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Abstract

We have previously described the preparation, purification and partial characterization of recombinant (rec) forms of rat luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the present study, the special functional features of these hormones were studied further, in vitro and in vivo, and compared with human recLH and recFSH, as well as with human urinary choriogonadotropin (hCG) and rat pituitary LH (NIDDK-RP3). In radioreceptor assay, the affinity of hCG binding to rat testis membranes was 5-fold higher than that of human recLH and 100-fold higher than that of rat recLH. In in vitro bioassay, using dispersed adult mouse interstitial cells or a mouse Leydig tumor cell line (BLT-1), hCG and human recLH were 10- to 20-fold more potent than rat recLH. Correspondingly, rat pituitary LH was about 10-fold less potent than rat recLH, and evoked a maximum testosterone response that was about half of that elicited by the other LH/CG preparations. Rat recFSH was about 10-fold less potent than human recFSH in stimulating cAMP production of a mouse Sertoli cell line (MSC-1) expressing the recombinant rat FSH [...]

Reference


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K Hakola, A-M Haavisto, D D Pierroz1, A Aebi1, A Rannikko, T Kirjavainen, M L Aubert1 and I Huhtaniemi

Department of Physiology, University of Turku, FIN-20520 Turku, Finland, and 1Division of Biology of Growth and Reproduction, Department of Pediatrics, University of Geneva Medical School, 1211 Geneva 14, Switzerland

Requests for offprints should be addressed to I Huhtaniemi

Abstract

We have previously described the preparation, purification and partial characterization of recombinant (rec) forms of rat luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the present study, the special functional features of these hormones were studied further, in vitro and in vivo, and compared with human recLH and recFSH, as well as with human urinary choriongonadotropin (hCG) and rat pituitary LH (NIDDK-RP3). In radioreceptor assay, the affinity of hCG binding to rat testis membranes was 5-fold higher than that of human recLH and 100-fold higher than that of rat recLH. In in vitro bioassay, using dispersed adult mouse interstitial cells or a mouse Leydig tumor cell line (BLT-1), hCG and human recLH were 10- to 20-fold more potent than rat recLH. Correspondingly, rat pituitary LH was about 10-fold less potent than rat recLH, and evoked a maximum testosterone response that was about half of that elicited by the other LH/CG preparations. Rat recFSH was about 10-fold less potent than human recFSH in stimulating cAMP production of a mouse Sertoli cell line (MSC-1) expressing the recombinant rat FSH receptor.

The circulating half-times (T1/2) of rat and human rec hormones were assessed after i.v. injections into adult male rats rendered gonadotropin-deficient by treatment with a gonadotropin-releasing hormone antagonist. A novel immunometric assay was used for the rat FSH measurements. In the one-component model the T1/2 values of rat and human recLH were 18·2 ± 1·9 min (n=7) and 44·6 ± 3·1 min (n=7) respectively and those of rat and human recFSH were 88·4 ± 10·7 min (n=6) and 55·0 ± 4·2 min (n=6) respectively; the two-component models revealed similar differences between the rec hormone preparations. Collectively, rat recLH was eliminated significantly faster from the circulation than human recLH (P<0·0001). In contrast, the elimination of rat recFSH was significantly slower than that of human recFSH (P=0·02).

In conclusion, rat recFSH and rat recLH display lower biopotencies per unit mass than the respective human hormones in vitro, and also in vivo for LH. This is paralleled by shorter T1/2 of rat recLH than the respective human hormone in the circulation, whereas human recFSH has a shorter T1/2 than human FSH. The special functional features of the rat rec gonadotropins emphasize the use of these preparations on studies of gonadotropin function in the rat, an important animal model for reproductive physiology.


