Autoradiographic Study of Binding and Internalization of Follicle-Stimulating Hormone in the Mouse Testis Minces *In Vitro*

AKITOSHI SHIMIZU, KAZUYOSHI TSUTSUI AND SEIICHIRO KAWASHIMA

*Zoological Institute, Faculty of Science, Hiroshima University,
Naka-ku, Hiroshima 730, Japan*

Abstract

The internalization of FSH-receptor complexes was demonstrated in mouse testis by means of light and electron microscopic autoradiography. Chopped testicular pieces were incubated with radioiodinated FSH (^{125}I -NIADDK-rat FSH-I-4) for 10, 20, 60 and 180 min. After incubation the pieces were fixed with glutaraldehyde containing tannic acid, and embedded in Spurr's resin. Semithin and ultrathin sections were cut for light and electron microscopic autoradiography, respectively. In light microscopic autoradiographs, silver grains were preferentially localized over Sertoli cells, regardless of incubation time. Sixty to 70% of the total number of grains were located over Sertoli cells which account for only about 4% of the total cell population of the seminiferous tubules. The majority of these grains correspond to the specific FSH binding sites, because few grains remained after incubation with an excess amount of unlabeled FSH. In electron microscopic autoradiographs, the half-distance (HD) value for the ^{125}I -labeled line source was about 216 nm in the present study. After 10 min of incubation, 56.6% of the total number of silver grains were located over the plasma membrane of Sertoli cells. In testicular pieces incubated for longer periods (20, 60 and 180 min), both the percentage and relative concentration of grains increased in the Golgi apparatus and lysosomes and decreased in the plasma membrane. These results suggest that [^{125}I]iodo-FSH first binds to FSH receptors on the plasma membrane of Sertoli cells, then FSH-receptor complexes are internalized. The increase in the number of grains over the lysosomes following longer incubation, indicates that internalized [^{125}I]iodo-FSH or FSH-receptor complexes are subjected to degradation.

Sertoli cells are the primary target of follicle-stimulating hormone (FSH) in the testis (Mancini *et al.*, 1967; Castro *et al.*, 1972; Fritz, 1978; Risbridger *et al.*, 1981).

FSH first binds to plasma membrane receptor of Sertoli cells and elicits various biochemical responses after the stimulation of adenyl cyclase producing cAMP (Fritz, 1978; Risbridger *et al.*, 1981). This responsiveness of Sertoli cells to FSH is generally dependent on the state of target cells and the concentration of FSH around the cells (Francis *et al.*, 1981; Jahnsen *et*

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Requests for reprints to: Seiichiro Kawashima
Zoological Institute, Faculty of Science, Hiroshima University, Naka-ku, Hiroshima 730, Japan.

al., 1981). Like many other peptide hormones (Tell *et al.*, 1978; Catt *et al.*, 1979), FSH is known to induce the desensitization of its own target cells. Several possible mechanisms may be considered for the desensitization. One such mechanism is the self-regulation of membrane receptor concentration. In fact, the reduction of testicular binding to FSH after FSH exposure has been demonstrated by several investigators (O'Shaughnessy *et al.*, 1978; Gnanaprakasam *et al.*, 1979; Jahnsen *et al.*, 1981). We have recently reported that hypophysectomy induced a significant increase in the number of FSH receptors in Sertoli cells in the mouse, and the hypophysectomy-induced increase was suppressed by injections of FSH (Tsutsui *et al.*, 1983, 1985).

These studies indicate the existence of down-regulation of FSH receptors in the testis, perhaps by the process of internalization of FSH-receptor complexes. Concerning the fate of receptor-bound FSH, Castro *et al.* (1970) reported that when ferritin-labeled FSH was injected into rats, the ferritin particles were observed over vesicles and dense bodies in Sertoli cells. However, Fawcett *et al.* (1970) found the ferritin injected alone also appeared in Sertoli cell vesicles. Therefore, it is not certain whether the intracellular ferritin represents internalized FSH-receptor complexes. By means of autoradiography, Orth and Christensen (1977) reported the binding of [125 I]iodo-FSH to the plasma membrane of Sertoli cells, but they did not show the internalization of labeled FSH or FSH-receptor complexes. The present study was designed to obtain evidence of the internalization of FSH or FSH-receptor complexes in mouse Sertoli cells by light and electron microscopic autoradiography.

