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Abstract
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Dose and Time Relationships of Intravenously Injected Rat Recombinant Luteinizing Hormone and Testicular Testosterone Secretion in the Male Rat

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ABSTRACT
The ability of hCG and LH to induce testosterone (T) secretion by Leydig cells is well documented. However, the influence of the pulsatile nature of LH secretion, with varying frequency and amplitude, on T production in vivo is less clear. In our earlier studies on the relationship between pulsatile LH release and T secretion in adult male rats, no simple causality was observed. The recent availability of rat recombinant (rec) LH prompted us to study the effects of one and of three i.v. pulses of different doses of rat recLH on T secretion in adult male rats rendered hypogonadotropic by treatment with the GnRH antagonist cetrorelix. One or three supraphysiological pulses of 1.0 μg of rat recLH produced similar maximal T responses. In contrast, high physiological LH pulses (0.1 μg) produced discrete T-response peaks, whereas multiple low pulses of LH (0.03 μg) were needed before a T response was achieved. The T stimulation was greatly diminished after an LH pulse of 0.1 μg if rats had been treated on the previous day with pulses of 0.03 vs. 0.1 μg rat recLH, apparently because of prolonged LH deprivation and lack of Leydig cell priming due to the GnRH antagonist treatment. The novel preparation of rat recLH provides a physiologically relevant tool for studying the complex relationship between pulsatile LH release and T secretion in male rats.

INTRODUCTION
The fact that LH and hCG induce testosterone (T) secretion by binding to the LH/CG receptors in Leydig cells is well documented [1, 2]. LH is also required to maintain the structure and specialized functions of Leydig cells in the long term in vivo [3, 4]. On the other hand, large doses of LH and hCG are known to desensitize Leydig cell androgen production and to down-regulate their LH receptors [1]. Endogenous LH is secreted in pulsatile fashion with varying frequency and amplitude, presumably also stimulating T secretion in a pulsatile manner [5]. In most species there is a close temporal relationship between the individual LH pulses and the peaks of T response, but in humans and rats the relationship is less clear because of the episodic secretion of both hormones [6, 7]. Hence, the exact relationship between single LH pulses and T responses remains to be elucidated.

The pulsatility of endogenous LH secretion can be monitored through serial blood sampling [6]. In addition, carefully monitored exogenous LH pulses can be injected into subjects lacking endogenous pulsatility in order to observe their gonadal responses [8]. In this study, both methods were used to study the relationship between LH pulses and the testicular T response in adult male rats. Since the structure, biopotency, and in vivo kinetics of rat LH are different from those of the human hormones, it is important to use homologous hormones to improve the physiological relevance of the observations. We have recently prepared and characterized a recombinant (rec) form of rat LH produced in Chinese hamster ovary cells [9]. The terminal carbohydrate moieties of rat recLH are sialylated, in contrast to those of pituitary LH, which are sulfated [10]. Nevertheless, the half-life of LH and hCG in rat circulation is 18.2 min (unpublished results), i.e., similar to that of rat pituitary LH, 18.6 min [11]. In addition, since it was important to mimic the low physiological LH levels of the male rat in these studies, our novel supersensitive immunofluorometric assay [12] proved useful. The assay allows LH detection from serum samples of 25 μl with a sensitivity of 0.02 μg/L (NIH rLH RP-2; Bethesda, MD).

In this study, we extended our findings on the complex relationship of endogenous LH and T pulses to the effects of one or three exogenous pulses of rat recLH at different doses on serum T in male rats rendered hypogonadotropic by GnRH antagonist (cetrorelix) treatment. The rats were chronically catheterized and attached to an automated repetitive blood-microsampling device to allow monitoring the LH and T levels at 7- to 10-min intervals for several hours.

MATERIALS AND METHODS
Adult male Sprague-Dawley rats (age 2 mo) were used. The light period was adjusted to 14L:10D. The temperature was controlled (21–23°C), and the animals had free access to tap water and standard pelleted animal food.

Appropriate permissions for the experiments were obtained from the local Ethical Committee on Animal Experimentation.

