In Vivo Bioactivities and Kinetic Parameters of Rat Luteinizing Hormone Components: Discrepancy Between In Vitro and In Vivo Assays

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Abstract. Rat pituitary LH shows an electrical charge heterogeneity owing to their carbohydrate moiety. The biological potencies of these isoelectric components increase with increasing pi when examined by in vitro testosterone production in isolated rat Leydig cells. The present study was carried out to clarify if this tendency was also true with in vivo action and to estimate their kinetic parameters. Seven isoelectric components of LH, i.e. X (pi 7.9), A (8.4), B (8.8), C (9.1), D (9.3), E (9.6) and F (9.8), were administered to cannulated adult male rats. Blood samples were serially collected over a 5 h period from the conscious animal, and rat LH and testosterone were assayed by RIA. The doses of a single component showed a linear relationship with the areas under the curve (AUC) of the blood LH. The testosterone AUC also correlated well with LH AUC. It was found that the components with lower pis had longer half-lives and larger LH AUC, and smaller total body clearance rates with highly significant correlation coefficients. The in vivo bioactivities of the components expressed as testosterone AUC increased with decreasing pis, indicating that the in vivo bioactivity was much influenced by the total body clearance rate. The intrinsic activities of the components were calculated as ratios of testosterone AUC to LH AUC where the effect of the clearance rate (or biological half-life) was eliminated. The intrinsic activities of components were not significantly different among the components except component X which showed a smaller activity. These data indicated that, with the exception of component X, an LH component with a lower pi has a longer biological half-life, resulting in a higher in vivo potency, quite contrary to the tendency of in vitro potency.

Key words: Charge heterogeneity, Rat LH, In vivo activity

LH is a glycoprotein hormone consisting of two non-covalently linked subunits, α and β. The α-subunit contains two N-linked oligosaccharides, and the β-subunit contains one such oligosaccharide [1]. Depending on the differences in isoelectric points (pis), rat pituitary LH can be separated into several components. This charge heterogeneity has been mainly attributed to the degree of terminal sulfation and sialylation in oligosaccharide chains [2, 3] which reflects the presence of various intermediate forms during the post-transcriptional oligosaccharide processing [4–6]. It is known that desialylation promotes the rapid in vivo clearance [7]. On the other hand, another elimination system involving hepatic receptors for sulfated oligosaccharides has also been shown [8–11].

The populations of these LH components differ according to the physiological states of the animals, such as sex, estrous cycles and castration [12].

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When the biological potencies of these components were examined by an in vitro assay method with testosterone production by isolated Leydig cells as a marker, we found that the potency increased with increasing pI [13]. It was also found that the receptor-binding activity of these components increases with increasing pI [14].

The present experiment was conducted to clarify if this tendency was also true of their in vivo action, and to estimate kinetic parameters of these components which might influence the activity.

Materials and Methods

Animals

Male Wistar rats, 8 weeks of age, were purchased from Charles River Japan Inc., Yokohama, Kanagawa Pref., and served as LH recipients. Each animal was kept in a polycarbonate cage with wood shavings on the floor. The animal room was air-conditioned at 23 ± 1°C and humidity 60 ± 5%, and lighted for 14 h per day, from 0600 to 2000 h. The rats were fed ad libitum with a laboratory ration and clean fresh water. One hundred and eighty male rats of the same strain and same age to serve as pituitary donors were also purchased from the same source and killed immediately after arrival.

Preparation of LH components

The pituitary glands obtained from the donors were divided into 3 portions. A portion of the glands (approximately 60) was homogenized in 10 ml of glass-distilled water, frozen and thawed, then centrifuged at 23,000 × g for 20 min to remove insoluble fragments. The supernatant fluid was subjected to preparative isoelectric focusing with a column (LKB-Produkter AB, Bromma, Sweden), size 110 ml (8100-1), as described previously [12]. As carrier ampholites, Ampholines pH 9–11, pH 7–9 and pH 5–7 (Pharmacia Biotech) were mixed to make up a pH gradient from pH 7 to 11. A density gradient from 5 to 50% sorbitol was formed for stabilization of the isoelectric focusing solution. Isoelectric focusing was run for 72 h at 2–4°C, the voltage applied being increased stepwise from 600 to 1,450 V. After focusing, 2.5 ml fractions were collected, and the fractions containing the LH components were identified by radioimmunoassay. Other portions of the glands were also separated similarly, and the fractions containing same components were pooled. Then 1.5 ml saline containing 0.5% rat serum albumin was added to each 2.5 ml fraction, followed by dialysis against glass-distilled water in the cold for 2 days and then lyophilization. The lyophilized materials were stored at −30°C until use.

Cannulation of the animals

At approximately 10 weeks of age, the adult rats (374.53 ± 2.43 g, mean ± SEM, n=66) were cannulated from the jugular vein into the right ventricle with SILASCON Medical Grade Tubing, 0.5 mm inner diameter and 1.0 mm outer diameter, (Kaneka Medix Co., Japan) under sodium pentobarbital (0.04 mg/g body weight ) anesthetic [15]. The end of the tubing was taken out through the skin and fixed behind the neck of the animal. The cannula was filled with physiological saline containing 10 U heparin/ml. The cannulated animals were allowed to recover for a minimum period of 20 h.