Materials and Methods

Animals

Male C57BL/6NCrj mice maintained in this laboratory were used. They were housed in a temperature-controlled room under daily photoperiods of 12 h-light (0600–1800 h) and 12 h-dark with free access to laboratory chow (CA-1, Japan Clea Inc.) and tap water.

Tracer

Highly purified rat FSH (NIADDK-rat FSH-I-4, biological potency: approximately $150 \times$ NIH-FSH-S1, LH contamination: less than $0.002 \times$ NIH-LH-S1) was radioiodinated with 131 I (Na 131 I, Radiochemical Centre, Amersham) by the lactoperoxidase method (Tsutsui *et al.*, 1978, 1980). In brief, 5 μ l Na 131 I (40 μ Ci/ μ l) was added to a reaction tube containing 25 μ l of rat FSH-I-4 (0.1 μ g/ μ l) dissolved in 0.2 M phosphate buffer at pH 7.0, then 5 μ l lactoperoxidase (0.33 units, 0.1 μ g/ml, Sigma Chemical Co.) and 5 μ l H $_2$ O $_2$ (7.5 μ g/ml) both dissolved in the same buffer were added. Reaction proceeded at room temperature for 1.5 min, and the reaction mixture was purified with Sephadex G-75. The specific radioactivity of the radioiodinated rat FSH ([131 I]iodo-FSH) was about 35 μ Ci/ μ g.

Autoradiography

For the autoradiographic study, the testes were removed from 35-day-old mice killed by cervical dislocation. After the removal of the tunica albuginea in Medium 199, the tissue was weighed on a torsion balance and cut into about 1 mg pieces. The testis pieces were preincubated in Medium 199 at 37°C for 30 min. Five to six preincubated pieces were weighed again after blotting on a filter paper, and transferred to a plastic reaction tube. To the tube was added 100 μ l [131 I]iodo-FSH (1.7 μ g/ml) with or without an excess amount of unlabeled porcine FSH (1 mg, NIH-FSH-P2, biological potency: $0.69 \times$ NIH-FSH-S1). After incubation at 37°C for 10, 20, 60 and 180 min with constant agitation, the medium was discarded and testis pieces were briefly washed by passing through eight changes of ice-cold Medium 199 in order to remove unbound [131 I]iodo-FSH.

Procedures for autoradiography were the same as described by Mizuhira *et al.* (1978). Briefly, testis pieces were fixed in 2.5% glutaraldehyde in 1/14 M phosphate buffer at pH 7.2 containing

0.5% tannic acid for about 4 h and further fixed in 2.5% glutaraldehyde in the same buffer at pH 6.8 containing 2.0% tannic acid for 2 h, and washed in the same buffer at pH 7.2 and post-fixed in 1.0% OsO_4 for 2 h. All fixation processes were performed at 4°C. After fixation, testis pieces were dehydrated in a graded ethanol series and embedded in Spurr's resin. After polymerization of Spurr's resin, the radioactivity of the embedded testis pieces was counted with an autowell gamma counter.

For light microscopic autoradiography, semithin (1 μm) sections were cut with a Pöter-Blum MT-1 ultramicrotome, stained with 1% toluidine blue in 1% borax and coated with 0.5% collodion dissolved in ethanol/ether (v/v, 1:1) to prevent the emulsion from directly contacting the sections.

For electron microscopic autoradiography, silver-gold interference color sections were prepared and stained with uranyl acetate and coated with carbon vapor.

Semithin and ultrathin sections were coated with Sakura NR-M2 emulsion (Konishiroku Photo Ind. Co., Ltd.) by dipping and touch methods, respectively. After exposure at 4°C for 7 to 14 days, the emulsion was developed with Konidol-X (Konishiroku Photo Ind. Co., Ltd.) at 20°C for 3.5 min. For electron microscopic autoradiography, ultrathin sections were post-stained with lead monoxide, coated again with carbon vapor to protect the silver grains from evaporation by the beam current and examined with a Hitachi HU-11D-S electron microscope. Some additional observations were done with a JEOL JEM-1200EX electron microscope.

