A Quantitative Immunohistochemical Study on the Pituitary LH Gonadotrophs in the Female Afghan Pika after Copulation

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Abstract

The female Afghan pika is known as a species of copulatory ovulators exhibiting persistent estrus unless copulatory stimuli are employed. The plasma LH concentration rose quickly after copulation, peaked at 6 h and descended thereafter until 12 h. Before copulation (estrus), immunoreactive LH-containing cells of the pituitary in this species were filled with numerous large electron dense secretory granules, but the Golgi apparatus and the granulated endoplasmic reticulum were not well developed. One hour later the stainability of the cells was faint, their secretory granules were small in size and in number, and were of low electron density. These alterations were the most predominant at 12 h after copulation. No exocytotic extrusion of secretory granules was observed throughout the experiment.

Female Afghan pika (Ochotona rufescens rufescens) recently available as a laboratory animal exhibits persistent estrus when kept under long-day conditions (Puget, 1973 a, b, c), and performs copulatory ovulation. Because of its unique multi-ovulation and multi-luteinization characteristics (Sazuki, 1984), it may be expected that some abrupt and dramatic morphological changes in gonadotrophs take place in association with gonadotrophin release after copulation.

In earlier papers, we described the ultrastructural and immunohistochemical characteristics of gonadotrophs and other pituitary cells in the female Afghan pika (Nakamura et al., 1986 b), and in female rats (Nakamura et al., 1985), and have shown that the immunostainability and immunoreactive population of LH cells, and mean diameter and population of their secretory granules may be suitable morphological indices of their secretory function.

In this study, we quantitatively explored immunohistochemical and ultrastructural changes in LH cells of the pika in correlation with the changes in the plasma LH concentration during the post-coital 24 hrs.

Materials and Methods

Animals

Adult (about 150 day-old) female Afghan pikas (Ochotona rufescens rufescens) were kept in a room with controlled temperature and lighting (lights on 0500–1900 h) and allowed food (CIEA

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Radioimmunoassay
Systemic blood was collected by heart puncture just prior to the autopsy and centrifuged at 4°C to separate the plasma from the blood cells. The plasma was stored at −20°C until assayed. The plasma concentration of LH was measured using the NIAMDD-rat-LH radioimmunoassay kit. The antiserum used was anti rat LH s-6. The reference preparation used as the standard was rat LH-RP-1. The preparation for iodination was rat LH-I-5.

Immunohistochemistry
The excised anterior pituitary glands were cut sagittally into two halves. For light-microscopic immunohistochemistry, one half was fixed in formol-sublimate solution (3: 7) for 16 h at 4°C. After dehydration in ethanol, they were embedded in Paraplast. Sections (2 μm thick) were prepared with a Porter type II ultratome and supplied for immunostaining. For electron-microscopic observation, the other half of each gland was cut into small pieces with a razor blade and fixed in a 1% glutaraldehyde solution in 0.1M phosphate buffer, pH 7.4, for 2 h at 4°C, then washed with 0.1M phosphate buffer containing 2% sucrose. The tissues were post-fixed in 1% OsO₄ made up with Millonig's buffer for 2h at 4°C. After dehydration in ascending concentrations of cold ethanol and treatment with propyleneoxide, they were infiltrated and embedded in Epon-Araldite. An ultrathin section, 0.05 μm thick, was available for EM observation, and the adjacent thick section, 2 μm thick, was immunostained with antihuman LHβ serum after removal of Epon for LM observation by treating with 14% sodium methylate (NaOCH₃) in methanol for 15 min, followed by immersion in 2% hydrogen peroxide (H₂O₂) for 30 min at room temperature. The superimposition technique was applied by the procedure of Yoshimura and Nogami (1981). The ultrathin sections were counterstained with uranyl acetate and lead citrate, and observed with a JEOL 100 B electron microscope.

