Different Profiles of Isoelectric Avian Luteinizing Hormone Components in Biological Activity and Immunoreactivity

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Abstract

Chicken LH components from the glycoprotein fraction of the anterior pituitary glands have been separated to isoelectric homogeneity by means of isoelectric focusing, and investigated for their biological activities in vitro. The activities of these components were measured with LH receptor binding, cyclic AMP accumulation and testosterone production in rat Leydig cells at equal doses expressed as immunoreactivity of IRC-2 (Gunma). All the components were active in the heterologous assay systems, although the relative potency expressed as the ratios of biological activity to immunoreactivity (B:I) differed significantly among the components. Component I, the amount of which is the largest (40—50%), with the most alkaline isoelectric point (pI) showed the highest B:I ratio, and the B:I ratio decreased with decreasing pI in the same way as in rat LH components (Wakabayashi, 1980; Hattori et al., 1983). Therefore, pituitary LH from photostimulated male quail, where the relative amount of component I was increased, was estimated to have higher B:I ratios than that from short-day (SD) treated male quail. Furthermore, the differences in activities among the components obtained by the three assays were in the following order: testosterone production>cyclic AMP accumulation>receptor binding, suggesting that the hormonal actions of components with higher B:I ratios (I, II and III) are efficiently amplified in the biological response to the final step. In the incubation of pituitary glands with hypothalamic extracts, component I in the pituitary glands from the long-day (LD) treated group was mostly decreased after the incubation, while all the components were decreased in parallel in the SD-treated group. The results suggest that the LH component releasable to GnRH changes in endocrine status though component I with the highest B:I ratio is relatively releasable in both SD- and LD-treated groups.

Pituitary glycoprotein hormones consist of a mixture of several isoelectric components in animal species and man (Braselton and McShan, 1970; Reichert, 1971; Yoshida and Ishii, 1973; Roos et al., 1975; Tamura-Takahashi and Ui, 1977; Wakabayashi, 1977; Yora and Ui, 1978; Hattori and Wakabayashi, 1979; Weise et al., 1979; Blum and Gupta, 1980). In previous reports from our laboratory, rat pituitary luteinizing hormone (LH) separated into 7 isoelectric components which had different ratios of in vitro biological activity to immunoreactivity (B:I), suggesting that the discrepancy between the bioassay and immunoassay of glycoprotein hormones is mainly responsible for the electrical heterogeneity (Wakabayashi, 1977, 1980; Hattori et al., 1983). Similar studies on human gonadotropins were reported by Diczfalusy and coworkers (Robertson and Diczfalusy, 1977; Robertson et al., 1977; Van Damme et al., 1977; Zaidi et al., 1982a, b). Avian LH also consists of several components in the pituitary glands.
of chicken, Japanese quail, guinea fowl and duck (Hattori and Wakabayashi, 1979; Wakabayashi, 1980). However, their biological activities remain obscure.

In the present study, the biological activity of each component separated from the chicken pituitary glands was characterized by functional characteristics such as receptor binding, cyclic AMP accumulation and testosterone production in vitro using rat Leydig cells.

**Materials and Methods**

**Birds**

Male Japanese quail (Coturnix coturnix japonica) were purchased from a commercial breeder at the age of 5 weeks and subjected to a photoperiod of 8L:16D (short-day (SD), lights on from 0900 to 1700) or 16L:8D (long-day (LD), lights on from 0500 to 2100) for 3 weeks before the experiments.

**Preparation of chicken LH components**

The chicken anterior pituitary glands (approximately 350) obtained from a slaughterhouse were homogenized in 30 ml of 0.01 M phosphate-buffered saline pH 7.5 (PBS) with a Teflon-glass homogenizer. After freeze-thawing and centrifugation at 23,000 g for 15 min, the glycoprotein fraction was extracted from the supernatant with ammonium acetate-ethanol (Hartree, 1966). The glycoprotein fraction was dissolved in 10 ml of 0.018% NaCl, and subjected to preparative isoelectric focusing with a column (LKB-Produkter AB, Bromma, Sweden), size 110 ml (8100-1), in an amount of 1.17 mg LH expressed as immunoreactivity of IRC-2 (Gunma), together with horse heart cytochrome c (pI=10.2) and horse skeletal muscle myoglobin (pI=7.5) (Sigma) as calibration proteins. The calibration proteins were purified to have homogenous pI using flat-bed isoelectric focusing. After focusing, the solution was eluted at a flow rate of 100 µl per min, and 450 µl fractions were collected. The pH measurement was carried out simultaneously during fractionation with a micro pH electrode (Fuji SE 1600GC). The fractions were diluted with PBS containing 0.1% gelatin and assayed for LH by radioimmunoassay.