Experiment 1
Six intact rats (250–270 g) were cannulated to the right jugular vein, and the catheter was extended to the right atrium. The day before the experiment, the cannulated rats were connected to the automated repetitive blood-microsampling apparatus originally described by Clark et al. [13] and modified by us [14]. On the next day, venous blood collection was performed every 7 min for a total of 5 h 36 min in order to produce physiological secretory patterns.

Experiment 2
Rats (weight 290–320 g) were treated with the GnRH antagonist cetrorelix acetate (SB-75; Asta Medica AG,
Frankfurt, Germany) in isotonic Ringer solution supplemented with 0.1% BSA (Ringer/0.1% BSA), twice: at 17 and 1 h before the experiment (100 μg/rat in 0.1 ml), i.m. and s.c., respectively. Groups of 3, 2, and 3 rats were injected with 3, 1, or 0.1 μg of highly purified rat recLH [9] in Ringer/0.1% BSA (0.1 ml i.v.), and cannulae were flushed with 0.4 ml Ringer/heparin after the i.v. injections. Blood samples of 0.3 ml were collected from the cannulae before and at 30 min, 1, 2, 3, 4, and 5 h after the LH injections. The same amounts of Ringer supplemented with heparin were injected i.v. into the rat after each blood collection.

**Experiment 3**

Rats (weight 238–261 g) were treated i.m. with cetrorelix acetate (SB-75) in Ringer/0.1% BSA twice, 2 days and 1 day before the experiment, with 100 μg and 200 μg/rat in 0.1 ml, respectively. The day before the experiment, the cannulated animals were connected to the sample collector (see above). Blood samples of 60 μl were collected between 0938 and 1748 h at 10-min intervals. Groups of 5 rats were treated three times (at 70, 135, and 190 min) with 0.1-ml i.v. injections of 0.1 μg or 0.03 μg of rat recLH in Ringer/0.1% BSA and flushed with 0.5 ml (Ringer/heparin). At the end of the sample collection, the rats were treated i.m. with cetrorelix acetate (SB-75; 200 μg/rat). On the next day, the same rats received one injection (at 1000 h) of 0.1 μg of rat recLH, followed by flushing with 0.5 ml of Ringer/heparin. Blood samples (60 μl) were collected between 0945 and 1740 h every 10 min as described above.

**LH and T Measurements**

Serum samples were separated by centrifugation and stored at −20°C until hormone measurements. Rat LH was measured using the time-resolved immunofluorometric assay (Delfia; Wallac OY, Turku, Finland) as described previously [12] with the exception that rat recLH was used as the standard. T was measured from serum by RIA after diethyl ether extraction [15].

**Statistical Analysis of the Data**

The area under curve (AUC) calculations and statistical analyses were carried out with a Macintosh version of Microsoft Excel 7.0 (Microsoft Corporation, Redmond, WA) and Statview 4.51 (Abacus Concepts, Inc., Berkeley, CA).
RESULTS

Experiment 1

The relationship between the endogenous LH secretion and T response was studied in intact adult male rats. Four typical secretory profiles are shown in Figure 1. As shown in a recent study with more extensive material (unpublished results), no clear relationship between the endogenous LH pulses and T secretion was observed. There was a tendency of T peaks to follow the LH peaks, but it was not very clear. A single LH peak did not seem to cause an immediate T response.

Experiment 2

To study the relationship of exogenous LH pulses and T secretion, the endogenous LH secretion of the male rats was first blocked by GnRH antagonist treatment. The basal level of LH in all rats treated in this way was <0.1 μg/L. Thereafter, the dose-response relationships between different doses of rat recLH (3, 1, and 0.1 μg) and T responses were studied (Fig. 2). The samples were manually collected at 0, 30, 60, 120, 180, 240, and 300 min. Rat recLH injections of 3 and 1 μg i.v. produced maximal LH peaks of 259 ± 24 and 76.1 ± 2.6 μg/L at 30 min (Fig. 2, upper) but similar maximum T responses of 43.3 ± 3.8 and 38.4 ± 0.8 nM at 60 min, respectively (Fig. 2, lower). Rat recLH injection of 0.1 μg i.v. produced a maximum LH level of 7.8 ± 0.7 μg/L at 30 min and a 50% lower maximal T response of 15.0 ± 1.9 nM at 60–120 min, which appeared to peak somewhat later than after the two higher doses.

