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Pulsatile Secretion of LH and FSH in Prepubertal and Early Pubertal Boys Revealed by Ultrasensitive Time-Resolved Immunofluorometric Assays

LEO DUNKEL, HENRIK ALFTHAN, ULF-HOAKAN STENMAN, PÄIVI TAPANAINEN, AND JAAKKO PERHEENTUPA

Children's Hospital, University of Helsinki [L.D., J.P], Departments I and II of Obstetrics and Gynecology, University of Helsinki, Helsinki; [H.A., U-H.S] and Department of Pediatrics, University of Oulu, Oulu, Finland [P.T.]

ABSTRACT. Pulsatile secretion of LH and FSH was examined in 10 prepubertal (aged 4.5–12.9 y) and seven early pubertal (aged 12.8–14.5 y) boys with ultrasensitive (0.019 and 0.014 IU/L time-resolved immunofluorometric assays. Plasma LH and FSH levels were measured every 15 or 20 min for 6 h during the day and night. The lowest mean LH level in a prepubertal boy was 0.02 IU/L and in eight other prepubertal boys mean LH levels were less than 0.4 IU/L. In early pubertal boys the mean LH levels ranged from 0.3 to 6.5 IU/L. The difference in mean FSH level between prepubertal (0.61 IU/L) and early pubertal boys (1.85 IU/L) was smaller than the difference in LH level. All boys had significant LH and FSH pulses. The LH interpulse interval was 135 ± 86 min (mean ± SD) and 76 ± 65 min for the prepubertal and pubertal boys, respectively ($p < 0.01$). For FSH, the respective values were 150 ± 122 and 221 ± 157 min (p = NS). The mean LH pulse amplitudes were 11-fold greater in the early pubertal boys than in the prepubertal boys, whereas the mean FSH pulse amplitudes were similar between the two groups. The present method shows that the mean LH levels in prepubertal boys are much lower, and the increase during puberty larger, than previously reported. The increase is apparently due to increased pulse frequency and amplitude. The increase in mean FSH level is smaller and evidently not caused by an increase in pulse frequency or pulse amplitude. *(Pediatr Res* 27:215–219, 1990)

Abbreviations

CV, coefficient of variation
IFMA, immunofluorometric assay
GnRH, gonadotropin-releasing hormone

In pubertal children and adults LH, and presumably GnRH are secreted episodically (1–3). However, some controversy still exists about the pulsatile secretion of LH in prepubertal children (4–10). Penny *et al.* (4) demonstrated three LH pulses during 4-h sampling periods in three late prepubertal children, and Jakacki *et al.* (5) observed 1 LH pulse every 3 h in eight of 14 prepubertal children aged 3 to 13.8 y. In a recent study by Wennink *et al.* (6) in three late prepubertal boys no daytime and one to two nocturnal LH pulses were observed during 6-h sampling periods by sensitive immunoradiometric assay employing two MAb to LH. However, in several other studies no clear pulsatile secretion of LH was observed before the onset of puberty (7–10). Pulsatile secretion of FSH has not been demonstrated in prepubertal children, and until recently it was poorly characterized even in adults (11).

Highly sensitive methods for assay of LH and FSH recently showed that the levels in prepubertal girls are much lower than earlier assumed (12). It is therefore possible that most assays used earlier were not sufficiently sensitive to detect gonadotropin pulses because of the low prepubertal concentrations. In our study we demonstrate pulsatile secretion of LH and FSH in prepubertal boys by ultrasensitive time-resolved immunofluorometric assays.

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Correspondence and reprint requests Dr. L. Dunkel, Children's Hospital, University of Helsinki, SF-00290 Helsinki, Finland.

