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Changes in serum inhibin, activin and follistatin concentrations during puberty in girls*

Carol M.Foster[1,2,5], David J.Phillips[3], Tammy Wyman[2], Lee W.Evans[4], Nigel P.Groome[4] and Vasantha Padmanabhan[1,2]

[1]Department of Pediatrics/Division of Endocrinology and [2]Reproductive Sciences Program, University of Michigan, Ann Arbor, Michigan, USA, [3]Institute of Reproduction and Development, Monash University, Clayton, Victoria, Australia and [4]Oxford Brookes University, Oxford, UK

[5]To whom correspondence should be addressed at: D1205 MPB Box 0718, Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109, USA

Serum concentrations of inhibin A, inhibin B, activin A and follistatin were determined using two-site enzyme-linked immunosorbent assays (ELISA) during pubertal ovarian development in 28 girls and five follicular phase women. Blood obtained every 15 to 20 min overnight was pooled for peptide determination. Serum inhibin A concentrations increased in mid puberty, exhibiting positive correlations with bone age ($r = 0.527$, $P = 0.0016$) and oestradiol concentrations ($r = 0.581$, $P = 0.0005$). Inhibin B concentrations peaked in mid puberty and declined thereafter, but remained greater than concentrations seen in prepubertal girls, and correlating positively with oestradiol ($r = 0.362$, $P = 0.046$) and follicle stimulating hormone (FSH) concentrations ($r = 0.369$, $P = 0.038$). Total activin A concentrations did not vary significantly across pubertal stages. Total follistatin concentrations, determined by radioimmunoassay, decreased with advancing puberty, exhibiting negative correlations with bone age ($r = –0.634$, $P = 0.0001$) and oestradiol concentration ($r = –0.687$, $P = 0.0001$). Follistatin concentrations determined by an ELISA specific for follistatin 288 were greatest in mid-pubertal girls, but concentrations in late puberty were less than those in early puberty. The free follistatin assay indicated that all circulating follistatin was activin-bound. These results suggest that significant changes in serum concentrations of FSH-regulatory peptides accompany the onset of puberty.

Key words: activin A/follistatin/girls/inhibin A and B/puberty

Introduction

Gonadotrophins are secreted episodically under the influence of gonadotrophin-releasing hormone (GnRH), but sex steroid negative feedback influences GnRH release and/or pituitary sensitivity to the GnRH stimulus depending on the relative state of pubertal maturation (Penny *et al.*, 1977; Foster *et al.*, 1989; Cemeroglu *et al.*, 1996; Kletter *et al.*, 1997). Additional peptides, such as inhibins, activins and follistatins, initially localized to the gonads but now known to be produced in other tissues as well, also appear to participate in the regulation of follicle stimulating hormone (FSH) secretion (Ying, 1988; DePaulo *et al.*, 1991). Several studies have demonstrated that the activins are stimulators of pituitary FSH secretion, while inhibins decrease FSH secretion (Ying, 1988; DePaolo *et al.*, 1991; Xu *et al.*, 1995; Draper *et al.*, 1998). Follistatin also decreases FSH secretion by binding activin, thereby preventing the interaction of activin with its receptor (Cataldo *et al.*, 1994; de Winter *et al.*, 1996).

The recent advent of specific, two-site immunoassays for inhibin A and B and activin A has made it possible to measure these regulatory peptides in the peripheral circulation. Initial studies utilizing these assays have suggested an endocrine role for inhibin A and B in the control of FSH secretion during the menstrual cycle in combination with oestradiol (Groome *et al.*, 1994, 1996; Welt *et al.*, 1997; Hayes *et al.*, 1998). Perimenopausal women have an age-related increase in serum FSH concentration that precedes a decline in serum oestradiol concentration (Yen, 1991). In these women, activin A concentrations increase while inhibin B concentrations decrease, suggesting that the sum change in concentrations of positive regulators of FSH (activin and GnRH), and negative regulators of FSH (oestradiol, inhibin and follistatin) may together contribute to the increases in FSH secretion seen in ageing (Reame *et al.*, 1998).