Introduction

The two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as their receptors, show high structural homology between different mammalian species. The cDNAs and genomic genes of the rat and human FSH and LH (see Gharib et al. 1990 for review), and their respective receptors (McFarland et al. 1990, Sprengel et al. 1990, Minegishi et al. 1990, 1991, Koo et al. 1991, Heckert et al. 1992) have recently been cloned. The homology at the DNA level of the rat and human gonadotropins and their receptors is 70–90% (Gharib et al. 1990, McFarland et al. 1990, Minegishi et al. 1990). However, the receptor binding of rat and human FSH and LH to the homologous and heterologous receptors vary more than could be predicted from the structural differences of these molecules. The human FSH receptor preferably recognizes human FSH to the respective LH (Tilly et al. 1992, Mulder et al. 1994), whereas the human LH receptor does not bind the rat LH at all (Jia et al. 1991). In contrast, the rat LH receptor preferably binds LH or choriongonadotropin (CG) of human origin (Huhtaniemi & Catt 1981, Jia et al. 1991). These differences suggest that it may be important to use homologous
Recombinant forms of rat and human LH and FSH

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Materials and Methods

Hormone preparations

The highly purified recombinant (rec) forms of rat LH (95% purity, 1100 IU/mg by in vitro bioassay, in relation to human recLH; Hakola et al. 1997b) and FSH (98% purity, 8820 IU/mg by in vitro bioassay, Hakola et al. 1997a) and of human FSH (Org 32489, >95% purity, 11 500 IU/mg by bioassay; batch 65), were donated by Organon (Organon International BV, Oss, The Netherlands). Highly purified human recLH (99.9% purity, 15 900 IU/mg by in vitro bioassay, batch C31) was generously gifted of Ares-Serono (Geneva, Switzerland), and highly purified urinary hCG (NIH CR 121, 11 500 IU/mg) was a generous gift of NICDH (NIH, Bethesda, MD, USA). Rat luteinizing hormone (NIDDK-rLH-RP3) and follicle-stimulating hormone (NIDDK-rFSH-RP2), both of pituitary origin, were used as reference preparations, and were obtained from Dr A F Parlow (Harbour-UCLA Medical Center, Torrance, CA, USA). The gonadotropin-releasing hormone (GnrRH) antagonist used, Cetrorelix acetate (SB-75), was donated by Asta Medica AG (Frankfurt, Germany).

Animals

Adult male rats (2–4 months of age) of the Sprague–Dawley strain and adult mice of the NMRI strain were used. The temperature of the vivarium was controlled (21–23 °C) and the light period was adjusted to 14 h light and 10 h darkness. The animals had free access to standard pelleted laboratory animal food and tap-water. The study was approved by the local Ethical Committee of Animal Experimentation.

LH receptor binding-inhibition assay

This assay was based on displacement of radioiodinated hCG from rat testicular membrane preparations by noniodinated LH/CG preparations, as described earlier (Huhtaniemi & Catt 1981). The assays were repeated three times with three replicate samples.

In vitro bioassay of LH

The in vitro bioassay was based on stimulation of cAMP and testosterone production in dispersed mouse testis interstitial cells as described earlier by van Damme et al. (1974) and modified by Ding and Huhtaniemi (1989). The cell stimulations were repeated three times with three replicate samples.

Stimulation tests with the murine Leydig tumor cell line (BLT-1)