MATERIALS AND METHODS

Subjects. Gonadotropin secretion was studied by frequent sampling of plasma in 17 boys (Table 1). Ten prepubertal boys, aged 4.5–12.9 y, were regarded as endocrinologically normal. Two of them had unilaterally incomplete testicular descent, with the testes palpable in the inquinal region. Three boys had constitutionally delayed growth with normal growth hormone secretion (nocturnal growth hormone peaks above 22 mU/L). One of the boys had delayed pubertal development (patient 10), with no testis enlargement at the age of 12.9 y. This boy has been followed subsequently, and has had spontaneous pubertal development excluding the possibility of gonadotropin deficiency. The other five boys were healthy by physical examination. Seven boys, aged 12.8–14.5 y, were in early puberty (genital stage G2) (13). Two boys had constitutionally delayed growth (nocturnal growth hormone peaks of more than 25 mU/L). The other five boys appeared healthy by physical examination. All 17 boys had followed their "growth channels," *i.e.* had not deviated in growth rate since infancy. Ht at the time of the study was evaluated in SD scores compared with normal Finnish children. The five boys with constitutionally delayed growth had relative ht between −2.1 and −2.5 SD for age. The other 11 boys had relative ht above −1.5 SD. None of the boys had received androgen therapy before the study.

Protocol. The studies were conducted at the Children's Hospital, University of Helsinki and at the University Hospital of Oulu. Informed consent was obtained from a parent and assent from the patient. The protocol was approved by the Ethical Committees of the hospitals.

On the study day the boys were allowed normal activity from rising until 2100 h. At that time they went to bed, and the room

Table 1. *Clinical characteristics of patients*

Patient	Bone age* (y)	Age (y)	Diagnosis	Pubertal stage†	Testosterone (nmol/L)‡ Day	Night	Sampling frequency (min)
1	4.3	4.5	Normal	I		0.12	5
2	5.0	5.3	ITD	I	0.2	0.42	15
3	5.0	5.5	ITD	I		0.05	15
4	7.0	7.2	Normal	I		0.11	5
5	8.5	9.0	Normal	I		0.21	15
6	9.5	9.5	CDG	I	0.26	0.38	15
7	10.0	10.3	Normal	I		0.1	20
8	11.0	10.8	Normal	I		0.05	20
9	9.0	11.4	CDG	I	0.20	0.4	15
10	8.5	12.9	CDG	I	0.31	0.22	15
11	11.0	12.8	Normal	II	0.23	0.4	15
12	13.0	12.8	Normal	II		4.9	20
13	13.0	12.9	Normal	II		1.7	20
14	11.0	13.0	Normal	II		5.3	20
15	11.8	13.3	CDG	II	0.15	0.6	15
16	11.0	13.5	Normal	II		5.5	20
17	12.5	14.5	CDG	II	0.4	1.3	15

* Determined by the method of Greulich and Pyle (21). ITD, incomplete testicular descent; CDG, constitutional delay of growth.
† Estimated from pubic hair distribution and genital development by the method of Tanner (13).
‡ Divide by 3.5 to convert to ng/L.

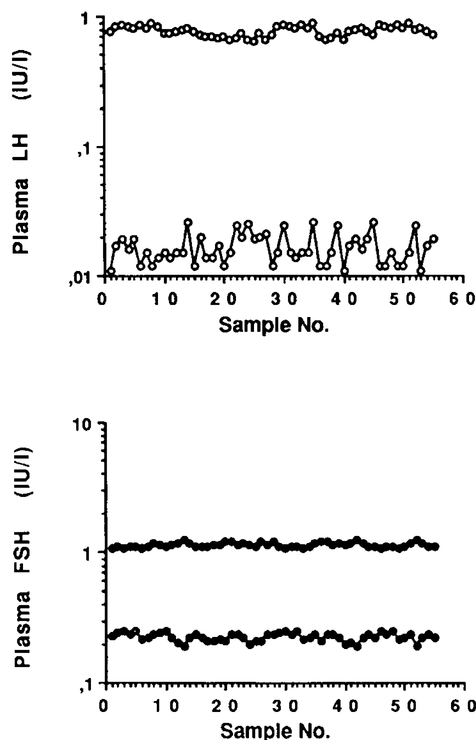

Fig. 1. Plasma LH and FSH concentrations in 55 aliquots from two different plasma pools were assayed in duplicate. No peaks were detected when the results were analyzed with the Munro program.