Whether gonadal peptides regulate FSH secretion during pubertal maturation is unclear. Recent studies have shown that inhibin B concentrations are greater in prepubertal boys than in prepubertal girls, suggesting that the greater FSH concentrations seen in prepubertal girls could be related to less inhibin availability from the developing ovary (Hayes *et al.*, 1998). Prepubertal girls, unlike prepubertal boys, readily release FSH into the circulation in response to GnRH administration (Lee, 1985; Foster *et al.*, 1992). Ovarian size increases throughout the prepubertal period and into puberty as a consequence of increased stroma, growth of individual follicles and growth of number of follicles (Peters *et al.*, 1978). Thus, the developing gonad could produce increasing concentrations of gonadal peptides that in turn could affect FSH secretion. Since activin and follistatin are produced in the pituitary (Ying, 1988; DePaolo *et al.*, 1991), there is also the possibility that changes in the peripheral concentrations of activin and follistatin may reflect the development of an intra-pituitary feedback system

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Table I. Subject characteristics of the girls

Subject number	Age (years)	Bone age (years)	Pubertal diagnosis[a]	Final diagnosis[b]	Spontaneous menses[b]
1	5.6	3.6	I	SS	LTF
2	9.3	5.8	I	IGHD	LTF
3	8.0	7.0	I	SS	Y
4	9.0	7.0	I	FSS	LTF
5	13.0	7.0	I	IGHD	Y
6	12.3	7.8	II	SS	LTF
7	8.2	8.3	II	CEP	LTF
8	11.2	9.5	II	IGHD	Y
9	12.5	10.0	II	SS	LTF
10	13.3	10.0	II	CGD	LTF
11	13.8	10.0	II	CGD	Y
12	14.0	10.0	II	CGD	Y
13	12.5	10.7	II	FSS	LTF
14	11.8	11.0	II	FSS	LTF
15	12.6	11.0	II	IGHD	Y
16	12.8	11.0	II	IUGR/SS	Y
17	13.2	11.0	II	CGD/FSS	LTF
18	15.5	13.0	II	CGD	LTF
19	12.6	11.0	III	CGD	Y
20	13.8	11.0	III	CGD	Y
21	13.3	11.8	III	FSS/CGD	Y
22	12.5	13.0	III	NL	Y
23	15.0	12.5	IV	FTS/CGD	Y
24	15.9	13.0	IV	CGD	Y
25	13.7	13.3	IV	FSS	Y
26	15.9	16.0	IV	NL	Y
27	16.2	16.0	IV	NL	Y
28	15.0	17.0	IV	NL	Y

[a]According to the method of Tanner (Tanner, 1978).
[b]CEP = constitutional early puberty; CGD = constitutional growth delay; FSS = familial short stature; FTS = familial tall stature; IGHD = isolated growth hormone deficiency; IUGR = intrauterine growth retardation; LTF = lost to follow-up; NL = normal subject; SS = short stature; Y = yes (spontaneous menses).

that regulates FSH secretion (Bilezikjian *et al.*, 1994). This study was designed to determine whether the gonadal peptides, activin A, inhibin A, inhibin B and follistatin, are correlated with maturation markers in girls. Such a correlation would suggest that gonadal peptides could contribute to the changes in FSH concentration that occur at puberty.

Materials and methods

Subjects

Twenty-eight healthy girls were referred for study because of short or tall stature, adolescent delay, or early puberty. All cases of early puberty were constitutional. Subject characteristics are shown in Table I. Previously published methods were used to determine stages of puberty (Tanner, 1978) and bone age (Greulich and Pyle, 1955). Body mass indices did not vary among groups and were appropriate for bone age (data not shown). All of the prepubertal girls studied subsequently had spontaneous onset of pubertal development. Seventeen of the 28 have established regular menstrual cycles; the remainder have been lost to follow-up. Four girls (two in stage I and two in stage II puberty) had a peak growth hormone (GH) concentration <8 ng/ml after two provocative stimuli and were treated with exogenous GH after this study. Each of these girls had spontaneous onset of puberty, and three have regular menstrual cycles. In addition, five adult women who were in the follicular phase of the menstrual cycle were also studied and are grouped as stage V puberty. Subjects were studied within 5 days of the onset of menses. Each was interviewed about their menstrual history before inclusion in the study. Those receiving medication, including hormone supplements, were excluded, as were subjects with irregular cycles. The girls had frequent blood sampling as part of other research and/or diagnostic protocols. Gonadotrophin data for 10 girls have been reported elsewhere (Cemeroglu *et al.*, 1998).

