EFFECTS OF LUTEINIZING HORMONE-RELEASING HORMONE ON INDUCTION OF DNA POLYMERASE $\alpha$ ACTIVITY AND DNA SYNTHESIS IN HYPOPHYSECTOMIZED IMMATURE RAT OVARIES

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ABSTRACT
To gain some understanding as to how the luteinizing hormone-releasing hormone (LH-RH) is involved in cell proliferation and division in ovaries, deoxyribonucleic acid (DNA) polymerase activity and DNA content of ovarian follicles from LH-RH-treated hypophysectomized rats were measured. Six hours following LH-RH administration (0.2-200 µg/rat), DNA polymerase $\alpha$ activity per ovary and the specific activity (activity per DNA content) were found to have increased significantly ($P<0.01$) in a dose-dependent manner. Thereafter (for 12 h following the treatment), ovarian DNA content increased significantly ($P<0.01$) with 20 or 200 µg of LH-RH. No significant change could be detected in the DNA polymerase $\beta$ activity as resulting from hormone treatment. From these results, LH-RH appears responsible for inducing DNA polymerase $\alpha$ activity and sequential DNA synthesis during cell proliferation in immature rat ovaries.

The hypothalamic gonadotropin releasing hormone (GnRH) has been reported as having receptors in granulosa, thecal and luteal cells at all stages of cellular differentiation (5), and to both directly stimulate and inhibit ovarian functions (2). However, very few studies have been performed on the effects of this hormone on DNA synthesis and cell division, and no reports are available that would demonstrate additional metabolic pathways leading to DNA replication. The authors recently showed evidence pointing to the possibility that the follicle-stimulating hormone (FSH) induces DNA polymerase $\alpha$ activity required for DNA replication (3) and DNA synthesis in immature rat ovaries (6, 7). If this is actually the case, LH-RH would likely be related in some way to the induction of ovary DNA polymerase $\alpha$ activity during follicle growth.

In the present in vivo study, when immature hypophysectomized rats were treated subcutaneously with LH-RH, significant increases in DNA polymerase $\alpha$ activity or sequential DNA synthesis occurred. The authors thus consider that LH-RH induces DNA polymerase $\alpha$ activity prior to DNA synthesis for cellular proliferation during ovarian follicle growth.

MATERIALS AND METHODS
LH-RH (synthetic LH-RH, gonadorelin in hydrochloride; AY-24, 031-A-26, prepared at Ayerst Research Laboratories, Montreal, Canada) was kindly provided by Drs S. Raiti
Immature female rats of the Wistar-Imamichi strain, obtained from the Imamichi Institute for Animal Reproduction (Saitama, Japan), were maintained in an environment illuminated daily from 0800 to 2000. The rats were hypophysectomized at 21 days of age via the parapharyngeal approach. They were subsequently treated with LH-RH (0.2, 2, 20 or 200 µg/0.2 ml of 0.9% saline/rat, subcutaneously) at 25 days of age, i.e., 4 days following the hypophysectomy. Standard purine chow (CRF, Charles River Co., WA, U.S.A.) and water were always available, but following hypophysectomy, an aqueous solution of 5% glucose was used as the source of drinking water. The animals were killed by decapitation under mild ether anesthesia at 3, 6 or 12 h following LH-RH treatment. The control group received saline alone. Following autopsy, the ovaries were quickly removed and the oviducts and surrounding tissue were stripped off. Large follicles (more than 0.25 mm in diameter) were removed from the isolated ovaries under a dissecting microscope and used for enzyme and DNA measurement. DNA polymerase α (a replicative enzyme in eukaryotes) and β (a repair enzyme) activity was determined from the extent to which [3H]deoxythymidine-monophosphate had been incorporated into the acid-insoluble fraction with activated DNA as a template. DNA content was determined according to Usuki and Shioda (6). The DNA polymerase activity and DNA content were expressed as pmol deoxyribonucleotide 5’-monophosphate (dnMP) incorporated into the DNA fraction in 20 min at 37°C/mg protein and µg/mg protein, respectively (7).

Protein content was determined by the method of Lowry et al. (4); with bovine serum albumin as the standard.

The estradiol-17β (E2) level in the serum at 3, 6 and 12 h following LH-RH treatment was determined by radioimmunoassay. Blood samples were obtained at autopsy and centrifuged at 3,000 rpm for 10 min at 4°C. The sera were frozen at -20°C until assayed for E2. Rabbit antiserum to 6-oxoestradiol-17β-6-oxime-BSA (bovine serum albumin; Teikoku-zoki Co., Tokyo, Japan) was used for the assay of E2.

