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Phasic Changes in Immunocytochemical Stainability of Pituitary Luteinizing Hormone Cells Associated with their Ultrastructural Changes during Estrous Cycle in the Rat

KAORU SUZUKI, MASAFUMI SAKUMA, HARUO NOGAMI AND FUJIO YOSHIMURA

Department of Anatomy, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105

Abstract

Changes in the immunoreactivity of pituitary luteinizing hormone (LH) cells and in their fine structure were studied in 4-day-cyclic female rats along with the radioimmunoassay of pituitary and serum LH.

Pituitary LH increased during diestrus (DE) and in early proestrus (PE) to a maximal level at noon of PE, followed by a marked decrease by 2100 h PE. Serum LH stayed at low levels in estrus (E) and in DE, while they displayed a significant increase at PE. Light microscopic immunocytochemistry distinguished intensely and weakly stained cells using rat LHα antiserum. The populations of intensely stained cells were 80% at PE, 30% at E and 75% at DE. This suggests that all of the LH cells do not secrete LH Synchronously on the afternoon of PE. Immunoreactivity of LH cells was related to the amount of secretory granules stored in the cells as determined by the superimposition technique. Analysis of the LH storage site by the protein A-gold method confirmed that the small secretory granules, which accumulated in LH cells at DE or PE, certainly contain LH. At least two LH cell types were distinguished: one is the oval or polygonal cell with flattened rER numerous mitochondria, abundant small secretory granules (about 200 nm), a well developed Golgi complex, and a round nucleus. The other has similar structural characteristics along with large secretory granules which are more than 300 nm in diameter. At noon of PE almost all of the LH cells were the first type while the second ones were mainly found at DE or E. The relationship of these LH cell types to the male gonadotrophs is discussed.

The morphology of the rat pituitary gonadotrophs has been studied using electron microscopy, immunocytochemistry or a combination of these two techniques (Kurosumi and Oota, 1968; Kurosumi, 1974; Tougard et al., 1973, 1980; Tixier-Vidal et al., 1975; Yoshimura et al., 1977, 1981); however, many of the previous studies were conducted on male rat pituitaries presumably because of the lack of distinct functional changes in gonadotropin secretion in this sex. It is well established that the serum luteinizing hormone (LH) concentration increases remarkably at proestrus, while it stays at
basal levels in the other phases of the estrous cycle (Blake, 1976). This transient increase in serum LH on the afternoon of proestrus results in remarkable depression of pituitary gonadotropin storage, and this may be followed by the subsequent gonadotropin synthesis by the next surge (Blake, 1980). Pituitary gonadotrophs seem to undergo distinct morphologic change according to these functional changes during the sexual cycle. Moriarty (1976) described the change in the LH and follicle stimulating hormone (FSH) storage pattern in cyclic female gonadotrophs as well as changes in the proportions of the populations of three morphologically different gonadotrophs. Blake (1980), who reported the ultrastructural changes in rat LH gonadotrophs around the LH surge using electron microscopic immunocytochemistry, observed marked degranulation of LH gonadotrophs during late proestrus. The present study was carried out to characterize in detail the morphologic changes in LH gonadotrophs of the cyclic female during the estrous cycle, using immunocytochemistry, protein A-gold method, and superimposition by electron microscopy and immunocytochemistry. Special attention was paid to the ultrastructural changes in LH immunoreactive cells before and after the proestrous LH surge, comparing these findings with the changes in serum and pituitary LH concentrations monitored by RIA.

Materials and Methods

Wistar-Imamichi female rats, 45 days of age, were purchased from Imamichi Inst. Animal Reprod. (Oomiya, Japan), and housed in light (light on from 0800 to 2000 h) and temperature (22°C) controlled rooms for at least 10 days. Rats with a 4-day cycle were killed at noon of each stage of the estrous cycle and at 2100 h of proestrus. The blood samples were collected and the sera removed and stored at −70°C until assay. Pituitaries were removed immediately and either homogenized in phosphate buffered saline (PBS, 154 mM NaCl/20 mM phosphate buffer, pH 7.4) or processed for morphological study.

