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Basal and Gonadotropin-Releasing Hormone-Releasable Serum Follicle-Stimulating Hormone Charge Isoform Distribution and In Vitro Biological-to-Immunological Ratio in Male Puberty

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Follicle-stimulating hormone is synthesized and secreted as a mixture of heterogeneous isoforms that differ from each other in carbohydrate structure, biological potency, and plasma half-life. The relative abundance of the FSH isoforms will depend on the endocrine status of the donor at the time of sample collection. In the present study, we attempted to define the impact of the changing endocrine milieu characteristic of male puberty on the charge heterogeneity and plasma half-life of the serum FSH isoforms released under endogenous and exogenous GnRH drives, and examined whether such a varying hormone milieu modifies the capability of the circulating hormone to trigger intracellular signal transduction at the human FSH receptor level. Forty healthy male subjects at Tanner stages (Ts) 1 to 5 were sampled at 10 min intervals for 10 h. Serum from successive samples collected across 2–4 h intervals containing FSH released under basal, low-dose (10 µg), and high-dose (90 µg) exogenous GnRH-stimulated conditions was subjected to preparative chromatofocusing and tested for bioactivity employing a homologous cell in vitro bioassay system. Deconvolution analysis was applied to estimate the apparent endogenous FSH plasma half-life in samples obtained after administration of low-dose exogenous GnRH. Under all conditions studied, serum FSH charge isoforms were distributed along a pH range of 7.0 to less than 3.0. Comparisons across the different Tanner stages revealed a significant and selective increase in the ratio of FSH isoforms with elution pH values <4.50 relative to those with values ≥4.50 at Ts-2. At Ts-3, this ratio returned to that present at Ts-1, to decline thereafter during the ensuing pubertal stages. Serum bioactive FSH concentrations progressively increased (from 3.72 ± 1.3 to 16.2 ± 5.3 IU/L) throughout puberty, and in all conditions bioactive FSH concentrations exceeded those detected by a specific radioimmunoassay. The biological to immunological (B:I) FSH ratio at baseline was significantly ($p < 0.05$) lower at Ts-1 and Ts-2 (1.33 ± 0.30 and 1.62 ± 0.34, respectively) than at more advanced stages of pubertal development (2.28 ± 0.20, 2.96 ± 0.38, and 2.77 ± 0.63 at Ts-3-, 4-, and -5, respectively) Similar differences were detected in samples containing FSH molecules released after low- and high-dose GnRH administration. The apparent endogenous FSH half-life of the deconvolved GnRH-induced FSH pulses was similar in the five study groups. These results demonstrate that the transition from infancy to sexual maturity in men is accompanied by qualitative changes in the circulating FSH isoform mixture. Although the changes in FSH glycosylation occurring throughout puberty are not of sufficient magnitude to alter the survival of the gonadotropin in the circulation, they allow preferential secretion of bioactive FSH. The enrichment of the circulating mix of FSH isoforms with highly bioactive variants throughout spontaneous puberty may potentially favor the development of spermatogenesis and acquisition of reproductive competence.

Key Words: Follicle-stimulating hormone; gonadotropin-releasing hormone; gonadotropins; puberty; isoforms; bioactive FSH.

Introduction

Follicle-stimulating hormone (FSH) is one of the master anterior pituitary hormones that regulate gonadal function. As with other members of the glycoprotein family of hormones, both α and β subunits of FSH contain various asparagine-linked oligosaccharide structures that play a key role

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in determining several functional properties of the hormone, including subunit assembly, intracellular sorting, metabolic clearance, and regulation of potency *(1,2)*. Multiple, post-translationally determined glycosylation variants of FSH have been isolated from anterior pituitary extracts and serum of several animal species, including humans *(3,4)*. In human FSH, nearly 95% is variably acidic owing to the variable proportion of negatively charged, terminally positioned sialic acid residues *(1,5)*, which allows one to separate its different isoforms by charge-based procedures *(3)*. Follicle-stimulating isoforms conform a dynamic population of molecules that differ from each other not only in charge, but also in metabolic clearance rate, receptor binding, and in vitro biological activity *(1,3,4)*. Although the regulation of glycoprotein hormone glycosylation is a complex process, evidence derived from studies in experimental animals and in humans indicates that changes in sex steroid hormone concentrations exposure play an important role in determining the relative abundance of gonadotropin isoforms within the pituitary *(1,3,6,7)*.

The onset of puberty in boys is associated with striking physical and behavioral changes. During this physiological process, the circulating concentrations of gonadotropins and gonadal hormones and the pituitary sensitivity to GnRH increase progressively *(8–11)*. A number of studies indicate that in addition to these physiological changes, puberty is also associated with biochemical changes in the type of gonadotropins synthesized by the anterior pituitary gland, which may be relevant to initiate and/or mantain the multitude of gonadal functions mediated by both hormones during this maturational process *(3,6,12,13)*. For example, in male rats and in humans, significant qualitative changes of intrapituitary FSH have been detected during sexual development *(14,15)*; in these studies, a shift to more acidic FSH isoforms was identified. Nevertheless, despite evidence suggesting that the endocrine milieu regulates the proportion of the FSH isoforms within the pituitary *(3,4,14,16)*, limited information is available regarding the regulation of FSH heterogeneity in the circulation, particularly during male puberty, which is more relevant to better define the potential impact of such changes in the molecular structure of the gonadotropin on testicular function. Studies in humans attempting to document the occurrence of qualitative changes in serum FSH isoform distribution during male puberty have yielded some conflicting results. Phillips et al. *(12)* found a significant shift to more acidic FSH isoforms in boys at puberal stage II, without further changes later in puberty, whereas other studies failed to document qualitative changes in circulating FSH**,** as inferred by examining the bioactive to immunoreactive (B:I) ratio of this gonadotropin *(17,18)*.

