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Spontaneous Gonadotropin and Testosterone Concentration Profiles in Prepubertal and Pubertal Boys: Temporal Relationship between Luteinizing Hormone and Testosterone

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ABSTRACT. To investigate the detailed pattern of change in circulating gonadotropin and testosterone concentrations around the onset of puberty and to determine the temporal relationship between the gonadotropin and testosterone secretion, plasma gonadotropin and testosterone were measured at 20-min intervals for 24 h in 21 normal short boys. The obtained plasma hormone concentrations were analyzed by Cluster pulse detection algorithm, cosinor analysis, and cross-correlation analysis. The 21 subjects were divided into the prepubertal (n = 16) and early pubertal (n = 5) groups. All subjects showed nocturnal LH and FSH pulses and had significant circadian LH and FSH rhythms. Except for six boys of prepubertal group, all subjects showed nocturnal testosterone pulses and had significant circadian testosterone rhythms. The acrophase (clocktime of maximal value) of circadian testosterone rhythm was 0308–0428 h. Cross-correlation analysis demonstrated significant positive cross-correlations between LH and testosterone that were maximum at a testosterone lag of 60–120 min. Further, to eliminate intrinsic autocorrelations within the LH and testosterone time series, we filtered the data before subjecting them to the cross-correlation analysis. As a result, significant positive cross-correlations were found at a testosterone lag of 40 min in 10 peripubertal boys. We conclude that testosterone concentration profiles are pulsatile and show marked circadian rhythm well before the onset of puberty. LH and testosterone time series are significantly coupled when testosterone lags LH by about 40 min. This time lag might correspond to the time for synthesizing and secreting testosterone in Leydig cells after binding of LH to the Leydig cell receptors. (*Pediatr Res* 34: 229–236, 1993)

Previous studies have shown that gonadotropin secretion is pulsatile and shows marked circadian rhythm because of nocturnal augmentation of gonadotropin secretion well before the onset of puberty and that this nocturnal secretion is dramatically increased with puberty (1–11). This initial neuroendocrinologic phase of puberty occurs before the clinical onset of puberty and is responsible for stimulating the Leydig cells to synthesize and secrete testosterone. Therefore, investigation of the detailed pattern of change in circulating gonadotropin and testosterone concentrations around the onset of puberty is essential to understand neuroendocrinologic mechanisms relating to the onset of puberty. Also, improvements in physiologic replacement therapy of testosterone for the patients with hypogonadism may be obtained from the detailed study in the spontaneous secretory dynamics of testosterone around the onset of puberty.

Few studies have examined both gonadotropin and testosterone concentration profiles simultaneously around the onset of puberty. Boyar *et al.* (12) and Judd *et al.* (13) have reported that plasma testosterone concentrations were significantly higher during nocturnal sleep compared with daytime waking in pubertal boys. However, they were unable to demonstrate a nocturnal testosterone elevation in prepubertal boys. Parker *et al.* (1) and Gordon *et al.* (14) have found a significant increase in testosterone concentrations during nocturnal sleep in prepubertal boys. These earlier studies suggest that not only plasma LH concentrations but also plasma testosterone concentrations could increase during night before the clinical onset of puberty. In these earlier studies, however, sampling duration was relatively limited and mathematically established methods were not applied for the analysis of hormone time series.

The present study was undertaken to investigate the detailed pattern of change in circulating testosterone concentrations around the onset of puberty and to determine the temporal relationship between the gonadotropin and testosterone secretion. Plasma LH, FSH, and testosterone were measured at 20-min intervals for 24 h in 21 prepubertal and early pubertal boys. The obtained plasma hormone concentrations were analyzed by Cluster pulse detection algorithm, cosinor analysis, and cross-correlation analysis.

MATERIALS AND METHODS

Study subjects. Twenty-one short but otherwise normal Japanese boys, aged 8.0 to 14.8 y, participated in this study. All the boys were referred to the Kobe Children's Hospital for evaluation of short stature. All 21 boys had adequate growth hormone secretion during provocative tests using insulin, arginine, or levodopa (peak level > 10 μg/L for two or more tests) and had normal adrenal and thyroid status. There was no evidence of systemic disease, malnutrition, or psychosomatic disturbances, and none of the boys had received long-term medication. Pubertal development was assessed according to Tanner's criteria (15), and bone age was evaluated according to the standards of Greulich and Pyle (16). The subjects were divided into two study groups as follows: *1*) prepubertal group (n = 16) with no evidence of onset of puberty on physical examination in all aspects; and *2*) early pubertal group (n = 5) with evidence of onset of puberty on physical examination but pubic hair development no greater than Tanner stage II. All the prepubertal boys were followed up

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for 1 y after the investigation to assess subsequent clinical progress. The prepubertal group was subdivided into three additional groups. The prepubertal A group consisted of six boys who had testes with greatest diameter less than 20 mm at the time of study and had shown no evidence of pubertal onset during the follow-up period. The prepubertal B group consisted of five boys who had testes with greatest diameter of 21–26 mm and had shown no evidence of pubertal onset during the follow-up period. The prepubertal C group consisted of five boys who had testes with greatest diameter of 21–26 mm and had developed into early puberty during the follow-up period. Clinical characteristics of the study subjects are summarized in Table 1. Informed consent was obtained from all children, parents, or both. The protocol was approved by the ethical committee of the Kobe Children's Hospital.