The cross-reactivities of the E2-antisera with progesterone, 17α-hydroxyprogesterone, testosterone and androst-4-ene-3,17-dione were <0.08, <0.08, 0.29 and 0.44%, respectively.

[2,4,6,7-3H]-E2 (specific activity 130 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) was used as the assay and recovery reagent. The serum was extracted with 8 ml of ethyl ether and evaporated under N2 and air in a heating block at 40°C.

After evaporation of the ethyl ether, the residue was chromatographed on a Sephadex LH-20 (0.5 g) column in benzene and methanol (85:15, v/v). E2 antiserum was added to each of the separated fractions. E2 antiseraum was diluted 1:50,000 in borate buffer (0.05 M; pH 8.0) containing BSA (0.06%), human gamma-globulin (0.05%) and 1.8 × 10^{-7} Ci [2,4,6,7-3H(N)]-E2.

The samples were incubated for 30 min at 20°C. Blanks and standard samples of E2 (0, 20, 50, 100, 200, 400, 800 and 1,000 pg) were assayed in duplicate together with the experimental samples. Bound and free hormones were separated by incubation for 10 min with 0.5 ml saturated ammonium sulphate and centrifugation for 10 min at 3,000 rpm at 4°C.

The supernatant fractions were collected and liquid scintillation counting was then carried out using scintillation fluid (a mixture of 0.3% PPO and 0.03% dimethyl POPOP in toluene and Insta-Gel; Packard Instruments, Downers Grove, IL, U.S.A.; 1:1, v/v, 10 ml). Water blanks were close to zero for E2. Accuracy was assessed by adding known quantities of E2 (0, 20, 50, 100, 200 and 500 pg/ml) to the serum and assaying the extracts. The intersay and intraassay coefficients of E2 were 7.6 and 12.6%, respectively. Linear regression analysis of the results gave the equation y = 1.03x + 0.5 (r = 0.99, P < 0.01) for E2, where y = amount recovered and x = amount added. The mean extraction efficiencies of E2 were 84.3 ± 8.3%. The assay sensitivity of E2 was 10 pg/ml. Data were expressed as pg/ml.

All chemical reagents were of analytical grade and organic solvents were distilled prior to use. The Student's t-test was used for statistical analysis and significant differences were
determined at a level of $P<0.05$.

RESULTS

In untreated or saline-treated hypophysectomized rat ovaries, no significant change in DNA polymerase $a$ or $\beta$ activity, DNA content or specific activity (activity/µg of ovarian DNA) was observed during 12 h following hypophysectomy (Fig. 1). The time course of change in ovarian DNA polymerase activity and DNA synthesis caused by LH-RH (0.2, 2, 20 or 200 µg/rat) were followed in hypophysectomized rats (Fig. 2). DNA polymerase $a$

Fig. 1 Changes in DNA polymerase activity, DNA content and specific activity in immature rat ovaries following hypophysectomy. DNA polymerase activity and DNA content were measured at the times indicated as described in Materials and Methods. DNA polymerase $a$ activity (○, untreated; ●, saline-treated); DNA polymerase $\beta$ activity (△, untreated; ▲, saline-treated); DNA content (□, untreated; ■, saline-treated); specific activity of DNA polymerase $a$ (○, untreated; ●, saline-treated); specific activity of DNA polymerase $\beta$ (△, untreated; ▲, saline-treated). Each value is the mean±SEM (vertical bars) of six observations. There were no statistical differences in DNA polymerase activity, DNA content or specific activity between untreated and saline-treated hypophysectomized rat ovaries.

