Dynamic Change in Charge Heterogeneity of Pituitary FSH throughout the Estrous Cycle in Female Rats

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Abstract

Anterior pituitaries were removed from female rats at various stages during the estrous cycle and FSH was fractionated by isoelectric focusing (IEF). Fourteen FSH components were observed during the estrous cycle and twelve of them were distributed between pH 3.71 and 6.66. IEF profiles of FSH in the pituitaries varied with the stage in the estrous cycle. Especially at the time of serum FSH surge on the day of proestrus, most of the components decreased, while only a highly alkaline component showed an increase. When these FSH components were separated and their nature was examined by radioreceptor assay (RRA), radioimmunoassay (RIA) and gel filtration, differences were observed among these components in the RRA/RIA ratio and gel filtration profile. As a general tendency, the RRA/RIA ratio of the components became greater while the apparent molecular size became smaller, as their pI became higher. However, some highly acidic components showed a biphasic elution pattern and the most acidic one eluted the latest on gel filtration, suggesting that these components may be heterogeneous in terms of molecular size. The FSH concentration in sera collected at different stages in the estrous cycle was measured by both RRA and RIA. The RRA/RIA ratio was high when the serum immunoreactive FSH was low, and low during the FSH surge.

From these findings, it is concluded that the quality of FSH molecules present in the anterior pituitary gland changes dynamically throughout the estrous cycle, especially during the period of serum FSH surge. Furthermore it is suggested that the type of FSH secreted from it also varies according to the stage in the estrous cycle.

Follicle-stimulating hormone (FSH) is known to exist in multiple forms in the anterior pituitary gland. Polymorphism of FSH in electrical charge has been demonstrated by an isoelectric focusing or chromatofocusing technique (Zaidi et al., 1981; Robertson et al., 1982; Ulloa-Aguirre and Chappel, 1982; Chappel et al., 1984; Blum et al., 1985) and that in molecular size has been assessed by gel filtration (Peckham and Knobil, 1976; Blum and Gupta, 1980; Foulds and Robertson, 1983; Blum and Gupta, 1985). Sialic acid content in FSH molecule has been shown to account for these heterogeneous features (Peckham and Knobil, 1976; Ulloa-Aguirre et al., 1984;
Blum et al., 1985). The profile of heterogeneity changes according to the endocrine status of the animals (Peckham and Knobil, 1972; Blum and Gupta, 1980; Robertson et al., 1982; Galle et al., 1983). Cameron and Chappel (1985) suggested that the form of FSH varies according to the stage in the estrous cycle in hamsters.

In female rats, it is well known that the serum FSH level fluctuates periodically with the estrous cycle, showing a large surge from the afternoon of proestrus to the morning of estrus with a simultaneous decrease in pituitary FSH (Taya and Igarashi, 1973; Elias and Blake, 1981).

In the present study, we examined in detail the profile of FSH isohormones present in the anterior pituitaries throughout the estrous cycle, especially focusing on the period around the serum FSH surge, as considerable secretion and synthesis of FSH were expected. We also examined the receptor binding activity and apparent molecular size of these isohormones. Furthermore, in order to estimate the physiological significance of the FSH isohormones, the receptor binding activity of FSH present in circulation were compared with immunochemical reactivity at various stages of the estrous cycle.

**Materials and Methods**

**Animals and sample preparation**

Female rats of Wistar-Imamichi strain were purchased from the Institute of Animal Breeding (Ohmiya, Saitama Pref., Japan) at 7 weeks of age and housed in a light- (light on 0500–1900 h) and temperature- (23°C) controlled room with free access to water and food. A vaginal smear test was carried out every morning and the animals exhibiting at least two consecutive 4-day estrous cycle were used.

Groups of rats (20 animals/group) were decapitated every 3 hours from 1800 h on the day of proestrus (P) to 0600 h on the day of estrus (E), and thereafter at 0900 h and 1500 h each day. Trunk blood was collected and allowed to clot. Serum samples were stored at −20°C until radioimmunoassay (RIA). The anterior pituitaries were removed immediately after decapitation, snap-frozen on dry ice and stored at −80°C.

