Heterogeneity in the bioactivity of LH secreted by peripubertal rats

A. J. Leigh1, N. L. Poyser2, R. C. Bonney3, S. A. Whitehead4 and C. A. Wilson5*

1Department of Veterinary Basic Sciences, Royal Veterinary College, London NW1 OUTF, UK; 2Department of Biomedical Sciences, University of Edinburgh Medical School, Edinburgh EH8 9JZ, UK; 3Unit of Metabolic Medicine, Imperial College School of Medicine, St Mary’s Hospital, London W2 1PG, UK; and Departments of 4Physiology and 5Obstetrics and Gynaecology, St George’s Hospital Medical School, London SW17 ORE, UK

Treatment of immature rats with 5 iu equine chorionic gonadotrophin (eCG) on day 25 typically stimulates a preovulatory surge of LH on day 27 and ovulation on day 28. In rats weighing > 60 g at the time of treatment, an LH surge and ovulation occurred in 75% of the animals but, in rats weighing < 60 g, only 13% ovulated even though 69% showed an LH surge. Previous findings have shown that exogenous LH can stimulate ovulation in the rats < 60 g, indicating that the anovulation was not due to ovarian immaturity, but rather to an abnormal form of LH. Thus, it was important to determine whether the bioactivity of LH released at the time of the surge differs in rats < 60 g compared with rats > 60 g. Experiments showed that LH from both groups of eCG-treated animals were equipotent in stimulating testosterone production from incubated Leydig cells and progesterone production from cultured granulosa cells. Similarly the surge of progesterone in vivo, which occurs co-incident with the LH surge, was of similar magnitude in both groups of animals. Since prostaglandin synthesis increases at the time of ovulation and is also stimulated by LH, it was investigated whether the activity of ovarian phospholipase A2, the rate limiting enzyme in prostaglandin synthesis, and ovarian prostaglandin E2 concentrations differed in the animals > 60 g and < 60 g. Phospholipase A2 activities were similar in both groups of animals at the time of the LH surge, as were the prostaglandin E2 concentrations. However, in all animals that ovulated (15/20 in rats > 60 g and 2/15 in rats < 60 g), there was a threefold increase in ovarian prostaglandin E2 concentrations. The results show that, in underweight animals, the bioactivity of LH, in terms of its ability to stimulate steroidogenesis and phospholipase A2 activity, is similar to that released by animals > 60 g; however, the LH produced by the underweight animals fails to induce ovulation by failing to increase, either directly or indirectly, prostaglandin E2 production. Comparison of the profiles of plasma LH collected at the time of the LH surge on an anionic ion exchange column indicates that the LH from rats < 60 g possesses significantly less of the neutral or basic glycoform of LH than that from rats > 60 g. This finding provides a further index that the biopotency of LH produced by underweight animals is different from that of rats > 60 g.

Introduction

Over the prepubertal period in female rats (at about day 21 until just before vaginal opening on days 36–40), gonadotrophin and ovarian steroid secretions are low (Ojeda et al., 1986). Administration of a single dose of equine chorionic gonadotrophin (eCG; pregnant mares’ serum gonadotrophin) from day 25 onwards stimulates the immature ovary to secrete oestradiol and this reaches a sufficiently high circulatory concentration 48 h later to exert a positive feedback effect. An endogenous LH surge occurs 54 h after the eCG administration resulting in ovulation (Wilson et al., 1974). In previous experiments, these events occurred in > 80% of rats weighing > 60 g on the day of the eCG injection; however, in rats weighing < 60 g, ovulation occurred in only 14% despite normal preovulatory plasma concentrations of oestradiol and an immunoactive LH surge in most of this group (Wilson and Endersby, 1979; Wilson et al., 1983; Buckingham and Wilson, 1985). Ovulation failure is not due to ovarian immaturity in the rats < 60 g since they ovulate in response to exogenous LH (Wilson et al., 1985).