In the present study, we attempted to define the impact of the changing endocrine milieu characteristic of male puberty on the charge heterogeneity and plasma half-life of the circulating mixture of FSH isoforms synthesized and secreted by the pituitary gland, and examined whether such a varying hormone milieu may modify the capability of the circulating hormone to trigger intracellular signal transduction at the human FSH receptor level. To accomplish this purpose, normal prepubertal, pubertal, and adult male volunteers were blood sampled at frequent intervals before and after administration of GnRH, and the changes in pH distribution profile and biopotency of FSH released under these conditions were then analyzed by preparative chromatofocusing and by a homologous cell in vitro bioassay system. As in previous studies *(19,20)*, we reasoned that consecutive administration of a low (10 μg) and a high (90 μg) GnRH dose would facilitate the identification and analysis of potency of the structurally heterogeneous population of molecules discharged from an intracellular readily releasable pool as well as those forms of this gonadotropin that may be newly synthesized under the influence of the particular endocrine milieu prevailing at each given stage of puberty *(21,22)*. Finally, the impact of the qualitative changes in FSH released after low-dose GnRH administration on the plasma half-life of the hormone was determined employing multiparameter deconvolution analysis.

Results

Baseline Serum Gonadotropin, Testosterone (T), and Estradiol (E_2) Levels, and Secretory FSH Response to Exogenous GnRH

As Table 1 shows, baseline serum gonadotropin levels progresively increased throughout the different stages of puberty. The first significant increase in serum FSH concentrations was observed early in puberty; in Ts-2 subjects, the immunoactive levels of this gonadotropin doubled those exhibited by prepubertal children. Serum T and E_2 concentrations also increased in a progressive manner throughout male puberty, attaining their maximal levels during the Ts-4 of pubertal development (Table 1).

In all subjects, a significant rise in serum FSH levels was observed after the administration of both the low and high GnRH doses (Fig. 1). Maximal FSH concentrations occurred 20–140 min (median, 40 min in Ts-1, -3 and -4; 30 min in Ts-2 and -5) after the low GnRH pulse and between 20 and 120 min (median 60 min in Ts-1 and Ts-2; 70 min in Ts-3; and 30 and 40 min in Ts-4 and -5, respectively) after the high GnRH dose. The magnitude of the FSH response in terms of area under the FSH curve (*a*FSH*c*) of the GnRH-induced FSH bursts progressively increased as puberty advanced, being higher in Ts-4 and Ts-5 subjects than in Ts-1 and Ts-3 boys. At Ts-2, however, the *a*FSH*c* after the high GnRH dose was similar to that observed in more advanced stages of puberty (Table 2). Although administration of the 90 μg GnRH dose elicited higher FSH responses than the lower dose, the differences between the two responses did not reach statistical significance.

Table 1
Mean ± SEM of Serum E_2, T, LH,
and FSH Concentrations in Subjects at Tanner Stages 1 to 5

	Baseline serum hormone concentrations			
Tanner stage	Estradiol (pmol/L)	Testosterone (nmol/L)	LH (IU/L)	FSH (IFMA) (IU/L)
1	80.9 ± 9.9[a]	1.5 ± 0.2[a]	1.2 ± 0.4[a]	4.52 ± 0.6[a]
2	96.7 ± 11.5[a]	8.6 ± 1.8[b,c]	2.2 ± 0.4[b,c]	8.14 ± 1.39[b,c]
3	116.7 ± 24.6[a,b]	8.5 ± 2.2[b,c]	2.3 ± 0.5[b,c]	6.35 ± 1.0[a,b]
4	141.7 ± 13.0[b]	19.5 ± 1.6[d]	3.7 ± 0.7[b,d]	10.16 ± 1.1[c]
5	159.5 ± 15.4[b]	22.7 ± 1.8[d]	5.6 ± 0.9[d]	9.91 ± 1.49[c]

Means identified by different superscript letters in the same column are significantly ($p < 0.05$) different.