Each pituitary sample was sonicated 3 times for 10 sec each time with a MicrosonTM Ultrasonic Cell Disruptor, Model MS-50 (HEATSYSTEMS-ULTRASONICS, INC., New York, U.S.A.) in 1 ml of a 3:2 mixture of 10% (W/V) ammonium acetate, pH 5.1 and ethanol, and kept at −20°C overnight. The homogenates were centrifuged at 15,000 × g for 15 min. Fifty µl of each supernatant fluid was removed and measured for FSH content by RIA. The residual supernatant fluids in each group were pooled, dialyzed against distilled water and lyophilized, then subjected to isoelectric focusing.

In another experiment, groups of rats (9–10 animals/group) were killed at 0900 h and 2100 h on P, 0300 h on E, and 0900 h on diestrus I (DI) and diestrus II (DII), and trunk blood was collected. Serum samples were stored at −20°C until RIA and radioreceptor assay (RRA).

**Isoelectric focusing (IEF)**

A small preparative column (size 25 ml) was used (Wakabayashi and Hattori, 1978). The linear pH gradient from 4 to 6 was made by mixing carrier ampholites with pH ranges of 4–6, 5–7 and 3.5–10 (Ampholine, LKB Produkter AB, Bromma, Sweden) in a ratio of 3:1:1 at a final concentration of 1.28% in sorbitol density gradient (5–50%).

The lyophilized sample was dissolved in a small volume of distilled water and added to the focusing solution, then applied on the column. The focusing was run at 4°C on the following schedule; 400 V for the 1st day, 800 V for the 2nd day and 1100 V for the 3rd and 4th days. After focusing, 0.45 ml fractions were collected and pH measured simultaneously with a flow-through micro pH electrode (SE-1700GC, Fuji Kagaku Keisoku Co. Ltd., Mitaka, Tokyo). The fractions were diluted to 1:20 with 0.1 M Tris-HCl buffer, pH 8.0 containing 1% bovine serum albumin (BSA) and assayed for FSH content by RIA.

**Radioimmunoassay (RIA)**

Immunoreactive rat FSH was measured by radioimmunoassay using a rat FSH RIA kit, kindly provided by the National Hormone and Pituitary Program, consisting of NIDDK rat
FSH-I-6, NIDDK rat FSH RP-2 and NIDDK anti-rat FSH serum S-11. FSH-I-6 was radioiodinated with $^{131}$I by the chrolamine-T method (Hunter and Greenwood, 1962) with minor modifications. Double antibody RIA was carried out according to the method of Monroe et al. (1968) with minor modifications. A goat anti-rabbit $\gamma$-globulin serum, prepared in our laboratory, was used as the second antibody.

In the experiment for estimation of the RRA/RIA ratio, FSH components prepared by IEF and serum samples were assayed with FSH-I-6 instead of FSH-RP-2 as standard.