Study protocol. The subjects were admitted to the Kobe Children's Hospital the evening before the study and an i.v. heparin lock needle was placed in a forearm vein at about 1800 h for venous sampling. Blood samples were drawn for LH, FSH, and testosterone measurement starting at 1000 h and at 20-min intervals thereafter for a 24-h period. The total amount of blood collected was less than 5% of the calculated blood volume. No side effects were observed in any subjects. Blood samples were kept on ice for a maximum of 20 min before centrifugation. Plasma was stored at −20°C for 1 mo until analysis. The subjects were instructed to carry out their normal activities but to avoid taking naps during the day or engaging in exercise. Three standard meals were given at 0800, 1200, and 1700 h.

Hormone assay. Plasma LH and FSH concentrations were measured in duplicate by a time-resolved immunofluorometric assay using a DELFIA LH kit and an FSH kit (Pharmacia, Turku, Finland). The assay standards were calibrated against the WHO Second International Reference of Pituitary LH 80/552 for LH and the Second International Reference of Pituitary FSH/LH 78/549 for FSH. The intraassay coefficients of variation were less than 7.8% in the range of 0.09–20.8 IU/L for LH assay and less than 11.0% in the range of 0.07–21.0 IU/L for FSH assay. The interassay coefficients of variation were less than 7.0% in the range of 3.4–59.6 IU/L for LH assay and less than 5.5% in the range of 5.1–27.1 IU/L for FSH assay. The detection limit of the assay for LH was 0.02 IU/L and that for FSH was 0.03 IU/L as defined by mean + 3 SD of 10 replicates of the zero standard provided by the manufacturer.

Plasma testosterone concentrations were measured in duplicate by a solid phase RIA using a TOTAL TESTOSTERONE kit (Diagnostic Products Corporation, Los Angeles, CA). This kit is a solid-phase RIA designed for the measurements of testosterone in unextracted serum or plasma. The intraassay coefficients of variation with testosterone concentrations of 0.70, 2.50, 12.21, and 24.92 nmol/L were 3.5, 6.7, 6.2, and 5.1%, respectively. The interassay coefficients of variation with testosterone concentrations of 0.70, 2.52, 11.57, and 26.34 nmol/L were 6.9, 7.7, 6.9, and 7.1%, respectively. The detection limit of the assay for testosterone was 0.17 nmol/L as defined by mean + 2 SD of 10 replicates of the zero standard provided by the manufacturer. Values below the detection limit were assigned the value of the detection limit. All samples from one patient were run in one assay to eliminate interassay variance. The conversion factor for testosterone from ng/dL to nmol/L is 0.03467.

Table 1. *Clinical characteristics of study subjects**

Study group	*n*	CA (y)	BA (y)†	Testicular size (mm)‡
Prepubertal boys				
A	6	9.4 ± 0.4 (8.0–10.7)	5.8 ± 0.7 (2.5–8.0)	18.3 ± 0.6 (16–20)
B	5	12.2 ± 0.2 (11.9–13.1)	8.6 ± 0.6 (6.5–10.5)	23.6 ± 0.5 (22–25)
C	5	11.5 ± 0.5 (10.0–13.0)	9.9 ± 0.4 (9.0–11.0)	23.9 ± 0.7 (22–26)
Early pubertal boys	5	13.2 ± 0.4 (11.9–14.8)	11.1 ± 0.6 (10.0–13.5)	30.1 ± 0.7 (28–33)

* Values are means ± SEM, with ranges in parentheses. CA, chronologic age; BA, bone age.

† Estimated by method of Greulich and Pyle (16).

‡ Greatest diameter; no differences in values were detected between prepubertal B and prepubertal C groups ($p = 0.77$).

Pulse analysis. Pulses within the LH, FSH, and testosterone data series during the nocturnal 8 h were evaluated using Cluster analysis (17). Nocturnal hormone series corresponded to the period 2200–0600 h. For LH and FSH pulse detection, a 1 × 1 (test nadir 1, test peak 1) cluster size was used, and the *t* statistics were set at 2.5 for both the upstroke and downstroke phases of the pulse, so the false-positive rate for LH and FSH would be less than 2% and 5%, respectively, on signal-free noise generated by assaying 100 replicates of a single serum pool. For testosterone pulse detection, a 1 × 1 cluster size was used, and the *t* statistics were set at 2.25 for both the upstroke and downstroke phases of the pulse, so the false-positive rate would be less than 5% on signal-free noise generated by assaying 100 replicates of a single serum pool.

Analysis of 24-h rhythms. The LH, FSH, and testosterone concentration time series were analyzed for 24-h rhythms by cosinor analysis (18). The acrophase (time of maximum value within the 24-h rhythm), mesor (mean value about which the cosine rhythm varies), and amplitude (difference between maximum value and mesor) were estimated for each study group.

Statistical analysis. Because of the skewed distribution of the LH, FSH, and testosterone concentrations, statistical treatment was performed after logarithmic transformation. Therefore, with the exception of the peak frequency, the peak interval, the clocktime of acrophase, and the relative amplitude of 24-h rhythms, the data presented are geometric means, with 95% confidence intervals. Statistical comparisons among groups were made using one-way analysis of variance, followed by multiple *t* tests with the Bonferroni adjustment. Correlations between the maximum concentrations of LH and FSH in 24-h time series and that of testosterone were examined by linear regression analysis. A *p* value of less than 0.05 was considered statistically significant.