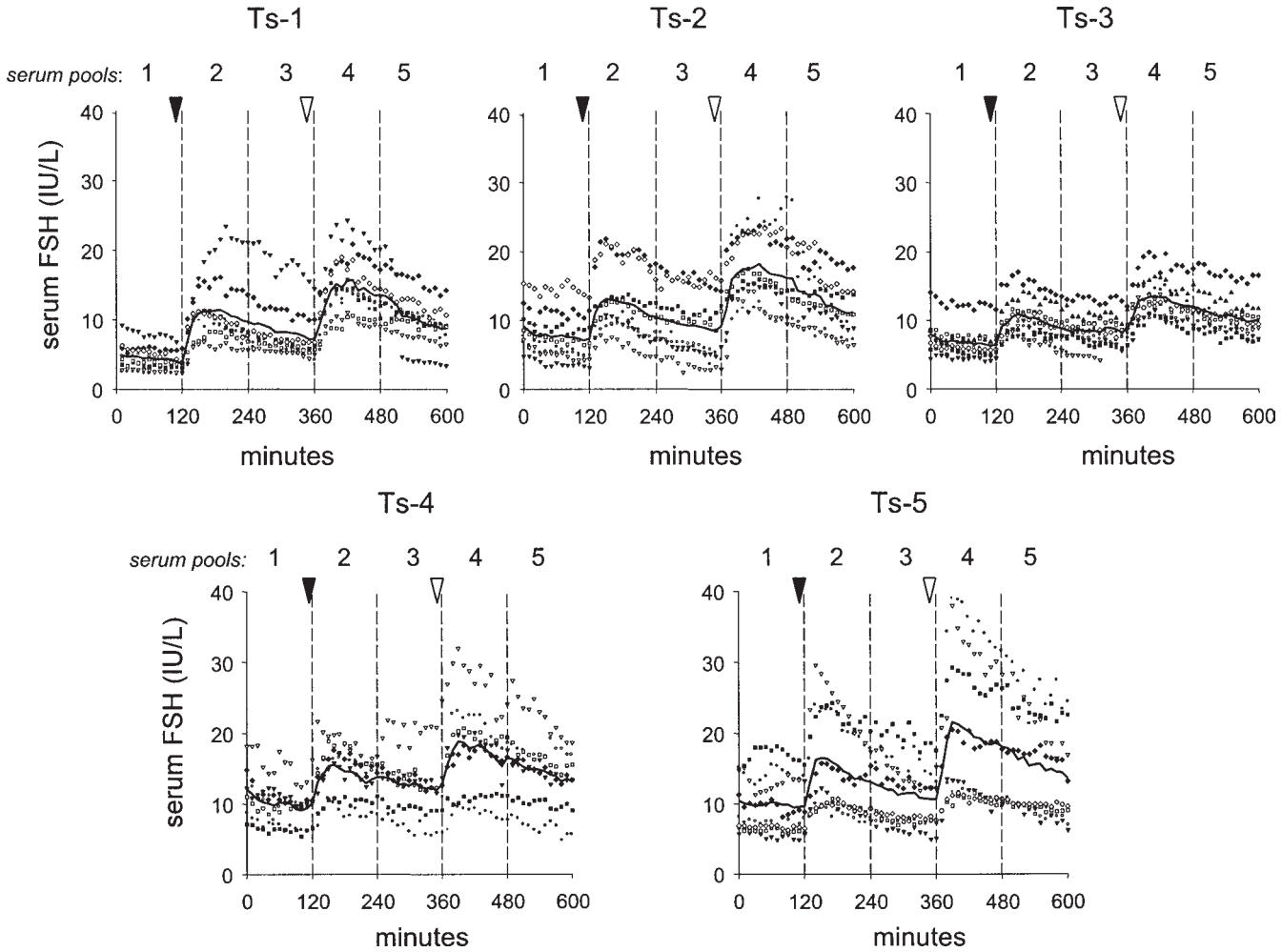

Fig. 1. Serum FSH concentration responses to 10 μg (*black arrowheads in each graph*) and 90 μg (*white arrowhead*) iv injections of exogenous GnRH during stages 1 to 5 of pubertal development. Each symbol represents an individual subject. The continuous lines represent the group means. Aliquots of samples from each 120-min study period (1 to 5, delineated by the vertical broken lines) were pooled and analyzed for FSH charge isoform distribution and in vitro biological activity as described in Material and Methods.

Table 2
Areas Under the FSH Curve (*a*FSH*c*) and Maximal FSH Increase (ΔFSH) after Low- and High-Dose GnRH Administration in Subjects at Ts-1 to -5[†]

	Study condition				
	Basal	GnRH 10 μg		GnRH 90 μg	
Tanner stage	*a*FSH*c* (IU/L/2h)	*a*FSH*c* (IU/L/4h)	ΔFSH (IU/L)	*a*FSH*c* (IU/L/4h)	ΔFSH (IU/L)
1	543 ± 75[a]	1041 ± 117[a]	7.8 ± 3.9	1345 ± 105[a]	9.1 ± 2.4
2	928 ± 151[b]	1174 ± 135[a]	5.9 ± 2.4	1628 ± 132[b]	10.3 ± 5.3[*]
3	818 ± 91[b]	1013 ± 63[a]	4.6 ± 1.2	1274 ± 72[a]	5.8 ± 1.1[*]
4	1211 ± 137[c]	1486 ± 113[b]	5.9 ± 1.8	1757 ± 167[b]	7.4 ± 3.4
5	1188 ± 180[c]	1445 ± 158[b]	7.2 ± 4.4	1920 ± 238[b]	11.3 ± 8.9

Means identified by different superscript letters in the same column are significantly ($p < 0.05$) different.

*$p < 0.05$ vs GnRH 10 μg.

[†]Values are the means ± SEM.

Serum FSH Isoform Distribution

Analysis of serum specimens by preparative chromatofocusing disclosed the presence of immunoactive FSH within a pH range of 7.0 to 3.0, as well as in those fractions recovered after the addition of 1.0 mol/L NaCl to the chromatofocusing columns (pH <3.0, salt peak) (Fig. 2). Although the percent recovery in each pH boundary varied according to the stage of puberty, the majority (>80%) of FSH immunoactivity was detected in fractions with elution pH values <4.50. To evaluate statistical differences in the patterns of serum FSH isoform distribution among the five pubertal stages, each chromatofocusing pattern was divided into two pH regions, considering 4.50 as the demarcating pH value. This value was based on previous findings that pituitary FSH isoforms with pH values below 4.5 exhibit longer plasma half-lives and lower in vitro biological potencies than their less acidic analogs as disclosed by heterologous cell bioassay systems *(23)*. Changes in FSH isoform distribution were then expressed as the ratio of the amounts of FSH isoforms with elution pH values below 4.50 relative to those at or above this pH value. Comparisons across the different Tanner stages revealed a significant ($p < 0.05$) and selective increase in this ratio [resulting from an increase in the relative abundance of more acidic isoforms (pH <4.50)] at the Ts-2 of puberty during all study conditions (i.e., basal, low-dose, and high-dose GnRH administration) (Fig. 3). At Ts-3, this ratio returned to that present at Ts-1, to decline thereafter during the ensuing pubertal stages (Fig. 3). In Ts-1 children and Ts-2 and Ts-3 boys, a significant increase in less acidic (pH ≥ 4.50) isoforms was observed after administration of both GnRH doses. Interestingly, in sexually mature individuals (i.e., Ts-4 and -5 subjects), acute administration of GnRH at low or high doses was unable to modify the distribution profile of serum FSH isoforms present at baseline.