**Radioreceptor assay (RRA)**

Preparation of the receptor fraction and radioreceptor assay were carried out according to the method of Wakabayashi et al. (1980) and Minegishi et al. (1980). Immature female rats (25 days) were subcutaneously injected with 50 IU of pregnant mare serum gonadotropin (PMSG; Peamex, Sankyo Co. Ltd., Tokyo) and killed after 3 days. Their ovaries were homogenized with a teflon-glass homogenizer at 4°C in 40 mM Tris-HCl buffer, pH 7.5 containing 5 mM MgCl$_2$, 0.01% merthiolate and 1% BSA (Tris-MgCl$_2$-BSA buffer) at a concentration of 200 mg tissue/ml. The homogenate was filtered through nylon guaze and the filtrate was centrifuged at 1,700 g for 10 min. The precipitate was then suspended in Tris-MgCl$_2$-BSA buffer at a concentration of 50 mg or 100 mg tissue-equivalent/ml and used as the receptor preparation in RRA for FSH components or for serum samples, respectively. $^{125}$I-labelled FSH (rat FSH-I-6) was prepared by the lactoperoxidase method described by Miyachi et al. (1972) with minor modification. Radioreceptor assay was carried out as follows using polypropylene tubes (12×75 mm). For FSH components, 100 $\mu$l of Tris-MgCl$_2$-BSA buffer, 100 $\mu$l of the sample or standard (rat FSH-I-6) dissolved in Tris-MgCl$_2$-BSA buffer, 100 $\mu$l of the receptor preparation, and 100 $\mu$l of $^{125}$I-FSH were used. The tubes were shaked at 90 cycles/min in an incubator at 37°C for 90 min. For serum samples, Tris-MgCl$_2$-BSA buffer was replaced by hormone-free serum containing 0.01% merthiolate in the standard system, and the incubation period was 4 h. After the incubation the tubes were chilled in ice water and added with 500 $\mu$l of ice-cold Tris-MgCl$_2$-BSA buffer, then centrifuged at 1,700×g for 15 min. The supernatants were decanted off and the pellets were counted in an automatic gamma counter. Non-specific binding of $^{125}$I-FSH was assessed by adding 50 IU PMSG instead of the standard.

**Gel filtration**

After isoelectric focusing, the FSH components were separated and chromatofigred.
Fig. 2. Isoelectric focusing profiles of FSH in the anterior pituitaries collected at various times on the proestrous day (P). FSH in the fractions was determined by radioimmunoassay. The open circle (○) row in each panel indicates the pH. The numbers and letters above each peak represent the species of FSH isohormones as shown in Table 1.
on a 1.6×86 cm column of Ultrogel AcA 44 (LKB) at 4°C. About 1 ml samples containing 5 μg or more FSH were applied to the column and eluted with 50 mM Tris-HCl, pH 7.2 containing 0.5 M NaCl and 0.05% NaN₃ at a flow rate of 8.4 ml/hr. Fractions of 1.0 ml were collected and measured for FSH content by RIA. BSA, ovalbumin and α-chymotrypsinogen A, type II (Sigma Chemical Co., St. Louis, MO) were added in each run as internal markers.

**Statistical analysis**

Student's t-test or Cochran-Cox test were used to compare the means of the RRA/RIA ratio of serum FSH.

**Results**

**Changes in serum and pituitary FSH levels during the estrous cycle**

Serum and pituitary immunoreactive FSH at various stages during the estrous cycle are shown in Fig. 1. Serum FSH was low at 0900 h on the day of proestrus, rapidly rose and reached a peak at 1800 h on the same day. Thereafter serum FSH decreased gradually and remained low from 1500 h on estrus to 1500 h on diestrus I. The levels

![Isoelectric focusing profiles of FSH in the anterior pituitaries collected at various times on the estrous (E) day. For further details see legend to Figure 2.](image-url)
dropped further on the day of diestrus II.

Pituitary immunoreactive FSH abruptly declined at 1800 h on the day of proestrus coincidentally with the serum FSH surge. Thereafter pituitary FSH rapidly increased, but after 0300 h on the day of estrus, the rate of increase became slow. FSH reached its maximum at 1500 h on diestrus II.

**Isoelectric focusing profiles of pituitary FSH during the estrous cycle**

Isoelectric focusing profiles of pituitary FSH during the estrous cycle are shown in Figs. 2, 3 and 4. In the female rats, at least 14 FSH components were observed, the amounts of which fluctuated during the estrous cycle. At some stages, some components disappeared (e.g. components 1, 3 and 8). The isoelectric points (pIs) for these components are shown in Table 1. As for components A and B, in a preliminary experiment, they showed a single peak with pl