LH is a heterodimeric glycoprotein with three glycoconjugate attachments that confer bioactivity and net
molecular charge to the molecule. These carbohydrate moieties are themselves heterogeneous, giving rise to a number of structures designated glycoforms (Jeffcoate, 1993) with different pI values associated with different bioactivity (Wilson et al., 1990; Lambert et al., 1998). Attempts have been made to elucidate the factor(s) controlling the form of LH secreted in the two groups of immature rats by comparing endocrine and physical characteristics of animals > 60 g and < 60 g. Significant differences between the two groups include lower plasma corticosterone and growth hormone concentrations, lower core body temperature and decreased oxygen consumption in the rats < 60 g (Everard et al., 1983; Wilson et al., 1983), indicating that one or more of these factors may contribute to the modulation of LH structure, and thence bioactivity in the pituitary gland and circulation.

Investigations into the bioactivity of the LH in the two groups of rats revealed that only the LH secreted by the > 60 g rats induced a positive response in the cytochemical bioassay, which depends on the ability of LH to deplete ovarian ascorbic acid (Buckingham and Wilson, 1985). The lighter rats appeared to secrete a form of LH incapable of depleting ovarian ascorbic acid or inducing ovulation. Among the principal factors regulating the rupture of the follicle are the prostaglandins (Poyser, 1987). In the present study, the ability of the endogenous LH surge to stimulate ovarian phospholipase A₂ (PLA₂) activity and ovarian concentrations of prostaglandin E₂ (PGE₂) was compared in eCG-treated rats > 60 g and < 60 g. PLA₂ is the enzyme responsible for releasing the prostaglandin precursor, arachidonic acid, from bound stores which is the rate-limiting step in the prostaglandin synthetic pathway (Irvine, 1982). LH stimulates the activity of ovarian PLA₂ in both adult rats and immature eCG-treated rats > 60 g (Bonney and Wilson, 1993) and also stimulates ovarian PG synthetase (Hedin et al., 1987; Sirois and Richards, 1992) and prostaglandin production (Bauminger and Lindner, 1975; Clark et al., 1978). It is the synthesis of PGE₂ that is preferentially stimulated (Brown and Poyser, 1984).

Since differences in LH bioactivity are related to the overall charge of LH (Wilson et al., 1990; Lambert et al., 1998), the present study compared the ion-exchange profiles of the plasma LH obtained at the expected time of the preovulatory surge from eCG-treated rats < 60 g and > 60 g.

### Materials and Methods

#### Animals and samples

Immature female Wistar rats bred at St George’s Hospital Medical School were maintained in a fixed lighting system of 12 h light: 12 h dark (lights on 07:00 h). On day 25 after birth, the rats were weighed and injected s.c. with 5 iu eCG (Folligon; Intervet Ltd, Cambridge) in a volume of 0.1 ml saline vehicle. A group of rats > 60 g was given 0.1 ml saline s.c. as a control. Groups of rats were decapitated, trunk blood was collected and ovaries were removed on day 27 at 10:00 h and 19:00 h and on day 28 at 03:30 h and 10:00 h. The times were chosen for the following reasons: day 27 at 10:00 h is a time of basal release of LH; day 27 at 19:00 h is the expected time of the preovulatory LH surge; day 28 at 03:30 h is near the expected time of follicular rupture; and by day 28 at 10:00 h, ovulation has been completed and ova can be noted in the oviducts.

The blood was centrifuged at 400 g at 4°C for 15 min and plasma was stored at −20°C. The ovaries were cleared of all adhering fat and immediately frozen in liquid nitrogen and stored at −80°C until assessed for either PLA₂ activity or PGE₂ content.

The experiments were performed on different groups of animals, which were variable in size due partly to the difficulty in obtaining sufficient numbers of rats weighing less than 60 g. The small volume of plasma obtained from these immature animals was also a limiting factor. The fact that each Table provides data from different groups of animals explains the differences in absolute concentrations of plasma LH found in each experiment, which is presumably due to biological variation in the amount of LH released and the time of the peak of the LH surge. However, it is clear when levels are ‘basal’ or ‘surge-like’.