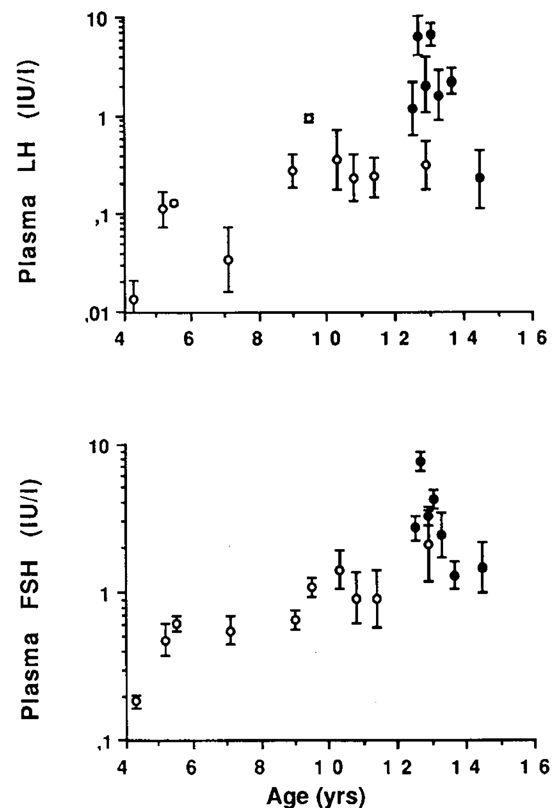

Fig. 2. Mean ± 1 SD plasma LH and FSH levels measured from frequent sampling in prepubertal (*open circles*) and early pubertal (*solid circles*) boys in relation to chronologic age.

lights were turned off. Sleep was monitored visually by trained nursing personnel. An indwelling i.v. cannula was inserted 30 min before the beginning of sampling. Blood samples were obtained every 5 min (two patients), every 15 min (nine patients), or every 20 min (six patients) for 6–8 h at night, and in 7 subjects for 6 h both during the day and at night (Table 1). Plasma LH and FSH were measured in all samples; testosterone was measured at the beginning of the sampling period. All samples from each boy were analyzed in the same assay.

Hormone measurements. Plasma testosterone was measured by RIA after separation of steroid fractions on a Lipidex-5000 microcolumn as previously described (14). Plasma LH and FSH concentrations were measured by time-resolved immunofluorometric assays, using reagents from LKB-Wallac (Turku, Finland). Three MAb were used in two different combinations. For the LH assay, antibodies directed toward the β-chain of LH, and for the FSH assay, antibodies directed toward the β-chain of FSH, were immobilized on the walls of microtiter strip wells. The indicator antibody, specific for the α-chain of FSH or LH, was