**In vitro bioassay**

The biological activity of LH was measured by LH receptor binding, cyclic AMP accumulation and testosterone production in vitro in rat Leydig cells. Leydig cells were prepared from decapsulated testes of adult rats by collagenase digestion (Dufau et al., 1974). The LH receptor binding was carried out in a plastic tube (1.1×5.5 cm) (Catt and Dufau, 1972). Each tube contained Leydig cells equivalent to approximately 1/5 of a testis, 125I-labeled rat LH (NIAMDD rat LH for radioiodination supplied by Dr. A. F. Parlow, Rat Pituitary Hormone Program, NIAMDD, NIH) prepared with lactoperoxidase (Miyachi et al., 1972) and LH preparations in 0.5 ml of 40 mM Tris-HCl buffer (pH 7.4) containing 1% BSA. The tubes were incubated at 37°C for 3 hr, centrifuged and radioactivity in the precipitates was counted with a gamma counter. Non-specific binding was determined in the presence of 50 IU human chorionic gonadotropin (hCG). Cyclic AMP accumulation by Leydig cells was carried out in siliconized glass tubes (1.5×10 cm) (Mendelson et al., 1975). Leydig cells equivalent to approximately a half of a testis were incubated in 1 ml of Medium 199 containing 0.1% BSA and 0.5 mM theophylline with LH preparations at 37°C for 2 hr. After centrifugation at 1,500×g for 15 min, the supernatants were assayed for cyclic AMP with a radioimmunoassay kit (Yamas Shoyu Co., Choshi, Chiba, Japan). Testosterone production by Leydig cells was performed in a polystyrene tube (2.5×10 cm) (Dufau et al., 1974). Leydig cells equivalent to approximately 1/4 of a testis were incubated in 2 ml of Medium 199 containing 0.1% BSA and 0.5 mM theophylline with LH preparations at 37°C for 2 hr. Then the tubes were chilled in an ice bath and centrifuged at 1,500×g for 15 min. The supernatants were directly assayed for testosterone by means of a double antibody radioimmunoassay using 125I-labeled testosterone-3-carboxymethylxime-tyrosine methylster as tracer (Ismail et al., 1972). The biological activity was calculated with the conventional statistical method for parallel line assays. The results were expressed as the equivalent of avian LH (IRC-2 (Wakabayashi and Hattori, 1978). Each LH component was applied to the column in an amount equivalent to 1 µg or less, expressed as immunoreactivity of IRC-2 (Gunma), together with horse heart cytochrome c (pI=10.2) and horse skeletal muscle myoglobin (pI=7.5) (Sigma) as calibration proteins. The calibration proteins were purified to have homogenous pI using flat-bed isoelectric focusing. After focusing, the solution was eluted at a flow rate of 100 µl per min, and 450 µl fractions were collected. The pH measurement was carried out simultaneously during fractionation with a micro pH electrode (Fuji SE 1600GC). The fractions were diluted with PBS containing 0.1% gelatin and assayed for LH by radioimmunoassay.
Isoelectric focusing of the pituitary gland extracts

The pituitary glands were obtained from SD- or LD-treated male quail. PBS extracts were prepared as described previously (Hattori and Wakabayashi, 1979). A small preparative column of isoelectric focusing was used under conditions identical to those described above.

Incubation of the pituitary glands with hypothalamic extracts

The pituitary glands from SD- or LD-treated male quail were incubated in Krebs-Henseleit-glucose buffer, pH 7.2-7.3, with the 0.1 N HCl-ethanol extracts of quail hypothalami (Hattori et al., 1980). The pituitary glands (10 tissues per flask) were preincubated in 1 ml of the medium at 37°C under an atmosphere of 95% O₂ and 5% CO₂ for 10 min. After removing the medium, they were further incubated in a medium containing hypothalamic extracts under the same CO₂-O₂ atmosphere for 2 hr. The glands were then removed for LH analysis by isoelectric focusing. The media were centrifuged at 23,000×g for 15 min, and the supernatants were assayed for LH by radioimmunoassay.

Avian LH radioimmunoassay.

Chicken LH was measured by a double antibody radioimmunoassay as described previously (Hattori and Wakabayashi, 1979).

