The physicochemical, immunological and biological properties of rat pituitary and plasma LH

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A comparison was made between the properties of LH derived from female rat pituitary glands and plasma. Samples were collected from adult intact rats 5 h before or at the time of the pro-oestrous preovulatory LH surge; 27-day-old rats untreated or given 5 iu pregnant mares' serum gonadotrophin (PMSG) s.c. on day 25, which induced LH release 54 h later and adult ovariectomized rats untreated or primed with either 5 µg oestradiol benzoate s.c. or 5 µg oestadiol benzoate followed 48 h later by 0.5 mg progesterone s.c., which induced LH release 4–6 h later. All pituitary LH samples were totally bound to an anionic ion-exchange resin (DE52), while only a small proportion of the plasma LH was bound. Only 0–10% plasma LH obtained from intact, ovariectomized (with and without steroids) and untreated immature rats was bound, while a greater proportion of bound LH (36%) was noted in rats treated with PMSG. Gel filtration indicated only slight differences between pituitary and plasma LH, the former eluting marginally earlier than whole plasma and the unbound and bound plasma forms derived after separation by DE52 resin. Affinity chromatography (Concanavalin A and *Glycine max*imus) showed that LH from both sources possesses high mannose oligosaccharides and that plasma LH does not bear terminal *N*-acetyl galactosamine residues, although 20% of the pituitary form does. Plasma obtained from pro-oestrous rats had greater bioactivity than had pituitary LH in stimulating testosterone from Leydig cells and progesterone from granulosa cells *in vitro*, and inducing ovulation in immature rats *in vivo*. Leydig cell bioassays for LH in fractions obtained from ion-exchange separation indicate that steroidogenic activity of unbound plasma LH is greater than bound pituitary LH when they were collected at times of enhanced release. When release was inhibited (oestrogen-primed ovariectomized rats or immature rats), the steroidogenic activity of plasma and pituitary LH were similar and an acidic steroidogenic component was present in the plasma that was not recognized immunogenically as LH. In summary, pituitary LH undergoes a conversion on release into the plasma that involves a change in binding characteristics on an ion-exchange resin. In conditions when LH release is enhanced there is an increase in bioactivity of plasma LH owing to modification either by steroids or some other plasma factor(s) that perhaps influence the structure of LH directly or by steroids acting indirectly to alter GnRH release, which then modifies LH structure. These structural changes are minor and probably involve alterations in the glycosyl attachments.

Introduction

Campbell and co-workers (1978a, b) were the first to suggest that LH stored in the pituitary gland differs from circulatory LH. They based this hypothesis upon the difference in clearance rates between rat pituitary and plasma LH, the latter possessing a shorter circulatory half-life. Reddy and Menon (1981) supported this with more direct evidence, showing that there were two forms of LH in the rat pituitary gland which

had different molecular weights, and that only the smaller, more active form was released into the plasma.

LH bioactivity depends on noncovalent subunit bonding and the attachment of appropriate glycoconjugate moieties, which consist of complex oligosaccharides with the potential to bind sialic acid or sulfate groups or both in chain-terminating positions (Keel and Grotjan, 1989; Wilson *et al.*, 1990). The potential for variation in the molecular weight of LH is thought to reside in the oligosaccharide glycoconjugates (Chowdhury *et al.*, 1982), although aggregation of LH molecules may partially account for heavier pituitary LH forms, since these heavier forms can be eliminated by ultracentrifugation (Keel

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and Grotjan, 1989). Variation in oligosaccharide structure and, in particular, the degree of terminal sialiation or sulfation confers net molecular charge heterogeneity; isoelectric focusing and chromatofocusing techniques will therefore separate the pituitary- and plasma-derived LH glycoforms according to their individual isoelectric points (pIs). Other investigators have reported between four and eight differentially charged glycoforms of rat and human LH in pituitary extracts and plasma using isoelectric focusing, while chromatofocusing and fast-phase liquid chromatography indicate the existence of 12–14 glycoforms with charge variance (Wakabayashi, 1977; Stockell-Hartree *et al.*, 1985; Keel and Grotjan, 1989). Bioassays of human pituitary LH *in vitro* have indicated that there is a direct relationship between pI and bioactivity, and that the basic forms are more active (Lichtenberg *et al.*, 1982).

It has been suggested that there is an alteration in LH structure on its release into the circulation, either in association with discharge from its 'storage pool' (Campbell *et al.*, 1978a, b; Sardanons *et al.*, 1987) or due to one or more agents present in the circulation that effect post-secretory alteration (Reddy and Menon, 1981; Wilson *et al.*, 1985). As it is the plasma LH glycoforms that mediate actions at the target organ, their structures are of primary significance.

We have compared the properties of rat pituitary- and plasma-derived LH using ion-exchange, gel filtration and affinity chromatography and have confirmed that a change in glycoform composition occurs when LH is released from the pituitary gland into the circulation, and that this is paralleled by changes in bioactivity. The possibility that the endocrine milieu influences this change was investigated by using different animal models to test whether circulatory agents influence the relative distribution of the different glycoforms.

Methods

Animals

Female Wistar rats bred at St George's Hospital Medical School, London were housed under a photoperiod of 12 h light:12 h dark (lights on at 07:00 h). Rats showing at least three regular oestrous cycles were either used intact or were ovariectomized under anaesthesia with halothane (Fluothane: ICI Pharmaceuticals Ltd, Macclesfield) and nitrous oxide, and allowed a recovery and stabilization period of 3 weeks before use. All adult rats were aged between 10 and 15 weeks, and weighed 250–300 g. Immature rats providing pituitary and plasma samples were 25 days old, and those used to assess the bioactivity of the samples were 22–23 days old.