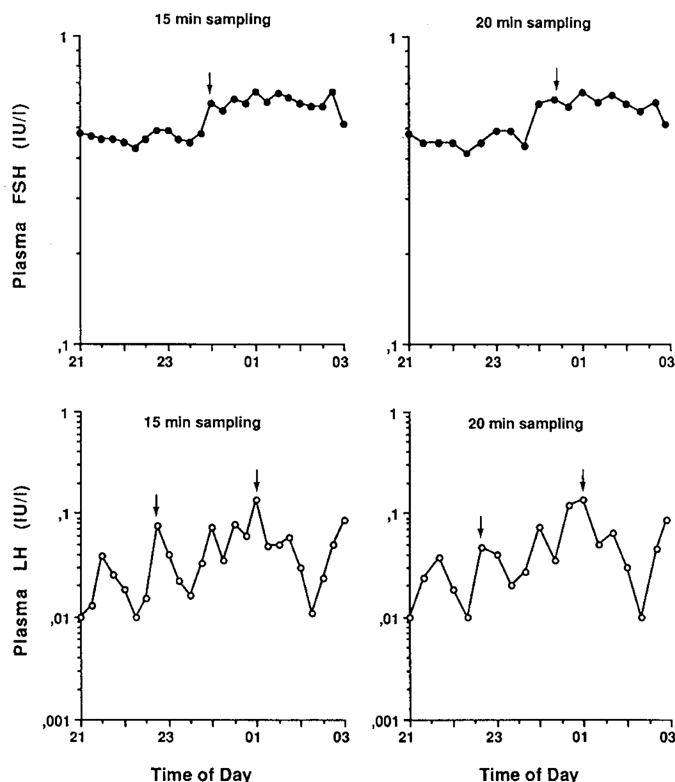

Fig. 3. Time courses of plasma LH (*open circles*) and FSH (*solid circles*) concentration in one boy indicating the effect of sampling interval on gonadotropin profiles. Samples were taken every 5 min, in the *left panels* values are given for the samples taken every 15 min and in the *right panels* values are given for the samples taken every 20 min. *Arrows* denote significant pulses.

labeled with a europium chelate (15). Of the sample, 25 μL were diluted with 200 μL of assay buffer and pipetted into the wells. After incubation for 16–20 h at room temperature, the wells were washed three times and 25 ng europium-labeled antibody in 200 μL assay buffer was added. The wells were washed after incubation for 40 min and 200 μL of enhancement solution added. The fluorescence was measured for 1 s/sample in an Arcus 1230 fluorometer (LKB-Wallac, Turku, Finland). The LH assay also measures human chorionic gonadotropin, but other glycoproteins (*e.g.* the α-subunit) do not react to a measurable degree in the assay. The dose-response curves were parallel to the standard. The elution pattern of FSH in prepubertal serum was indistinguishable from menopausal urinary FSH. The LH standards were calibrated against WHO IRP 68/40, and FSH standards against the second IRP of pituitary FSH/LH (78/549). The assay sensitivity for LH was 0.019 IU/L and for FSH 0.014 IU/L, as defined by mean + 2 SD of 12 replicates of a 0 sample. Plasma of two patients with hypogonadotropic hypogonadism gave results indistinguishable from the 0 samples.

The intraassay CV was calcuated by measuring 10 replicates at five and six different concentrations of LH and FSH, respectively. The CV ranged from 2.1 to 8.5% for FSH and from 3.1 to 13.9% for LH at the different concentrations. The results were used for linear interpolation of CV for various concentrations of LH or FSH and for calculation of the assay SD coefficients for the pulse analysis program.

Pulse analysis. LH and FSH pulse analysis was performed by using a computerized pulse analysis program, Munro (Zaristow Software, East Lothian, Scotland), developed by Philip L. Taylor. The program identifies secretory peaks by ht and duration from a smoothed baseline, using the assay SD as a scale factor. Munro is an adaptation of the Pulsar program developed by Merriam and Wachter (16), with an essential difference only in the calculation of the baseline. The baseline is generated by linear interpolation between the nadirs, followed by smoothing using a moving average. The remaining stages of the Munro algorithm are identical with the Pulsar program. As the baseline in the Munro program is calculated from the nadirs rather than from the moving average of the data, Munro can deal with data containing pulses whose width and amplitude are very variable. This we considered essential in the analysis of FSH pulses, which appeared to be wider than LH pulses.

The cut-off parameters G1-5 of the Munro program were set to 3.98, 2.4, 1.7, 1.2, and 0.98 times the intraassay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate the moving average was set to 150 min, *i.e.* 10 data points wide. The program did not, with these settings, detect any peaks when 55 consecutive samples from each of two different plasma pools was assayed. The results of the assays of the two plasma pools, with LH levels around 0.03 and 0.8 IU/L, and FSH levels around 0.22 and 1.1 IU/L, respectively, are shown in Figure 1. Thus, use of a special program for minimizing false positive error in pulse detection was not deemed necessary (17). Missing values comprised less than 0.5% of the total samples and were left blank. When the LH and FSH peaks occurred in the same or successive samples, the peaks were considered to be concordant. The interpulse interval was defined as the time period between consecutive peaks.