#### Radioimmunoassay for LH

LH was measured using 10 µl of plasma or 50 µl of chromatographic fractions in duplicate. Reagents were provided by National Hormone and Pituitary Program (Baltimore, MD) and were NIH-rLH-RP³ standard and NIH-rLH-S10 antibody. The sensitivity of the assay was 1 ng ml⁻¹ and the intra- and inter-assay coefficients of variation were 8 and 10.5%, respectively. The presence of 5 mmol NH₄HCO₃ l⁻¹ (pH 7.4) or 100 mmol NaCl l⁻¹ did not interfere with the assay.

#### Bioassay for LH

The mouse Leydig cell bioassay was carried out according to the modified method of van Damme et al. (1974) as described by Leigh et al. (1994). Intra- and inter-assay coefficients of variation were 8.7 and 11.4% for the overall bioassay and 8.0 and 9.7% for the testosterone radioimmunoassay, and the sensitivity was 1 ng ml⁻¹.

#### Measurement of progesterone

Secretion of progesterone in vivo was assessed by measuring the hormone by radioimmunoassay in plasma collected at the expected time of the LH surge (day 27, at 19:00 h). The assay was carried out according to the method of Hodges et al. (1983) using reagents provided by the World Health Organisation. The intra- and interassay coefficients of variation were 8.9 and 9.6%, respectively.

The details of the measurement of the secretion of progesterone in vitro from granulosa cells are as described in Leigh et al. (1994). Plasma samples of 10 µl were added to 1 ml of the granulosa cell cultures and incubated for 48 h at 37°C in a humidified atmosphere of 95% O₂:5% CO₂. Samples were stored at −20°C for progesterone radioimmunoassay.
Measurement of ovarian phospholipase A₂ activity

Phospholipase A₂ activity was measured in crude cytosol preparations of rat ovarian tissue using a double isotope assay based on the liberation of oleic acid from 1-palmitoyl-2-[¹⁴C]-oleoyl phosphatidyl choline according to the method reported in detail by Bonney and Wilson (1993).

Ovarian prostaglandin E₂

Each pair of ovaries was homogenized in ethanol and the prostaglandins extracted according to the method of Poyser (1988). The amount of PGE₂ present in each extract was then measured by radioimmunoassay using antibodies raised in the Department of Pharmacology, University of Edinburgh; their crossreactivities have been reported by Poyser (1987). The inter- and intra-assay coefficients of variation were < 9% for each assay. The detection limit was 10–30 pg PGE₂ per assay tube.

Ion-exchange chromatography

Pooled plasma samples (equal volume from each of 10–14 rats) were collected at 19.00 h on day 27 (the expected time of the LH surge). The final volumes and concentrations of the pools were 4.9 ± 0.20 ml and 23.4 ± 5.4 ng ml⁻¹ (n = 9 pools) from the eCG-treated rats < 60 g and 5.5 ± 3.5 ml and 29.6 ± 11.6 ng ml⁻¹ (n = 10 pools) from the eCG-treated rats > 60 g. The pools were dialysed (Pierce and Warrener, Chester; MWCo 12-14000) against 1 l of 5 mmol ammonium hydrogen carbonate (NH₄HCO₃) l⁻¹ (pH 7.4, running buffer) overnight at 4°C and then applied to a column of DE52 resin (diethyl aminoethyl cellulose; Whatman Biosystems Ltd., Maidstone; 50 ml bed volume) at 10 ml h⁻¹. The sample was washed through with one bed volume of freshly prepared running buffer and 2 ml fractions were collected. After 30–40 fractions had been collected, a 0–100 mmol sodium chloride (NaCl) 1⁻¹ gradient in running buffer was applied and 100–120 fractions were collected. All fractions were assayed for LH by radioimmunoassay. Recovery after dialysis was usually complete and loss was never > 10%. Recovery after chromatography was 74.8 ± 6.3% (n = 9) for rats < 60 g and 85.0 ± 5.7% (n = 10) for rats > 60 g.