Treatments and times of sample collection

Intact rats were killed on the day of pro-oestrus either 5 h before the expected preovulatory LH surge (14:00 h) when pituitary glands were collected, or at the expected time of the LH surge (19:00 h) when pituitary glands and trunk blood were collected. Immature rats received either 5 iu pregnant mares' serum gonadotrophin (PMSG; Folligon: Intervet (UK) Ltd, Cambridge) 0.1 ml saline per rat s.c. or 0.1 ml saline per rat at 25 days of age. These rats were killed at 19:00 h on day 27

(time of the anticipated LH surge induced by PMSG). Adult ovariectomized rats were treated s.c. with either 5 µg oestradiol benzoate per rat in corn oil vehicle (Sigma Chemical Co., Poole, Dorset) or with 5 µg oestradiol benzoate per rat followed 48 h later by an injection s.c. of 0.5 mg progesterone per rat (Sigma Chemical Co.) or 0.1 ml vehicle per rat. All ovariectomized rats were killed 54 h after treatment with oil or oestradiol benzoate (i.e. 4 h after the injection of progesterone at the expected time of the steroid-induced LH surge).

Samples

All animals were killed by decapitation. Trunk blood was collected into heparinized polypropylene tubes and immediately centrifuged at 400 g at 4°C for 15 min, and supernatant plasma was stored at –20°C. Plasma obtained from steroid-treated ovariectomized rats was mixed with 5% purified charcoal (BDH Lab. Supplies, Merck Ltd, Lutterworth, Leics) for 30 min at room temperature; the mixture was then centrifuged at 400 g for 15 min and the supernatant was decanted from the charcoal pellet. This procedure removed the steroids present in the sample. Pituitary glands were removed and homogenized in 1 ml of 0.5 mol phosphate-buffered saline l⁻¹ containing 100 iu Trasylol ml⁻¹ (Bayer, Leverkusen) at pH 7.4. The homogenate was then centrifuged for 5 min and the supernatant stored at –20°C.

Chromatographic techniques

Ion-exchange chromatography. Pooled plasma (8–12 ml, consisting of samples of equal volume obtained from 3–4 rats), or 0.2 ml of pituitary extract (i.e. the equivalent of a fifth of a pituitary) were dialysed overnight (Spectrum Medical Industries Inc., CA, MWCO 12 000–14 000) against 1.0 l of 5 mmol ammonium hydrogen carbonate l⁻¹ (NH₄HCO₃ at pH 7.4 at 4°C). The samples were drawn onto a column of DE52 (diethylaminoethyl cellulose; Whatman Biosystems Ltd, Maidstone) bed volume 50 ml, at a rate of 10 ml h⁻¹. The column was flushed with 5 mmol NH₄HCO₃ l⁻¹, pH 7.4, at a rate of 10 ml h⁻¹ and 2 ml fractions were collected every 12 min. At fraction 30, a salt gradient (0–200 mmol NaCl l⁻¹) in 5 mmol NH₄HCO₃ l⁻¹, pH 7.4 was applied and developed for the duration of fractionation. A total of 100 or 120 fractions of 2 ml each were collected. Aliquots (50 µl) from each fraction were assayed in duplicate by radioimmunoassay and, in some cases, by mouse Leydig cell bioassay. It was found that a start sample containing more than 60 ng LH provided sufficient LH for measurement after fractionation. Recovery was nearly 100% for pituitary LH and between 60% and 90% for plasma LH, according to both radioimmunoassay and Leydig cell bioassay (Table 1). The reduced recovery of the plasma LH appeared to be due to a tightly absorbed proportion of LH that eluted only after application of 1 mol NaCl l⁻¹. This concentration of salt did not interfere with the immunoassay but was toxic to the Leydig cells. These fractions need to be de-salted to assess the bioactivity of this tightly bound component.

Gel filtration. A Sephadex G100 column (Pharmacia Ltd, Milton Keynes), 1 cm × 100 cm, was equilibrated in 0.01 mol

Tris-HCl running buffer l^{-1} , pH 7.4. Plasma samples and pooled ion-exchange fractions were lyophilized and reconstituted in 0.5 ml of running buffer, and then dialysed overnight against 1.0 l running buffer. Pituitary extract (0.2 ml) was dialysed similarly. Samples were applied to the column at a rate of 10 ml h^{-1} and fractions collected (2.0 ml in 12 min). BSA (67 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12 kDa) were used as molecular size markers and after they had eluted absorption measurements were taken at 280 nm. Recovery was between 85 and 100%.

Affinity chromatography. All samples consisted of pooled fractions obtained from ion-exchange separation. These were dialysed overnight against 1.0 l of running buffer (0.1 mol Tris-HCl l^{-1} , pH 7.4, 0.14 mol NaCl l^{-1} , 1% BSA, 0.1% Thiomersal, 1 mmol $MnCl_2$ l^{-1} , 1 mmol $CaCl_2$ l^{-1} , 1 mmol $MgCl_2$ l^{-1}). Samples were applied at a rate of 2 ml h^{-1} to a Concanavalin-A-sepharose (Con A) column, bed volume 5 ml, which was connected in series to a Soya bean agglutinin-sepharose *Glycine max* (Glycine max) column, bed volume 10 ml (lectins obtained from Sigma Chemical Co.).