In Vitro FSH Bioactivity

Five serum FSH pools from each subject (pools 1–5 in Fig. 1) were tested at three or four dose levels for FSH bioactivity employing a homologous cell in vitro bioassay system. Incubation of HEK-293 cells expressing the recombinant human FSH receptor with increasing amounts of LER-907 or the serum pools from each set of samples induced significant and parallel dose-dependent cAMP accumulation (Fig. 4). Serum bioactive FSH concentrations (pooled over the 2-h baseline interval) averaged 3.72 ± 1.3 (Ts-1), 6.2 ± 3.6 (Ts-2), 9.0 ± 2.5 (Ts-3), 18.6 ± 9.0 (Ts-4), and 16.2 ± 5.3 (Ts-5) IU/L. Bioactive FSH concentrations were higher after GnRH than at baseline in all study groups (bioactive FSH in serum pool 2 = 9.8 ± 3.5, 10.3 ± 6.3, 17.0 ± 4.5; 24.2 ± 13.0, and 20.8 ± 6.5 IU/L and serum pool 4 = 20.8 ± 6.5, 14.2 ± 7.3, 17.7 ± 4.8, 30.9 ± 16.1, and 25.0 ± 7.2 IU/L, Ts-1 to -5, respectively), and in all conditions bioactive FSH concentrations exceeded those detected by the RIA. Comparisons among the resulting B:I FSH ratios across puberty, revealed that the basal FSH ratio was significantly ($p < 0.05$) lower at Ts-1 than at more advanced stages of pubertal development (Table 3). Samples from boys in Ts-2 also exhibited lower B:I FSH ratios than subjects in Ts-3, -4, and -5, whereas the ratios among these latter groups did not differ from each other. Similar differences were detected in serum pools 2 and 5 (i.e., in samples containing FSH molecules released immediately after low-dose GnRH administration, as well as those that remained in circulation 2 h after high-dose GnRH injection), and when the complete exogenous GnRH-provoked FSH pulses (i.e., pools 2 plus 3 and 4 plus 5) were jointly considered (Table 3). In all pubertal stages, the B:I FSH ratios at baseline (determined in pool 1) were similar to those exhibited by the pools of samples obtained after the low- and high-dose GnRH challenges, with few exceptions: pool 4 (collected from samples

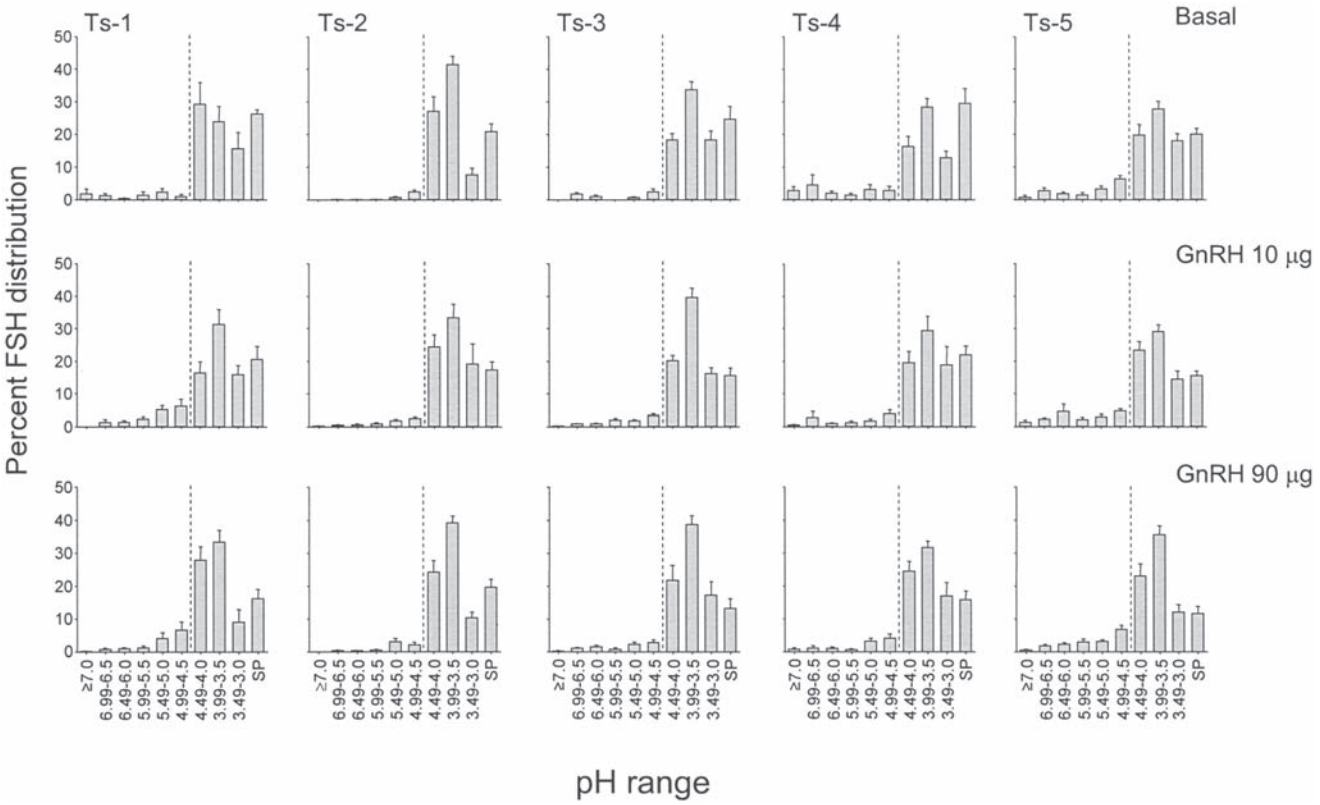

Fig. 2. Mean distribution pH profiles of serum FSH from healthy subjects at Tanner stages 1 to 5 of puberal development. After fractionation of serum pools 1, 2-3, and 4-5 (see Fig. 1) by chromatofocusing, the FSH content of each fraction was measured by RIA to determine the percentages eluting in various fractions. **Upper panels:** baseline periods; **middle panels:** low-dose GnRH-stimulated study periods; **lower panels:** high-dose GnRH-stimulated periods. Vertical broken lines separate isoforms with pH values <4.5 from those with pH ≥ 4.5. SP: salt peak.