Cross-correlation analysis. Cross-correlation analysis was used to assess significant correlations between the LH and testosterone time series and between the LH and FSH time series. The cross-correlation coefficient (r_k) measures the correlation between two time series at a distance (lag) k time units apart (19, 20). The corresponding standard error of the correlation coefficient for a series of N samples at lag k can be estimated as $(N - k)^{-1/2}$ (21). Significant cross-correlations can be inferred when r_k values exceed zero by more than twice the appropriate standard error of the time lag.

It is obvious that cross-correlations can be a helpful tool for checking the dependencies between two time series. However, when a series, *e.g.* LH or testosterone time series, is highly autocorrelated, the cross-correlation function between the raw data of two time series can be difficult to interpret and even can be misleading (19). Therefore, to obtain valuable information from cross-correlations, we prewhitened (or filtered) the data before calculating the cross-correlations. This prewhitening process consists of three steps. First, to eliminate all variations in each series that can be explained by its past data, we fit the appropriate univariate models for each series involved. Then, at the second stage, we obtain residuals of the models by calculating the differences between the observed and the fitted values. These residuals are defined as the prewhitened data. Finally, we evaluate the nature of the relationship that exists between the prewhitened series (19).

RESULTS

Except for LH values in the 36 samples from one boy of the prepubertal A group, all LH and FSH values in the samples from 21 boys were well within the detectable range of the assay. In all 21 subjects, the mean plasma LH and FSH concentrations during the nocturnal 8 h (clocktime, 2200–0600 h) were significantly higher than those during the daytime 8 h (1000–1800 h) ($p < 0.0001$), suggesting an increasing hormonal concentration after onset of sleep. In all six subjects of the prepubertal A group, the plasma testosterone concentrations remained lower than the detection limit throughout the 24-h study period and did not show any increase after the onset of sleep. In the other 15 subjects including 10 prepubertal subjects, the plasma testosterone concentrations tended to increase after the onset of sleep, and in 13 of these subjects the mean concentrations during the nocturnal 8 h were significantly higher than those during the daytime 8 h ($p < 0.0001$). Representative profiles of LH, FSH, and testosterone from respective groups are shown in Figure 1.

Table 2 shows the 24-h concentration profiles of LH, FSH, and testosterone in the four study groups. The mean 24-h concentrations of LH in the prepubertal A group were significantly lower than those in all other groups ($p < 0.01$), and the values in the early pubertal group were significantly higher than those in the prepubertal A ($p < 0.01$) and prepubertal B groups ($p < 0.05$). The mean 24-h concentrations of FSH in the early pubertal group were significantly higher than those in the prepubertal A group ($p < 0.05$); however, no differences in the values were detected among the prepubertal B, prepubertal C, and early pubertal groups. The mean 24-h concentrations of testosterone tended to increase with development of sexual maturation; however, no differences in the value were statistically detected between the prepubertal A and prepubertal B groups.

To assess the influence of gonadotropin secretion on testosterone secretion, we first determined the correlations between the maximum 24-h spontaneous concentrations of gonadotropin and those of testosterone by linear regression analysis. The maximum 24-h concentrations of LH were highly correlated with those of testosterone ($r = 0.87$, $p = 0.00001$). In contrast, there were no significant correlations between the maximum concentrations of FSH and those of testosterone. Therefore, subsequent analyses of the correlation between gonadotropin and testosterone were restricted to that between LH and testosterone.

Pulse characteristics. The results of characterization of LH, FSH, and testosterone concentration series using Cluster analysis are shown in Table 3. All subjects showed nocturnal spontaneous LH and FSH pulses. In the prepubertal A group, the LH pulse frequency tended to be lower and the pulse interval tended to be longer than those in all other groups; however, there was no significant difference among the four groups. The LH pulse amplitude was significantly lower in the prepubertal A group than in all other groups ($p < 0.01$). Although the LH pulse amplitude in the early pubertal group tended to be higher compared with those in the prepubertal B and prepubertal C groups, no differences in the values were detected among the prepubertal B, prepubertal C, and early pubertal groups. No differences in the FSH pulse frequency, pulse interval, and pulse amplitude were detected among the prepubertal A, prepubertal B, prepubertal C, and early pubertal groups.

All subjects in the prepubertal A group had no detectable testosterone pulsations because their plasma testosterone concentrations remained lower than assay sensitivity throughout the 24-h study period. All subjects in the other three groups showed nocturnal spontaneous testosterone pulses. No differences in the testosterone pulse frequency and pulse interval were detected among the prepubertal B, prepubertal C, and early pubertal groups. The testosterone pulse amplitude was statistically distinguishable among three groups ($p < 0.05$) and increased with the development of sexual maturation.

Twenty-four-h rhythms. The cosinor analysis was performed to evaluate 24-h rhythms in LH, FSH, and testosterone concentration series. The group mean cosinor functions for each group are illustrated in Figure 2.

All study subjects had significant LH 24-h rhythms. As shown in Table 4, the acrophase of the LH 24-h rhythm remained constant around the onset of puberty. The amplitude of the LH 24-h rhythm was significantly lower in the prepubertal A group than in all other groups ($p < 0.01$). The amplitude in the early pubertal group was higher than those in the prepubertal A ($p < 0.01$) and prepubertal B groups ($p < 0.05$) but did not differ statistically from the prepubertal C values, perhaps because the number of subjects was too small to identify statistical differences between the groups. The amplitude in the prepubertal B group did not differ statistically from the values in the prepubertal C group.

All study subjects also had significant FSH 24-h rhythms. No differences in the amplitude and acrophase of the FSH 24-h rhythm were detected among four groups. The acrophase of FSH lagged behind that of LH by about 2.0–2.5 h.