In vivo experiments

In vivo experiments were carried out with a Tsumura Free Moving System (Cannula Swivel TCS 2–21; Tsumura Co., Tokyo, Japan). The hormone dissolved in 500 ul of physiological saline containing 1% rat serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo) or vehicle (1% RSA-saline) was administered to the rats through the cannula. Blood samples (100 ul) were collected through the cannula directly before (expressed as 0 min) and 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after the administration, and thereafter at 30 min intervals for another 4 h. The samples were immediately mixed with 400 ul PBS, pH 7.5, containing 1% BSA and 0.05% sodium azide, then centrifuged and the supernatant fluids were kept frozen until LH and testosterone assays.

A preliminary dose response study was conducted with component B (125 ng, 250 ng, 500 ng, 1000 ng, 2000 ng), and the experiments were carried out with various components at a fixed dose of 500 ng.
Radioimmunoassay

The LH level was determined by double antibody RIA [16], with an NIDDK assay kit (NIDDK LH I-9 for radioiodination, NIDDK LH RP-3 as the standard and NIDDK anti-LH S-11 as the antiserum) with the 2nd antibody prepared in our laboratory (HAC-RBA2-03GTP86). The sensitivity and intra- and inter-assay coefficients of variation were 0.125 ng/ml, 4.77% and 6.01%, respectively. The testosterone level was measured with a DPC total testosterone kit (Nippon DPC Corporation, Tokyo, Japan). The sensitivity and intra- and inter-assay coefficients of variation were 0.04 ng/ml, 6.15%, and 4.27%, respectively.

Analyses of data

Kinetic parameters of LH components were estimated after subtraction of the basal LH level (0 min), and the area under the curve (AUC) was estimated by the trapezoidal method [17]. The circulating half-life was calculated as 0.693/k, where k is the elimination rate constant estimated as the slope of the log-linear regression. Total body clearance (CLtot) was determined from the ratio of the dose administered to the AUC.

To estimate in vivo gonadotropic activity, testosterone AUC was calculated by the trapezoidal method followed by subtraction of the basal AUC value obtained with vehicle administration. To eliminate the influence of biological degradation and excretion, intrinsic testosterone producing activity was calculated as the ratio of testosterone AUC to LH AUC.

Results

Separation of LH components by isoelectric focusing

The preparative IEF of rat pituitary LH showed similar elution profiles throughout 3 trials, as shown in Fig. 1. Seven isoelectric components were obtained, i.e. X (pI 7.9), A (8.4), B (8.8), C (9.1), D (9.3), E (9.6) and F (9.8) which corresponded well with the data in the former report [12]. These seven isoelectric components were subjected to the in vivo potency estimation.

Dose response study

The results of the dose-response study are summarized in Fig. 2. Fig. 2A shows disappearance profiles of LH component B. Blood LH levels decline rapidly after intravenous administration through the cannula. Blood testosterone levels after LH administration are shown in Fig. 2B. The blood testosterone level increased dose-dependently. The relationship between LH AUC and the doses of
LH component B is shown in Fig. 2C. The LH AUC increased linearly depending on the dose of LH component B. Fig. 2D shows a dose dependent increase in blood testosterone in response to LH component B administration. C) LH AUC and doses of LH component B, D) Testosterone AUC and doses of LH component. AUC, area under the curve.

Kinetic parameters

Fig. 3 shows the blood LH levels after intravenous administration of LH components. From these data, the kinetic parameters were calculated and shown in Table 1. As shown in Fig. 3, the disappearance profiles differed depending on the component. The estimated half-life of a LH component was shorter, and LH AUC was smaller, and the total body clearance rate was greater when it has a higher pI. Fig. 4 shows the scatter diagram of kinetic parameters plotted against pls of the components. Each kinetic parameter correlated highly significantly with pl, i.e. half-life \( r = -0.82, P<0.001 \) (Fig. 4A); LH AUC \( r = -0.92, P<0.0001 \) (Fig. 4B); and \( CL_{tot} = 0.85, P<0.0001 \) (Fig. 4C).

### Table 1. Kinetic parameters of rat LH components

<table>
<thead>
<tr>
<th>Component</th>
<th>pl</th>
<th>half-life (min)</th>
<th>LH AUC (ng·min/ml)</th>
<th>CL_{tot} (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>7.9</td>
<td>17.78 ± 0.31</td>
<td>445.50 ± 21.63</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>A</td>
<td>8.4</td>
<td>15.67 ± 0.70</td>
<td>415.91 ± 7.82</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>B</td>
<td>8.8</td>
<td>15.02 ± 0.54</td>
<td>356.16 ± 9.77</td>
<td>1.41 ± 0.04</td>
</tr>
<tr>
<td>C</td>
<td>9.1</td>
<td>14.38 ± 0.47</td>
<td>331.31 ± 22.00</td>
<td>1.54 ± 0.10</td>
</tr>
<tr>
<td>D</td>
<td>9.3</td>
<td>11.42 ± 0.85</td>
<td>248.12 ± 17.52</td>
<td>2.06 ± 0.14</td>
</tr>
<tr>
<td>E</td>
<td>9.6</td>
<td>13.43 ± 0.27</td>
<td>210.31 ± 6.33</td>
<td>2.39 ± 0.07</td>
</tr>
<tr>
<td>F</td>
<td>9.8</td>
<td>9.90 ± 0.36</td>
<td>160.72 ± 8.26</td>
<td>3.15 ± 0.17</td>
</tr>
</tbody>
</table>

