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RENOTROPIC ACTIVITY OF LUTROPIN: DIRECT STIMULATION OF DNA SYNTHESIS OF CULTURED RAT RENAL CORTICAL CELLS*

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Two differently purified ovine lutropin (LH) preparations were studied to see if they stimulated [^{3}H] thymidine incorporation into cultured rat renal cortical cells. Both preparations showed a dose-dependent (0.11-1 ng/ml), time-dependent (peaking at 18 h), serum-dependent (7.5% of fetal calf serum or 10% castrated-hypo-physectomized rat serum) renotropic effect. Ovine TSH and FSH failed to mimic this specific, renotropic effect. We concluded that LH directly stimulates renal DNA synthesis in cooperation with other serum factor's.

Hypophysectomy causes renal atrophy(1,2) and inhibits compensatory renal growth following unilateral nephrectomy(3,4), suggesting that the pituitary factor plays an important role in renal growth. We demonstrated that ovine lutropin (oLH) preparation increased [^{3}H] thymidine incorporation into the renal DNA of the castrated-hypophysectomized rat (in vivo)(5). Radioautographic studies showed that an oLH preparation stimulated DNA synthesis of the proximal tubules and endothelial cells of the outer medulla(6). The analysis of amino acid sequences showed that the purified preparation (G100-fr.3) consisted of heterogeneous LH isoforms; G100-fr.3 lost its renotropic activity when dissociated

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from α and ß subunits, and regained it when reassociated with these subunits(7).

These findings made it clear that renotropic activity was an intrinsic LH-isoform property.

We studied whether LH isoforms directly stimulate renal DNA synthesis.

Materials and Methods

A highly purified renotropic fraction (G100-fr.3) was prepared from an oLH fraction(8) by chromatography on carboxymethyl-cellulose (Whatman) with a discontinuous ionic strength gradient, Concanavalin A-Sepharose (Pharmacia Fine Chemicals), and Sephadex G-100(7). Briefly, the oLH fraction was added to a carboxymethyl-cellulose column (2.6 × 25 cm) equilibrated with 10 mM of sodium phosphate buffer, pH 6.0. The column was developed with this buffer for about 100 ml, then the pH was increased to 7.5 (200 ml); the active fraction was finally eluted when 0.3 M of NaCl was added. This fraction was dialyzed against water, lyophilized, dissolved in 10 mM of Tris-HCl, pH 7.5, containing 0.3 M of NaCl, and added to a Concanavalin A-Sepharose column (1.8 × 19 cm) which was then equilibrated and developed for about 50 ml with the same buffer. The active fraction was eluted by additing 0.2 M of α-methyl-mannoside to the buffer. Following dialysis against water and lyophiliza- tion, the renotropic fraction was chromatographed on a Sephadex G-100 column (2.2 × 200 cm) which was equilibrated and developed with 1% ammonium bicarbonate. The final yield was about 400 mg from 1 kg of lyophilized ovine pituitary powder (Waitaki International Ltd., Christchurch, New Zealand).

Highly purified oLH (NIAMDD-oLH-24), oFSH (NIH-FSH-S9), and oTSH (NIAMDD-oTSH-9) were kindly provided by the National Hormone and Pituitary Program through the courtesy of Dr. S. Raiti (Baltimore, MD).

Two pairs of kidneys were obtained from 4 week-old two male Sprague-Dawley rats anesthetized with pentobarbital.

After decapsulation, the kidneys were cut in half and the inner medulla mechanically removed with a razor blade. Resulting chips of kidney were minced and suspended in 10 ml of Hanks' solution containing 1500 ng/ml of Dispase (Sanko Junyaku Ltd., Tokyo). After gentle mixing by a magnetic stirring bar at 20°C for 20 min, the suspension was filtrated through nylon mesh (No. 80) and centrifuged at 100 g for 5 min.

The centrifugate, enriched in tubular fragments, was suspended in 5 ml of Ham's F-10 and further fractionated using discontinuous Ficoll gradient as Scholer and Edelman reported(9). Briefly, Ficoll (polyscore, mol wt 400,000, Sigma) was freshly dissolve of in Ham's F-10 in concentrations of 1, 2, 6, 8 and 12% (wt/wt, pH 7.4), corresponding to a density of 1.013-1.053 g/ml. A discontinuous gradient consisting of 30 ml layers was generated in a 200 ml beaker (ID 52 mm) and five ml of the pool suspension were carefully loaded on the gradient. When the particle front reached the 8%/12% interphase, 20 ml, 50 ml, and two 30 ml fractions were collected from the top. Fraction 4, enriched in proximal segments(9), was centrifuged and the fragments washed three times and resuspended in Ham's F-10 medium containing 10% fetal calf serum (FCS) (10^5 cells/ml).