With the exception of the prepubertal A group, all study subjects had significant testosterone 24-h rhythms. No significant differences in the acrophase of the testosterone 24-h rhythm were detected among the prepubertal B, prepubertal C, and early pubertal groups. The acrophase of testosterone lagged behind that of LH by about 1.5–3 h. The amplitude of the testosterone 24-h rhythm was statistically distinguishable among the three groups ($p < 0.05$) and increased with the development of sexual maturation.

The relative amplitude of 24-h rhythms was estimated as the percentage ratio of amplitude to mesor (Table 4). No differences in the relative amplitude of the LH 24-h rhythm were detected among the prepubertal A, prepubertal B, prepubertal C, and early pubertal groups. Similarly, no differences in the relative amplitude of FSH were detected among the four groups; however, the values for FSH were significantly lower than those for LH (paired t test, $p < 0.0001$). The relative amplitude of testosterone of the prepubertal B, prepubertal C, and early pubertal groups was 61%, 129%, and 118%, respectively, and the value of the prepubertal C group was significantly higher than that of the prepubertal B group ($p < 0.01$).

Cross-correlations between LH and testosterone and between LH and FSH. As shown in Figure 1, visual inspection suggested that plasma LH and testosterone concentration profiles fluctuated in an approximately parallel manner over 24 h. This observation was statistically evaluated by cross-correlation analysis. The raw data from 15 boys, who had significant circadian LH and testosterone rhythms, were subjected to cross-correlation analysis. As a result, significant positive cross-correlations between the LH and testosterone were found in all 15 boys. As summarized in Figure 3, the prepubertal B, prepubertal C, and early pubertal groups had significant median cross-correlations. The maximum cross-correlations in the prepubertal B, prepubertal C, and early pubertal groups were found at lags of 120, 60, and 60 min between LH and testosterone with the coefficients of 0.81, 0.88, and 0.84, respectively (Table 5). Moreover, significant negative cross-correlations were found in 14 of 15 boys when testosterone preceded LH. As shown in Figure 3, the prepubertal B, prepubertal C, and early pubertal groups had significant median negative cross-correlations when testosterone preceded LH by 400, 520, and 540 min, respectively. These observations imply that whenever plasma testosterone concentration increases, plasma LH concentration will decrease 400–540 min later and the decrease in plasma testosterone concentration will always be followed by an increase in plasma LH concentration 400–540 min later.

It is possible that two time series that are not related show high spurious cross-correlation if each time series is highly autocorrelated. Therefore, prewhitened data were subjected to cross-correlation analysis (see Materials and Methods). As shown in