Analysis of autoradiographs

To calculate the percentage of silver grains laying on Sertoli cells at the light microscopic level, 40 seminiferous tubules were randomly chosen at each incubation time. For an electron microscopic autoradiographical study, the half distance (HD) value for ^{131}I -labeled line source was measured by the method of Salpeter *et al.* (1977). As the radioactive line source, 25% gelatin containing [^{131}I]iodo-FSH (1.7 $\mu\text{g}/\text{ml}$) was used. The radioactive line source was tightly sandwiched between either resin blocks or testis pieces which were similarly fixed, dehydrated and embedded in resin as described above. Ultrathin sections cut at right angles to the radioactive line source were used for electron

microscopic autoradiographs. The perpendicular distance from the midplane of the radioactive line source to the developed grains was measured.

In electron microscopic autoradiographs, more than 450 silver grains at each incubation time were counted on photographs (final magnification, $\times 30,000$) and the true grain counts in each cellular compartment were calculated according to the method of Nadler (1971). Around each silver grain, a resolution boundary circle 12 mm in diameter was drawn to localize the source of radioactivity. If there was only one kind of cellular compartment in the circle, such a grain was designated as an 'exclusive grain'. On the other hand, if there were multiple kinds of cellular compartments within the circle, such a grain was called a 'shared grain'. On the assumption that a shared grain was produced by the radiation emitted from the compartments within the circle and the radiation was statistically proportional to the content of the radioactivity of the compartments, the total count of shared grains in the photographs was assigned to each cellular compartment. The true grain count for each compartment was the sum of exclusive and assigned shared grains, and was expressed as the percentage of the total number of grains. In addition, the relative concentration of grains in each cellular compartment was calculated by dividing the percentage of its grain count by the percentage of the area occupied by each compartment.

Results

Radioactivity of testis pieces

Fig. 1 illustrates the radioactivity of testis pieces as a function of incubation time. Specific [^{131}I]iodo-FSH binding to testis pieces after 180 min of incubation was 6.3 times more than the level after 10 min of incubation (380 vs. 60 cpm/mg tissue).

Light microscopic autoradiograph

Silver grains were preferentially localized over Sertoli cells regardless of incubation time (Fig. 2a, b). About 60 to 70% of the total number of grains in the seminiferous

Fig. 1. Effects of incubation time on the radioactivity of testicular pieces. Testicular pieces (five or six in a reaction tube) and [^{131}I]iodo-FSH at the concentration of 5.6×10^{-8} M were incubated at 37°C with (non-specific binding) or without (total binding) in excess amount of unlabeled FSH. The specific binding was calculated as the difference between total binding and non-specific binding. Testicular pieces were fixed and embedded in Spurr's resin. Each point depicts the mean of two determinations.

Table 1. Percentage of silver grains on Sertoli cells

Incubation time (min)	Silver grains (%)
10	$63.3 \pm 5.9^*$
20	69.7 ± 5.4
60	68.7 ± 4.6
180	60.6 ± 6.8

* mean $\pm$ S. E.

Testis pieces were incubated at 37°C with [^{131}I]iodo-FSH at a concentration of 5.6×10^{-8} M. The total number of silver grains and number of grains over Sertoli cells in 40 seminiferous tubules were counted at each incubation time.

ferous tubules were localized over Sertoli cells which account for only about 4% of the total cell population of the seminiferous tubules (Table 1). When an excess amount of unlabeled FSH (1 mg NIH-FSH-P2) was added to the incubation medium containing [^{131}I]iodo-FSH, the concentration of silver grains was almost totally abolished and the background level of silver grains appeared to be unaffected (Fig. 2c).

Grain distribution from the line source

The HD value for the ^{131}I -labeled line source sandwiched between resin blocks was about 875 nm (Fig. 3a) and that sandwiched between testis pieces was 216 nm (Fig. 3b). As shown in Fig. 3b, the majority of grains (77.0%) were within 400 nm from the line source. Therefore, a silver grain, whose distance from the line source was less than 400 nm, was classified as derived from the line source. Therefore, if a resolution boundary circle 12 mm in diameter is drawn around each silver grain in electron microphotographs, the circle will enclose the true radioactive source with a probability of 77.0%.