Once the sample had been applied, the Con A column was flushed with running buffer (0.1 mol Tris-HCl l^{-1} , pH 7.4) at 2 ml h^{-1} for at least 10 fractions, followed by an addition of 200 mmol mannose l^{-1} (methyl α -D-glucopyranoside; Sigma Chemical Co.) in running buffer at 10 ml h^{-1} . At the time of applying the mannose, the *Glycine max* column was connected in series to the Con A column and at fraction number 60 the mannose buffer was replaced by 200 mmol D-galactose l^{-1} in running buffer, also applied at 10 ml h^{-1} (Sigma Chemical Co.). In all cases 120 fractions of 2 ml were collected. Recovery from the Con A-Glycine max system was $59 \pm 8\%$ ($n = 6$). This level of recovery is to be expected at the scale of separation used (Pharmacia, 1991).

Assays for LH

Radioimmunoassay. LH concentrations were measured in duplicate in 50 μ l aliquots of the chromatographic fractions, in 10 μ l of plasma and pituitary extract (the pituitary extract was diluted 1:500), using reagents provided by the National Hormone and Pituitary Program (Baltimore, MD) consisting of the NIH-rLH-RP3 standard and NIH-rLH-S10 antibody. The sensitivity of the assay was 1.25 ng ml^{-1} and the intra-assay and interassay coefficients of variance were 8% ($n = 20$) and 10.5% ($n = 6$ for each dose), respectively, at three dosages: 25, 100 and 400 ng. The presence of the buffers used in the various chromatographic separations (NaCl, 5–200 mmol l^{-1} and 1 mol l^{-1} ; galactose, 200 mmol l^{-1} ; and mannose, 200 mmol l^{-1}) did not interfere with the assay, and standard curves were superimposable in the absence and presence of these solutions.

The Leydig (interstitial) cell bioassay. The assay was modified from the method of van Damme *et al.* (1974). Samples of plasma (50 μ l) and pituitary extract (50 μ l, diluted 1:500), or 50 μ l of fractions from the anionic exchange separation were assayed in duplicate in well plates (96 wells, 250 μ l capacity; Becton Dickinson, Lincoln Park, NJ). Leydig cells were obtained

from 21–24-day-old male B6 mice (bred at St George's Hospital Medical School), and 150 μ l of a suspension containing 5×10^6 cells ml^{-1} was added to each well and incubated for 3 h at 37°C in an oxygenated environment. The cells were then killed by placing the well plates at $-80^\circ C$. Comparison of data from assays performed in parallel with the method described by van Damme *et al.* (1974), in which cells were killed at 100°C, showed consistently similar results. Samples of the incubate (50 μ l) were assayed in duplicate for testosterone using testosterone antibody No. 505 and [^{125}I]testosterone (generously supplied by the MRC Reproductive Biology Unit, Edinburgh). The intra-assay and interassay coefficients of variance were 8.7 and 11.4%, respectively, and the sensitivity was 1 ng ml^{-1} .

Stimulation of progesterone production by LH

Dissected rat ovarian follicles (≥ 400 μ m diameter) were placed in 1 $\times$ minimum essential medium containing 20 mmol Hepes buffer l^{-1} , 100 U penicillin ml^{-1} and 100 μ g streptomycin sulfate ml^{-1} . The follicles were ruptured and the granulosa cells dispersed by passing the cellular suspension through a 25-gauge needle and recovered by centrifugation. They were washed twice in McCoy's 5A culture medium containing 25 mmol Hepes l^{-1} , 2 mmol L-glutamate l^{-1} and 0.01% BSA (all from Sigma Chemical Co.), and then plated out in 1 ml aliquots at a concentration of 3×10^5 cells ml^{-1} . Cell viability was $>95\%$, as determined by the trypan blue exclusion test. Plasma (10 μ l) or pituitary extract (50 μ l, diluted 1:500) was added to 1 ml of the cell cultures and incubated for 48 h at 37°C in a humidified atmosphere of 95% O_2 : 5% CO_2 . Samples were stored at $-20^\circ C$ for progesterone radioimmunoassay (Immunodiagnostic Systems Ltd, Tyne and Wear). This assay has been validated for measuring progesterone in both plasma and culture medium.

Ovulatory activity of LH

PMSG induces an endogenous LH surge 54 h after administration, followed by ovulation the next day in immature rats. However, this occurs only after the rats have achieved a certain degree of maturity (Buckingham and Wilson, 1985); in our present Wistar rat colony this occurs on day 25 when the rats have a bodymass of more than 55 g. In younger lighter rats, PMSG is less effective and using 22–23-day-old females weighing less than 55 g, administration of PMSG induces only a small proportion to ovulate with no rise in immunoactive plasma LH at the expected time of the surge in the non-ovulating animals (untreated rats on day 25 at 19:00 h, 1.80 ± 0.2 ($n = 10$); PMSG-treated rats, 2.66 ± 0.4 ($n = 16$) ng LH $ml^{-1} \pm SEM$).

In these experiments, therefore, 22–23-day-old female rats weighing between 45 and 54 g were injected s.c. with 5 iu PMSG; 52–54 h later they received an injection i.v. into the tail vein of one of the following: 0.5 ml saline, 0.2–1.0 ml plasma (collected at 19:00 h from pro-oestrous adult rats and dialysed against 1 l isotonic saline for 24 h), or 0.5 ml of similarly dialysed and diluted extracts of pituitaries (collected at 14:00 h on the day of pro-oestrus). All the samples had been assayed

Table 1. Proportion of unbound and bound LH in samples from rats applied to an anionic ion-exchange resin (DE52)