Fig. 2 Changes in DNA polymerase activity (A), DNA content (B) and specific activity (C) caused by LH-RH in hypophysectomized immature rat ovaries. Hypophysectomized rats were administered 0.2–200 µg/rat of LH-RH at 25 days of age and DNA polymerase $a$ activity and DNA content were then measured as described in the text. Only saline was administered to the control. The specific activity of DNA polymerase $a$ was expressed as DNA polymerase $a$ activity per DNA content of an ovary. Each value is the mean±SEM (vertical bars) of six observations. DNA polymerase $a$ activity (○, LH-RH-treated; ●, saline-treated); DNA polymerase $\beta$ activity (△, LH-RH-treated; ▲, saline-treated); DNA content (□, LH-RH-treated; ■, saline-treated); specific activity of DNA polymerase $a$ (○, LH-RH-treated; ●, saline-treated); specific activity of DNA polymerase $\beta$ (△, LH-RH-treated; ▲, saline-treated); 0.2, 2, 20 and 200 µg correspond to dose of LH-RH used. *significantly different from the control, $P<0.05$; **significantly different from the control, $P<0.01$; ***significantly different from the control, $P<0.001$. 
activity increased gradually following 0.2–200 μg/rat LH-RH treatment, reaching a peak 6 h after treatment and then showed a plateau or slight decrease (Fig. 2A). The ovarian DNA content began to increase from 6 h following LH-RH treatment, becoming significant (P<0.01) at 12 h after 20–200 μg/rat LH-RH treatment (Fig. 2B). In Fig. 2C, the specific activity of DNA polymerase α has also increased significantly (P<0.01) from 6 h following LH-RH treatment. More effective stimulation was obtained with 20–200 μg/rat of LH-RH than with 0.2–2 μg/rat. However, there was no significant difference in DNA polymerase α activity, DNA content or specific activity between 20 and 200 μg/rat of LH-RH. DNA polymerase β activity or specific activity of DNA polymerase β did not change at these doses (Fig. 2, A and C).

DNA polymerase α activity in the follicular extracts used in this experiment is additive; neither activators nor inhibitors could be detected in the extracts (Fig. 3). To investigate the effects of serum E2 on ovarian polymerase α activity, the E2 level was measured. Serum E2 levels at 3, 6 and 12 h following LH-RH treatment (0.2–200 μg/rat) ranged from 65.0 to 109.2 pg/ml, these levels being not statistically significant compared with those in the control group (87.2±3.5; mean±SEM).

DISCUSSION

Although many studies have demonstrated the direct stimulatory effect of LH-RH itself on ovarian functions, very little work has been done in humans and rats in regard to the direct effects of LH-RH on cellular proliferation during ovarian follicular growth. Recently, Usuki and Shioda (6), and Usuki and Usuki (7) found FSH or estrogen to increase DNA polymerase α, an essential enzyme for DNA replication in eukaryotic cells (3), activity per cell in vivo in the rat ovary and this increase in activity to be accompanied by DNA synthesis. In the present in vivo study, DNA polymerase α activity, which tends to decrease following hypophysectomy in intact rat ovaries (6), was enhanced significantly by LH-RH treatment, indicating LH-RH to possibly increase DNA polymerase α activity in vivo in immature rat ovaries.

An activator of the enzyme may not be involved in this increase, since enzyme activity was found to be additive when LH-RH-treated and -untreated ovarian extracts were mixed and assayed for activity. This increase in activity caused by LH-RH thus apparently arises from increase in the enzyme molecule itself.

Nuclear DNA content per ovary increased significantly during 12 h following LH-RH treatment but did not change with increased induction of DNA polymerase α.

The specific activity of DNA polymerase α (DNA polymerase α activity per μg DNA) also increased with DNA polymerase α activity. These findings confirm the observation in the previous study (6) that the level of DNA polymerase α activity is controlled at the cellular level in the ovary.

The present study used ovarian follicles, making it difficult to determine ovarian cells responsible for induction of DNA polymerase α activity. This is because ovarian GnRH receptors are widely distributed in luteal, thecal and granulosa cells at all stages of cellular differentiation (5). The cellular site of action of GnRH remains to be determined.

Dorrington et al. (1) reported LH-RH to
independently stimulate estrogen production in granulosa cells in culture. However, in our in vivo experiment, the serum E$_2$ level showed no significant change compared with that in the hormone-untreated controls, indicating LH-RH to possibly not have indirectly enhanced DNA polymerase $\alpha$ activity or DNA content via E$_2$ production. The mechanism involved in the latter also remains to be elucidated.

Since enzyme induction should be a prerequisite for DNA synthesis during ovarian growth (6), these findings give some grounds for considering that LH-RH functions to induce DNA polymerase $\alpha$ prior to DNA synthesis in the ovary. The physiological significance of the direct ovarian action of LH-RH is also a very intriguing problem that should be investigated, in regard to both basic and clinical aspects. The results of such a study may provide unique models for understanding the mechanism of LH-RH action in cellular proliferation during ovarian follicular growth.

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