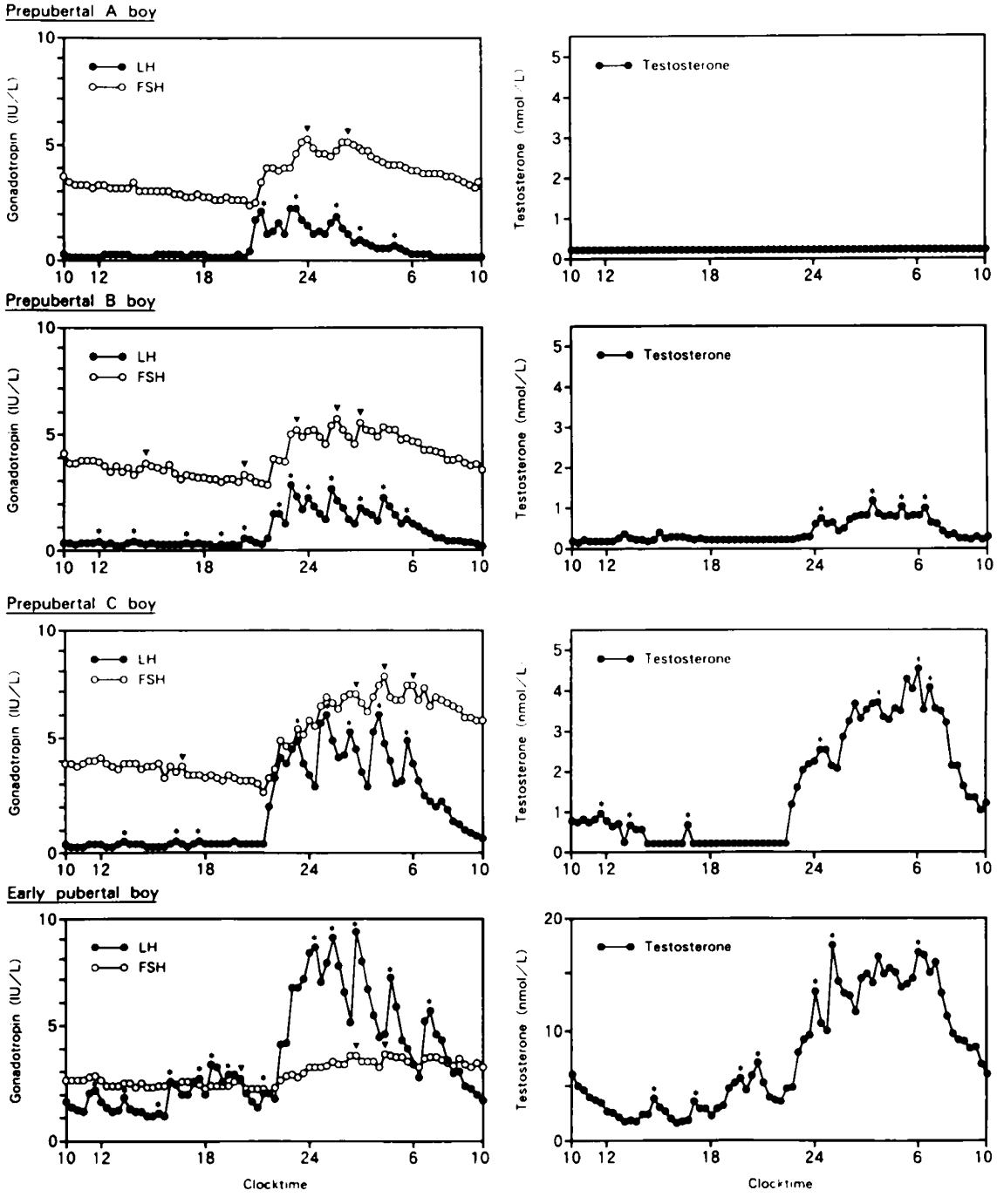

Fig. 1. Representative concentration profiles of LH, FSH, and testosterone from respective groups. Blood samples were drawn for LH, FSH, and testosterone measurement at 20-min intervals for 24 h starting at 1000 h. *Asterisks* (LH and testosterone) and *arrowheads* (FSH) indicate significant pulses detected by Cluster analysis (17). Note different scales on the vertical axes.

Figure 4, the prepubertal C and early pubertal groups had significant median positive cross-correlations between LH and testosterone time series with maximum correlation coefficients of 0.32 and 0.31 at 40- and 40-min lags, respectively. In contrast, the prepubertal B group had no significant median cross-correlations. None of the groups had significant negative cross-correlations (Fig. 4).

To search for possible relationships between LH and FSH concentrations, cross-correlation analysis was applied to the LH and FSH time series from 21 boys. By analyzing the raw data of LH and FSH time series, the significant median cross-correlations between LH and FSH were found in the prepubertal A, prepubertal B, prepubertal C, and early pubertal groups with maximum coefficients of 0.86, 0.82, 0.84, and 0.83 at lags of 20, 80, 80, and 80 min, respectively. Cross-correlation analysis on the prewhitened data demonstrated that all 21 boys had significant positive cross-correlations at 0-min lag or 20-min lag between LH and FSH. The prepubertal A, prepubertal B, prepubertal C, and early pubertal groups had significant median positive cross-correlations between LH and FSH time series with maximum correlation coefficients of 0.48, 0.58, 0.48, and 0.61 at 0-min lag, respectively.

DISCUSSION

It is well known that gonadotropin secretion is pulsatile and shows marked circadian rhythm because of nocturnal augmentation of gonadotropin secretion well before the onset of puberty

Table 2. *Twenty-four-h concentration profiles of LH, FSH, and testosterone from four study groups**

Study group	24-h mean concentrations		
	LH (IU/L)	FSH (IU/L)	Testosterone (nmol/L)
Prepubertal boys			
A	0.19 (0.09–0.41)	1.38[a] (0.72–2.63)	0.17[a] (0.17–0.17)
B	0.84[a] (0.53–1.33)	2.62[a,b] (1.19–5.78)	0.28[a] (0.21–0.39)
C	1.74[a,b] (1.31–2.31)	2.15[a,b] (1.22–3.79)	0.95[b] (0.46–1.97)
Early pubertal boys	3.09[b] (2.36–4.05)	4.25[b] (2.59–6.96)	4.92[c] (3.36–7.20)

* Values are means, with 95% confidence intervals in parentheses. Comparisons among groups were made using one-way analysis of variance, followed by multiple t tests with the Bonferroni adjustment. Any value followed by a superscript differs significantly ($p < 0.05$) from all other values in the same column not followed by the same superscript.

(1–11). Parker *et al.* (1) and Gordon *et al.* (14) have found an elevation in testosterone concentrations during nocturnal sleep in prepubertal boys. These earlier studies suggested that circadian rhythm might exist in testosterone secretion similarly as in the LH secretion before the onset of puberty. In these studies, however, the duration of blood sampling was relatively limited and mathematically established methods were not applied for analyzing the LH and testosterone time series. On the other hand, in the present study using mathematically established methods of analysis and longer duration of observation, we have investigated the detailed pattern of change in circulating gonadotropin and testosterone concentrations around the onset of puberty.

Regarding LH pulsatility evaluated by using Cluster analysis, the findings obtained in this study were in close agreement with those in previous studies (4, 8, 11). LH pulse amplitude tended to increase with advancing pubertal stage, whereas LH pulse frequency and interval showed little change. This implies that an alteration in circulating LH concentrations during puberty is through a change in LH pulse amplitude rather than pulse frequency (4, 8, 11). However, controversy about a change in LH pulse frequency during puberty exists (7, 10).

Pulse analysis of FSH concentration profiles has been difficult because of the slow metabolic clearance of FSH from the peripheral circulation and the relatively low amplitude of FSH pulses. Therefore, little is known about the pulse properties of FSH in prepubertal and early pubertal boys (7–10). With the ultrasensitive assay, we could detect nocturnal spontaneous FSH pulses in all subjects. Although the mean concentrations of FSH in the early pubertal group were significantly higher than those in the prepubertal A group, no differences in the pulse frequency, pulse interval, and pulse amplitude were detected among the four groups. These findings were in close agreement with those in previous studies (7, 8, 10) and suggest that basal secretion of FSH from the pituitary increases with the development of sexual maturation (7). Deconvolution analysis (22) may allow a more direct study of FSH secretory dynamics around the onset of puberty.