The results are the mean ± SEM (n=6). LH AUC, area under the curve of LH; CL_{tot}, total body clearance.
In vivo gonadotropic activities

A bolus injection of each LH component caused a rapid rise in blood testosterone which was already seen 5 min after the injection, then decreased rather quickly during the next 1 h, and gradually returned to basal levels during the following 3 h (Fig. 5). From the profile of the blood testosterone levels, we calculated testosterone AUC, and used it as a parameter which expresses the in vivo potency of a component. We also calculated the ratio of testosterone AUC to LH AUC in order to compare the intrinsic potencies of the components by eliminating the influence of their half-lives. The in vivo potency expressed by testosterone AUC, except component X, increased with decreasing pl (Fig. 6), and correlated well with pl (Fig. 8A, \( r = -0.83, P < 0.001 \)). Component A, the pl of which is 8.4, showed the most potent activity of all. Intrinsic testosterone producing potencies were not significantly different among the components with the exception of component X as shown in Figs. 7 and 8B. LH AUC and testosterone AUC were highly correlated as shown in Fig. 8C (\( r = 0.81, P < 0.001 \)).

Discussion

Our present study revealed the kinetic parameters and in vivo bioactivities of seven LH isoelectric components in adult male rats. The disappearance patterns of the blood LH components following bolus injections showed that LH components with lower pls had longer half-lives than those with higher pls. AUC of LH components decreased with increasing pl, and the total body clearance rate also increased with increasing pl. LH is mainly distributed to and broken down in the kidneys [18] and liver [18, 19]. Desulfated bovine LH was reported to be cleared rapidly from the circulation [11]. Desialylation also promotes the rapid in vivo clearance [7]. Both
desialylation and desulfation cause the appearance of galactose on the surface of the oligosaccharide moiety, and such glycoproteins are easily trapped by asialoglycoprotein receptor in the liver [11]. The present results seemed to be consistent with this kind of clearance mechanism, but from our previous studies [4-6], it is possible that the naturally occurring LH components with highly
alkaline pIs have high-mannose type oligosaccharide chains, i.e. the molecules which appear in the early steps of glycoprotein processing. It is more likely that these highly alkaline components are trapped by the mannose receptor in the liver [20]. The percentages of these highly alkaline components are higher in female rats than in males [12]. In female rats, the pituitary LH content greatly decreases in the evening of the proestrous day, and it is recovered until the morning of the next proestrous day, indicating that LH is renewed every 4 days. Owing to such clearance mechanisms, after secretion from the pituitary gland, LH components with low alkaline pIs stay longer in the circulation than those with higher alkaline pIs, resulting in more potent in vivo bioactivity as revealed by the testosterone AUC.

In recent endocrinological studies, most experiments have been carried out with in vitro techniques. We have also estimated the biological potencies in our studies on the charge heterogeneity of LH with in vitro testosterone production by dispersed rat or mouse Leydig Cells [21-23]. We [12,14] and other investigators [24-26], have found that the less alkaline anterior pituitary LH components exhibit a lower ratio of biological activity (in vitro) to immunological activity (RIA). A similar tendency was also reported for the LH components of the chicken [27]. The present in vivo study indicated a quite opposite tendency: that LH components with lower alkaline pIs had stronger potencies. This discrepancy between in vitro and in vivo findings is mostly explained by the difference in the biological half-lives of the components. The influence of the half-life seemed to overcome the difference of in vitro potencies, but we found another discrepancy. We calculated the in vivo intrinsic testosterone producing activities from the ratio of testosterone AUC to LH AUC where the influence of the clearance rate should be eliminated, and the activity to stimulate the testicular interstitial cells should be estimated. The results indicated that intrinsic testosterone producing activities were nearly the same in all components except component X which must have a different nature from the other components. The reason for this discrepancy between in vitro activity and intrinsic activity obtained in in vivo experiments is not yet thoroughly understood. In our previous studies, we measured LH components by radioreceptor assay, and compared the results with those of RIA [14]. We found that RRA/RIA ratios were higher in the components with higher pIs. This fact may indicate that the difference in in vitro potencies is due to the difference in the affinities of the components for the receptor. But usually the receptor-binding activity is studied in the absence of serum components, and so is the in vitro testosterone production. This is a situation which is quite different from that of in vivo studies. It is well known that the existence of serum components, i.e. the addition of a large volume of serum to the incubation system, strongly inhibits the binding of a hormone to the receptor. This may explain the difference between intrinsic testosterone producing activities in vivo and in vitro, though this problem requires further study.

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