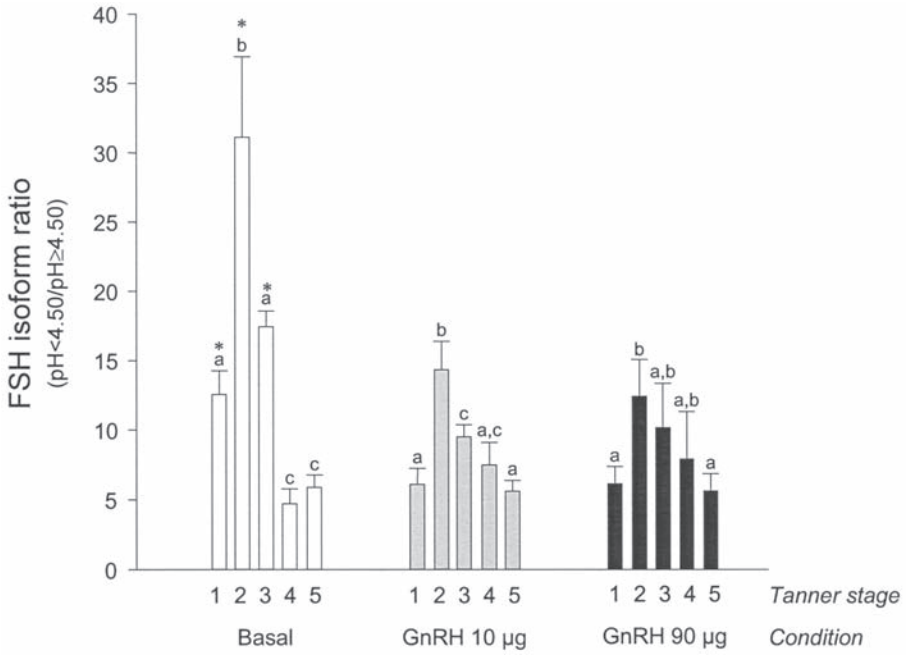

Fig. 3. The ratio (mean ± SEM) of serum FSH isoforms with pH values <4.50 relative to those with pH values ≥ 4.5. Different letters above bars indicate the existence of significant ($p < 0.05$) differences within each study condition. *$p < 0.05$ vs GnRH 10 µg and GnRH 90 µg.

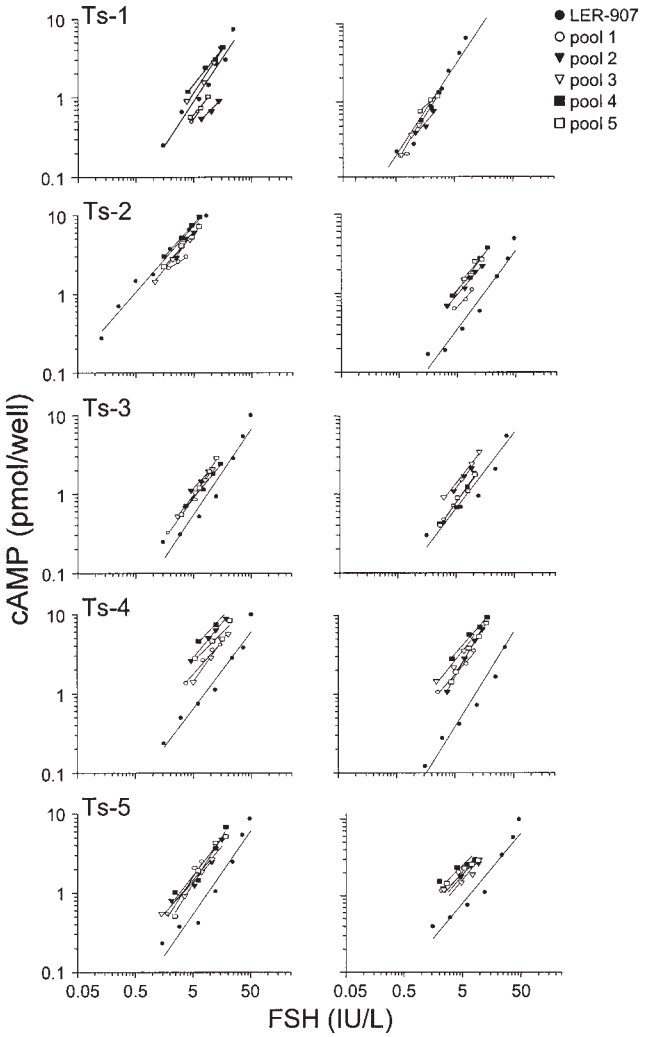

Fig. 4. Impact of increasing concentrations of FSH in serum pools 1 to 5 (symbols) from two subjets at each Tanner stage to elicit cAMP production by HEK-293 cells transfected with the full-length recombinant human FSH receptor. The dose is expressed in terms of the LER-907 standard as measured by RIA of each serum pool (25–100 μL).

obtained 10–120 min after high-dose GnRH administration) from Ts-1 children and pools 2 and 3 (collected from samples obtained 10–120 and 130–240 min after the low-dose GnRH challenge) from Ts-3 boys.

Apparent Plasma Half-Life of FSH Released in Response to Low-Dose GnRH Administration

Deconvolution analysis was employed to analyze whether the changes in FSH isoform distribution occurring throughout pubertal development were of sufficient magnitude to modify the half-life of the circulating gonadotropin released in response to a submaximally effective dose of exogenous GnRH. Although the apparent endogenous FSH half-life of the deconvolved GnRH-induced FSH pulse tended to increase during the more advanced stages of pubertal development, the values were not significantly different from those present in prepubertal or early pubertal children (Ts-1, 202 ± 30; Ts-2, 179 ± 46; Ts-3, 350 ± 74; Ts-4, 342 ± 75; Ts-5, 375 ± 91 min).