Electron microscopic autoradiograph

Under the electron microscope, Sertoli cells show a characteristic profile. They extend radially from the basement membrane of the seminiferous epithelium toward the lumen of the tubules and engulf various stages of germ cells. Their nuclei are indented and euchromatic. Most of silver grains were encountered over Sertoli cells

Fig. 2. Light microscopic autoradiographs of mouse testis pieces incubated with [131 I]iodo-FSH alone (a, b) or in combination with an excess amount of unlabeled FSH (1.0 mg NIH-FSH-P2) (c) for 60 min. Fig. 2a, Several concentrations of silver grains are visible over Sertoli cells of the seminiferous tubule. Fig. 2b, A part of Fig. 2a, magnified. Fig. 2c, Very few silver grains are visible. Bar: 10 μ m.

and only few grains were visible over other cells of the tubules (Fig. 4). The relative concentration of grains over Sertoli cells was 10-fold greater than over other cell types during the entire course of incubation. When testis pieces were incubated with [131 I]iodo-FSH plus an excess amount of unlabeled FSH, silver grains were almost completely eliminated from Sertoli cells.

The results of quantitative analysis during the incubation time are shown in Table 2.

After 10 min of incubation about 55% of the total number of grains were encountered over the plasma membrane and the relative concentration of grains over the plasma membrane was decidedly higher than that over other cellular compartments. Fig. 5a shows a silver grain over the plasma membrane. After longer incubation periods (20 and 60 min), the percentage of silver grains over the Golgi apparatus and lysosomes was increased from the value after 10 min

Fig. 3. Distribution of grains in electron microscopic autoradiographs from radioactive line source. The line source was sandwiched between either resin blocks (Fig. 3a) or testis pieces (Fig. 3b). The number of grains was plotted against the distance from the line source as a grain distribution histogram and an integrated form. HDs of grain distribution from the line source sandwiched between either resin blocks or testis pieces were about 875 nm and 216 nm, respectively. Note the abscissa scale difference between panels a and b.

Table 2. Percentage and relative concentration (RC) of silver grains over various cellular compartments of Sertoli cells after incubation of testicular pieces with [^{131}I]iodo-FSH

Cellular compartment	Incubation time (min)							
	10		20		60		180	
	%	RC	%	RC	%	RC	%	RC
Plasma membrane	54.6	12.4	32.4	7.4	24.9	5.7	12.4	2.8
Golgi apparatus	1.9	0.8	5.3	2.3	8.7	3.8	13.7	6.0
Lysosomes	0.2	0.2	0.6	0.4	1.3	0.8	3.9	2.4
Mitochondria	10.9	0.9	12.9	1.0	14.2	1.1	14.5	1.1
Endoplasmic reticulum	28.7	1.1	36.7	1.4	39.3	1.4	39.6	1.5
Nucleus	0.9	0.1	1.7	0.2	3.1	0.3	2.1	0.2
Cytoplasmic matrix	2.7	0.1	10.4	0.2	8.5	0.2	13.7	0.3

of incubation. The relative concentration of grains over plasma membrane after 20 or 60 min of incubation was less than that after 10 min, but was still more than those of other cellular compartments. The relative concentration of silver grains over the Golgi apparatus and lysosomes after 20 or 60 min

of incubation was higher than that after 10 min of incubation. Silver grains associated with endocytotic vesicles were also encountered (Fig. 5b). These vesicles were often of a receptosome-like shape (Fig. 5c). This may represent a process of translocation of FSH-receptor complexes to intracel-

Fig. 4. Low magnification electron microscopic autoradiograph of a mouse testis piece incubated with [125 I]iodo-FSH for 60 min. The three silver grains circumscribed are all located over the cytoplasm of a Sertoli cell (C). N: nucleus of Sertoli cell, SC: spermatocytes, SG: spermatogonia, BM: basement membrane. Bar: 2 μ m.

