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Influence of luteinizing hormone-releasing hormone (LHRH) treatment on cellular proliferation in the rat anterior pituitary

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Summary. This study was designed to gain insight into the action of LHRH on the control of cellular proliferation in the anterior pituitary. The fraction of cells labelled with bromodeoxyuridine (S-phase cells) was studied in cytospin preparations of anterior pituitary cells taken from control male and female rats and from rats treated with daily doses of 2 μg/100 g body weight of LHRH (7 days), with doses of 40 μg of LHRH given on alternate days for 14 days (7 doses) or, finally, treated with daily doses of 50 ng of busereline acetate (14 days). Treatment with LHRH for 14 days resulted in a significant increase in the fraction of S-phase cells. However, neither the blockade of gonadotrophin secretion with busereline acetate nor its stimulation with LHRH for seven days resulted in a significant change in the proliferative activity of anterior pituitary cells. This action was independent of sex. No significant changes were seen in the proportions of pituitary gonadotrops of the different study groups. Regardless of the treatment-group very few cells doubly-immunostained for BrdU and LH were found. It is concluded that LHRH may stimulate cellular proliferation in the anterior pituitary, but further studies are needed to define which cells are involved in this action.

Key words: Cell proliferation, Pituitary gland, LHRH, Rat

Introduction

The physiological regulation of pituitary growth depends upon complex interaction of hypothalamic, intrapituitary and circulating peptides. Several of the hypothalamic peptides which regulate pituitary function such as thyrotrophin-releasing hormone (TRH) (Kunert-Radeck and Pawlikowsky, 1975), corticotrophin-releasing hormone (CRF) (McNicol et al., 1988) growth hormone-releasing factor (GRF) (Billestrup et al., 1986) and dopamine (Stepien et al., 1978) are known to have mitogenic actions. However, the action of luteinizing hormone-releasing hormone (LHRH) on the proliferative activity of anterior pituitary cells is controversial (Carbajo et al., 1990).

It is well known that depending on the pattern of administration, LHRH either stimulates or inhibits the release of gonadotrophins (Schuiling et al., 1984). The potential effects of LHRH on the proliferative activity of anterior pituitary cells might also depend on its pattern of administration. We therefore studied the effects on the proliferative activity of rat anterior pituitary cells of different dosage patterns of LHRH and those of busereline acetate, a potent synthetic analogue of the LHRH, in an attempt to gain insight into the action of LHRH, on the control of cellular proliferation in the anterior pituitary.

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Materials and methods

Animals

Thirty-day-old Sprague-Dawley rats were caged separately and kept in our animal facilities with food and water available *ad libitum* in controlled lighting (on at 08:00 h; off at 20:00 h). Three different groups of animals (4 males and 4 females each) were treated as follows: one group received a daily intramuscular injection of 2 μg of LHRH/100 g of body weight dissolved in 0.1 ml of saline (injection time 09:00 h) for seven days; another group was made up of animals that received 40 μg of LHRH in 0.1 ml of saline on alternate days for 14 days (seven doses) and, finally, a third group of animals was subcutaneously injected twice daily with 50 ng of busereline acetate (Suprefact®, Behring), an LHRH analogue, for 14 days. Three other groups of animals (4 males and 4 females each) were used as controls and received injections of saline with the same pattern as their LHRH-treated counterparts.

Labelling of S-phase cells with bromodeoxyuridine

On the last day of treatment all animals received an intraperitoneal injection of 50 mg/kg of bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO, USA) at 12:00 and were killed 60 minutes later by cervical dislocation.

Cytological preparation

The pituitary was removed and the intermediate lobe discarded. Anterior pituitaries from animals of the same group were pooled and enzymatically dispersed into single cells as described elsewhere (Carbajo et al., 1992). Aliquots of 100 µl containing approximately 4 x 10^4 cells from cell suspensions of the different groups of the study were centrifuged onto microscope slides using a Shandon Cytocentrifuge (1100 r.p.m.). Cytospins were allowed to dry at room temperature and frozen (-20 °C) until used.

Immunocytochemistry for bromodeoxyuridine

At least five cytospins per group were allowed to thaw, fixed for 5 min in 70% ethanol, washed in distilled water and rinsed in phosphate-buffered saline (PBS; 0.1M, pH 7.4). DNA was denatured by incubating cytospins for 45 min in 95% formamide in 2X standard sodium citrate (SSC) at 60 °C. Next, samples were incubated with monoclonal antibody to BrdU (1:20; Dakopatts, Glostrup, Denmark) for 60 minutes at room temperature followed by incubation with 1:50 dilution of peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 45 minutes. The final reaction product was revealed with 3,3'-diaminobenzidine (Sigma) (DAB, 15 mg in 100 ml of 0.05M Tris-hydrochloric buffer plus 100 µl of 0.3% H_2O_2). Preparations were lightly counterstained with Mayer haematoxylin and mounted with DPX.