The BLT-1 cells (Kananen et al. 1996) are derived from a mouse Leydig cell tumor originating from a transgenic mouse expressing a 6 kb mouse inhibit α-subunit promoter/Simian virus 40 T-antigen (SV40 Tag) fusion gene. The cell line expresses the LH receptor and produces cAMP and progesterone in response to LH stimulation. The culture medium was Dulbecco’s modified Eagle’s medium/F12 (1:1, with 0.365 g/l l-glutamine) (Life Technologies, GIBCO BRL, Glasgow, UK) supplemented with 10% fetal calf serum (FCS, AutoGen Bioclear, Calne, Wilts, UK), 4.5 g/l glucose, 20 mmol/l HEPES, 0.1 g/l gentamycin (Biological Industries, Bet-HaEmek, Israel) and 1.25 mg/l fungizone (GIBCO BRL). BLT-1 cells were inoculated to 24-well plates (Greiner Labortechnik, Frickenhausen, Germany) at 10⁵ cells/well in 0.5 ml complete culture medium, on the day before the experiment, and cultured at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The next day the cells were washed with PBS (GIBCO BRL), and 0.5 ml culture medium without FCS was added, containing 0.2 mmol/l 3-isobutyl-1-methylxanthine (MIX; Aldrich-Chemie, Steinheim, Germany), hCG, human recLH, rat recLH or no hormones. In the dose–response experiments, 0.1 ml culture medium was removed after 1-h culture, diluted 1:1 with 2 mmol/l theophylline, boiled for 5 min, and stored at −20 °C for cAMP measurements (see below). After an 8-h culture, the rest of the media were collected, boiled for 5 min, and stored at −20 °C for progesterone measurements (see below). In the time–response experiments, the concentrations of the above hormones were 3 μg/l and the culture time varied from 1 h to 48 h.
stimulations were repeated three times with four replicate samples in the dose–response experiments, and six replicate samples were used in the time–response experiments.

Stimulation tests with the murine Sertoli tumor cell line (MSC-1)

The MSC-1 cells are derived from a mouse Sertoli cell tumor originating from transgenic mice carrying a fusion gene of the human anti-Müllerian hormone promoter sequences linked to the SV40 Tag gene (Peschon et al. 1992). The cell line used was stably transfected with the rat FSH receptor cDNA, and displayed FSH binding and FSH-responsive cAMP production (Eskola et al. 1998). The culture medium used was as above without fungizone. MSC-1 cells were inoculated to 24-well plates (Greiner) at 50 000 cells/well in 0·5 ml culture medium on the day before the experiment, and cultured at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The next day, 0·5 ml fresh culture medium was added containing 0·2 mmol/l MIX and human recFSH, rat recFSH or no hormones. After 4 h, the culture media were collected and treated as above for the cAMP measurements. The MSC-1 cell stimulations were repeated three times with four replicate samples.

In vivo experiments

Adult male rats (weighing 233–265 g) of the Sprague–Dawley strain were used (6–7 rats per group). The day before the experiment a cannula was inserted into the right jugular vein of the rats. The rats were pretreated with Cetrorelix acetate in 5% mannitol (100 µg/rat) the day before, and 1 h before the experiments. When measuring FSH half-times, an additional dose of Cetrorelix (100 µg/rat) was given 8 h after the FSH injections. Thereafter, the rats received a single intravenous injection of rat recLH (10 µg/rat), human recLH (1 µg/rat), rat recFSH (10 µg/rat) or human recFSH (1 µg/rat) in Ringer solution+0·1% BSA (0·1 ml/rat), to allow measurements of circulatory half-times of these hormones. The blood samples (0·2 ml) were collected through the indwelling venous jugular catheter, followed by flushing of the catheter with 0·4 ml Ringer/heparin. Serial blood samples were collected in this fashion prior to the gonadotropin injections, and at times 7·5, 15, 30, 60, 90, 120, 240 and 300 min for LH and 0·5, 1, 3, 5, 7, 9, 12, 24 h for FSH half-time (T₁/₂) measurements. The blood samples were allowed to clot, centrifuged, and the serum was stored at −20 °C until the hormone measurements.