<table>
<thead>
<tr>
<th>FSH Components</th>
<th>pI (n)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>3.68a</td>
</tr>
<tr>
<td>1</td>
<td>3.71±0.08 (5)</td>
</tr>
<tr>
<td>2</td>
<td>3.87±0.06 (13)</td>
</tr>
<tr>
<td>3</td>
<td>4.05±0.08 (9)</td>
</tr>
<tr>
<td>4</td>
<td>4.17±0.06 (13)</td>
</tr>
<tr>
<td>5</td>
<td>4.47±0.06 (13)</td>
</tr>
<tr>
<td>6</td>
<td>4.79±0.06 (12)</td>
</tr>
<tr>
<td>7</td>
<td>5.06±0.06 (13)</td>
</tr>
<tr>
<td>8</td>
<td>5.31±0.04 (9)</td>
</tr>
<tr>
<td>9</td>
<td>5.44±0.07 (13)</td>
</tr>
<tr>
<td>10</td>
<td>5.74±0.07 (13)</td>
</tr>
<tr>
<td>11</td>
<td>6.05±0.11 (13)</td>
</tr>
<tr>
<td>12</td>
<td>6.66±0.10 (12)</td>
</tr>
<tr>
<td>B</td>
<td>8.09a</td>
</tr>
</tbody>
</table>

a: pIs were estimated by IEF with different pH range
n: Number of observations

**Fig. 4.** Isoelectric focusing profiles of FSH in the anterior pituitaries collected at 0900 h and 1500 h on diestrus I (DI) and diestrus II (DII), respectively. For further details see legend to Figure 2.
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of 8.09 and 3.68, respectively, when applied on IEF in a different pH range. On the proestrous day, before the FSH surge, most of the components were apparent and acidic components below pH 4.2 (components, A-4) were predominant. At 1800 h, simultaneously with the FSH surge, most of the components decreased to be trace amounts. On the other hand, only component B showed an increase at this time. This increase became eminent at 2100 h and stayed on until 0600 h of the estrous day. This phenomenon was specific for this period, since no increase in this component was observed at any other stage of the estrous cycle. Many other components started to increase from 2100 h on the proestrous day and became obvious by 0300 h on the estrous day. At 1500 h on the estrous day, acidic components below pH 4.2 became predominant again. On days diestrus I and diestrus II, the second decrease followed by restoration was observed in the acidic region below pH 4.2, and the relative amounts of components 5 to 7 increased from 0900 h on diestrus I to 1500 h on diestrus II. In addition, at 1500 h on diestrus II, when the pituitary FSH level was fully restored, and at the time of the pre-FSH surge (0900 h) on the proestrous day, large amounts of components A, 2 and 4 were observed.

At several stages in the estrous cycle, IEF was repeated two or three times, and the IEF profiles were found to be reproducible.

The ratio of receptor binding to immunological reactivity of pituitary FSH components and serum FSH

Eleven major components in the pituitary were separated and measured for FSH concentration simultaneously by RRA and RIA. The RRA/RIA ratios of these components are shown in Fig. 5. The ratio became higher as the pI of the component became higher.

FSH in serum samples collected at different stages of the estrous cycle were measured by both RRA and RIA. The assay values and RRA/RIA ratios are presented in Table 2. The RRA/RIA ratio of serum FSH differed according to the stage of the estrous cycle. The ratio was high at P 0900 h and DII 0900 h when the serum FSH level was very low, while it became

![Fig. 5. The receptor binding/radioimmunoassay activity ratio (RRA/RIA ratio) of the FSH components. Vertical bars indicate the mean ± S.E.M. (n=4. But in case of component A and 4, n=3).](image)
Table 2. Assay values for serum FSH measured by both radioreceptor assay (RRA) and radioimmunoassay (RIA), and RRA/RIA ratio. Serum samples were collected at different stages of the estrous cycle. Values are the mean±S.E.M.; n=number of animals.