Protocol

The Institutional Review Board of the University of Michigan approved the research protocol. Informed written consent was obtained before the study from the adult volunteers, and from a parent of each child. Minor children aged over 7 years also gave their written assent. Subjects were admitted to the Clinical Research Center of the University of Michigan. An indwelling i.v. catheter was placed in a forearm vein by 18:00 h on the night of the study and was kept patent with a dilute solution of heparin. To confirm that the nighttime elevations in luteinizing hormone (LH) concentration conformed to those expected for pubertal stage, blood was obtained every 15 min from either 20:00 or 22:00 h until 08:00 h on the following day. Lights were turned off at 22:00 h and on at 06:00 h. To obtain an integrated measure of peripheral FSH-regulatory peptide tone, an aliquot of each sample was pooled for determination of activin A, inhibin A and inhibin B, total and free follistatin, LH, FSH and oestradiol concentrations.

Hormone assays

Activin A and inhibins A and B were determined using two-site enzyme-linked immunosorbent assays (ELISA) (Serotec, Raleigh, NC, USA). The sensitivities of the assays were 0.2 ng/ml, 5 pg/ml and 16 pg/ml for activin A, inhibin A and inhibin B respectively. The samples were measured in one assay in triplicate. The intra-assay coefficients of variation were 5, 8 and 6% for activin A, inhibin A and inhibin B respectively.

Total follistatin concentrations were determined using a heterologous radioimmunoassay described previously (O'Connor *et al.*, 1999) which employs dissociating reagents [20% Tween 20, 10% sodium deoxycholate and 0.4% sodium dodecyl sulphate (SDS)], to remove the interference of bound activin. The rabbit polyclonal antiserum used in this assay was raised against 35 kDa bovine follistatin. In the assay, recombinant human follistatin (rhFS) 288 was used as both tracer and standard. Cross-reactivity was 100% for rhFS 288 and 36% for rhFS 315 (O'Connor *et al.*, 1999). In the presence of dissociating reagents, addition of increasing amounts of activin did not alter the amount of measured follistatin (O'Connor *et al.*, 1999). The assay sensitivity was 1.6 ng/ml, and the intra-assay coefficient of variation 12.3%. Samples of human male and female serum diluted parallel in the assay, and the recovery of added follistatin (3.125–100 ng/ml) ranged from 103 to 110% (O'Connor *et al.*, 1999). Follistatin 288 concentrations were measured using a recently developed two-site ELISA (Evans *et al*., 1998). The assay sensitivity was 37 pg/ml and the intra-assay coefficient of variation 17%. This assay cross-reacts 9.9% with follistatin 315 (Evans *et al*., 1998). Free follistatin was determined by a second-generation two-site chemiluminescent assay that utilizes two monoclonal antibodies generated against non-overlapping epitopes of human follistatin as described previously (McConnell *et al*., 1998). The assay sensitivity was 0.8 ng/ml, and the intra-assay coefficient of variation <4%.

LH and FSH were determined by immunofluorometric assay using commercially available kits (Wallac, Gaithersburg, MD, USA). The assay sensitivity was 0.05 IU/l for LH and FSH. The intra-assay coefficients of variation were 3.1% for LH and 3.9% for FSH.