Statistical analyses. Because of positive skewness of intraindividual LH and FSH levels, mean values were calculated after logarithmic transformation. Differences between the groups were analyzed by Student's t test. p levels <0.05 were considered significant. Discordance between LH and FSH pulses were assessed using the χ^2 test.

RESULTS

Mean nocturnal plasma LH and FSH levels. In the prepubertal boys the mean plasma LH levels were very low. The lowest individual mean value was 0.02 IU/L (patient 1, age 4.5 y); four other boys, aged 5–11 y, had values less than 0.4 IU/L (Fig. 2). One of the prepubertal boys, aged 9 y, had a mean LH level of 0.87 IU/L. In the early pubertal boys the mean LH levels ranged from 0.3 to 6.5 IU/L. The difference in mean FSH levels between prepubertal and pubertal boys was smaller than that of LH levels. The lowest prepubertal FSH level was 0.19 IU/L and the highest pubertal FSH level was 4.0 IU/L. The mean FSH levels correlated with age: $r = 0.67$, $p < 0.05$ for all boys, and $r = 0.77$, $p < 0.01$ for the prepubertal boys.

Nocturnal LH and FSH pulses. To assess whether the 15-min sampling frequency might yield different information than that obtained from the 20-min sampling frequency, plasma was collected at 5-min intervals in two boys at night (patients 1 and 4). Samples obtained every 15 and 20 min were used for further analysis (Fig. 3). Increasing the sampling interval from 15 to 20 min had little effect on shape of the curves and no effect on the number of significant pulses in the two boys studied.

All the boys had significant LH and FSH pulses. In the prepubertal boys the highest LH levels were less than 1.2 IU/L (Fig. 4). In the early pubertal boys the highest levels ranged from 3.5 to 11 IU/L (Fig. 4). The highest FSH levels were 0.6–2.1 IU/L in the prepubertal boys and 2.1–3.9 IU/L in the early pubertal boys.

Significant synchronism was observed between LH and FSH secretion. The synchronism was clear even in the prepubertal boys. In the prepubertal boys, of the 45 LH and 28 FSH pulses observed, 15 occurred concordantly, *i.e.* within 15–20 min ($p < 0.01$). In the pubertal boys the values were: 36 LH pulses, 12 FSH pulses, and nine concordant pulses ($p < 0.01$).

The mean nocturnal LH pulse intervals were shorter in early puberty than in prepuberty (Fig. 5). The mean LH pulse amplitudes were also greater in early puberty than in prepuberty (Fig.