Hormone and cAMP measurements

Rat LH was measured using the time-resolved immunofluorimetric assay (IFMA, Delfia, Wallac OY, Turku, Finland) as described before (Haavisto et al. 1993) with the exception that rat recLH was used as standard. Human LH (hLH Spec, Wallac OY) and rat and human FSH (hFSH, Wallac OY) from the rat sera were measured using the time-resolved immunofluorimetric assay principle (Delfia) described previously by Lövgren et al. (1984). A rat FSH immunofluorimetric assay (IFMA) was established and carried out as follows. After screening numerous monoclonal antibodies (MAB) against rat FSH, two antibodies against human FSH were found to display sufficient binding. The MAB against hFSH β-subunit (no. 6602, Medix, Kauniainen, Finland) and the MAB against hFSH α-subunit (8D10, Wallac OY) were used as capture and tracer antibodies respectively. The capture antibody was biotinylated (e.g. Haavisto et al. 1993) and it was attached (0·5 µg/well; 2·5 mg/l) to streptavidin–coated microtiter wells (Delfia) for 30 min at 20 °C under continuous shaking, using a Delfia Plateshaker (Wallac OY). After washing twice with Delfia platewasher (Wallac OY), 25 µl rat FSH standard (NIDDK-rFSH-RP2) or sample were incubated with 0·3 µg/well (1·3 mg/l) europium–labeled monoclonal antibodies (MAB) against rat FSH, two anti-human FSH were found to display su

Statistical analyses

The half-time (T₁/₂) values of LH and FSH elimination from rat circulation after i.v. injection were estimated by using the least-squares method. Both one- and two-component models were used. The elimination function \[\ln[1-E(t)]=e^{-\frac{t}{\ln(2)/HL}}\] of the one-component model is: \[E(t)=e^{-\frac{t}{\ln(2)/HL}}\] and of the two-component model is: \[E(t)=F\times e^{-\frac{t}{\ln(2)/HL}}+\]
+(1 − F) × e^{−t × \ln(2)/HL_2}, where t is the time difference from the start of the measurements (t_0), HL_1 and HL_2 are the two half-lives of elimination, and F and 1 − F their fractional distributions respectively.

The data are expressed as means ± s.e.m. The statistical analyses were carried out by a Macintosh version of the StatView and SuperANOVA programs (Abacus Concepts, Inc., Berkeley, CA, USA) using 1 Factor ANOVA, followed by Duncan’s new multiple range and Fisher’s protected LSD post-hoc tests, or unpaired Student’s t-test in the case of two groups. A P value less than 0.05 was considered statistically significant.

Results

**LH receptor binding-inhibition assay**

In the radioreceptor assay using rat testis membranes, hCG binding displayed affinity that was about 3-fold higher than that of human recLH (P=0.0002) and about 100-fold higher than that of rat recLH, in physiological salt concentrations (Fig. 1).

In vitro bioassay of LH using mouse interstitial cells and BLT-1 cells

In the in vitro bioassay using mouse interstitial cells, human recLH and hCG evoked cAMP and testosterone responses in a dose-dependent manner (Fig. 2A,B). The human hormone evoked similar maximum responses of cAMP and testosterone, about 4 nmol/l and 50 nmol/l respectively. The maximum testosterone response to rat recLH was also similar, but the highest dose of this hormone was not high enough to saturate the cAMP response. Rat pituitary LH (RP3) was clearly less effective than the other
preparations, even the highest dose only marginally stimulated cAMP production, and the maximal testosterone response remained at about 40% of that of the other hormone preparations tested. Human recLH and hCG were equipotent in the in vitro bioassay, and rat recLH was about 30-fold less potent. The potency of RP3 could not be accurately compared with the other hormones, due to the low level of cAMP and testosterone response even at the highest concentrations tested. The mean ED$_{50}$ values were similar for hCG and human recLH, 0.27 and 0.33 µg/l respectively. The ED$_{50}$ value was 7.9 ± 5.5 µg/l (n=3) for rat recLH.

The capacities of rat recLH, human recLH and hCG to stimulate cAMP and progesterone production in murine Leydig tumor cells (BLT-1) were also determined. The cell line expresses the LH receptor, and produces cAMP and progesterone in response to LH stimulation (Kananen et al. 1996). All the three LH/CG forms tested stimulated cAMP and progesterone production in a dose–dependent manner and their maximum responses were similar (data not shown). As in primary interstitial cells, hCG and human recLH were over ten times more potent than rat recLH.