Female rat model	Pituitary LH			LH in fractions			Plasma LH			LH in fractions		
	LH ^a ($\mu\text{g ml}^{-1}$)	Number of experiments ^b	% Recovery	% Unbound	% Bound	LH ^a (ng ml^{-1})	Number of experiments ^c	% Recovery	% Unbound	% Bound	% Unbound	% Bound
Pro-oestrus (14:00 h)	3.90 $\pm$ 0.85 (4)	4	93.50 $\pm$ 5.90	0	100	—	—	—	—	—	—	—
Pro-oestrus (19:00 h)	3.80 $\pm$ 0.70 (3)	3	98.00 $\pm$ 1.10	0	100	16.10 $\pm$ 1.86 (24)	8	61.00 $\pm$ 4.50	89.40 $\pm$ 4.85	10.50 $\pm$ 4.90	89.40 $\pm$ 4.85	10.50 $\pm$ 4.90
Ovariectomized	6.31 $\pm$ 1.60 (4)	2	100	0	100	11.40 $\pm$ 1.70 (23)	6	90.20 $\pm$ 7.25	88.98 $\pm$ 4.58	9.02 $\pm$ 4.13	88.98 $\pm$ 4.58	9.02 $\pm$ 4.13
Ovariectomized + OB	—	—	—	—	—	4.10 $\pm$ 0.50 (25)	7	89.50 $\pm$ 10.50	91.34 $\pm$ 6.30	8.64 $\pm$ 6.30	91.34 $\pm$ 6.30	8.64 $\pm$ 6.30
Ovariectomized + OB + P	—	—	—	—	—	27.80 $\pm$ 2.99 (25)	6	86.70 $\pm$ 13.20	91.03 $\pm$ 1.47	8.97 $\pm$ 1.47	91.03 $\pm$ 1.47	8.97 $\pm$ 1.47
27 days old	3.10 $\pm$ 0.60 (3)	3	100	0	100	1.63 $\pm$ 0.10 (90)	3 ^d	69.00 $\pm$ 16.00	100	0	100	0
27 days old + PMSG	3.50 $\pm$ 0.90 (3)	3	100	0	100	20.97 $\pm$ 3.96 (55)	19	80.00 $\pm$ 7.70	63.80 $\pm$ 5.30	36.20 $\pm$ 5.20*	63.80 $\pm$ 5.30	36.20 $\pm$ 5.20*

Values are means $\pm$ SEM.^aLH concentration (expressed as the NIH-rLH-RP3 standard) in individual samples before dialysis and pooling (pituitary concentrations of the undiluted extracts).^bEach experiment was carried out on individual pituitaries.^cEach experiment was carried out on equal volumes of plasma pooled from three or four rats.^dEach experiment was carried out on equal volumes of plasma pooled from 30 rats.* $P < 0.01$ compared with the other groups (ANOVA and Gabriel's test).

OB: oestradiol benzoate; P: progesterone; PMSG: pregnant mares' serum gonadotrophin.

Table 2. Comparison of the bioactivity of pro-oestrous pituitary and plasma LH in rats

Pro-oestrous samples	Immunogenic LH ($\mu\text{g ml}^{-1}$)	LH applied to cells (ng)	Testosterone ($\mu\text{mol l}^{-1} \text{ ng}^{-1} \text{ LH}$)	Progesterone ($\text{nmol l}^{-1} \text{ ng}^{-1} \text{ LH}$)
Plasma at 19:00 h (7) ^a	$24.1 \times 10^3 \pm 4.9$	0.24 ± 0.06	169.6 ± 55.3	113.0 ± 14.7
Pituitaries at 19:00 h (5) ^b	5.39 ± 0.64	0.54 ± 0.06	68.25 ± 10.7	54.78 ± 4.9
Pituitaries at 14:00 h (5) ^b	6.19 ± 1.16	0.62 ± 0.12	60.05 ± 10.8	56.64 ± 11.56
Ratio of plasma activity:pituitary activity	—	—	2.65:1	2.03:1

Values are means $\pm$ SEM.

^aPlasma LH was applied at 10 μl .

^bPituitary extracts were applied after 1:500 dilution at 50 μl .

for LH by radioimmunoassay and the variable volumes of plasma and dilutions of pituitary extract were used to administer graded doses of LH expressed in terms of the NIH-LH-RP3 standard. The day after the LH injection, the animals were killed and the oviducts inspected for the presence of eggs.

Chromatographic recoveries

Chromatography results are presented in relation to the sample applied to the column. The effect of the various procedures carried out before the samples were applied (i.e. dialysis, lyophilization and charcoal treatment) have not been taken into account, and it is possible that they may have had a selective effect on LH forms or have damaged a bioactive moiety. Recovery of the immunoactive LH after dialysis against the three chromatographic buffers (i.e. 5 mmol NH_4HCO_3 l^{-1} , 0.01 mol Tris-HCl l^{-1} and the lectin Tris buffer) was noted and was usually complete, with never more than a 10% loss in some plasma samples.

Statistical analyses

Comparison of the percentage proportion of LH binding to the anion-exchange column (Table 1) and the bioactivity of LH samples (Table 2) was made by ANOVA followed by Gabriel's multiple comparison test between groups of unequal size. Correlation between LH concentration and stimulation of progesterone was assessed by Pearson's test, and comparison of the number of ovulating rats after administration of the LH samples by Fisher's tables.