Immunostaining was carried out according to the Avidin-Biotin-Proxidase complex (ABC) method (Su-Ming et al., 1981), using an ABC kit from Vector (California USA). The all sections were incubated with anti-human LHβ serum (a gift from Dr. A. F. Parlow, NIH, Bethesda, Maryland, U. S. A.) at 30°C for 2 hr. The Specificity of this antiserum was confirmed by adsorption test as described in an earlier paper of Nakamura et al. (1986 b). In order to colorize all the sections, they were incubated at 25°C for 5 min in DAB solution (0.05 M Tris HCl buffer containing 0.004% 3, 3' -diaminobenzidine tetrahydrochloride, and 0.003% H₂O₂, pH 7.4).

The percentage of intensely or weakly immunostained LH cells was counted in light microscopy of Paraplast sections (a total of about 200 immunostained LH cells in each animal).

In addition, the intensity of immunoreactivity of all the 120 LH cells in three Paraplast sections in each hour group were measured quantitatively with microspectrophotometry using a multipurpose microspectrophotometer (MMSP-Tu; Olympus Co., Tokyo, Japan) with 3 μm of spot size at 453 nm wavelength which was the maximum extinction wavelength of DAB (Fahimi and Herzog, 1973). The validity of this quantitative measurement using microspectrophotometer was already reported by some investigators (Shirasawa et al., 1983; Terlou et al., 1983; Nakamura et al., 1985).

For ultrastructural observation, we examined the first 8 immunostained cells randomly selected from an adjacent section. Enlarged electronmicrographs, of which the area was equivalent to approximately 70 cm² at a magnification of ×50000, were used for the measurement of size and number of secretory granules as reported previously by Nakamura et al. (1985). Thus, 24 immunostained LH cells per group were employed for quantitative measurements.

Results
Immunoreactivity of LH cells
Before copulation or ovariectomy, both the population of intensely stained LH cells and their staining intensity were high (Fig. 1A), but after copulation (Fig. 1B) or ovar-
Fig. 1. LH cells immunoreactive for anti-human LH in the female Afghan pikas. Arrows show the intensely immunostained cells. ×530

A. normal before copulation. They are generally not uniform in shape.

B. 1 h after copulation. Immunostainability of most LH cells is low, but intensely stained ones remain (arrow).

C. ovariectomized on Day 60 and killed on Day 120. The immunoreactivity of many LH cells is low as seen 1 h after copulation.
iectomy (Fig. 1C), their number diminished and their staining intensity was lowered although the total number (about 200) of LH cells per section remained unchanged after copulation. The optical density (OD) of the immunostained LH cells was measured for the objective criterion of immunostainability. When the OD of about 100 immunoreactive LH cells were measured at random at 1 h after copulation, the bimodal cell population based on absorbance at a wavelength of 453 nm was observed, that is, two peaks at 0.2 and 0.35 in OD, as shown in Fig. 2. The existence of two peaks strongly suggested the presence of two kinds of LH cells consisting of weakly and
intensely immunostained cells.

The intensities of immunostainability of LH cells were measured to compare the pre- and post-copulation periods. Sixty weakly immunostained cells randomly chosen from all groups were given a value, nearly 0.2 in OD (Fig. 3). As to the intensity of intensely stained LH cells, a high pre-copulatory value, about 0.55 in OD, was significantly reduced by copulation, but it still remained

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Fig. 4. Ultrastructure of LH cells in female pikas before copulation (A), 1 h after copulation (B) and ovariectomized (C). ×6100. Insets show immunostained LH cells on the adjacent thick sections. ×1300. A, shows an intensely stained LH cell laden with large secretory granules; B, shows LH cells with remarkable deprivation of the secretory granules; C, shows conspicuous degranulation; G, the Golgi apparatus.
Electron-microscopical observations

Before copulation (Fig. 4A), most LH cell possessed the numerous electron dense spherical secretory granules and occasional moderately dilated rough endoplasmic reticulum (RER). One hour after copulation, however, the secretory granules decreased considerably in number and in electron density and Golgi apparatus and dilated RER appeared prominently (Fig. 4B). Despite the loss of secretory granules, there was no evidence of exocytosis of secretory granules throughout the period after copulation (1–24 h). In LH cells of the ovariectomized female pikas (Fig. 4C), Golgi apparatus and RER cisternae were enlarged and a few of the smallest, low electron dense secretory granules remained. Both copulation and ovariectomy deprived secretory granules of LH cells, and reduced their size and electron density.