Immunocytochemistry

Sections from paraplast or Epon embedded tissues were stained immunocytochemically for rat LHβ according to the methods described previously (Yoshimura et al., 1981; Nogami and Yoshimura, 1982). Dilutions of antiserum, from 500 to 16000 fold, were applied to control sections from pituitary glands of proestrus to find the appropriate staining conditions. Antiserum to rat LHβ (kindly supplied by Dr. A. F. Parlow, NIH) was used throughout this study, which specificity was tested by RIA and absorption tests, the results of which have already been published elsewhere (Yoshimura et al., 1981).

Radioimmunoassay

Serum and pituitary LH concentrations were determined by RIA using the materials supplied by NIADDK. Protein concentrations were determined by the method of Lowry et al (1951), using bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Protein A-gold technique

Pituitary glands were fixed in 1% glutaraldehyde, and embedded in Epon-araldite. Ultrathin sections were placed on nickel grids which were floated on droplets of anti-rat LHβ serum diluted 1:2000 with PBS containing 0.5% bovine serum albumin at 30°C for 2 hr. The grids were washed with PBS and floated again on a droplet of protein A-gold complex prepared by the method of Roth et al. (1978) at 30°C for 45–60 min. The sections were washed successively with PBS and distilled water. The specificity of the procedure was assessed by the following controls; 1) incubation of the section with the antiserum previously absorbed with its homologous antigen (NIAMDD rat LH I-5), followed by the application of the protein A-gold; 2) omission of the antiserum treatment.

Cell count

Frontal sections (3-5 sections/rat) obtained from pituitaries at various stages of the estrous cycle were immunostained for LHβ, and were submitted for cell count. Over 2000 cells were counted in each phase (n=3–5) dividing each into the two cell populations of intensely and
weakly stained cells.
The statistical significance of the data was determined by Student's t-test.

Results

Serum and pituitary LH levels were low during estrous (E) and through diestrous (DE), while serum LH was elevated at 2100 h on proestrous (PE). Pituitary LH increased at noon of PE, and was followed by a marked decline by the late evening (Fig. 1).

Immunocytochemical staining using rat LHβ antiserum (1:4000) of both paraplast and Epon sections at the light microscopic level clearly distinguished the two classes of LH cells based on staining intensity (Fig. 2). The percentages of intensely stained cells compared to the total LH cells were 80.0 ± 1.7% (PE), 29.8 ± 2.0% (E) and 74.7 ± 4.8% (DE), respectively (Fig. 3). This means that the weakly stained cells share a certain population even at PE. The numbers of LH cells stained per section (mean ± SEM were 1024 ± 74 (PE, n = 5), 912 ± 88 (E, n = 3), 1174 ± 38 (DI and DII, n = 3) respectively, with no significant difference between groups (P > 0.1).

Fine structure of gonadotrophs during the estrous cycle
1200 h Proestrous

Many of the LH cells were polygonal or oval in shape containing numerous secretory granules and profiles of rough endoplasmic reticulum (rER) (Fig. 4). The secretory granules seemed to be uniform in size and reacted strongly to the antiserum. The LH cells with long cytoplasmic processes were occasionally found in the gland (Fig. 5). Secretory granules accumulated at one end of the processes which faced a blood capillary.

2100 Proestrous

During late PE, the LH cells were found to retain a few secretory granules, corresponding to a deline in immunoreactivity. Figure 6 shows one of these cells which contains many mitochondria, a well developed rER, but few secretory granules. The Golgi complex was prominent and consisted of dilated sacs. In contrast, small numbers of LH cells were found to contain numerous secretory granules at this time (Fig. 7). These cells were ovoid in shape, and were characterized by having flattened rER, small and abundant granules, an intense reaction to the antiserum. They seemed to
Fig. 2. Paraplast (a–c) and Epon (d, e) sections of pituitaries from females at PE (a, d), E (b, e), and DE (c) stained immunocytochemically for rat LHβ. Note the difference in the staining intensity of LH cells at each stage. a–c, ×400; d, e, ×500.