Veldhuis *et al.* (21) have assessed the testosterone concentration profile in normal adult men using Cluster analysis and identified significant pulsations in testosterone time series. They have reported that testosterone pulses occurred at mean (±SEM) pulse intervals of 112 ± 14 min with the mean amplitude of 8.4 ± 0.9 nmol/L. We also used Cluster analysis for evaluation of testosterone pulsatility in the prepubertal and early pubertal boys. As far as we know, the present study is the first one in which the testosterone concentration profiles in prepubertal boys were subjected to a mathematically based pulse detection method. We could identify significant pulsations in testosterone time series even in the prepubertal boys. The testosterone pulse amplitude significantly increased with advancing pubertal stage although the changes in the pulse frequency and interval were negligible. In comparison to the testosterone pulsatility in men reported by Veldhuis *et al.* (21), the pulse amplitude in the prepubertal boys was considerably low. In contrast, there seemed to be little difference in pulse interval between prepubertal boys reported here and adult men reported by Veldhuis *et al.* (21). Although we could not draw a conclusion from the present study because of the small numbers of subjects, it was considered possible that an alteration in circulating testosterone concentrations accompanying the sexual maturation might be caused by a change in pulse amplitude rather than frequency.

The cosinor analysis revealed that the FSH and testosterone concentration time series also showed significant circadian rhythm similar to that of LH well before the onset of puberty. Although the absolute amplitude value of the LH circadian rhythm increased about 3.1-fold from the prepubertal B group to the early pubertal group, the degree of increase in testosterone was 37-fold, indicating that the change in secretory dynamics accompanying the onset of puberty was greater in testosterone than in LH. Moreover, the mean 24-h concentrations (Table 2), the pulse amplitude (Table 3), and the amplitude of the 24-h rhythm (Table 4) of testosterone in the prepubertal C group differed statistically from the values in the prepubertal B group, although the values of LH in the prepubertal C group were

Table 3. *Pulse properties of nocturnal 8-h concentration profiles of LH, FSH, and testosterone from four study groups**

Study group	LH			FSH			Testosterone		
	Pulse frequency (per 8 h)	Pulse interval (min)	Pulse amplitude (IU/L)	Pulse frequency (per 8 h)	Pulse interval (min)	Pulse amplitude (IU/L)	Pulse frequency (per 8 h)	Pulse interval (min)	Pulse amplitude (nmol/L)
Prepubertal boys									
A	3.3 (2.0–4.6)	129 (100–158)	0.29[a] (0.16–0.54)	2.8 (1.7–4.0)	150 (105–195)	0.73 (0.32–1.66)			
B	4.4 (2.5–6.3)	102 (63–141)	1.46 (0.81–2.63)	2.6 (2.0–3.2)	140 (106–174)	1.11 (0.58–2.12)	2.0 (0.6–3.4)	140 (86–194)	0.34 (0.21–0.55)
C	5.6 (4.3–6.9)	93 (70–115)	1.87 (1.34–2.60)	2.6 (0.9–4.3)	115 (71–159)	1.03 (0.39–2.73)	2.2 (1.0–3.4)	127 (40–213)	1.77[a] (0.80–3.91)
Early pubertal boys	4.4 (3.8–5.0)	87 (73–100)	3.27 (2.13–5.00)	2.0 (0.6–3.4)	131 (75–187)	1.08 (0.40–2.89)	3.2 (2.7–3.7)	135 (71–198)	4.98[b] (3.15–7.86)

* All LH, FSH, and testosterone pulse properties were evaluated using Cluster analysis (17). The prepubertal A group had no detectable testosterone pulsations. Values are means, with 95% confidence intervals in parentheses. Comparisons among groups were made using one-way analysis of variance, followed by multiple t tests with the Bonferroni adjustment. Any value followed by a superscript differs significantly ($p < 0.05$) from all other values in the same column not followed by the same superscript.