**Stimulation tests with the murine Sertoli tumor cell line (MSC-1)**

The MSC-1 cell line permanently transfected with the rat FSH receptor cDNA was used to measure the cAMP response to stimulation with rat recFSH and human recFSH. Both hormones stimulated cAMP production in a dose–dependent manner (Fig. 3). Human recFSH was about 10-fold more potent than the respective rat hormone. The maximum stimulation was about 200-fold from the control level (no hormone added) with both hormones.
In vivo experiments

The T₁ values in rat circulation were determined after 10 µg and 1 µg i.v. injections of rat and human recLH respectively, in gonadotropin antagonist-treated rats. The maximum concentrations, at 7·5 min after the injections, were 3650 ± 420 µg/l for rat recLH, and 866 ± 36 µg/l for human recLH. The T₁ values were 18·2 ± 1·9 min (n=7) and 44·6 ± 3·1 min (n=7) for rat recLH and human recLH respectively (Fig. 4A), and the difference between the two hormones was significant (P<0·0001). In two-component models, the first half-times were 12·7 ± 3·5 min and 10·6 ± 2·2 min and the second half-times were 43·7 ± 11·8 min and 88·7 ± 5·7 for rat recLH and human recLH respectively. Both recLH preparations showed similar maximum testosterone responses, 40–50 nmol/l, 90 min after the LH injections (Fig. 4B,C). It was found that the testosterone levels were unaltered between 90 and 300 min after the human LH injection, whereas a significant decrease was observed at the same time in the rat LH-injected animals.

The T₁ values of rat and human recFSH in rat circulation were determined after 10 µg and 1 µg i.v. injections respectively, in gonadotropin antagonist-treated rats (Fig. 5). The maximum concentrations, at 30 min after the injections, were 1450 ± 118 µg/l (n=6) for rat recFSH, and 422 ± 19 IU/l (n=6) for human recFSH. The T₁ values were 88·4 ± 10·7 min (n=6) and 55·0 ± 4·2 min (n=6) for rat recFSH and human recFSH respectively, and the difference between the two hormones was significant (P<0·02). In two-component models, the fast phases were 64·0 ± 9·7 min and 38·0 ± 5·3 min and the slow phases were 10·0 ± 2·9 h and 4·7 ± 1·3 h, for rat and human recFSH respectively.

Discussion

In this study, we have compared in vitro and in vivo the functions of rat recLH and recFSH with the respective human hormones, as well as with urinary hCG and rat pituitary LH. In addition, a novel immunofluorimetric assay for rat FSH is described.

The receptor binding affinity of human recLH was threefold lower than that of hCG, but the in vitro bioactivities of the two hormones were similar. There is no apparent explanation for this finding, but it may be due to the fact that the bioactivity dose–response occurs at low level of receptor saturation, whereas the radioreceptor assay uses higher receptor occupancy. Any interaction between free and occupied receptors may then be critically dependent on the characteristics of the ligand molecule. This is even clearer with rat recLH, which consistently shows shallower binding-inhibition curves than the human hormones. Moreover, we have observed previously that the receptor binding of rat LH is greatly influenced by the salt concentrations in the assay buffer, as compared with hCG (Hakola et al. 1997b). It seems that the binding affinities of human LH and CG are less influenced by salt concentrations of the assay buffer compared with rat and ovine LH (Huhtaniemi & Catt 1981).

The 10-fold difference in biopotency of rat recLH and the human hormones (hCG and recLH), and their similar maximum responses, were confirmed in the mouse testis interstitial cell and mouse Leydig tumor cell assays. Similarly, human recFSH was about 10-fold more potent than the respective rat hormone. In the in vitro bioassay of LH, the maximum steroidogenic response to rat pituitary LH (RP3) was less than half that to the other LH/CG preparations. This might be due to possible impurities, or deglycosylated forms of the hormone (Sairam 1989), some of which might act as competitive LH antagonists. No accurate ED₅₀ value could therefore be calculated for RP3. Rat pituitary LH (RP3) is a widely used standard for measuring rat LH. The present data show that it is apparently a suboptimal choice for this purpose, and may not reflect accurately the bioactive component of the LH immunoreactivity to be measured.