Fig. 3. Changes in the percentages of the intensely stained cells during rat estrous cycle (mean ± SEM). n = 5 (PE), n = 3 (E and DE).

Fig. 4–9. Superimposition technique of immunocytochemically stained sections (inset) and electron micrograph.
Fig. 4. LH cells at 1200 h proestrous with intense immunostainability. These cells contain numerous secretory granules and flattened rER. ×5000.

Fig. 5. Irregular shaped LH cells with a cytoplasmic process found in proestrous. Secretory granules are accumulated at the end of the cytoplasmic process facing a blood capillary, and are sparsely populated in other parts of the cell. ×5500.
be the same kind of cells as those which were commonly observed at PE noon (Fig. 4).

**Estrous**

At this stage, the number of degranulated LH cells decreased when compared with those observed at 2100 h PE. Degranulated LH cells (Fig. 8) were often polygonal and less ovoid in shape, containing a few secretory granules (100–120 nm in diameter). The rER was flattened and Golgi complex well developed. This cell type resembled degranulated LH cells found at late PE.

**Diestrous**

LH cells recovered their immunoreactivity considerably by this time, with an increase in the number of secretory granules per cell profile (Fig. 9). The predominant cell type during DE is ovoid or polygonal in shape and contains abundant secretory granules, flattened rER, and a well developed Golgi apparatus. During this period, many LH cells were found to have both large (more than 300 nm in diameter) and small (150–200 nm in diameter) secretory granules.

**Protein A-gold technique**

LH storage in the gonadotrophs was localized by the protein A-gold complex on sections fixed with glutaraldehyde (Fig. 10). The labeling of gold particles was found exclusively over the gonadotrophs with minimal background labeling over the other cell types or over the nuclei. On detailed examination, the gold particles tended to be more densely concentrated over the small granules than over the large secretory granules, and were also scattered in the cytosol (Figs. 10a and b).

**Discussion**

In the present study, dilution of the first specific antiserum was carefully controlled to distinguish both intensely and weakly stained cells. Intensity of coloration by the peroxidase reaction seems to depend to a certain extent on the amount of antigen fixed on the section, provided that the antiserum is used at the appropriate dilution (Shirasawa et al., 1983). Therefore, the difference in staining intensity between LH cells observed in this study may reflect the differences in the LH content in individual cells. We observed a remarkable decline in immunoreactivity of LH cells after the LH surge, however, the total LH cell population did not show any significant change throughout the sexual cycle. These date are compatible with the cell count data of Dada et al. (1983) by light microscopic immunocytochemistry. This suggests that the cells with reduced immunoreactivity in late PE might have discharged their LH store at the surge. It is noteworthy that the decline in the staining intensity did not occur in all of LH cells but only in some. The percentage of intensively stained cells was reduced by 50% during the surge, and about 30% of the total LH cells display intense staining even at late PE. These results, taken together with the fact that the weakly stained cells share up to 20% at PE noon, suggest that not all of the LH cells always secrete LH synchronously at the time of the preovulatory surge. A possible alternative explanation is that some cells may be able to synthesize LH after the discharge in a short time, and thus restore their immunostainability by late PE. The decrease observed in immunoreactivity of LH cells after the surge and subsequent restoration coincides well with the changes in pituitary LH concentration. Superimposition using electron microscopy and immunocytochemistry showed that the decrease in LH cell immunoreactivity may be due to a reduction in the number of secretory granules. Intensely stained cells consistently contain a large number of granules at any stage of the sexual cycle.
Fig. 6. Degranulated LH cell with weak immunoreactivity appeared at 2100 h proestrous. ×7200.