Discussion

Based on frequent blood sampling and chromatofocusing of the resultant serum pools from male individuals at different stages of pubertal development, we here demonstrate that puberty is accompanied by modest, but otherwise significant, changes in the pH distribution of circulating FSH isoforms. Two distinctly different shifts in pH distribution of serum FSH isoforms were evident across puberty; the first occurred during early puberty (i.e., at Ts-2), a time at which a significant increase in more acidic (pH < 4.5) isoforms was detected, whereas a second shift toward less acidic (pH ≥ 4.5) variants was found during late puberty. The finding of changes in charge distribution of serum FSH isoforms during male sexual maturation is in agreement with a previous study showing a similar shift to more acidic/sialylated variants during early stages of puberty *(12)*; however, in this latter study the median charge of FSH circulating at Ts-5 returned to values similar to those present at mid-puberty (Ts-3), and serum FSH isoforms were invariably more basic at Ts-1 than at later stages of pubertal development. Differences in the charged-based procedures employed to separate the various FSH isoforms may account for such apparent discrepancies *(3)*.

Employing a homologous cell in vitro bioassay system and a polyclonal antibody-based immunoassay to monitor changes in the B:I activity ratio of circulating FSH during pubertal development, we found that bioactive FSH and the B:I ratio of the gonadotropin molecules released either under the endogenous GnRH drive (i.e., in baseline conditions) or after pituitary exposure to submaximal and supraphysiological doses of GnRH progressively increased throughout pubertal development. This suggests that the changing hormonal milieu characteristic of this physiological process may favor the ability of the FSH molecule to trigger a biological response at the target cell level. The finding that the B:I FSH ratio in pooled samples containing high concentrations of the gonadotropin (as those collected after stimulation with exogenous GnRH) was similar to that detected in basal conditions does not support the possibility that differences in immuno- and/or bioassay sensitivities may underlie the changes in B:I ratios detected across puberty *(24–26)*. Although the results from several studies suggest that differences in glycosylation may affect the reactivity of glycoproteins toward certain antibodies (and, consequently, alter the B:I ratio of the hormone) *(27–30)*, we and others have found that, for the naturally occurring FSH variants, the potential discrepancies among different quantitative immunoassays (polyclonal, monoclonal, and polyclonal/monoclonal) do not impact significantly on the in vitro B:I ratio of the human FSH glycoforms *(31,32)*.

Table 3
Biological to Immunological FSH Ratio in the Various Serum Pools (See Fig. 1) from Subjects at Ts-1 to -5[†]

	Basal	GnRH 10 μg			GnRH 90 μg		
Tanner stage	Pool 1	Pool 2	Pool 3	Pools 2+3	Pool 4	Pool 5	Pools 4+5
1	1.33 ± 0.30^{a}	1.49 ± 0.34^{a}	1.76 ± 0.43^{a}	1.62 ± 0.27^{a}	$2.12 \pm 0.32^{a*}$	$1.35 \pm 0.29^{a**}$	1.73 ± 0.23^{a}
2	$1.62 \pm 0.34^{a,b}$	1.73 ± 0.44^{b}	2.27 ± 0.85^{a}	2.00 ± 0.47^{a}	2.76 ± 0.94^{a}	1.66 ± 0.45^{b}	2.21 ± 0.52^{a}
3	$2.28 \pm 0.20^{b,c}$	3.01 ± 0.11^{b}	3.11 ± 0.37^{a}	$3.06 \pm 0.19^{b*}$	2.82 ± 0.42^{a}	2.73 ± 0.35^{c}	2.78 ± 0.27^{b}
4	2.96 ± 0.38^{c}	3.05 ± 0.41^{b}	3.16 ± 0.49^{a}	3.10 ± 0.31^{b}	3.56 ± 0.60^{a}	3.06 ± 0.49^{c}	3.33 ± 0.37^{b}
5	2.77 ± 0.63^{c}	2.61 ± 0.51^{b}	2.72 ± 0.56^{a}	2.76 ± 0.37^{b}	2.79 ± 0.51^{a}	2.65 ± 0.46^{b}	2.81 ± 0.33^{b}

Means identified by different superscript letters in the same column are significantly ($p < 0.05$) different.
*$p < 0.05$ vs serum pool 1.
**$p < 0.05$ vs serum pool 4.
[†]Values are the means ± SEM.

The pubertal changes in serum FSH B:I activity ratio detected herein contrast with previous studies *(17,18)* in which immunoactive FSH levels progressively increased with pubertal development in boys, whereas bioactive FSH concentrations remained constant despite significant increases in sex steroid concentrations. Although in these studies the failure to detect changes in the FSH B:I ratio during puberty may be related to the use of an heterologous cell system that employs a different end point to assess the in vitro response *(33)*, we have found that excluding the more basic FSH variant [which behaves as an antagonist of FSH action in heterologous but not in homologous cell assay systems *(34,35)*, and that was virtually absent in the serum specimens analyzed in the present study], there is a good correlation between the estrogen and cAMP responses provoked by the pituitary FSH glycoforms in homologous and heterologous cell assay systems *(36)*. This observation suggests that differences in sampling procedures (single vs frequent sampling) and sample analysis (single vs pooled samples), may rather underlie the apparent discrepancies between these studies.

It has consistently been found that more acidic/sialylated pituitary FSH isoforms are less potent in provoking androgen aromatization and tissue-type plasminogen activator enzyme activity than their less sialylated isoforms *(1,3,4)*, although it is not known whether this difference is due to variations in sialylation alone. In this regard, it was interesting to find that in the sexually mature individuals (Ts-4 and -5) the shift toward less acidic serum FSH isoforms correlated with the presence of increased in vitro B:I ratios, whereas in Ts-3 boys this ratio was significantly higher than in prepubertal children despite similar serum FSH pH distribution profiles. This finding strongly suggests that variations in terminal sialylation cannot be the sole explanation for the differences in in vitro B:I FSH ratio detected throughout puberty and that factors other than sialylation may be involved in the increased capacity of the circulating FSH molecules to evoke signal transduction in mid- and late puberty *(37,38)*. The finding that the changes in FSH sialylation occurring across puberty are not of sufficient magnitude to alter the survival of this gonadotropin in circulation further supports this view.