Table 1. Comparison of the effect of 5 iu eCG given subcutaneously to 25-day-old female rats < 60 g and > 60 g on the induction of an LH surge and ovulation

<table>
<thead>
<tr>
<th>Weight of rats on day 25 (g)</th>
<th>Number of rats with an LH surge &gt; 10 ng ml⁻¹ on day 27 at 19:00 h</th>
<th>Peak LH concentration (ng ml⁻¹) (n)</th>
<th>Number of rats ovulating on day 28</th>
<th>Number of eggs per ovulating rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 60 g (68.4 ± 1.4)</td>
<td>17/23 (74%)</td>
<td>23.3 ± 5.3 (17)</td>
<td>15/20 (75%)</td>
<td>8.8 ± 1.1</td>
</tr>
<tr>
<td>&lt; 60 g (56.0 ± 0.6)</td>
<td>9/13 (69%)</td>
<td>21.1 ± 5.4 (9)</td>
<td>2/15 (13%)*</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.

*P < 0.001 compared with > 60 g group (chi-squared test).
granulosa cells (Table 2). The preovulatory LH surge is known to induce the concomitant increase in plasma progesterone in female rats (Hillensjo et al., 1976; Goff and Henderson, 1979) and, in the two groups of eCG-treated animals investigated in the present study, the concentrations of plasma progesterone measured at the expected time of the LH surge were similar (Table 3).

**Effect of plasma LH in eCG-treated rats < 60 g and > 60 g on ovarian prostaglandin production**

A comparison was made of the increase in ovarian PLA₂ activity in the eCG-treated rats < 60 g and > 60 g at the time of the preovulatory LH surge (day 27 at 19:00 h). The increase was similar in both groups (Table 4), indicating that the respective endogenous LH surges had a similar potency in inducing PLA₂ activity.

A similar comparison of ovarian PGE₂ content in the eCG-treated rats < 60 g and > 60 g was also made: concentrations of LH in the plasma and PGE₂ concentrations in the ovary were assessed at intervals after eCG treatment, including after rupture of the follicles had taken place. Thus, a correlation of the occurrence of ovulation and stimulation of PGE₂ production could be made. There was no change in ovarian PGE₂ stores on the morning or evening of day 27, nor at 03:30 h on day 28 (Table 5). However, a significant increase was seen later, at 10:00 h on day 28, but only in those animals in which ovulation had occurred. This increase occurred irrespective of body weight, although ovulation occurred rarely in the underweight animals.

**Comparison of the anionic ion-exchange profile of plasma obtained from eCG-treated rats > 60 g and < 60 g**

Application of plasma LH to an anionic ion-exchange column showed that the LH separated into an unbound form that passed straight through the ion-exchange resin in the first 4–20 fractions. The rest of the LH was bound to the resin, and the onset of its elution only appeared when the salt gradient reached a concentration of 17.75 ± 3.5 mmol NaCl l⁻¹ for the plasma from rats < 60 g, and 23.3 ± 4.2 mmol NaCl l⁻¹ for the plasma from rats > 60 g. A representative profile of the bound form of LH in the samples from rats < 60 g compared with those from rats > 60 g is shown (Fig. 1). The ratio of unbound:bound forms of LH was significantly greater (P < 0.05) in the plasma obtained from rats > 60 g (71.0 ± 5.5% unbound; 29.0 ± 5.5% bound; ratio unbound:bound, 2.66 ± 0.05, n = 10) compared with plasma from rats < 60 g (55.9 ± 8.9% unbound; 44.0 ± 8.9% bound; unbound:bound, 1.44 ± 0.39, n = 9). This difference was not due to different concentrations of LH in the plasma samples that were applied to the columns, as there was no correlation between concentration and the unbound:bound ratio (R = 0.46, P = 0.15) and the mean concentration in the plasma from rats < 60 g and > 60 g was very similar.
Discussion