Fig. 4. Low magnification electron microscopic autoradiograph of a mouse testis piece incubated with [125 I]iodo-FSH for 60 min. The three silver grains circumscribed are all located over the cytoplasm of

lular compartments. After 180 min of incubation, the percentage and relative concentration of silver grains over the plasma membrane were both decreased to about

23% of those after 10 min of incubation, and were no longer the highest among the cellular compartments studied. However, those over the Golgi apparatus and lyso-

Fig. 5. Electron microscopic autoradiographs of Sertoli cells in the mouse testis pieces incubated with [^{131}I]iodo-FSH. Autoradiographs a-e are arranged to show a presumptive process of internalization of [^{131}I]iodo-FSH. Labeled FSH first binds to the plasma membrane of Sertoli cell and the membrane is invaginated (a). The invaginated part is pinched off to form a small vesicle (b). Small vesicles are often of a receptosome-like shape (c). Finally, these vesicles transfer their contents to the Golgi apparatus (d) and lysosomes (e). Bar: a, b: 1 μm , c, d, e: 0.5 μm .

somes were markedly increased (Table 2, Fig. 5d, e), during the entire course of incubation.

In the mitochondria, the endoplasmic reticulum, the nucleus and cytoplasmic matrix, neither percentage nor relative concentration of silver grains showed appreciable changes as a function of incubation time. The relative concentrations of grains over the nucleus and cytoplasmic matrix were the lowest among cellular compartments

Discussion

The present autoradiographic study showed that [^{131}I]iodo-FSH binds preferentially to Sertoli cells in mouse testis pieces *in vitro*. The binding was specific to the FSH receptor, because labeling was reduced to

background levels when an excess amount of unlabeled FSH was added to the incubation medium along with [^{131}I]iodo-FSH. The present results are in harmony with the findings of previous investigators who demonstrated that Sertoli cells are the primary target of FSH in the rat testis (Mancini *et al.*, 1967; Castro *et al.*, 1972; Fritz, 1978; Risbridger *et al.*, 1981). Orth and Christensen (1977) mentioned the uptake of labeled FSH by interstitial tissue macrophages, but in our preparations the frequency of silver grains over the macrophages appeared to be the same as the background level.

In the present experiments, the fixative containing tannic acid was used for the two reasons already given by Mizuhira *et al.* (1978). First, tannic acid is a good fixative for proteins and polypeptides when the aldehyde group coexists, and it prevents the loss of labeled hormones bound to receptors during autoradiographical procedures (Mizuhira *et al.*, 1978). Second, in the processes of post-fixation and staining, testis pieces prefixed with tannic acid can incorporate a greater amount of heavy metals such as osmium and uranium than those treated with a fixative containing no tannic acid. Since such heavy metals in the tissue absorb β -rays, the resolution of autoradiographs was improved by the use of tannic acid (Mizuhira *et al.*, 1978). In the present study, the HD value from the ^{131}I -labeled line source was about 216 nm when the line source was sandwiched between testis pieces which were fixed with tannic acid, stained with heavy metals and coated with carbon vapor, while the HD value from the line source sandwiched between resin blocks which were not subjected to such treatments was about 875 nm.

The distribution and frequency of silver grains were different among cellular compartments and changed with the incubation time. Cellular compartments can be classified into three types: 1) those that are significantly labeled (relative concentration

of silver grains >1.0) and show a distinct kinetic pattern of labeling with incubation time (plasma membrane, Golgi apparatus, lysosomes) (By definition (Nadler, 1971) a relative concentration of silver grains greater than 1 represents significant labeling.), 2) those that are significantly labeled but show no change in the level of labeling with incubation time (mitochondria, endoplasmic reticulum), and 3) those that are not significantly labeled (relative concentration of silver grains <1.0 : nucleus, cytoplasmic matrix).

As far as changes in the relative concentration of silver grains are concerned, the present data indicate that [^{131}I]iodo-FSH binds first to the plasma membrane, and with time the ligand or ligand-receptor complexes become increasingly more associated with the Golgi apparatus and lysosomes. These results strongly suggest the internalization of FSH or FSH-receptor complexes in the mouse Sertoli cells.

The relative concentration of silver grains over both the nucleus and cytoplasmic matrix never showed significant labeling at any time during incubation. Such labeling might represent artifact background reactions. Other cellular compartments, mitochondria and endoplasmic reticulum, showed a significant level of relative concentrations of silver grains, but failed to show incubation time-dependent changes. It is not known why they showed significant labeling from early in incubation.