The biochemical mechanisms subserving the changes in serum FSH charge isoform distribution throughout male puberty and the increased capability of mid- and late puberal FSH to evoke receptor activation and intracellar signaling are unclear. The observation that the distribution of circulating gonadotropin isoforms may vary in a significant manner in other physiological conditions, such as the menstrual cycle *(20,39–41)*, suggests that the alterations in the molecular structure of FSH during male puberty may be regulated by changes in hypothalamic inputs (GnRH) and/or end products from the gonads. In agreement with previous studies *(6,7)*, we found that administration of low- or high-dose exogenous GnRH significantly increased the release of FSH isoforms with elution pH values ≥4.5 in prepubertal, early pubertal, and midpubertal boys; however, GnRH administration to sexually mature individuals did not modify the pH distribution nor the B:I ratio of circulating FSH. This latter finding strongly suggests that GnRH does not play a significant role in regulating FSH glycosylation, and that its apparent effect on FSH heterogeneity during early and midpuberty is rather quantitative inducing the release of all forms present in the pituitary, thereby facilitating the detection of less acidic, short-lived FSH isoforms. Although some studies have documented changes in FSH charge isoform distribution after exposure to a supraphysiological dose of exogenous GnRH *(42,43)*, pulsatile administration of this releasing peptide failed to alter the pattern of pH isoform distribution of intrapituitary and/or secreted FSH *(19,44)*. Thus, discrepant findings may reflect differences in experimental paradigms. The recognition in experimental animals and in humans that sex steroid hormones (mainly E_2 and T) modify the charge isoform distribution of intrapituitary and

secreted FSH *(38,40,45–48)* and that estrogen administration reduces the activity and mRNA expression of some enzymes involved in terminal sialylation and sulfation of pituitary gonadotropins *(1,49,50)*, collectively suggests that progressive exposure of the pituitary to these steroids may provoke changes in FSH glycosylation and thereby increase the efficiency of the gonadotropin signal to evoke a measurable effect at the target cell level.

In summary, the transition from infancy to sexual maturity in men is accompanied by qualitative changes in the circulating FSH isoform mixture. Although the changes in FSH glycosylation occurring during male sexual maturation are not of sufficient magnitude to alter the survival of the gonadotropin in the circulation, they allow preferential secretion of bioactive FSH. The enrichment of the circulating mix of FSH isoforms with highly bioactive variants throughout spontaneous puberty may potentially favor the development of spermatogenesis and acquisition of reproductive competence.

Material and Methods

Subjects and Study Design

Forty healthy male subjects at Tanner stage (Ts) 1 to 5 [classified on the basis of testicular volume *(51)*], with median ages of 9.5 yr (Ts-1, range 8–12 yr, $n = 8$), 11.0 yr (Ts-2, range 10–14 yr, $n = 8$), 13.0 yr (Ts-3, range 11–16 yr, $n = 9$), 14.0 yr (Ts-4, range 12–16 yr, $n = 7$), and 24.0 yr (Ts-5, range 23–28 yr, $n = 8$) agreed to participate in the study. Approval from the human ethics committee of the institute and informed written consent from the adult volunteers or the parents of the children were obtained. All subjects were in good general health, with no known endocrinopathies, exhibited a normal weight for their corresponding height, and were not taking medications. Volunteers were admitted to the metabolic research ward at 07:00 h and an indwelling heparinized iv catheter was placed in an antecubital vein. Starting at 08:00 h, blood samples were obtained every 10 min for 10 h; at the beginning of the third hour, subjects received a rapid iv bolus of 10 μg GnRH (Serono de Mexico, S.A. de C.V., Mexico) and, 4 h later, a second GnRH bolus containing 90 μg of the decapeptide. Subjects were recumbent during the study and consumed light controlled meals at 09:00 h and 14:00 h. Blood samples were allowed to clot at room temperature for 30 min, then were centrifuged at 1000*g*. Sera were separated into three aliquots and stored frozen at −20°C until assay.

FSH, LH, T, and E_2 Immunoassays

Each subject's set of samples was processed in duplicate for FSH determinations in a single assay run. Serum FSH levels were determined by time-resolved immunofluorometric assay (IFMA) (Delfia, Wallac, Turku, Finland), employing an antibody directed against the β-subunit of FSH, a second antibody directed against an epitope on the α-subunit *(52)*, and the reference preparation LER-907 [National Hormone and Pituitary Program (NHPP), Torrance, CA] as the standard. The sensitivity of the assay was 0.013 mIU/well (0.52 IU/L) as expressed in terms of the 2nd International Reference Preparation of human menopausal gonadotropins (2nd IRP-HMG; 1 mg LER 907= 53 IU). The intra- and interassay coefficients of variation were determined using commercial quality control samples (Immunoassay Tri-level control, Diagnostic Products Corporation, Los Angeles, CA) placed at the beginning, middle, and end of each series of samples assayed; these coefficients ranged from 3–5% and 6–8%, respectively. Baseline samples from each study period were also analyzed for LH, E_2, and T content by specific radioimmunoassays (RIA). The RIA of LH was performed employing ^{125}I-labeled LH-I3 as the tracer, the reference preparation LER-907 as the standard, and the anti-human LH-3, at a final dilution of 1:800 000 as the antiserum *(53)*. Cross-reactivity of this antiserum with highly purified FSH, GH, and PRL is <0.2%. The sensitivity of the assay was 0.7 IU/L (2nd IRP-HMG; 1 mg LER 907= 277 IU). All LH RIA reagents were generously provided by the NHPP. The intra- and interassay coefficients of variation were determined using multiple replicates ($n = 3$ per dose) of a serum pool collected from postmenopausal women, assayed at dose levels that displaced the corresponding tracer from the antibody at 10–20%, 45–60%, and 75–90% total binding; these coefficients ranged from 6–8% and 7–12%, respectively. Serum T and E_2 concentrations were determined in each sample collected during the baseline period by RIA after solvent extraction (recoveries >90% for both sex steroids), using antisera kindly provided by the WHO Matched Reagent Programme (Geneve, Switzerland) as previously described *(20)*. Intra- and interassay coefficients of variation for both assays (at 45–55% total binding) were less than 8% and less than 11%, respectively.