Results

Ion-exchange chromatography

LH extracted from pituitaries of pro-oestrous (at 14:00 h or 19:00 h), ovariectomized or 27-day-old female rats and applied to a DE52 anionic ion-exchange resin were all totally bound and eluted only following application of a 0–200 mmol NaCl l^{-1} gradient (see Table 1). The ratio of the steroidogenic biocompetence (B) to the immunoreactive (I) amount of LH (i.e. B:I) was 0.2:1, 0.8:1 and 0.8:1 in three different profiles of

pro-oestrous pituitary glands (the first is shown in Fig. 1). In contrast, when plasma LH was applied to the anion-exchange resin, most of it was detected in fractions collected before application of the salt gradient, and so appeared to be a form that did not bind to the resin. Figure 2 illustrates one such profile and Table 1 shows that in eight pro-oestrous plasma LH profiles $89.40 \pm 4.85\%$ of the total recovered was unbound. To verify that the detection of unbound LH was not due to saturation of column-binding capacity by other plasma proteins, pooled fractions containing the 'unbound' form were passed through a fresh column and found to remain unbound. The immuno- and bioassay results of the LH present in the fraction were compared and, again in contrast to the pituitary LH, plasma LH was found to have greater steroidogenic than immunogenic activity, with a B:I ratio of 3:1, 1.5:1 and 5:1 in three profiles (the first is illustrated in Fig. 2).

Comparisons have also been made of bioassay and immunoassay results of the LH profiles obtained from the plasma of untreated immature female rats, ovariectomized adults and ovariectomized adults treated with either 5 μg oestradiol benzoate, which reduced circulating concentrations of LH in the animal, or 5 μg oestradiol benzoate followed by 0.5 mg progesterone, which induced an increase in LH release (see Table 1 for plasma LH concentrations). All of the ovariectomized adult plasma samples were treated with charcoal to remove endogenous steroids before they were applied to the anion-exchange column. The B:I ratio altered with the endocrine milieu; it was 0.8:1 in the immature plasma sample, 2:1 in the adult ovariectomized sample, 0.8:1 in the sample treated with oestradiol benzoate and 5:1 in the sample treated with oestradiol benzoate and progesterone. In the anion-exchange profiles obtained from the immature and ovariectomized group treated with oestradiol benzoate, in which LH release is at its lowest, a tightly bound steroidogenic component was revealed that was not detected by the radioimmunoassay (Fig. 3b, c).

Table 1 summarizes the proportion of unbound and bound LH (expressed as percentages of the total LH in the fractions) in plasma and pituitaries of female rats in a variety of endocrine conditions (i.e. intact adults on the day of pro-oestrus, untreated and steroid-treated ovariectomized adults, and untreated and PMSG-treated immature rats). All the pituitary LH anion-exchange profiles were similar, with 100% LH

Fig. 1. Immuno- and biogenic ion-exchange profiles of pro-oestrous pituitary LH in rats. An extract (0.2 ml) from a pro-oestrous rat pituitary was applied to the DE52 ion-exchange column after overnight dialysis. An NaCl gradient (200 mmol l^{-1}) was applied at fraction 40. LH in the fractions was detected by radioimmunoassay (●) and Leydig cell bioassay (□).

Fig. 2. Immuno- and biogenic ion-exchange profiles of pro-oestrous plasma LH in rats. Pooled pro-oestrous plasma collected at 19:00 h on the day of pro-oestrus was applied to the DE52 ion-exchange column after overnight dialysis. An NaCl gradient (200 mmol l^{-1}) was applied at fraction 30. LH in the fractions detected by radioimmunoassay (●) and Leydig cell bioassay (□).

binding. Plasma LH profiles of adult rats and untreated immature rats were also similar to each other, with no significant difference in the relative proportions of bound to unbound LH. Steroid treatment in ovariectomized rats, either exerting a negative feedback effect on LH release ($5 \mu\text{g}$ oestradiol benzoate alone) or a positive feedback effect ($5 \mu\text{g}$ oestradiol benzoate plus 0.5 mg progesterone), had no effect on the relative proportions. However, when immature rats

were treated with PMSG their plasma LH profile showed a significantly greater ($P < 0.01$) proportion of the bound form compared with the other groups.

Gel filtration

Gel filtration disclosed that pro-oestrous pituitary-derived

Fig. 3. Ion-exchange profiles of untreated immature female rat pituitary and plasma LH. (a) Immature rat pituitary extract was applied to the DE52 ion-exchange column after overnight dialysis. LH in the fractions was detected by radioimmunoassay. Immature rat plasma extract was applied to the DE52 column after overnight dialysis and LH in the fractions was detected (b) by radioimmunoassay and (c) by Leydig cell bioassay. A NaCl gradient (200 mmol l⁻¹) was applied at fraction 32 for pituitary LH and at fraction 40 for plasma LH.

LH eluted from the column marginally earlier than did pro-oestrous plasma-derived LH. Pooled anion-exchange fractions containing either unbound or bound LH (obtained after ion-exchange separation of plasma taken from PMSG-treated immature rats) eluted from the G100 column in slightly different regions, the unbound form co-eluting with the unfractionated pro-oestrous plasma peak and the bound form eluting a little later. Thus, the bound LH from a plasma source did not correspond to the pituitary-derived bound LH (see Fig. 4).

Affinity chromatography

Pooled fractions obtained after anion-exchange chromatography containing either unbound LH from pro-oestrous plasma or bound LH from pro-oestrous pituitary glands were applied to two lectin columns in series (Con A followed by *Glycine*

maximus). Figure 5 shows the mean of three profiles, where the LH in the fractions is expressed as a percentage of the total LH recovered. Both pituitary and plasma LH were totally adsorbed to Con A and eluted only after application of 200 mmol mannose buffer l⁻¹. The mean elution volume for onset and peak concentration following application of the mannose was 22.7 ± 0.7 (onset) and 31.0 ± 0.7 (peak) ml for pituitary LH ($n = 3$) and 19.7 ± 0.9 (onset) and 28.7 ± 0.7 (peak) ml for plasma LH ($n = 3$). Little or none of the plasma LH bound to the *Glycine maximus* column, but $18.8 \pm 5\%$ of the pituitary LH was eluted from this column after addition of 200 mmol galactose l⁻¹, suggesting that a terminal galactose residue is exposed in a proportion of the pituitary LH forms but not in those of the plasma.