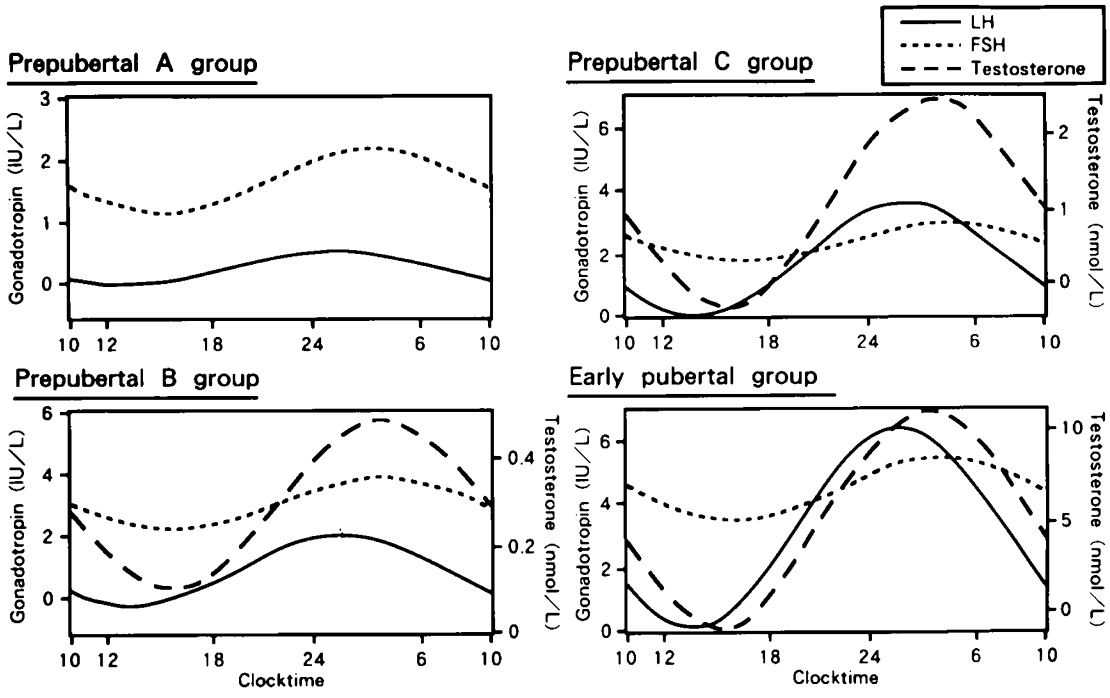

Fig. 2. The 24-h rhythm in LH, FSH, and testosterone concentration profiles. Each panel denotes the individual group mean cosinor functions for LH, FSH, and testosterone concentration time series. All study groups had significant circadian LH and FSH rhythms. With the exception of the prepubertal A group, all study groups had significant circadian testosterone rhythms. Note different scales on the vertical axes.

Table 4. *Properties of 24-h rhythms in LH, FSH, and testosterone concentration profiles from four study groups**

	LH			FSH			Testosterone		
Study group	Amplitude (IU/L)	Acrophase (h; clock time)	Relative amplitude (%)	Amplitude (IU/L)	Acrophase (h; clock time)	Relative amplitude (%)	Amplitude (nmol/L)	Acrophase (h; clock time)	Relative amplitude (%)
Prepubertal boys									
A	0.20	0117	105	0.42	0323	31			
	(0.09–0.47)	(0032–0201)	(93–117)	(0.22–0.83)	(0250–0356)	(27–35)			
B	0.99[a]	0132	117	0.71	0356	28	0.14	0428	61[a]
	(0.61–1.61)	(0048–0216)	(110–124)	(0.38–1.35)	(0308–0444)	(19–38)	(0.05–0.41)	(0220–0636)	(24–99)
C	1.70[a,b]	0152	100	0.41	0404	22	1.15[a]	0328	129[b]
	(1.10–2.62)	(0112–0232)	(75–125)	(0.14–1.23)	(0231–0537)	(8–35)	(0.49–2.70)	(0236–0420)	(107–151)
Early pubertal boys	3.07[b]	0128	99	0.92	0352	22	5.20[b]	0308	118[a,b]
	(2.89–4.12)	(0048–0208)	(87–112)	(0.53–1.59)	(0249–0445)	(18–25)	(2.57–10.54)	(0222–0354)	(68–167)

* Values are means, with 95% confidence intervals in parentheses. The prepubertal A group did not show any significant 24-h rhythm in testosterone concentration profiles. Relative amplitude was estimated as the percentage ratio of amplitude to mesor of 24-h rhythms. Comparisons among groups were made using one-way analysis of variance, followed by multiple t tests with the Bonferroni adjustment. Because the data points were all equidistant (20 min), the mesors were identical to the 24-h mean concentrations in Table 2 and therefore are not shown here. Any value followed by a superscript differs significantly ($p < 0.05$) from all other values in the same column not followed by the same superscript.

indistinguishable from those in the prepubertal B group. Therefore, it was difficult to explain the change in testosterone concentrations around the onset of puberty in terms of the change in LH concentration alone; sensitivity of the testis to the LH stimulation might change around the onset of puberty.

For the FSH 24-h rhythm, the relative amplitude of FSH 24-h rhythm was lower than that of LH 24-h rhythm. This might be due in large part to the long half-life of FSH, which could mask the 24-h rhythm of FSH (10).

The temporal relationship between the LH and testosterone time series was evaluated by cross-correlation analysis. By analyzing the raw data of LH and testosterone, the maximum cross-correlations between LH and testosterone were found in the prepubertal B, prepubertal C, and early pubertal groups at lags of 120, 60, and 60 min, respectively. This finding agreed with the result that Veldhuis *et al.* (21) obtained by analyzing the unmodified LH and testosterone profiles in men using the same method. Furthermore, we observed significant negative cross-correlations in the prepubertal B, prepubertal C, and early pubertal groups at the points when testosterone preceded LH by 400–540 min. This might suggest the presence of a negative feedback system for LH by testosterone in the boys around the onset of puberty; establishment of circadian rhythm for LH could be explained by this negative feedback system. However, the cross-correlation analysis that we used in the present study is essentially a method that allows analysis of information contained in the lag cross-correlations as opposed to the lead cross-correlations. Therefore, although the result of analysis suggests the presence of causal feedback, it is necessary to construct a more complicated causal feedback model to prove this point (19).

When the LH, testosterone, or both time series are autocorrelated, the cross-correlation function between the raw data of the two time series can be difficult to interpret and sometimes can be misleading. Therefore, we constructed appropriate models from the LH and testosterone time series and applied cross-correlation analysis to the resulting residual (prewhitened data), which was defined as the difference between the observed and

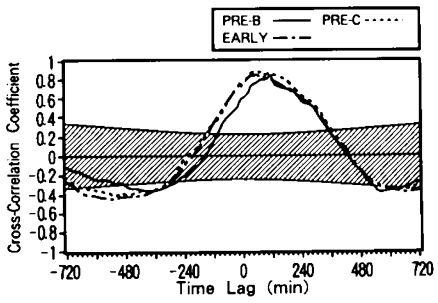

Fig. 3. Median cross-correlation coefficients between LH and testosterone time series in four study groups. The raw data of LH and testosterone time series were subjected to cross-correlation analysis. The 95% confidence limit for the null hypothesis, which assumes the absence of a significant correlation, is denoted by the *shaded region*. Therefore, cross-correlation values above the shaded region denote significant positive cross-correlation coefficients.