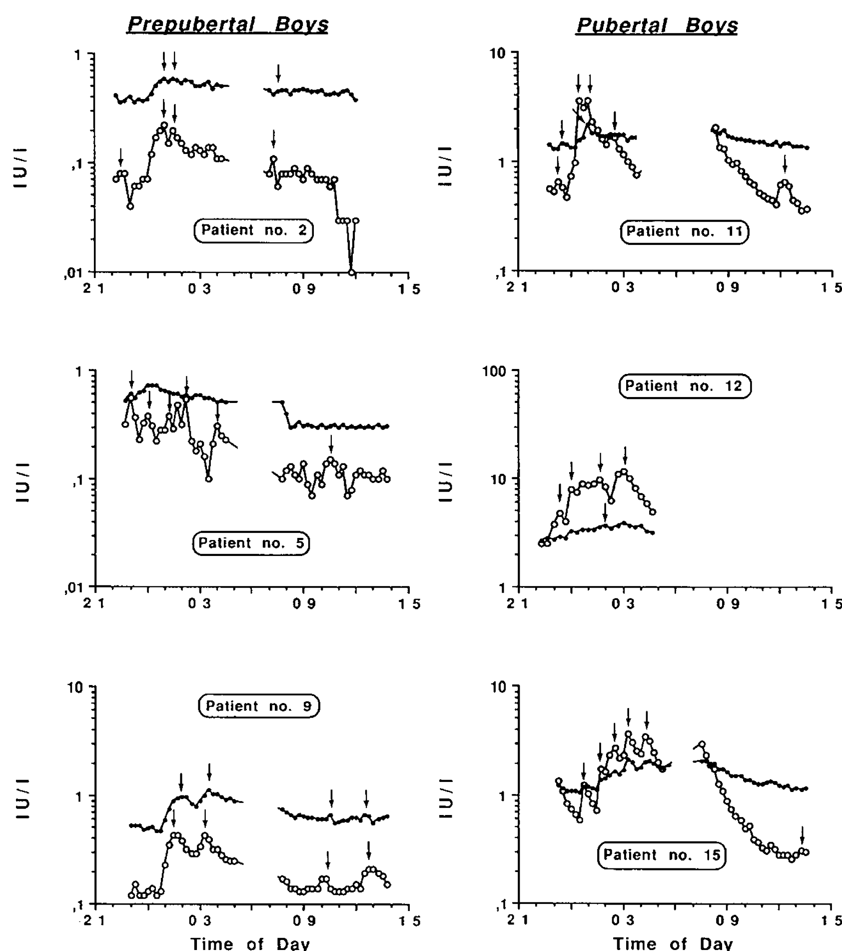

Fig. 4. Time courses of plasma LH (*open circles*) and FSH (*solid circles*) concentration in three prepubertal boys (*left panels*) and three early pubertal boys (*right panels*). *Arrows* denote significant pulses, for clarity, only LH pulses are shown for the patients 5 and 15. Note different scales on the y axis.

5). The mean FSH pulse intervals and the mean FSH pulse amplitudes were similar in pre- and early pubertal boys.

Day time versus night time sampling periods. In seven of the 17 boys, samples were taken both at night and in the daytime. Five of these seven had higher mean LH levels during the night. In one prepubertal boy and one pubertal boy this difference was not significant ($p > 0.05$), although in all seven boys the highest LH levels were at night. The difference between the nighttime and daytime levels was less evident for FSH than for LH. Two prepubertal boys had higher mean nighttime FSH levels, whereas the rest showed no significant difference, although all the boys had their highest levels at night. The mean interpulse interval was longer during the daytime both in prepubertal and in pubertal boys. In the seven boys whose pulses were studied both at night and during the day, 73% of all LH and 71% of all FSH pulses occurred at night. Further, in all boys the pulse amplitudes were higher at night.

DISCUSSION

With our method we could observe much lower plasma LH levels than previously reported for prepubertal boys (1, 4, 5). In boys less than 9 y old the levels ranged from 0.02 to 0.8 IU/L (mean 0.1 IU/L). Our group has previously reported equally low plasma LH levels for prepubertal girls and an abrupt increase in LH levels at the onset of puberty (12). Thus boys and girls appear to be similar in this respect. Two of the boys of our normal group had incomplete testicular descent, but their gonadotropin profiles did not differ from those of the other normal boys. Therefore it seems unlikely that these boys had had partial LH deficiency, which is occasionally seen in boys with incomplete testicular descent (18).

The low plasma LH levels could be observed because of the very high sensitivity of the assay used, *i.e.* about 50- to 100-fold that of RIA. In the previous study, a correlation between RIA and IFMA LH concentrations was reported (12). In the prepubertal age category the correlation was weak, as it was not possible to measure true prepubertal levels by RIA. The low levels obtained by IFMA were not explained by differences in calibration between IFMA and RIA. At higher LH concentrations the correlation between IFMA and RIA was good (12).

The great sensitivity of the present assay enabled us to detect LH and FSH pulses in prepuberty. This confirms findings by Penny *et al.* (4), Jakacki *et al.* (5), and Wennink *et al.* (6) of pulsatile LH secretion in prepubertal children. The mean nocturnal interpulse interval for LH in our normal prepubertal boys was 135 min, which is close to the value observed by Jakacki *et al.* (5) (120–180 min) and Wennink *et al.* (6) (180 min) and much longer than that found by Penny *et al.* (4) (80 min). The differences may be due to differences in the sensitivity of the assays used or in the criteria adopted for significant pulses. According to recent results of Hale *et al.* (19), the onset of puberty is associated with an increase in LH pulse frequency during the early hours of sleep, and our data support this finding. The nocturnal LH pulse interval in our early pubertal boys was the same as found by Hale *et al.* (19).