Bioactivity

Table 2 shows the LH bioactivity of pro-oestrous pituitary and plasma samples as assessed by testosterone stimulation from Leydig cells and progesterone stimulation from granulosa cells. Pituitary glands, whether collected in pro-oestrus at 14:00 h (before the LH surge) or at 19:00 h (at the time of the LH surge), had similar steroidogenic bioactivities to each other and were approximately half as active as plasma LH collected at 19:00 h. Plasma LH stimulated the production of testosterone (van Damme *et al.*, 1974) and progesterone ($r = 0.88$; $P < 0.02$) in a dose-dependent manner.

An immature rat model was used for testing the ovulatory activity of LH samples *in vivo*. Table 3 shows that PMSG followed by a dose of saline induced ovulation in only 22% of the 22–23-day-old rats weighing 49.8 ± 0.87 g. Administration of pituitary or plasma-derived LH samples *i.v.* over a range of doses revealed that the plasma LH was approximately five times more active, since it induced a significant number of ovulations compared with saline at 20 ng LH per rat, while a similar effect by pituitary LH was noted only at 100 ng LH per rat.

Discussion

LH consists of two noncovalently linked peptide chains, designated α and β subunits. There are two asparagine *N*-linked oligosaccharide moieties at positions 52 and 78 on the α subunit and one at position 13 or 30 (in the human) on the β subunit (Wilson *et al.*, 1990). Carbohydrate structures are *N*-linked to the β -amide nitrogen of asparagine (Asn) via an *N*-acetyl-D-glucosamine moiety (GlcNAc) and typically possess a common pentasaccharide core consisting of two GlcNAc and three mannose (Man) residues: Asn-GlcNAc-GlcNAc- β 1-Man- β 1-(Man α 1-6)-Man α 1-3. The GlcNAc nearest the Asn may be fucosylated. There are two or more sugar chains attached to the Man residues via GlcNAc and these can be very variable in structure since besides GlcNAc, Man and fucose they may contain *N*-acetyl-D-galactosamine (GalNAc) and *N*-acetylneuraminic acid (NeuNAc; sialic acid). The oligosaccharides are all electrochemically neutral except those bearing NeuNAc or sulfate groups on their terminal sugars; sulfated oligosaccharides predominate in LH. Heterogeneity of the sugar structures and branch-terminating radicals are thought to

Fig. 4. G100 profile of rat pituitary and plasma LH. Pooled ion-exchange fractions of plasma (□) were lyophilized and reconstituted in 0.5 ml of 0.01 mol Tris-HCl l⁻¹ (pH 7.4) and dialysed. These samples and 0.2 ml pituitary extracts (●) were applied to the Sephadex G100 column, and the presence of LH was detected in the fractions by radioimmunoassay. Unbound (■) and bound (○) plasma from the DE52 separation were obtained from 27-day-old female rats treated with pregnant mares' serum gonadotrophin, which contain significant amounts of both (see Table 1). CA: carbonic anhydrase; CC: cytochrome C.

Fig. 5. Binding profiles of pro-oestrous pituitary and plasma LH on Concanavalin A and *Glycine max* lectin columns. Pooled DE52 fractions containing either unbound plasma LH or bound pituitary LH were dialysed and applied to the Concanavalin A column. Mannose (200 mmol l⁻¹; solid line) was applied at fraction 10 (20 ml), at which point the Concanavalin A was linked to the *Glycine max* column. At fraction 60 (120 ml), galactose (200 mmol l⁻¹; dotted line) was applied. LH in the fractions was detected by radioimmunoassay. The graphs show the mean of three profiles of plasma LH expressed as a percentage of the total LH recovered (●) and the mean of three profiles of pituitary LH expressed as a percentage of the total LH recovered (□).

underlie heterogeneity in LH structure (Baenziger and Green, 1988; Grotjan, 1989).

Several pituitary and plasma LH glycoforms have been separated according to charge by isoelectric focusing or chromatofocusing techniques (Keel and Grotjan, 1989). Affinity chromatography using Con A has also revealed the existence

of rat pituitary and plasma LH glycoforms with different glycoconjugate oligomannose structures (Hattori *et al.*, 1988; Snyder *et al.*, 1989; Papandreou *et al.*, 1993). The relative proportion of gonadotrophin glycoforms, as determined by charge, that are present in the pituitary gland and plasma can alter with the endocrine state (for example, during the

Table 3. The effect of pituitary and plasma LH on ovulation in rats (22–23 days old) treated with pregnant mares' serum gonadotrophin

Dose of LH (ng per 60 g rat)	Number of rats ovulating	Mean number of eggs per ovulatory rat
Pituitary		
Saline	4/18 (22%)	4.75 $\pm$ 2.13
12.5	0/5 (0%)	None
25.0	1/7 (14%)	10
50.0	4/8 (50%)	6.5 $\pm$ 1.3
100.0	4/6 (67%)*	3.25 $\pm$ 0.6
200.0	7/9 (78%)**	6.1 $\pm$ 1.1
Plasma		
Saline	4/18 (22%)	4.75 $\pm$ 2.13
1.25	1/5 (20%)	10
2.5	1/5 (20%)	10
5.0	3/8 (37.5%)	7.7 $\pm$ 3.2
10.0	2/11 (18%)	4.0
20.0	6/8 (75%)**	5.3 $\pm$ 1.6