Immunocytochemistry to identify LH-producing cells

Once rinsed in PBS cytospin preparations were incubated with serum anti human luteinizing hormone (a-hLH; 1:00) (Dakopatts) for one hour at room temperature followed by incubation with guinea-pig anti-rabbit immunoglobulin (Ig)G at a dilution of 1:100 and, finally, with rabbit peroxidase anti-peroxidase (PA) (Dakopatts). The final reaction product was visualized with DAB. Some of these slides were further processed to reveal the incorporated BrDU, the final reaction product then being revealed with nickel-intensified DAB (black) (Vector Laboratories, Burlingame, CA, USA).

Data processing

Duplicates of all the groups of the experiment were carried out. A thousand cells were counted on each of five cytospins per group to calculate the BrdU labelling index (LI = number of nuclei labelled with BrdU divided by the total number of cells x 100). The proportion of anti-LH-positive cells was also calculated in five slides per group. Statistical analysis of proportions was carried out after inverse sine transformation by one-factor ANOVA at a level of significance of 95%. For two-group comparisons the Scheffe F-test was used.

Results

BrdU-labelled nuclei were easily identified on cytospin preparations from the different groups of our study (Fig. 1). This allowed for the quantification of S-phase cells and the subsequent calculation of a BrdU labelling index (BrdU-LI=BrdU-labelled cells per hundred cells). Numerical data are shown in Table 1. The BrdU-LI was similar in the three groups of control animals. No sex difference was found either in control or treated animals. The BrdU-LI of the group treated with 40 µg of LHRH on alternate days was greater than that of control animals. No difference was found between the controls and the other two groups of treated animals.

Double immunostaining for BrdU and LH resulted in the identification of both S-phase cells and LH producing cells on the same slide (Fig. 2a-e). The LH immunoreaction was stronger in cytospin preparations from the control animals than those from treated animals. The LH-immunoreaction was particularly weak in samples from animals treated with 40 µg of LHRH on alternate days. Regardless of the group, cells doubly-immunostained with anti-LH and anti-BrdU sera were rarely found (Fig. 2d). In all cases the proportion of LH-

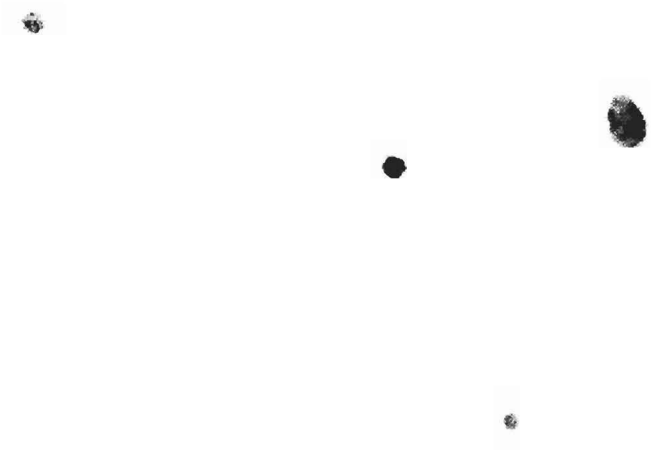
Table 1. Proportions (mean±SEM) of BrdU-labelled cells and LH-cells obtained from cytospin preparations corresponding to control rats and to rats treated with LHRH and busereline acetate.

	MALES		FEMALES	
	BrdU-LI	LH cells (%)	Brdu-LI	LH cells (%)
LHRH 2µg	1.8±0.1	5.9±0.4	1.9±0.3	6.9±0.5
Control	1.7±0.2	7.1±0.8	1.6±0.2	7.1±0.9
LHRH 40µg	2.6±0.2*	4.8±0.7	2.8±0.3*	6.5±1.0
Control	1.6±0.1	6.2±0.9	1.8±0.3	8.1±0.7
Busereline acetate	1.8±0.2	4.7±0.7	1.8±0.3	6.9±0.9
Control	1.6±0.3	6.9±0.5	1.8±0.2	7.6±0.8

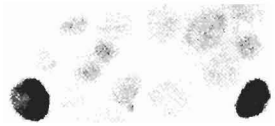
*: statistically significant difference (Scheffe F test).

Fig. 1. BrdU-labelled nuclei are easily identifiable on cytospin preparations of rat anterior pituitary cells. x 225

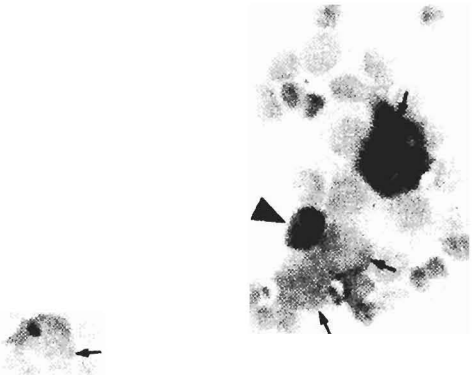
Fig. 2. Cytospin preparations doubly immunostained with anti-LH (light; arrows) and anti-BrdU (dark; arrowheads) sera taken from control animals **(a)** and animals treated either with daily doses of 2 µg of LHRH/100 g of body weight **(b)**, treated with 40 µg of LHRH on alternate days **(c-d)** or with busereline acetate **(e)**. Note that the LH immunoreaction is stronger in the control group than in the treated animals. Doubly immunostained cells (d) are rarely found. Figs. 2a,b,c and e, x 450; Fig. 2d. x 520



1



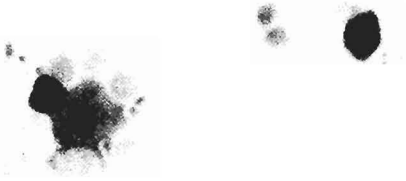
2c



2a

2d

2b



2e

immunoreactive cells tended to be smaller in treated animals than in their control counterparts (Table 1).