Table 5. *Time lags at which maximum uncorrected cross-correlations occur between LH and testosterone in prepubertal and early pubertal boys*

Study group	Lag (latency in min)	Cross-correlation coefficient*
Prepubertal boys		
B	120	0.81 (0.18–0.82)
C	60	0.88 (0.80–0.91)
Early pubertal boys	60	0.84 (0.81–0.91)

* Values are medians, with ranges in parentheses.

the fitted values (19). The result of the analysis differed slightly from that of cross-correlation analysis on the raw data. No significant positive cross-correlation was observed in the prepubertal B group, whereas significant positive cross-correlations were detected in the prepubertal C and early pubertal groups at 40-min lags between LH and testosterone. This time lag was considered to correspond to the time required for the synthesis and secretion of testosterone after binding of LH to the Leydig cell receptors. Veldhuis *et al.* (21) used stepwise autoregressive modeling to eliminate intrinsic autocorrelations within the LH and testosterone time series and detected significant positive cross-correlations at 10- to 20-min lags between LH and testosterone in men. The delays between the LH and testosterone time series in men in their report were shorter than those in the boys around the onset of puberty in our report. The difference in the time lag may arise from the following points: *1*) Depending on the degree of maturation of the testis, the time for synthesizing and secreting testosterone after binding of LH to the Leydig cell receptors is longer in the sexually less mature individuals; *2*) the method for eliminating intrinsic autocorrelations within the LH and testosterone time series differed between our present study and that of Veldhuis *et al.*; and *3*) difference in sampling frequency between two studies caused difference in time lag. To solve these problems, a study in which subjects at various stages from prepuberty to adulthood are analyzed at the same sampling frequency and by the same method must be conducted in the future.

Cross-correlation analysis on the prewhitened data of LH and FSH time series demonstrated that all four groups had significant median cross-correlations between LH and FSH at 0-min lag. This finding indicates that LH and FSH are released from gonadotrophs synchronously by a single releasing factor. However, there were differences in the acrophase of 24-h rhythm between LH and FSH. This might reflect the differences in the half-life or in the sensitivity of hormone secretion to the stimulation of gonadotropin-releasing hormone between LH and FSH, but another FSH-releasing factor might modulate FSH secretion.

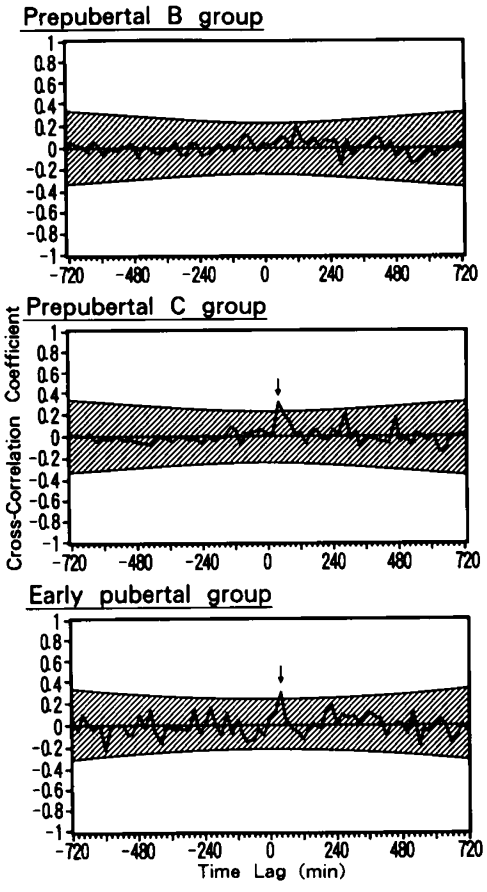

Fig. 4. Median cross-correlation coefficients between LH and testosterone time series in four study groups. The prewhitened data of LH and testosterone time series were subjected to cross-correlation analysis (see text for details). The 95% confidence limit for the null hypothesis, which assumes the absence of a significant correlation, is denoted by the *shaded region*. *Arrows* indicate significant positive cross-correlations.

We conclude that FSH and testosterone concentration profiles are pulsatile and show significant circadian rhythm well before the onset of puberty. LH and testosterone time series are significantly coupled when testosterone lags LH by about 40 min, at least within the restraints of our 20-min sampling intervals. This time lag might correspond to the time for synthesizing and secreting testosterone after binding of LH to the Leydig cell receptors, and the length of this time lag might reflect the degree of maturity of the testis. The findings obtained in the present study give important suggestions for understanding the process of sexual maturation in the hypothalamic-pituitary-gonadal axis. In addition, these findings can provide a model for improving physiologic replacement therapy of testosterone for patients with hypogonadism.

It is not known whether the findings obtained from normal short boys in the present study have general applicability to boys of normal stature. As sexual maturation might be delayed in normal short boys, the nocturnal testosterone concentrations in boys of normal stature might start to increase at an earlier stage of prepuberty than in normal short boys and the time lag between the LH and testosterone time series might be of shorter duration. To clarify these points, additional studies in boys of normal stature should be done in the future.

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