Fig. 7. LH cell at 2100 h proestrous with intense immunostainability. A large number of secretory granules of a single population are observed. ×8000.
Fig. 8. Three LH cells at noon of estrous. These cells displayed weak immunostaining, corresponding to the small number of granules in their cytoplasm. ×4000.

Fig. 9. LH cell found in a diestrous female containing many secretory granules of two sizes, a circularly arranged Golgi apparatus and parallel arrays of rER. ×4800.
Fig. 10. Localization of LHβ in a proestrous female gonadotroph by the protein A-gold method. Gold particles are exclusively localized over the LH cell (L) with minimal background labeling over the extracellular space or over the other cell types (a). b, High magnification of a portion of a. a, ×7350; b, 44000.
Analysis by protein A-gold technique confirmed the LH storage in small secretory granules. These observations are in good agreement with the description of Blake (1980) which indicates that the anterior pituitary gland LH concentration is directly related to the number of LH secretion granules during the rat estrous cycle. Our results are in contrast to those obtained by Polkowska and Jutisz (1979), who observed remarkable changes in the LH cell population in the gland during the sexual cycle and found that degranulation occurred in most of the gonadotrophs. The difference between these two studies is considered to be due in part to the differences in the sensitivity of immunostaining or in the time when the animals were killed.

The present electron microscopic study demonstrated remarkable changes in the amount of secretory granules during the sexual cycle. However, the basal structure of the LH cells seemed not to be altered. The LH cells in female rats are characterized fine structurally by flattened ER, numerous small secretory granules, many mitochondria, a well developed Golgi complex and round nuclei. The cells tended to be polygonal or oval in shape at PE as described by Blake (1980). Secretory granules were generally small in diameter, whereas the large ones were observed only during DE. These features are quite different from the common structure of male gonadotrophs described earlier. In male rats, LH cells are usually ovoid in shape, containing large (400–700 nm in diameter) and small (about 250 nm in diameter) secretory granules, and well developed vesiculated rER. The LH cells with only small granules found in PE may be identical in fine structure to the Type II gonadotroph of the classification of Kurosumi (1974), the Type B cell described by Tougard et al (1980), or the Type II/III, or Type IIIA basophil by Yoshimura et al. (1981). A cell type containing two classes of secretory granules and vesiculated rER is rare in the female while it is commonly found in the male pituitary. The cells containing large and small granules which are mainly observed at DE should be referred to as Type II/IIIB or IIIB as classified by Yoshimura et al. (1981), Type A (Tougard et al., 1980) or Type I gonadotroph (Kurosumi, 1974). Thus at least two types of LH cells were distinguished throughout the estrous cycle. Our results are in agreement with those of an earlier study by Moriatry (1976) suggesting that female rats have two distinctive LH cell types, but are inconsistent with the study of Blake (1980) where only a basic LH cell type was described. As the LH cell population seemed to be constant during the estrous cycle, it is conceivable that a single cell type may be able to produce both sizes of granules. This concept is compatible with the view that a gonadotroph may undergo morphological changes according to the functional state of the cell (Yoshimura and Harumiya, 1965; Yoshimura et al 1977; Tougard, 1980).

In this study, only LH cells were examined. Many investigators now seem to agree with the concept that LH and FSH are stored in the same cell, although cells with only LH or FSH are certainly detectable under various physiological conditions. In a recent publication from our laboratory, Nakamura et al (1985) suggest that gonadotrophs with a single hormone are very small in number, therefore our immunocytochemical staining for rat LHβ seems to be enough to detect almost all gonadotrophs on the section. It is possible that some weakly stained cells which are consistently observed at every phase of the estrous cycle may contain a certain amount of FSH, because LH and FSH content may differ from cell to cell (Inoue and Kurosumi, 1984).
References