De Greef et al. (1983) have reported previously a half-time of 18·6 min for rat LH after injection of adeno-hypophysial extracts in female rats. These data on LH are surprisingly similar to the T₁ values measured for rat recLH in our study (18·2 min). The carbohydrate side chain termini of the pituitary LH molecules are both sulfated and sialylated (Sairam 1989), whereas they are exclusively sialylated in the rec glycoprotein hormones synthesized by CHO cells (Smith et al. 1990). The terminal sulfation...
shortens the \( T_1 \) of the hormone, but this apparently is of
minor importance in rat LH because of the similarities in
the half-times of the two LH preparations. The half-time
of human recLH observed with the one-component model
was 44.6 ± 3.1 min, which is consistent with earlier re-
ports. Previously it was shown that the half-time of human
LH in human circulation was 47 min (Veldhuis et al. 1987)
and in rat circulation 53 and 48 min with LH samples from
females and males respectively (Haavisto et al. 1995).
Human recLH has been reported to have a serum immu-
noreactive half-time of 52 min in monkeys (Porchet et al.
1995). Rat recLH was eliminated significantly faster than
human recLH (\( P<0.0001 \)). The difference is apparently
due to differences of the amino acid structure, since both
LH forms were produced by the same CHO cells and
were evidently similarly glycosylated.

A larger difference was found in the circulatory \( T_1 \) of rat
FSH between the earlier findings on pituitary (40.5–5 min;
de Greef et al. 1983) and recFSH (88.4–4 min). The reason
for the difference is not apparent, since pituitary FSH and
recFSH should be structurally closer, due to the fact that
the carbohydrates of FSH are not sulfated in the same way
as those of LH. It is possible that the two hormone
preparations have different isoform compositions with
different mean rates of elimination. With regard to LH,
the lower bioactivity of rat LH was coupled with faster \( T_1 \)
value in the circulation of this hormone, as compared with
the respective human hormone. In contrast, whereas rat
recFSH had lower bioactivity (Hakola et al. 1997a) and
receptor affinity than the cognate human hormone, the \( T_1 \)
of the rat hormone was longer than that of the human
hormone. In accordance, the elimination half-times of
human recFSH were previously reported to be about 5.7 h
in rats, but about 30 h in dogs and humans (de Leeuw et
al. 1996), which makes human FSH a suboptimal choice to
study the physiology of FSH action in rats. Why human
FSH seems to be more rapidly eliminated from the rat
circulation than the homologous gonadotropins remains
obscure.

Finally, we report here a novel IFMA for rat FSH. It
was developed with the same principle as the rat LH
IFMA a few years ago (Haavisto et al. 1993). A pair of
heterologous LH antibodies was found with high affinity
and specificity to recognize rat LH, and in this way the
sensitivity of the conventional RIA was increased about
50-fold. With the rat FSH IFMA, the methodological
advancement was not as great, since the sensitivity of the
IFMA assay is only marginally better than that of the NIH
RIA. The new assay, however, offers several advantages
being faster (overnight vs 2–3 days), using no radio-
isotopes, requiring smaller sample volumes (25–50 vs
50–100 µl serum), and having practically unlimited shelf-
life of all reagents needed. Once set up, it is accessible
immediately, and does not require hazardous radioiodina-
tion of the \(^{125}\)IodoFSH tracers which are notoriously fast in
decaying.

In conclusion, we expanded our previous findings
(Hakola et al. 1997a,b) that rat recLH and rat recFSH are
functional in vitro and in vivo. The recombinant rat
hormones are pure, free of any crossreacting contaminans,
and their structure can be defined in detail. They have
similar half-lives in vivo as their pituitary counterparts.
They can also be produced in large quantities for in vivo
studies on pituitary–gonadal functions in rats. The impor-
tance of the use of homologous hormones in physiological
studies of the rat pituitary–gonadal functions is highlighted
by the profound functional differences that were found
between the rat and human gonadotropins in the present
study.

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