The accumulation of silver grains over the Golgi apparatus was similar to what has been reported for other labeled ligands, such as insulin (Bergeron *et al.*, 1979; Goldfine *et al.*, 1981; Goldstein *et al.*, 1981), prolactin (Josefsberg *et al.*, 1978), growth hormone (GH) (Barazzzone *et al.*, 1980), luteinizing hormone-releasing hormone (LHRH) (Pelletier *et al.*, 1982) and corticotropin-releasing factor (CRF) (Leroux *et al.*, 1984). So far, there has been no evidence of the existence of FSH receptors

in the Golgi fraction of Sertoli cells.

Recently receptor-mediated endocytosis of specific ligands was reported for low density lipoprotein (Goldstein *et al.*, 1979) and epidermal growth factor (Willingham *et al.*, 1980). After the clustering of ligand-receptor complexes in clathrin-coated pits, these complexes were internalized to form clathrin-coated (Goldstein *et al.*, 1979) or non-coated (Willingham *et al.*, 1980) intracellular vesicles. Especially non-coated vesicles, which are designated as receptosomes, selectively avoided fusion with lysosomes but moved toward the Golgi region (Willingham *et al.*, 1980). However, non-coated vesicles as well as clathrin-coated vesicles finally deliver their contents into the lysosomes (Goldstein *et al.*, 1979; Willingham *et al.*, 1980).

In the present study, silver grains were visible over receptosome-like small vesicles. However, because of their limited resolution in autoradiographs all grains overlying receptosome-like vesicles were recognized as 'shared grains'. By definition (Nadler, 1971), the relative concentration of silver grains over a cellular compartment which has no exclusive grains was calculated to be zero. Therefore, no evidence of the association of silver grains with receptosome-like vesicles was obtained in the present study. However, it seems likely that the internalization and accumulation of receptor-bound FSH in other cellular compartments occur through the mediation of small receptosome-like vesicles which are formed from the plasma membrane, as observed in other systems (Josefsberg *et al.*, 1978; Bergeron *et al.*, 1979; Barazzzone *et al.*, 1980; Goldfine *et al.*, 1981; Goldstein *et al.*, 1981; Pelletier *et al.*, 1982; Leroux *et al.*, 1984).

In the present experiments, we have observed the labeling over lysosomes. Similar autoradiographical observations have been reported for other hormones such as insulin (Bergeron *et al.*, 1979; Goldfine *et al.*, 1981), human chorionic gonadotropin (hCG) (Amsterdam *et al.*, 1979), GH

(Barazzzone *et al.*, 1980), LHRH (Pelletier *et al.*, 1982; Duello *et al.*, 1983) and CRF (Leroux *et al.*, 1984). It was also demonstrated that various [¹²⁵I]iodo-hormones were degraded to [¹²⁵I]-mono- or diiodo tyrosine after the internalization, and this degradation was inhibited by lysosomotropic agents, suggesting lysosome-mediated degradation of internalized hormones (Carpenter *et al.*, 1979; Ascoli *et al.*, 1978; Amsterdam *et al.*, 1979; Krupp *et al.*, 1982; Gorin *et al.*, 1984). Fletcher and Reichert (1984) reported that degradation of labeled human FSH was inhibited by lysosomotropic agents, chloroquine and NH₄Cl. Recently, we have observed similar inhibition in cultured mouse Sertoli cells by the same agents (unpublished observations). These findings altogether indicate that internalized labeled FSH or FSH-receptor complexes in lysosomes are degraded in lysosomes.

The internalization of labeled FSH and the down-regulation of FSH receptors in mouse testis (Tsutsui *et al.*, 1985) may be closely related to each other. However, to substantiate this hypothesis, the fate of FSH receptors needs to be clarified under the same experimental conditions, because the silver grains observed in the present study failed to disclose whether they were derived from free [¹³¹I]iodo-FSH or receptor-bound FSH.

In summary, the present results in mouse testicular pieces indicate that the sequence of events may be as follows: 1) The binding of FSH to FSH receptors on the plasma membrane of Sertoli cells, 2) the internalization of FSH or FSH-receptor complexes and 3) migration of FSH or FSH-receptor complexes to the Golgi apparatus and lysosomes. The incorporation of [¹³¹I]iodo-FSH to lysosomes suggests that the degradation of FSH may occur in the lysosomes.

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