

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Luteinizing hormone (LH) subunit

LH polypeptide

(RT-PCR)

LH subunit

LH-β subunit(LH<sub>β</sub>) exon

primer RT-PCR

306 bp band가

428 bp band가

hCG common α-subunit(C<sub>α</sub>) PCR

LH standard curve

curve가 sigmoid immunoreactive LH가

PMSG

LH 48 preovulatory LH surge

LH 24 48, 72

LH가

가

LH 가 , LH

(endocrine; LH) (autocrine paracrine; LH)

**Key Words** : LH, Gene expression, Rat ovary, RT-PCR, Tissue specificity, Intraovarian factor

**Table 1.** Sequences and positions of primers used in RT-PCR.

Set	Name	Sequences	Size (bp)
LH $\beta$ - I	P1(5'- primer)	GTGCCGGCCTGTCAACGCAAC	306
	P2(3'- primer)	CAGCTCATTGGTTGAGTCCTG	
LH $\beta$ - II	P3(5'- primer)	TGGAGCTCACTGACCACCATC	482
	P4(3'- primer)	GTACACTGTAGATGCCTGGCA	
C $\alpha$	P5(5'- primer)	GCTACAGAAGATATGCGGCTG	418
	P6(3'- primer)	CGACACTCAGTGCCATCGCAG	
*	dT	ATAGAATTC- dT <sub>25</sub>	for RT

Sequences are all 5' to 3' direction. All oligos were purchased from Korea Bioneer. Primer designs were carried out based on the reported cDNA sequences of rat LH $\beta$  and C $\alpha$ (Godine *et al.*, 1982; Jameson *et al.*, 1984; Zhang *et al.*, 1995b).

## Figure legend

**Figure 1.** Detection of the transcripts for rat LH subunits in the rat ovaries by RT-PCR. PCR and electrophoresis were carried out as described in Materials and Methods. **A;** Amplification of cDNA fragments coding the LH $\beta$  regions commonly found in the rat pituitary and testis. **B;** A set of primers corresponding the exon 1 of the rat testicular LH $\beta$  was used in this PCR. **C;** Amplification of cDNA fragments from the C $\alpha$  transcripts in pituitary, testis and ovary. Lanes I, 100 bp DNA size marker; II, pituitary; III, testis; IV, ovary; and V, negative control.

**Figure 2.** LH RIA parallelism with increasing amounts of LH standards and the ovarian tissue extracts, indicating the presence of immunoreactive LH-like molecules in the rat ovary. In this experiment, ovaries from immature rats (Day 25) were used.

**Figure 3.** Differential effect of PMSG on changes in serum LH levels and the ovarian LH contents. CON; sera or tissue extracts from immature rats (day 25). Bar represents the mean level ( $\pm$ S.E.) of repeated experiments (n=5 to 8).