Discussion

The action of the LHRH on the proliferative activity of anterior pituitary cells is controversial. Stepien (1981) reported a dose-dependent inhibitory effect of LHRH on (^{3}H)-thymidine uptake in cultured anterior pituitary cells. Inoue et al. (1986) found no change in the mitotic activity of pituitary gonadotropes from castrated rats after LHRH administration. More recently, Sakai et al. (1988) showed that, in castrated rats, both the increased serum levels of gonadotrophins and the increased mitotic activity of gonadotropes were significantly reduced by passive immunization to LHRH, and concluded that LHRH is an important factor for the stimulation of proliferation of pituitary gonadotropes in castrated rats. No definitive conclusions on the action of LHRH on cellular proliferation of the anterior pituitary can be drawn from the above-mentioned studies; this is consistent with the remarkable differences between data on cellular proliferation of the anterior pituitary obtained from in vivo and in vitro experiments (Carbajo-Pérez and Watanabe, 1990; Watanabe and Carbajo-Pérez, 1990; Carbajo et al., 1992).

The present work was designed to gain insight into the action of LHRH on the control of cellular proliferation in the anterior pituitary. Bearing in mind that this action might be linked to the effects of LHRH on gonadotrophin secretion, the fraction of BrdU-labelled cells was studied in cytospin preparations of anterior pituitary cells taken from animals treated with LHRH in three different ways. Firstly, a short-term treatment (7 days) with small doses of LHRH (2 μg/100 g of body weight) was used. As shown by Garner et al. (1990), this treatment should result in increased gonadotrophin secretion. However, the action of LHRH on the proliferative activity of anterior pituitary cells might require longer exposure times. Accordingly, another group of rats was treated with high doses of LHRH (40 μg) on alternate days for fourteen days, since this dosage has also been shown to increase gonadotrophin secretion (Carbajo et al., 1987). Finally, to know the effect of the blockage of gonadotrophin secretion on the cellular proliferation of the anterior pituitary, daily injections of 50 ng of busereline acetate, a dosage known to block gonadotrophin secretion (Sandow, 1980), were given for fourteen days. Since sex differences in pituitary LH storage and release appear in LHRH-treated rats (Watanabe, 1986), male and female rats were included in the different groups of treatment and studied separately.

In our study, neither the blockage of gonadotrophin secretion nor its stimulation with LHRH for a short period of time resulted in significant changes in the proliferative activity of anterior pituitary cells. However, sustained stimulation with LHRH for 14 days rendered a higher proportion of S-phase cells. Thus, it seems that any action of LHRH on the proliferative activity of the anterior pituitary would require a longer exposure to the stimulus than the actions of LHRH on the modulation of gonadotrophin secretion. This action was independent of sex since no differences were found between male and female rats in our study.

Pituitary gonadotropes are most probably responsible for the increased proliferative activity of the anterior pituitary found after LHRH treatment. However, this point cannot be confirmed from our results. The percentage of LH cells tended to be smaller in treated animals but no significant changes were found when the proportions of LH-immunoreactive cells from controls and LHRH-treated animals were compared. It should be mentioned that a clear reduction in the intensity of immunostaining was observed in the treated animals, this most probably being caused by a depletion of immunoreactive material from gonadotropes.

Quantification of cells doubly immunostained for anti-LH and anti-BrdU sera might have given accurate information on whether the increase in the proportion of proliferating cells was due to a specific increase in the proliferative activity of pituitary gonadotropes. However, regardless of the experimental group, very few doubly-immunostained cells were found. To record a number of doubly-immunostained cells large enough to be able to make a proper statistical evaluation, many more slides should have been processed. This raises an important methodological question. Study of cellular proliferation on cytospin preparations was decided on the basis that individual variation is minimized since the pituitaries from different animals are pooled and because the identification of doubly-immunostained cells is simpler than in tissue sections (Carbajo et al., 1981). This method has proved to be useful for calculating the global proliferative activity of anterior pituitary cells but has not been effective in the evaluation of variations in the proportions of proliferating gonadotropes. Double-immunostaining for BrdU and anterior pituitary hormones might be the method of first choice to study the proliferative activity of somatotropes or lactotropes (which are by far the largest cell populations of the anterior pituitary) although, in the light of our results, to study relatively small cell populations of the anterior pituitary such as gonadotropes, paraffin sections should be used since these permit the screening of greater areas and considerably increase the cellular population under study.

It is concluded that LHRH may stimulate cellular proliferation on the anterior pituitary but further studies are needed to define which cells are involved in this action and the extent to which this action may be of physiological significance.

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