Results

Preparation of chicken LH components

The glycoprotein fraction prepared from the chicken pituitary glands contained 5 or more LH isoelectric components as previously described (Hattori and Wakabayashi, 1979). The replacement curves by the components were parallel to the standard curve obtained with IRC-2 (Gunma) by radioimmunoassay. The components obtained as a result of isoelectric focusing were individually subjected to refocusing on the column and the fractions were assayed for LH by radioimmunoassay. Each component was prepared to isoelectric homogeneity except component IV which was contaminated with a small amount of component V. The peak of each component refocused had the

![Graph](image)

Fig. 1. Refocusing of LH components on isoelectric focusing. (top panel), Initial stage isoelectric focusing of glycoprotein fraction from the chicken pituitary glands using an LKB column (110 ml). ○, LH activity by radioimmunoassay. ●, pH. (other panels), Refocusing of individual LH components prepared on isoelectric focusing of top panel. ○, LH activity by radioimmunoassay.

Conditions are described in MATERIALS and METHODS.
same isoelectric point (pI) as that before focusing, with values of 10.07, 9.43, 9.10, 8.50 and 7.92, indicating that there were no significant changes in the electrical charge during the preparation of the components. The fact that each peak remained homogeneous after refocusing indicates that isoelectric components of chicken pituitary LH are not artifacts.

**Biological activities of the components**

The biological activities of these isoelectric components were measured by LH receptor binding, cyclic AMP accumulation and testosterone production *in vitro* in rat Leydig cells. Each component was assayed at equal doses expressed as the immunoreactivity of IRC-2 (Gunma). The relative potency is, therefore, expressed as the ratio of biological activity to immunoreactivity (B : I). The sensitivity of measurements for chicken LH components using rat Leydig cells was low compared with mammalian LH because of the species-specificity of hormonal action. However, it is possible to measure the biological activity of chicken LH components by these heterologous assays. As shown in Fig. 2, all the components were active and their dose-response lines were parallel to the standard line with IRC-2 (Gunma) in the three assay methods. In the assay of cyclic AMP accumulation, the response in a logarithmic scale was roughly parallel to the standard. The sensitivity of the assay with testosterone production was less than 100 ng which was about 5-fold more sensitive than the other two assays. It was noted that the B : I ratio significantly differed among the components in the three assays, as shown in Table 1. Component I with more alkaline pI gave a higher B : I ratio, and the B : I ratios of the components decreased with decreasing pI. The estimated B : I ratios of LH components also differed according to the assay systems. The assay by testosterone production gave the highest estimate to a com-
Table 1. The activities of chicken LH components in LH receptor binding, cyclic AMP and testosterone production with rat Leydig cells.

<table>
<thead>
<tr>
<th>Components</th>
<th>Receptor Binding</th>
<th>Relative Potency (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (9.8)</td>
<td>2.76 (2.33-3.41)</td>
<td>0.055 (2.95 (2.34-3.81)</td>
</tr>
<tr>
<td>II (9.4)</td>
<td>1.72 (1.48-1.97)</td>
<td>0.045 (2.47 (2.22-2.77)</td>
</tr>
<tr>
<td>III (9.0)</td>
<td>1.55 (1.41-1.69)</td>
<td>0.027 (2.38 (2.24-2.54)</td>
</tr>
<tr>
<td>IV (8.5)</td>
<td>0.63 (0.57-0.69)</td>
<td>0.030 (0.69 (0.62-0.77)</td>
</tr>
<tr>
<td>V (8.0)</td>
<td>0.36 (0.26-0.42)</td>
<td>0.050 (0.25 (0.24-0.26)</td>
</tr>
</tbody>
</table>

* All the assays were performed in 2×2 point assay design with IRC-2 (Gunma) as the standard preparation. The amount of each component was determined by radioimmunoassay, and relative potency is expressed as the ratio of biological activity to immunoreactivity for IRC-2 (Gunma).

ponent, and cyclic AMP accumulation and receptor binding gave the estimates followed in descending order. For example, a comparison of components I and V for the B : I ratios revealed that the former was 7-fold in receptor binding, 11-fold in cyclic AMP accumulation and 20-fold in testosterone production higher than the latter.