Aliquots of 350 µl of the suspension were plated in four 24-well dishes (A/S Nunc, Denmark) and incubated at 37°C in 5% CO_2-95% air. 48

hours after plating, the medium was removed and cells were incubated in Ham's F-10 medium without FCS for 24 hours. The medium was then changed to Ham's F-10 containing FCS and the sample. 18 hours after addition of the sample, [1,2-methyl-^{3}H] thymidine (101 Ci/mmol; New England Nuclear Corp., Boston, MA) was added to cultures at a final concentration of 1 μCi/ml. 2 hours later, cells were washed twice with cold Hanks' solution and incubated with 10% trichloracetic acid (TCA) for 15 minutes at 4°C. After subsequent washes with cold 10% TCA and ethanol-ether (3:1), precipitates were dissolved by additing 0.5 ml of 1 N NaOH. The solution, neutralized with 1 N HCl, was then moved to a scintillation vial with 5 ml of ACS II (Amarsham Corp., IL.) and its radioactivity counted in a scintillation counter.

In vitro LH activity was assayed by determining cyclic AMP accumulation in cultured rat leydig cells obtained by the method of Ramachandran and Sairam (10). The sample was incubated for 1 h with 2 mM of 3-isobutyl-1-methy-xanthine (Sigma Chem, Co.), and the reaction was stopped by immersing the dishes in boiling water for 5 min. The accumulated cyclic AMP was measured by commercial radioimmunoassay kit obtained from New England Nuclear (Boston, MA).

Student's t test was used for statistical analysis, and results were described by means ±SE.

Results

G100-fr.3 stimulated [^{3}H] thymidine uptake in the presence of 7.5% fetal calf serum (FCS) or 10% castrated-hypophysectomized rat serum (CHRS), but not in the presence of 2.5% FCS (Fig. 1).

In the presence of 7.5% FCS, [^{3}H] thymidine uptake reached the peak at 18 hours after the addition of 0.3 ng/ml of G100-fr.3 (Fig. 2).

Two LH preparations, G100-fr.3 and NIAMDD-oLH-24, were compared in vitro renotropic and gonadotropic activity (Fig. 3). No significant difference was observed in terms of renotropic activity, although G100-fr.3 showed weaker gonadotropic activity than NIAMDD-oLH-24.

No significant renotropic activity was observed in ovine pituitary glycoproteins oTSH and oFSH examined between 0.11-10 ng/ml under the same conditions under which G100-fr.3 showed significant activity (data not shown).

Discussion

Our major conclusion is that LH can directly stimulate DNA synthesis at low concentrations. This renotropic activity was dose-responsive and time-dependent. The fact that FSH and TSH did not show renotropic activity suggests the specificity of this assay. Relatively high