Only a small proportion of rats weighing < 60 g on the day of eCG treatment ovulated as opposed to > 70% of their heavier littermates. However, the eCG stimulated the release of an endogenous immunogenic preovulatory LH surge. Two possible causes for this phenomenon are the immaturity and, thus, the unresponsiveness of the ovaries in the rats < 60 g, and that the immunogenic LH released in these animals has an abnormal bioactivity. The former explanation is unlikely, since administration of exogenous LH can stimulate ovulation in eCG-treated rats < 60 g (Wilson et al., 1985). However, it is likely that the LH released in the rats < 60 g has a different bioactivity, since it is not detected by the cytochemical bioassay (Buckingham and Wilson, 1985). The form(s) of LH released by the underweight immature rat may not possess the glycoconjugate structures required to elicit the full range of biological responses. Thus, plasma LH taken from these animals does not induce ovulation, although it seems to possess full steroidogenic bioactivity (Wilson and Endersby, 1979; Buckingham and Wilson, 1985).

In the present study, these findings have been extended to show that the LH secreted in rats < 60 g and > 60 g is equipotent in stimulating both testosterone from Leydig cells and progesterone from granulosa cells in vitro and in vivo. The fact that the steroidogenically bioactive LH in rats < 60 g

Table 5. Ovarian prostaglandin E2 concentration (ng mg⁻¹ ovary) in rats < 60 g and > 60 g treated with 5 iu eCG on day 25

<table>
<thead>
<tr>
<th>Groups</th>
<th>Surge (LH &gt; 10 ng ml⁻¹)</th>
<th>No surge (LH &lt; 10 ng ml⁻¹)</th>
<th>Ovulation</th>
<th>No ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 60 g saline-treated rats</td>
<td>0.314 ± 0.023 (6)</td>
<td>0.298 ± 0.022 (5)</td>
<td>–</td>
<td>0.310 ± 0.050 (5)</td>
</tr>
<tr>
<td>&gt; 60 g eCG-treated rats</td>
<td>0.331 ± 0.038 (10)</td>
<td>0.280 ± 0.015 (18)</td>
<td>0.259 ± 0.011 (5)</td>
<td>0.306 ± 0.022 (7)</td>
</tr>
<tr>
<td>&lt; 60 g eCG-treated rats</td>
<td>0.273 ± 0.039 (5)</td>
<td>0.270 ± 0.021 (9)</td>
<td>0.223 ± 0.010 (4)</td>
<td>0.290 ± 0.019 (8)</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.

Ovarian prostaglandin E2 concentrations were measured at 10:00 and 19:00 h on day 27 and at 03:30 and 10:00 h on day 28 in rats < 60 g and > 60 g treated with 5 iu eCG on day 25.

*p < 0.0001 compared with all other times in the > 60 g eCG-treated rats (ANOVA and Gabriel’s test).

Fig. 1. Anionic ion-exchange profile of plasma LH taken at 19:00 h on day 27 from rats treated with 5 iu eCG on day 25. □, Plasma from rats > 60 g; ●, plasma from rats < 60 g. The dotted line represents the salt gradient, which was started at fraction 30.
did not induce ovulation indicates that it lacks the ability to stimulate the production of factors essential for follicular rupture. LH regulates several of the agents involved in ovulation induction, including the cytokines, such as interleukin 1 (Hurwitz et al., 1991; Brannstrom et al., 1993), catecholamines (Ben Jonathan et al., 1984; Flores et al., 1990), plasminogen activator (Reich et al., 1986) and the prostaglandins (Poyser, 1987).