Experiment 3

The T responses were thereafter evaluated after 1 or 3 pulse injections of 1 μg rat recLH, the latter 55 min apart, in adult male rats pretreated with cetrorelix acetate. The maximum T responses after 1 or 3 injections of 1 μg of rat recLH were similar: 56.1 ± 22 and 50.1 ± 9.4 nM, respectively, both obtained at 100 min (Fig. 3). The second and third LH peaks did not produce clear T peaks but rather slightly prolonged the T response. The AUCs of the induced LH peaks were 141 ± 16 and 328 ± 9 (p < 0.0001). The AUCs of the T responses, 6231 ± 1401 and 10180 ± 1621, were not significantly different. The LH levels of the control rat that received only gonadotropin antagonist treatment were <0.1 μg/L throughout the follow-up period (not shown).

Experiment 4

The relationship of smaller exogenous LH pulses and T responses was studied next. Three i.v. pulses of 0.1 (group
FIG. 4. Serum LH and T levels after three i.v. injections of 0.1 μg (group 1; n = 5; A) or 0.03 μg of rat recLH (group 2; n = 5; B). The responses of the same groups on the following day to an injection of 0.1 μg rat recLH are shown in C (group 1) and D (group 2).

1) or 0.03 μg (group 2) of rat recLH were administered on the first day of the experiment, and one i.v. pulse of 0.1 μg rat recLH was given to both groups on the following day. Both groups were pretreated with cetorelix acetate. Each one of the three pulses of 0.1 μg rat recLH seemed to produce a separate T peak. The maximum T response, 40.2 ± 7.5 nM, was achieved after the second LH peak at 226 min (Fig. 4A). In contrast, the T response was greatest, 5.1 ± 1.4 nM, at 247 min after the third pulse of 0.03 μg rat recLH. The T concentrations were clearly smaller after 0.03 μg rat recLH than after the higher LH doses (Fig. 4B). The AUCs of the LH curves, 123 ± 20 vs. 336 ± 49, and those of the T curves, 929 ± 271 vs. 5260 ± 665, were significantly smaller (p = 0.0009 and p = 0.009) after the three pulses of 0.03 μg than after 0.1 μg rat recLH i.v.

On the next day, both groups received a single i.v. injection of 0.1 μg rat recLH. The peaks of the LH concentrations were similar in the two groups, 4.2 ± 0.9 and 6.4 ± 1.1 μg/L, as were the AUCs of the induced LH responses, 137 ± 16 and 157 ± 22. However, the T responses displayed a clear difference. The maximum T concentrations were 24.3 ± 2.1 and 5.2 ± 1.2 nM, both at 105 min; and the AUCs of the T curves, 2850 ± 322 and 1240 ± 239, were significantly greater (p = 0.007) in the 0.1 μg group. In both groups, the T concentrations started rising spontaneously after about 250 min, when the T concentrations were lowest, despite the fact that the LH concentrations remained below 0.1 μg/L (Fig. 4, C and D).

DISCUSSION

In the present study we used two experimental strategies to study the relationship between pulsatile LH secretion and testicular T response. The first strategy was to take peripheral blood samples from intact male rats, and the second was to stimulate male rats, rendered hypogonadotropic by GnRH antagonist treatment, with i.v. injections of rat recLH. Previous studies have shown that the LH/T relationship is complex [6, 7, 16–18]. Since the causal relationships of the endogenous secretory patterns are difficult to interpret (Fig. 1, A–D), we decided to examine this function using defined exogenous pulses of LH.

Since the physiological LH secretion occurs in pulses of varying amplitude and frequency [7, 16], it is crucial for the physiological relevance of the results that the exogenous stimulus mimics the behavior of the endogenous hormone. Previously, most studies on gonadotropin-stimulated T production in the rat have been carried out using hCG, which has a half-life of several hours in men [19] and in rats [20]. Other heterologous mammalian hormones have also been used, e.g., ovine LH [21]. The half-life of human LH in human and rat circulation is 47–48 min [22, 23], and that of rat LH is 19 min in rat circulation [11]. Another variable is the affinity of the LH receptor for LH: the rat receptor binds human LH with about 100-fold higher affinity than rat LH [24, 25]. The recent availability of rat recLH prompted us to study its effects on T production in vivo.