5 iu PMSG was injected s.c. into all the rats on day 22 or 23 of age. All rats were autopsied three days later and the number of eggs in the oviducts noted. Two days after the PMSG injection, controls received 0.5 ml saline i.v.; they weighed 49.8 ± 0.87 g ($n = 18$) on day 22 or 23 and 61.8 ± 1.36 g at autopsy. Rats receiving plasma in volumes between 0.2 and 1.0 ml to provide 1.25–20 ng LH per 60 g rat weighed 51.8 ± 0.82 g ($n = 37$) on day 22 or 23, and 55.7 ± 0.80 g and 61.8 ± 0.70 g on the days of injection i.v. and autopsy, respectively. Rats receiving pituitary extract over a concentration range of 12.5–200 ng LH (0.5 ml) $^{-1}$ per 60 g rat bodymass weighed 50.6 ± 0.73 g ($n = 35$) on day 22 or 23 and 55.76 ± 0.17 g and 61.5 ± 0.75 g on the days of injections i.v. and autopsy, respectively.

* $P < 0.05$ and ** $P < 0.01$ denote a significant difference in the number of rats ovulating compared with the saline-treated group (Fisher's tables).

reproductive cycle or over the peripubertal period; Uchida and Suginami, 1984; Chappel and Ramaley, 1985; Wide, 1985) and in parallel there is a change in biological activity (Reiter *et al.*, 1982; Dufau and Veldhuis, 1987). Since more basic glycoforms are reported to be more biopotent in terms of steroidogenic capacity *in vitro*, it is likely that the proportion of acid radical terminating groups alters with the endocrine milieu. The relationship between the sugar structures and bioactivity has yet to be unequivocally elucidated.

In this report a comparison was made between the forms of LH in the pituitary gland and plasma. The anion-exchange technique using DE52 resin was originally intended as a preparatory step before investigating LH from the two tissue sources with lectin affinity chromatography. This anion-exchange method is inappropriate to effect separation of glycoforms with respect to specific glycosyl structures, but revealed that while pituitary LH was completely adsorbed to the resin, most of the plasma LH was not. It is unlikely that this is artefactual, since all samples were pre-dialysed to minimize the amount of proteins present with a lower molecular weight and unbound plasma LH remained unbound when such fractions were pooled and re-applied to a fresh column. It is also unlikely that pH differences accounted for the differential binding, since this was monitored carefully and maintained at pH 7.4 for all the samples.

The differential binding may be independent of charge but if it is related to differential charge, there may be a modification of the glycoconjugates with a reduction in acid radicals on release from the pituitary. LH released from rat pituitary glands *in vitro* is more basic than the stored pituitary LH (Baldwin *et al.*, 1986; Leigh *et al.*, 1991). The fact that LH undergoes some change in structure on secretion has been suggested by others who compared the affinity binding half-lives, molecular weight or charge of stored and released LH glycoforms both *in vivo* and *in vitro* (Campbell *et al.*, 1978a, b; Reddy and Menon, 1981; Baldwin *et al.*, 1986; Papandreou *et al.*, 1993). The role of endocrine control in this conversion was investigated by using several animal models to assess the involvement of gonadal steroids. Results showed that the presence of circulatory steroids did not affect LH glycoform composition: the proportions of unbound and bound plasma LH were not significantly different in intact pro-oestrous adult and untreated immature females, or in ovariectomized rats whether untreated or primed with steroids exerting either a negative or positive feedback effect. Similarly, there was no change in pituitary LH, which remained totally bound in all endocrine models.

Only treatment with PMSG induced a difference in the proportion of unbound and bound plasma LH, with a significant increase noticeable in the bound form compared with the plasma LH obtained from the other endocrine models. Gel filtration showed that this bound plasma was more similar to unbound plasma LH than to bound pituitary LH, implying that it had also undergone a degree of structural change on secretion.

Gel filtration of pituitary and plasma LH showed that they eluted closely (but not exactly) together, indicating that their molecular weights were not greatly different, thus eliminating the possibilities of aggregation, division into subunits or major structural changes. The behaviour of glycoproteins on Sephadex resin is not necessarily representative of molecular weight, since the glycosylation affects filtration characteristics in an unpredictable manner (Kobata, 1990). Thus, even though molecular size markers were used, no truly valid quantitation of molecular weight differences can be made. LH elution peaks in our profiles occurred after the BSA marker at a similar point to those reported elsewhere (Mukhopadhyay *et al.*, 1979; Baldwin *et al.*, 1986). However, the small difference in elution volume between the pituitary and plasma LH does suggest some discrete structural or conformational differences and that the plasma LH may undergo a conversion that decreases its molecular size very slightly as well as inducing an alteration in its net molecular charge. The bound form of LH noted in plasma (of which approximately 10% is bound) differs from the bound form of LH obtained from the pituitary gland, since it elutes from a gel filtration column marginally later than does whole-plasma LH.

LH from both pituitary gland and plasma sources contains N-linked glycosyl residues with inner core fucosylation, since both tissues yielded LH that bound to Con A and was eluted at similar points after application of 200 mmol mannose l^{-1} , the plasma form appearing approximately 3 ml earlier than the pituitary form. Further experiments using a mannose gradient will reveal whether the multiple glycoforms from the two sources can be separated according to their affinity to the lectin. Papandreou *et al.* (1993) indicate that this is possible: using two

concentrations of sugar they eluted human plasma and pituitary LH from Con A columns and found unbound, weakly and firmly bound forms, with a greater predominance of the unbound form in plasma LH. These authors and Snyder *et al.* (1989) associated the reduced affinity with an increase in negative charge, but others reported that lectin binding characteristics are independent of acid radicals (Hajdukovic *et al.*, 1988; Kobata and Yamashita, 1988).