Changes in the amounts of LH components of male quail pituitary glands

When the male quail maintained in SD were exposed to a long-day photoperiod of 16L : 8D, the amounts of LH components in the pituitary gland markedly increased at 3 weeks after the onset of photostimulation. As shown in Table 2, the relative amounts of component I increased mainly as compared with other components. However, there do not seem to be acute changes in the relative amounts of isoelectric components in the avian pituitary glands in in vivo experiments, i.e., castration and treatment with testosterone propionate (0.05 or 0.5 mg per day for 7 days). In the next experiments, the pituitary glands from SD- and LD-treated male quail were incubated with extracts of quail hypothalami, equivalent to 2 and 10 hypothalami. The LH amounts released to media by 2 hypothalamic extracts were 1.9-fold in the LD group and 7.8-fold in the SD group, indicating that the pituitary gland from SD-treated male quail is more sensitive to gonadotropin releasing hormone (GnRH) than that from LD-treated male quail. The PBS extracts of the pituitary glands incubated with hypothalamic extracts were subjected to isoelectric focusing and LH was measured by radioimmunoassay. As shown in Fig. 3, there were great differences in the profile of the reduction of LH components between the two groups. In the SD group, all the LH components decreased proportionally to their amounts in the pituitary gland. On the other hand, in the LD-treated group, component I was

Table 2. The relative amounts of isoelectric components in the pituitary glands from short-day and long-day treated male quail.

<table>
<thead>
<tr>
<th>Pituitary Glands</th>
<th>Components</th>
<th>I (9.8)</th>
<th>II (9.4)</th>
<th>III (9.0)</th>
<th>IV (8.5)</th>
<th>V/Acid (less than 8.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-Day (8L : 16D)</td>
<td>40.05*</td>
<td>19.34</td>
<td>17.12</td>
<td>9.69</td>
<td>13.78</td>
<td></td>
</tr>
<tr>
<td>Long-Day (16L : 8D)</td>
<td>51.22</td>
<td>16.78</td>
<td>13.96</td>
<td>5.23</td>
<td>12.79</td>
<td></td>
</tr>
</tbody>
</table>

* The values are expressed as percentages of the total amounts of LH components in the pituitary gland.
mainly decreased by incubation with 2 and 10 hypothalami.

**B:I ratios of pituitary LH from male quail**

As described above, the relative amounts of LH components in the pituitary glands of male quail do not always change in parallel. This fact indicates that the B:I ratios in pituitary LH from male quail differ. The biological activity of LH in the pituitary glands from SD- and LD-treated quail was measured by testosterone production in vitro in rat Leydig cells. Pituitary LH from the LD-treated group, which has a relatively high amount of component I, gave had much higher activity in the B:I than the SD-treated group (Table 3).

### Discussion

Considerable evidence has been accumulated indicating the electrical heterogeneity of pituitary glycoprotein hormones from animal species and man. We first found the presence of isoelectric LH components in the pituitary glands of chicken and Japanese quail (Hattori and Wakabayashi, 1979), and the peak of each component after re-focusing remained homogenous and there was no significant change in the pI value (Fig. 1), indicating that the components were not artifacts of the isoelectric focusing procedure. The biological activities of the

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Table 3. The biological activity of LH in the pituitary glands of male quail.

<table>
<thead>
<tr>
<th>Pituitary Glands</th>
<th>Relative Potency (95% CL)*</th>
<th>λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-Day (8L : 16D)</td>
<td>3.47 (3.31–3.64)</td>
<td>0.011</td>
</tr>
<tr>
<td>Long-Day (16L : 8D)</td>
<td>3.87 (3.71–4.03)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* The biological activity was determined by testosterone production in vitro in rat Leydig cells. The assays were performed in the same assay design as in Table 1. Relative potency is expressed as the ratio of biological activity to immunoreactivity for IRC-2 (Gunma).
BIOACTIVITIES OF AVIAN LH COMPONENTS

components separated from the glycoprotein fraction of the chicken pituitary glands were measured with a heterologous assay using rat Leydig cells. Testicular androgen secretion in birds is specific for LH but not FSH (Chase, 1982). Though the in vitro heterologous assays by receptor binding, cyclic AMP accumulation and testosterone production were less sensitive for measurement of avian LH preparations than the method using quail interstitial cells (Maung and Follett, 1977), evidence of a parallel between the avian standard preparation (IRC–2 (Gunma)) and the test preparation was obtained in each assay. Activity was also expressed as the B : I ratios, since the components were assayed at equal doses of IRC–2 (Gunma) immunoreactivity. According to the assay methods, interestingly, component I with more alkaline pl (9.8) displayed the highest B : I ratio and the ratio decreased with decreasing pl (Table 1). The characteristic profile of B : I ratios of chicken LH components showed a close agreement with that in rat LH components (Wakabayashi, 1980; Hattori et al., 1983). Zaidi et al. (1982) also reported that some purified human LH preparations with relatively high amounts of alkaline species (pl 8.5) had higher B : I ratios than those of other preparation. Differences in the B : I ratios of LH components from the chicken pituitary glands may not be responsible for other immunoreactive substances, if any, such as α and β subunits and metabolites. Gel filtration of each component on Sephadex G–100 revealed the presence of “big LH”, probably a dimeric form of native LH, though immunoreactive substances of smaller molecular size were not contained (Hattori and Wakabayashi, 1979). The relative amounts of “big LH” in these components were 15% for I, 32% for II, 40% for III and 30% for IV, suggesting that a physicochemical property of the isoelectric component might cause a difference in the B : I ratios. Thus, pituitary LH from animals and man consists of heterogenous molecular forms exibiting different B : I ratio. Furthermore, when the biological activity of the components was measured, differences among the estimates of the LH components by the assay with testosterone production were larger than those obtained with the assays involving receptor binding and cyclic AMP accumulation (Table 1). This observation indicates that the hormonal actions of components with higher B : I ratio are efficiently amplified in the biological response to the final step.