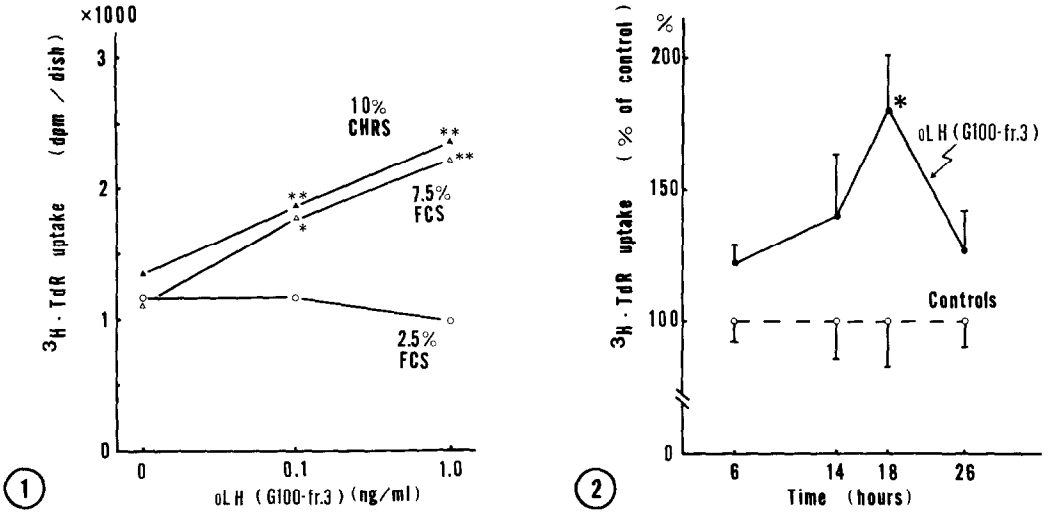

Fig. 1. Synergistic effect of G100-fr.3 and serum on [^{3}H] thymidine uptake.
[^{3}H] thymidine uptakes were determined at 18-20 h after addition of G100-fr.3 and serum.
FCS indicates fetal calf serum, and CHRS is obtained from rats who was castrated and hypophysectomized 12 days earlier. Each point indicates a mean of six dishes.
**, p< 0.01 vs controls.

Fig. 2. Time-course of [^{3}H] thymidine uptake after addition of G100-fr.3. Each point indicates a mean of six dishes.
*, p< 0.05 vs controls.

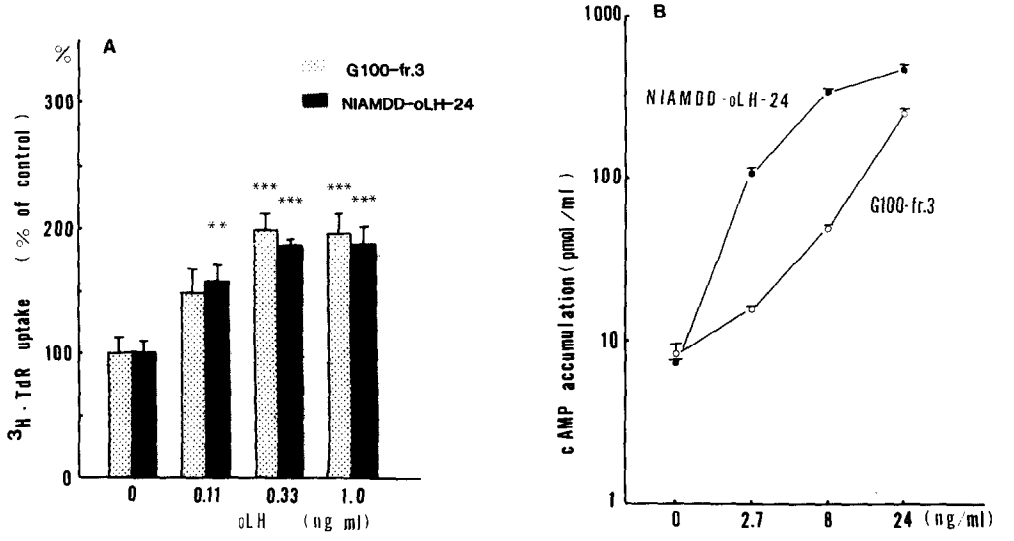

Fig. 3. Dose-responsiveness of G100-fr.3 and NIAMDD-oLH-24.
A) [^{3}H] thymidine uptake into cultured rat renal cortical cells. n=6
B) Cyclic AMP accumulation in cultured rat Leydig cells. n=3
**, P< 0.01, ***, P< 0.001 vs controls.

concentrations of FCS were needed to express the activity, indicating that LH and other serum factor works synergistically. Because castrated-hypophysectomized rat serum could be substituted for FCS, this cryptic factor(or factors) seems unlikely to be pituitary-derived, pituitary-factor-dependent, androgen, or androgen-dependent factor.

NIAMDD-oLH-24 demonstrated significant renotropic activity in the *in vitro* system but no activity in the *in vivo* bioassay (5). This discrepancy suggests that the metabolic clearance rate may be important, or that cultured cortical cells may react differently from cells *in situ*. In future, it may be possible to measure the difference *in vitro* renotropic potency between G100-fr.3 and NIAMDD-oLH-24 by using markers that represent LH's renotropic effect instead of [^{3}H] thymidine uptake. Both preparation showed different gonadotropic potency in the cultured Leydig cells.

Ours is the first demonstration of the direct renotropic effect of LH to our knowledge.

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