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Serum FSH [ng×(I-6)/ml]</th>
<th>RRA</th>
<th>RIA</th>
<th>RRA/RIA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 0900 h</td>
<td>10</td>
<td>20.65±0.54</td>
<td>4.85±0.34</td>
<td>4.41±0.26</td>
<td></td>
</tr>
<tr>
<td>P 2100 h</td>
<td>10</td>
<td>33.80±1.06</td>
<td>15.23±0.50</td>
<td>2.23±0.08</td>
<td></td>
</tr>
<tr>
<td>E 0300 h</td>
<td>10</td>
<td>39.41±1.00</td>
<td>15.92±0.47</td>
<td>2.49±0.08</td>
<td></td>
</tr>
<tr>
<td>DI 0900 h</td>
<td>10</td>
<td>26.90±0.74</td>
<td>7.77±0.56</td>
<td>3.57±0.18</td>
<td></td>
</tr>
<tr>
<td>DII 0900 h</td>
<td>9</td>
<td>22.06±0.81</td>
<td>5.91±0.18</td>
<td>3.77±0.20</td>
<td></td>
</tr>
</tbody>
</table>

a and b: p<0.001 and p<0.05, respectively, vs. P 2100 h.
b: p<0.001 vs. E 0300 h.
c: p<0.001 vs. P 0900 h.
d: p<0.001 vs. P 0900 h.

low at the FSH surge e.g. P 2100 h and E 0300 h.

**Gel filtration of the FSH components**

Fig. 6. shows the elution profiles of the FSH components on gel filtration. The more acidic components were eluted earlier and thus appeared to be of larger molecular size (Fig. 6 a and b). However, highly acidic components exhibited peculiar gel filtration profiles (Fig. 6 c). Biphasic elution patterns were observed in components A, 2 and 4, indicating that these components included, at least, two kinds of FSH species which differed in molecular size. The position of their second peak corresponded to that where component B was eluted. The first peak of component 2 was smaller and the second peak was greater than those of component 4. The first peak of component A was only a trace amount and the second peak was really the only noticeable peak.

**Discussion**

We confirmed the existence of multiple forms of FSH molecule in the anterior pituitary of female rats. Microheterogeneity of FSH has been demonstrated in the rat (Foulds and Robertson, 1983; Chappel et al., 1983; Blum et al., 1985), hamster (Ulloa-Aguirre and Chappel, 1982), monkey (Chappel et al., 1984) and man (Zaidi et al., 1981). Furthermore, it has been shown that the charge heterogeneity of FSH changes according to the sex, age and endocrine status (Robertson et al., 1982; Chappel et al., 1983; Cameron and Chappel, 1985; Galle et al., 1983).

In the present study, We observed a dynamic change in the electrofocusing profile of pituitary FSH throughout the estrous cycle in female rats. These results agree with a previous report (Cameron and Chappel, 1985). But, in previous studies, the observation was intermittent, so that the change in the profiles with the passage of time was not so clear. Our detailed examination revealed an interesting and dynamic change in the FSH isoformes. Our results demonstrated that the quality, as well as the quantity, of FSH molecule in the anterior pituitary changes during the estrous cycle. The most drastic change occurred at night on the proestrous day. Coincidently with the FSH surge, the majority of the components were decreased and almost disappeared except for component B. This fact indicated that most of the components were released altogether from the anterior pituitary at the time of
the FSH surge, but only component showed an increase in quantity in this period and this increase in component B could be seen until 0600 h on the estrous day. Since no increase was observed at any other stage of the estrous cycle, this rise was specific for this period. During this period, pituitary FSH content was rapidly restored, therefore,
presumably FSH molecules were vigorously synthesized in the anterior pituitary. Component B is therefore assumed to be an immature type of FSH which is in the course of a synthetic process, presumably glycosylation. Similarly to LH (Hattori et al., 1985), it has been also suggested for FSH that the degree of sialylation on carbohydrate moieties is responsible for the charge heterogeneity, and that each FSH component differs in sialic acid content and the components with higher pIs contain less sialic acid (Chappel et al., 1982; Ulloa-Aguirre et al., 1984; Blum et al., 1985). We also confirmed that desialylation of native FSH components by neuraminidase treatment shifted their pIs, mainly to 9.33 (unpublished data). Hence, component B seems to be scarcely sialylated. Although it is not clear whether this component is also released from the anterior pituitary like other components or released after the transition to other components by more sialylation, its increase in amount after FSH surge may reflect the biosynthetic process of the FSH isohormone.