The serum FSH levels in the prepubertal boys were clearly higher than the LH levels, and thus the difference between pre- and early puberty was smaller than for LH. The greater increase in the mean LH over the mean FSH levels at the onset of puberty

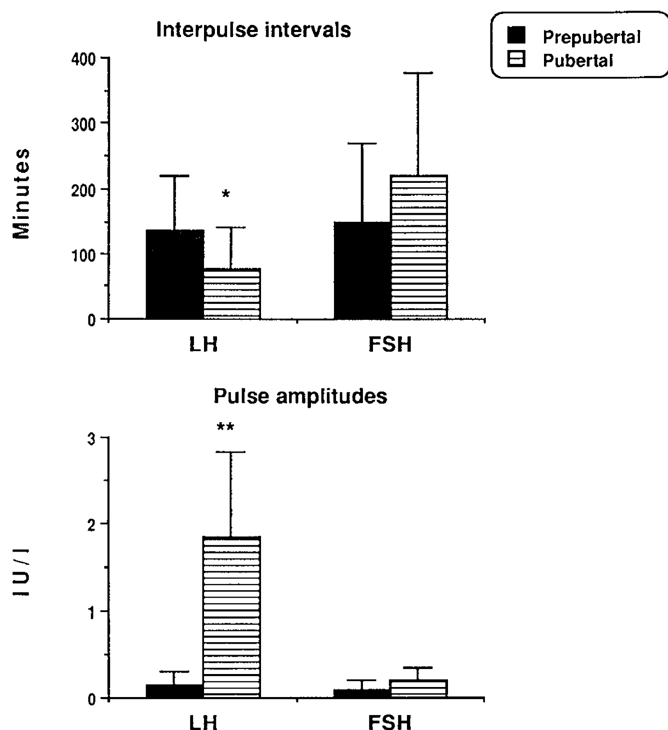

Fig. 5. Nocturnal LH and FSH interpulse intervals (*upper panel*), and pulse amplitudes (*lower panel*) in the prepubertal and early pubertal boys. Statistically significant differences between the two groups are indicated with *asterisks*: $*p < 0.05$, $**p < 0.01$.

may be related to the change in pulse frequency, as an increase in GnRH pulse frequency appears predominantly to increase the LH concentrations (20).

The onset of puberty has been considered to be heralded by an augmentation of LH pulse amplitude (8). Our results suggest that pituitary LH secretion is very sensitive to endogenous GnRH even before puberty. Thus the onset of puberty may be associated with an increase in GnRH secretion rather than an increase in sensitivity of the pituitary gonadotroph to GnRH. This agrees with the observation of Corley *et al.* (1) whose indirect estimations of GnRH pulse amplitudes in the hypophysial portal circulation of prepubertal and early pubertal children showed an increase in amplitude at the onset of puberty.

The pattern of FSH secretion in prepubertal and early pubertal boys has not been well characterized. We observed FSH pulses almost with the same frequency as LH pulses in prepubertal boys and 54 percent of the FSH pulses occurred concordantly with LH pulses. Although the available evidence suggests that LH and FSH are released concurrently by a single releasing factor, the synchronism that we observed is surprisingly high. The time interval between an exogenous GnRH bolus and peak gonadotropin concentration in the peripheral plasma is significantly longer for FSH than for LH (2). In FSH, in contrast to LH, the difference in plasma levels between pubertal and prepubertal boys did not result from increased pulse frequency or amplitude. This suggests that basal secretion of FSH from the pituitary increases at the onset of puberty.

In conclusion, assays of LH and FSH by ultrasensitive IFMA methods revealed that before the onset of puberty both gonadotropins are secreted in pulses. The increase in LH level at the onset of puberty appears to be due to an increase in pulse amplitude and frequency, whereas the increase in FSH level is due to other mechanisms than increased pulsatile secretion.

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