The two key enzymes required for prostaglandin synthesis are PLA$_2$, which is the rate-limiting enzyme and releases arachidonic acid from phospholipids (Irvine, 1982), and prostaglandin H synthase (PGHS), which converts arachidonic acid to PGH$_2$. The PGH$_2$ is subsequently converted to PGE$_2$ by PGE$_2$ isomerase. LH stimulates the activity of both PLA$_2$ (Bonney and Wilson, 1993) and PGHS (Hedin et al., 1984; Sirois and Richards, 1992). In the present study, LH secreted in rats < 60 g and > 60 g was equally active in stimulating PLA$_2$; an increase in PLA$_2$ activity occurred at the time of the enhanced LH secretion in both groups. Therefore, the absence of ovulation in the lighter rats was not due to the lack of activity of the enzyme producing the free precursor for prostaglandin production. However, ovarian PGE$_2$ content increased only in animals that ovulated. Ovulation is dependent on ovarian prostaglandin production (Bauminger and Lindner, 1975; Behrman, 1979; Lindner et al., 1980; Poyser, 1987). Thus, it may be that ovulation can only take place when PGE$_2$ concentrations are sufficiently increased. Under the influence of LH, ovarian prostaglandin concentrations increase in the afternoon of proestrus, with PGE$_2$ dominating the PGE$_2$:PGF ratio (Lemaire et al., 1975); in addition, Brown and Poyser (1984) reported that a significant increase in ovarian PGE$_2$ synthesizing capacity takes place in the early hours of the day of ovulation. In the present study, an increase in ovarian PGE$_2$ content occurred between 03:30 and 10:00 h in all the rats that ovulated. LH from most of the rats < 60 g was unable to induce ovulation perhaps because it was not able to increase PG production from arachidonic acid in the ovaries of these rats. PGHS, is the isoform of PGHS involved in ovulation in the ovary (Sirois and Richards, 1992) and it is possible that LH in the rats < 60 g was not able to stimulate the activity of ovarian PGHS. The possibility that other variables influence PG production cannot be ruled out.

Preliminary investigations were carried out to determine whether LH from the two groups of rats had different physicochemical properties that might underlie the differences in their bioactivity. It has long been established that LH bioactivity is correlated with charge; more basic LH forms are more potent, at least as far as steroidogenic activity in vitro is concerned (Wilson et al., 1990; Lambert et al., 1998). It is possible that such differences account for the ability of these forms of LH to stimulate prostaglandin production. In the present study, samples were assessed by ion-exchange liquid chromatography. The samples were passed through an anionic resin that binds acidic molecules, allowing basic and neutral components to pass through. The LH from the eCG-treated animals < 60 g and > 60 g separated into unbound and bound forms, which may be equivalent to more basic and acidic forms, respectively. This hypothesis is supported by the observations that LH in plasma from both adult and > 60 g immature rats consists mainly of an unbound form on an anion-exchange column, which binds acidic molecules (Leigh et al., 1994), whereas on a cationic resin, which binds basic molecules, the LH was eluted mainly in the bound form (A. J. Leigh and C. A. Wilson). Comparison of the LH obtained from rats < 60 g and rats > 60 g showed that rats < 60 g had significantly less of the unbound (neutral or basic) form of LH. This finding may correlate with the inability of the LH from rats < 60 g to induce ovulation and stimulate an increase in ovarian PGE$_2$ concentration. A comparison of the bound forms in the two groups also indicates that they are different; the LH of the < 60 g samples elutes earlier, perhaps because this form is at an intermediate stage and has not been fully converted from the inactive acidic structure to the bioactive neutral or basic form.

In summary, underweight immature rats secrete a form of LH that has steroidogenic bioactivity, but is incapable of stimulating ovarian PGE$_2$ production and inducing follicular rupture. A similar form of LH may be produced in girls during their post menarche period when, typically, their menstrual cycles are predominantly anovulatory (Spence, 1997). Similarly, women who are 91% of their ideal body weight are infertile, despite the occurrence of menstrual cycles. This infertility can occur in women returning to normal body weight after a sustained period of low body weight, such as that seen in anorexia and in female athletes (Bates et al., 1982).

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