On the other hand, highly acidic components, e.g. components A, 2 and 4 were predominant when the pituitary FSH level was very high, i.e. in the morning on the proestrous day and in the afternoon on diestrus II. These components may be a stored type of FSH and would be expected to contain numerous sialic acid moieties as a result of complete glycosylation, on the basis of the facts described above.

The differences were observed between FSH components in the RRA/RIA ratio and the profile on gel filtration. The more acidic components exhibited a lower ratio of receptor binding to immunological activity and larger molecular size. These results agree with previous reports (Ulloa-Aguirre et al., 1984; Foulds and Robertson, 1983; Blum et al., 1985). Those and present studies suggest that sialic acid residue has an unfavorable effect on the binding of the FSH molecule with its receptor and that multiplicity in molecular size is also related to its sialic acid content. Furthermore, FSH isohormones with higher pI values possess more potent bioactivity (Miller et al., 1983), suggesting that sialic acid may play a negative role in the biological action of FSH. However, sialic acid was shown to have the advantage that it elongates the half-life of glycoprotein in the circulation (Morell et al., 1971). The more acidic FSH components have a longer plasma half-life and are abundantly present in serum (Blum and Gupta, 1985). Our results on gel filtration of highly acidic components, i.e. components A, 2 and 4, were partly incompatible with those reports. If the heterogeneity in the charge and molecular size of FSH is exclusively dependent on its sialic acid content, these highly acidic components are expected to eluted earlier on gel filtration. However, components 2 an 4 had biphasic elution patterns and component A was eluted at the same position as component B (Fig. 6c). These results were probably not due to the dissociation of intact FSH into subunits, as they were eluted earlier than α subunit, nor due to the contamination of another component, since, when refocused, they exhibited a single peak with the same pI as they showed before. Therefore components 2 and 4 consist of at least two FSH subpopulations which are different in molecular size. Perhaps FSH molecules in the first peak of components 2 and 4 contain much sialic acid. On the other hand, in the second peak of those and component A, other negatively charged groups, which lower the pI without increasing the molecular size, may be involved. Recently, two glycoprotein hormones, LH and TSH, were demonstrated to be sulfated in their carbohydrate moieties (Parsons and Pierce, 1980; Hortin et al., 1981; Bedi et al., 1982; Gesundheit et al., 1986). Furthermore, the possibility was shown that human FSH may contain sulfate residue (Tolvo et al., 1982).
It was speculated that this negatively charged residue might play a role analogous to that of sialic acid in the structure and function of glycoprotein hormones. FSH molecules consisting of component A and the second peak of components 2 and 4, in this study, may possibly contain sulfate.

Receptor binding activity of FSH molecules in circulation also changed according to the stage in the estrous cycle. When serum FSH was at its basal level, the RRA/RIA ratio was high, but at the FSH surge it became low. In the anterior pituitary, highly acidic components became predominant prior to the surge and then most of the components were released at the time of the surge. Therefore, low receptor binding activity of circulating FSH during the surge might be due to a greater population of highly acidic components which have lower potency. On the other hand, high receptor binding activity during the period with a low serum level suggested that the components with higher potency might be secreted from the pituitary in this state, and if we say on the basis of the RRA, that the serum FSH levels are considerably high on diestrus I, and even on diestrus II. This seems to be favorable for the growth and the maturation of the follicles for the next ovulation. But, so far, it is unknown which components are secreted in these stages. Besides, the mechanism of accumulation of the acidic components prior to the surge is not clear. Nevertheless, these results indicated that the type of FSH secreted, as well as the amount, is also controlled in accordance with the estrous cycle.

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