Only about 1% of the LH receptor sites are known to be occupied by the ligand hormone during maximum stimulation of testicular steroidogenesis in vitro [26] and in vivo [27]. T stimulation cannot be further increased by a higher level of receptor occupancy [27]. This seems to be the case also upon stimulation with high doses of rat recLH (Figs. 2 and 3). A high dose of rat recLH (1.0 µg) induced a maximal T response that could not be further increased by a higher dose (3.0 µg); only the steroidogenic response was somewhat prolonged (Fig. 2, lower). This is in keeping with the previous findings on desensitization of the steroidogenic response following a pharmacological dose of the trophic hormone.

High doses of LH and hCG are known to cause desensitization of the Leydig cells, which is time and dose dependent (for a review, see [1]). However, whether this is a physiological regulatory mechanism of Leydig cell steroidogenesis in vivo has remained debatable [21]. In fact, a single injection of a high dose of 150 µg ovine LH, known to have short half-time of 5 min [28], desensitized the Leydig cell T-response less than did 100 IU (about 7 µg) of hCG in male rats [21], indicating that the desensitizing response may be inherent to stimulation with hormone preparations having an unphysiologically long rate of elimination. Moreover, twice-daily treatment with 6 or 12.5 µg of ovine LH for 6 days increased the testicular responsiveness to subsequent LH/hCG stimulations compared to that in nontreated control cells [21]. Low doses of hCG increased the Leydig cell responsiveness, which was associated with changes in enzyme activities in the smooth endoplasmic reticulum in relation to controls [29]. The low doses of rat recLH (3 × 0.03 µg) were also followed by decreased testicular responsiveness to LH stimulation (0.1 µg) on the following day as compared to that in rats treated with a higher dose of the hormone (3 × 0.1 µg). This finding suggests that the rats treated with the higher LH dose on Day 1 of the experiment had maintained the steroidogenic capacity of the Leydig cells, whereas those with more profound LH deprivation due to the GnRH antagonist treatment had lost the majority of it.

When the T responses to the three pulses of LH at the different dose levels were evaluated, interesting conclusions on the LH/T relationships could be drawn. If the LH dose was pharmacological, a loss of response to a subsequent stimulation was induced. If high physiological doses were used, each LH peak was followed by a discrete T response. However, if the doses were in the medium to low physiological range, multiple peaks of LH pulses were needed to evoke a T response. Continuous LH priming may be needed in order for the Leydig cell T production to maintain its responsiveness to LH stimulation as shown in Figure 4. The present findings provide a mechanistic explanation for the apparent lack of direct correlation of endogenous LH pulses with the T response. A single peak evokes a T response only if it is high enough, but multiple smaller LH peaks may in cumulative fashion be needed for the response. The time since the last prominent LH stimulus is an important determining factor of the magnitude of the T response to a given LH stimulus. Hence the functional state of the Leydig cells determines how they respond to the LH stimulus.

Several groups have reported a prolonged biphasic T response after a single injection of hCG in men [19, 30] and after an infusion of hCG in perfused Leydig cells [31]. In our experiment, after the LH stimulation with 0.1 µg rat recLH in both groups, the T concentrations started to rise spontaneously despite the fact that LH concentrations were low. Whether the phenomenon is related to that shown previously with hCG remains to be elucidated.

Besides LH, the Leydig cell development and function are affected by local factors produced by Sertoli cells and other testicular cells [32]. Their modulatory effects on LH-stimulated T secretion are still not well known. In addition, FSH might exert indirect effects on Leydig cell function, as has been shown with human recFSH in immature hypophysectomized rats [33, 34]. This effect may not be of major physiological significance, since in mice with disrupted FSHβ subunit gene and missing FSH production [35], as well as in men homozygous for an inactivating mutation in the FSH receptor [36], the LH-stimulated Leydig cell functions appear to be largely normal. Therefore, although also FSH secretion was suppressed in our GnRH antagonist-treated model, we consider the rat recLH effects observed to be physiologically relevant.

In conclusion, the present study emphasizes that homologous hormone preparations should be used when one studies the physiology of LH action in the rat, because of the different half-times in circulation and the affinity of the LH receptor of rat and human LH. Pure rat recLH can now be used for such studies.

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