Changes in the diameter of secretory granules

The Distribution pattern of the secretory granules indicates that the diameter is longest (Oh; mean 216.2 nm) before copulation (Fig. 5). After copulation, however, the diameter tended to decrease moderately and eventually reached the minimum value at 6 h (mean 183.5 nm) and rose again by 24 h (mean 202.3 nm). In the ovx pika, the diameter of secretory granules was the smallest (171.2 nm), compared to those in normal and copulated females.

Immunohistochemical and ultrastructural changes in LH cells in correlation with plasma LH concentration

After 1 h, the plasma LH concentration rose to twice as high as before copulation, and reached the maximum (about 3.5 times) at 6 h, but decreased again at 12 h (Fig. 6A). The intensely stained LH cells made up about 80% of the immunoreactive cells before copulation, but quickly decreased to about 20% in 6 h (Fig. 6B). Immunostainability of the cells (Fig. 6B) was expressed in terms of OD at 453 nm wavelength. The numerical value (the ratio of weakly stained cells × their averaged OD + the ratio of intensely stained cells × their averaged OD) was used for the index used to estimate the whole immunostainability of the LH cells in the gland of each animal. The whole im-
munostainability of LH cells decreased after copulation and the number of intensely stained LH cells also decreased. The index of total granule volume (V) was expressed in terms of the product of averaged diameter (2r) to the total number of secretory granules (n), using the formula \( V = n \times r^3 \times 10^{-8} \). As shown in Fig. 6C, the total granule number and volume decreased coincidently after copulation.

**Discussion**

In the female Afghan pika, quantitative expressions of immunohistochemical and ultrastructural findings seem to be useful in characterizing the LH gonadotrophs related to LH secretion. Terlou *et al.* (1983) successfully employed microspectrophotometry in quantitative measurement of the intensity
of immunostainability of gonadotrophs in juvenile rainbow trout. A close relationship between immunohistochemical or ultrastructural changes in LH cells and LH secretion from the anterior pituitary gland demonstrated during the estrous cycle of female rats (Blake, 1980; Nakamura et al., 1985).

We could not accurately determine the plasma LH concentration in the pika, because the antiserum to rat LH was used for the present radioimmunoassay. However, the values obtained in the present radioimmunoassay may be worth using in studying the LH surge and peri-ovulatory events. It was not found that the increase in the plasma FSH concentration accompanied the LH surge in the pika (Ota and Suzuki, unpublished data).

As already mentioned in our preceding paper (Nakamura et al., 1986 b) almost all LH cells react also with FSH antiserum in the normal (estrus) adult female of the Afghan pika and they are small in the size and irregular in shape, compared to those in the rat. Prior to copulation (Oh), most LH cells in the pika were highly immunoreactive and laden with the numerous large electron dense secretory granules. After copulation, the reduction in immunostainability, diameter, number and density of secretory granules in LH gonadotrophs were characteristics of morphological indications of LH surge. The reversed correlation of the diameter of secretory granules to the plasma LH concentration is noteworthy. The mean diameter of secretory granules decreased with the increase in the plasma LH concentration after copulation, and the diameter of the secretory granules was smallest in ovariectomized pika. From these, it is tempting to speculate that small secretory granules might appear when the LH release is enhanced, while large ones do when the hormone is stored in the cells. In other words, small secretory granules are the releasing form, while large ones are the stored form. This concept is supported by the observation in orchidectomized rat (Nakamura and Yoshimura, 1986 a).

The immunoreactivity of the LH cells and mean diameter and number of their secretory granules were strikingly diminished during the first 1 h after copulation, although the plasma LH level reached the maximum 6 h later. There is a time lag between the change in the morphology of LH gonadotrophs and that in the plasma LH level.

In the pika, LH cells after copulation are morphologically similar to LH cells after ovariectomy. After ovariectomy in rat, the release rate of LH was increased and the LH level in gonadotrophs was also increased (Garner and Blake, 1981). It is therefore assumed that also in pikas, the LH gonadotrophs, of which there are few secretory granules which are small in size, of low density and weak immunostainability, might be exerting an effect not only active LH release, but also its active synthesis by copulatory stimuli.

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