The relative amounts of isoelectric LH components in the pituitary glands of male Japanese quail were varied by photostimulation for 3 weeks (Table 2). Changes in the relative amounts of LH components were also observed in rat (Wakabayashi, 1977) and man (Strollo et al., 1981; Zaidi et al., 1982). In the rat pituitary glands, castration decreased the relative amounts of components with the highest B : I ratio and increased the relative amounts of components with lower B : I ratio, and resulted in a significant change in the B : I ratios in pituitary LH (Hattori et al., 1983). Moreover, the fact that molecular species of bioactive human LH were not in agreement between plasma and the pituitary gland (Zaidi et al., 1982) and that plasma from mid-cycle women contained high amounts of alkaline LH species (pl 8.5) (Strollo et al., 1981) indicates that LH species of rapid biosynthesis and release may be alkaline components. Similar finding for releasable LH species were obtained in the present study. In the incubation of the pituitary glands from SD- and LD-treated male quail with hypothalamic extracts, all the components in the pituitary glands of the SD-treated group decreased after incubation, while component I decreased mainly in the LD-treated group (Fig. 3). The ratios of radioreceptor assay to radioimmunoassay of LH released
from the pituitary glands after incubation with synthetic GnRH differed among the immature, premature and mature groups, and the ratio was higher in the mature group (Sharpe et al., 1975). The authors concluded that a change occurred in the LH molecule during stimulation of its release from the rat pituitary glands. However, we feel that there is a change in the relative amounts of pituitary LH components after incubation with GnRH but not in the LH molecule itself. Marked differences between in vitro biological assay and radioimmunoassay of serum LH in cycling women, men and postmenopausal women (Dufau et al., 1976 a, b) and in male rats (Solano et al., 1980) could be probably explained by the profile of the B:I ratios of LH species by isoelectric focusing.

Differences between total LH bioactivity in the pituitary glands from SD- and LD-treated male quail were significant but small compared with rat pituitary LH from male, female and castrated animals (Wakabayashi, 1977; Hattori et al., 1983). This seems to be due to a characteristic distribution of isoelectric LH components. Component I of chicken and quail LH corresponding to component F of rat LH is a major species. Component I occupies 40 to 50% of the total amount of LH, while the relative amount of component F is only less than 10% and greatly changes in endocrine status. In addition, the B:I ratios could vary according to the standard preparations used. An avian standard preparation IRC-2 (Gunma) roughly contains components I, II and III, and is similar to the isoelectric LH profile in the pituitary gland. In contrast to avian LH standard, a rat LH standard (RP-1) mainly contrasts of some components with a lower B:I ratio (Wakabayashi, 1977). It becomes necessary to improve a standard preparation possessing B:I ratios similar to those of pituitary and circulating LH, especially in rat LH. However, this is difficult since the LH species in the pituitary gland and serum vary according to the endocrine status, as noted above.

The role of sialic acid has been well documented in the in vivo biological activity of hCG. Van Hall et al. (1972) showed that progressive desialylation of hCG led to a reduced half-life of the hormone in plasma. There was indeed a correlation between the in vivo bioactivity and the sialic acid content of several isoelectric hCG components (Merz et al., 1974, Nwokoro et al., 1981), suggesting that sialic acid is responsible for the electrical heterogeneity in highly purified hCG. In addition, deglycosylation of hCG by anhydrous hydrogen fluoride caused a decrease in its cyclic AMP accumulation activity in vitro in rat interstitial cells, though the ability of the deglycosylated hCG to bind receptor was retained or increased (Manjunath and Sairam, 1982). Thus, carbohydrate moiety of glycoprotein hormones appears to play an important role in functional characteristics such as receptor binding, in vivo and in vitro biological responses. Whether carbohydrate moiety causes the difference in the B:I ratios among isoelectric LH components has to be investigated further.

Acknowledgments

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