The sugar specificity of the *Glycine maximus* column is galactosamine (or *N*-acetylated galactosamine). While plasma LH did not react with *Glycine maximus*, approximately 20% of the pituitary LH bound to the lectin. This indicates that some of the pituitary LH bears glycosyl attachments terminating in an exposed GalNAc residue, while in plasma LH the terminal grouping is absent; perhaps the pituitary LH form is still at an intermediate stage in the glycosylation biosynthetic pathway.

More basic forms of LH are reported to possess a greater steroidogenic bioactivity (Lichtenberg *et al.*, 1984; Hattori *et al.*, 1988) and studies *in vitro* have shown that pituitary glands release a more basic form of LH with a greater steroidogenic bioactivity *in vitro* than that stored in the gland (Mukhopadhyay *et al.*, 1979; Baldwin *et al.*, 1986). We have found that LH derived from pro-oestrous plasma is at least twice as active as pro-oestrous pituitary LH in its ability to stimulate testosterone production from Leydig cells and progesterone production from granulosa cells *in vitro*. LH derived from plasma was also more effective than pituitary LH in inducing ovulation *in vivo*, since 20 ng plasma LH and 100 ng pituitary LH (both given per 60 g bodymass) were the minimum doses showing a significant activity. Even taking bodymass into account, both these doses are considerably lower than the i.v. injection of 1 µg purified rat pituitary LH (NIDDK-rLH-I-7) per adult rat needed to induce ovulation in pentobarbitone-treated pro-oestrous rats (Ishikawa, 1992). The enhanced activity may be due to potentiating factors in whole pituitary extract. Similarly, the greater effect of plasma LH over pituitary LH as assessed both *in vitro* or *in vivo* may be due to factors present exclusively in adult plasma. However, up to 8% plasma does not interfere with the Leydig cell bioassay (Tsatsoulis *et al.*, 1990) and so discrepancies in activity of plasma and pituitary LH (at least on steroidogenesis) may well be due to structural differences.

Increased steroidogenic activity of plasma LH was noted only in endocrine states of enhanced LH release, such as on the evening of pro-oestrus and in ovariectomized rats treated with oestrogen and progesterone. When LH release was inhibited, as in untreated immature rats or in ovariectomized rats treated with oestrogen alone, steroidogenic biopotency of bound pituitary and unbound plasma LH were similar; however, this relative reduction in bioactivity of the unbound LH in these two models may be masked *in vivo* since after anion-exchange separation another agent appeared to be present in the plasma that was tightly bound to the DE52 resin, not recognized immunogenically as LH, but that had steroidogenic activity as measured by the Leydig cell bioassay. A monoclonal LH antibody has been used to detect a human genetic variant that was not recognized immunologically but that had normal steroidogenic bioactivity (Pettersson and Soderholm, 1991; Pettersson *et al.*, 1991). Similarly, we also detected a steroidogenically active form of LH in immature PMSG-

treated rats that was not recognized by a polyclonal antibody (NIAMMD-S3), although it could be detected by another polyclonal antibody (No. 15; antiserum to ovine LH) (Buckingham and Wilson, 1985). An alternative possibility is that this steroidogenic factor is not LH at all, but another negatively charged molecule.

Factors controlling the change in structure, charge and bioactivity of pituitary LH to that of plasma LH forms *in vivo* remain unidentified. We have shown that gonadal steroids do not appear to affect charge, as they do not alter pituitary and plasma LH anion-exchange profiles obtained from rats in different endocrine conditions. The change in bioactivity noted in unbound plasma LH at times of enhanced release may be due to the steroids acting directly at the pituitary level or due to their indirect effect at the hypothalamic level, enhancing gonadotrophin-releasing hormone (GnRH) release. This effect at the hypothalamic level has been suggested by Tsatsoulis *et al.* (1991) to explain the variation in B:I ratios of human plasma LH in different endocrine states and dysfunction, and by Ulloa-Aguirre *et al.* (1992) for changes in biological activity of rat plasma FSH. GnRH is known to modify glycosylation (Liu *et al.*, 1976; Vogel *et al.*, 1986) and sulfation (Sardanons *et al.*, 1987), both of which would affect steroidogenic bioactivity. GnRH also alters the binding to an anionic exchange resin of LH glycoforms released *in vitro* from perfused rat pituitary glands (Leigh *et al.*, 1991). Steroids can influence the bioactivity of LH released from rat pituitary glands *in vitro* (Mukhopadhyay *et al.*, 1979); they too may act at the pituitary level to influence enzymes involved in glycosylation (Liu and Jackson, 1977) or to stabilize mRNA encoding α and β subunits (Paul *et al.*, 1990), both ultimately affecting post-translational processing. Probably a combination of indirect hypothalamic and direct pituitary effects of steroids are involved.

In conclusion, these results show that pituitary LH undergoes a conversion on release into the plasma that involves a change in its binding characteristics to an anionic ion-exchange resin, and that this is independent of steroidal control, although it can be modified slightly by PMSG. In conditions of enhanced LH release, the steroidogenic biocompetence of plasma LH is greater than that of LH stored in the pituitary. In conditions of reduced LH release, the steroidogenic activities of immunogenic pituitary and plasma LH are similar, and another 'factor' with steroidogenic activity appears in the plasma that is not recognized immunogenically as LH. These changes appear to be endocrine dependent and may be due to a direct effect of gonadal steroids (or some other agents present in the circulation) at the pituitary level, or to an indirect effect exerted by first manipulating GnRH release. Only minor differences were seen between pituitary and plasma LH after separation by gel filtration or lectin affinity chromatography, showing that only subtle modifications of the structure of LH are responsible for the differences noted in the pituitary- and plasma-derived LH samples.

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