A specific double antibody RIA was performed to determine the FSH content in fractions collected during the chromatofocusing of serum samples (see below). This FSH RIA system employed ^{125}I-labeled FSH I-1 as the tracer (specific activity 60–70 μCi/μg protein), LER-907 as the standard, and anti-human FSH-6 (NHPP) at a final dilution of 1:250,000 as the antiserum *(34)*. This antiserum exhibits less than 0.1% cross-reactivity with highly purified human LH and PRL and undetectable reactivity with free alpha-subunit and GH. The sensitivity of the assay was 0.24 mIU/tube (2nd IRP-HMG).

Chromatofocusing of Serum Samples

Serum samples from all subjects were subjected to concentration by dialysis and freeze-dried; the FSH isoforms were then separated on the basis of charge as previously described (20). Briefly, for each individual series, samples corresponding to the 2-h baseline period and to the low and high GnRH-stimulated study periods (4-h each) were separately pooled, transferred to dialysis membrane tubing

(molecular weight cut-off, 12,000–14,000; Spectrum Medical Industries, Los Angeles, CA), dialyzed at 4°C for 24 h against deionized water, and thereafter against 0.01 *M* ammonium carbonate (pH 7.5), and freeze-dried. Each specimen was slowly redissolved in 5–8 mL eluent buffer [1:8 dilution of Polybuffer-74 (Pharmacia Fine Chemicals, Piscataway, NJ) in deionized water, pH 3.0], and a 4–6-mL aliquot of the suspension was then applied to the top of a 20 × 1-cm column of polybuffer exchange resin (PBE-94, Pharmacia) and chromatofocused at 4°C. When the column eluent reached pH 3.0, the eluent buffer was changed to a solution of 1 mol/L NaCl to displace those proteins bound at the lower-limiting pH (salt peak). Sets of fractions corresponding to ten 0.5 pH units were separately pooled, concentrated by dialysis and freeze-drying as described above, and stored frozen at –20°C until the RIA of FSH. On the day of the FSH RIA, each specimen was slowly redissolved (to approximately one tenth of its original volume) in deionized water, such that the majority of the dose levels fell on the linear portion of the FSH RIA standard curve. To avoid interassay variations, all pooled fractions from a single chromatofocusing separation were assayed in triplicate incubations in a single assay run.

In Vitro Bioassay of Human FSH

For each individual series of samples, those corresponding to the complete (2-h) baseline period and to the low and high GnRH-stimulated study periods were pooled separately, as shown in Fig. 1 (five pools per subject; pools 1 to 5 in Fig. 1), and assayed for cAMP production and immunoactive FSH content. The capacity of each pool to provoke cAMP production was tested by a homologous cell in vitro bioassay described previously *(19)*. This assay employs a human embryonic kidney-derived 293-cell line stably expressing the human FSH receptor cDNA *(31,36,54)*. Cells (5×10^4 cells/culture dish) were exposed to increasing doses (25–100 μL) of each serum pool or LER-907 to assess for parallelism with the standard curve. The minimal detectable response of the assay was 0.16 mIU LER-907/tube. All pools from a single subject were bioassayed in triplicate incubations in a single assay run. The inter- and intraassay coefficients of variation at the ED_{50} dose level were <16% and <11%, respectively. Aliquots (25–100 μL) of samples from each serum pool series bioassayed were additionally analyzed for immunoactive FSH content by the RIA procedure described above.

cAMP RIA

Total (intra- plus extracellular) cAMP levels were determined by RIA after acetylation of the samples and cAMP standards. The RIA of cAMP was performed as previously described *(36)*, employing the CV-27 cAMP antiserum (NIDDK) at a final dilution of 1:75 000. The sensitivity of the assay was 4 fmol/tube and the inter- and intraassay coefficients of variation ranged from 9 to 11% and from 4 to 7% respectively. The relative in vitro biological activity of FSH was calculated by interpolation. Data are expressed as the mean B:I activity ratio, the ratio of FSH activity exhibited by serum pools 1–5 in the in vitro bioassay relative to that yielded by the RIA, calculated after conversion of the results to IU/L of the 2nd IRP-HMG.

Deconvolution Analysis of GnRH-Induced FSH Pulses

Deconvolution analysis was applied to estimate the apparent endogenous FSH half-life in blood sampled at 10-min intervals after the administration of exogenous GnRH *(55–57)*. Because the relatively long plasma half-life of FSH *(58)* may potentially trail across from the first GnRH-evoked FSH peak into the second, this parameter was only determined from the low GnRH dose-provoked FSH pulse episode.

Statistical Analysis

The *a*FSH*c* were calculated by trapezoidal reconstruction. Maximal response to low- and high-dose exogenous GnRH (ΔFSH) was defined as the difference between the maximal FSH level and the mean of the basal FSH concentration (for the first exogenous GnRH-stimulated FSH peak) or the FSH level preceding the second GnRH dose (i.e., FSH levels at 360 min). Differences between means of the *a*FSH*c*, ΔFSH, B:I FSH ratios, apparent plasma half-life and baseline serum E_2, T, LH, and FSH concentrations between each Tanner stage were analyzed by ANOVA followed by Student's unpaired *t* tests, whereas differences across the same Tanner stage where determined by the Bonferroni protected Student's paired *t* test. Tests for parallelism among the slopes generated by the different serum pools and LER-907 in the in vitro FSH bioassay were performed following the method of De Lean et al. *(59)*. Values are reported as the mean ± SEM, unless specified. Probabilities <0.05 were considered statistically significant.

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