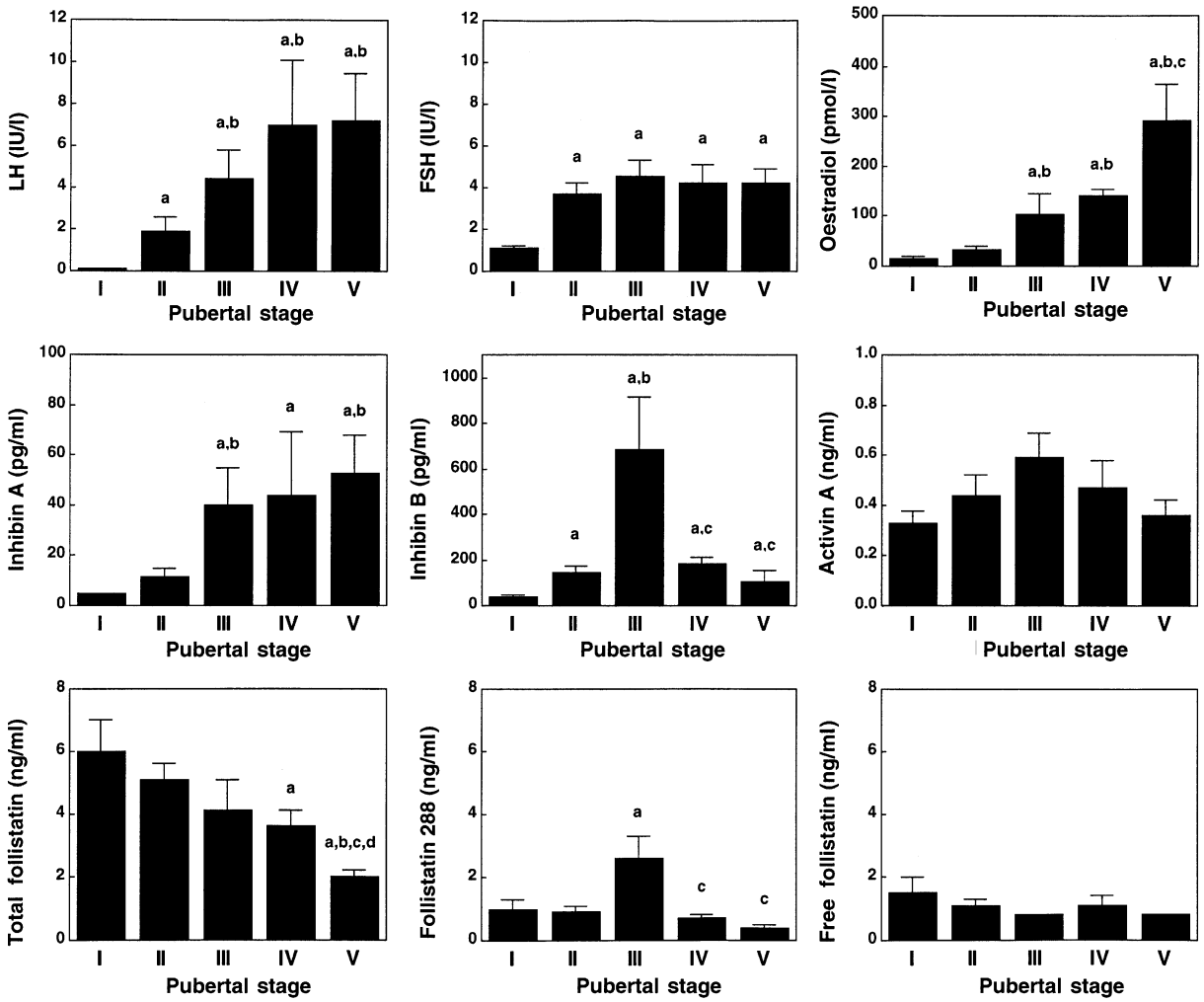

Figure 1. LH, FSH, oestradiol, inhibin A, inhibin B, activin A, and total, 288 and free follistatin concentrations in girls across the pubertal transition and in follicular phase women. Blood was obtained from 20:00 or 22:00 to 08:00 h and an aliquot of each sample combined to make a pool for assay. The numbers of subjects in each cell were: stage I, five girls; stage II, 13 girls; stage III, four girls except for inhibin B where only results from three girls were available; stage IV, six girls; stage V, five women. Data are represented as the mean ± SE. Letters above the bars indicate significant differences as follows: a, different with respect to stage I; b, different with respect to stage II; c, different with respect to stage III; d, different with respect to stage IV.

Statistical analyses

Hormone concentrations were transformed logarithmically before analysis to adjust for data heterogeneity. Differences in hormone concentrations between pubertal stages were assessed by one-way analysis of variance followed by a Duncan new multiple range test. Correlations were determined by regression. Adult volunteers did not have bone age determinations; their chronological age was assigned to the bone age value. In all cases, a P value of < 0.05 was considered significant.

Results

Gonadotrophin concentrations

As expected, mean serum LH and FSH concentrations each increased with advancing pubertal stage (Figure 1). The increase in LH was progressive across puberty through stage III. There was a robust correlation between bone age and LH concentration ($r = 0.656$, $P = 0.0001$; Table II). FSH concentrations increased between stages I and II but were relatively constant thereafter and were also positively correlated with bone age ($r = 0.444$, $P = 0.0096$; Table II).

Table II. Correlation coefficients between hormone or peptide concentrations and bone age

Hormone or peptide	Bone age (r)	FSH (r)	Oestradiol (r)
LH	0.656[a]	0.847[a]	
FSH	0.444[c]		
Oestradiol	0.767[a]	0.503[c]	
Inhibin A	0.527[c]	0.238	0.581[b]
Inhibin B	0.172	0.369[d]	0.362[d]
Activin A	−0.057	−0.039	−0.076
Total follistatin	0.634	0.217	0.687[a]
Follistatin 288	−0.321	−0.147	−0.192

[a]$P < 0.0001$; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$.

Inhibin A, inhibin B and activin A concentrations

Inhibin A concentrations increased between stage II and III puberty and remained at similar concentrations in stage IV and V puberty (Figure 1). Inhibin A concentrations exhibited a positive correlation with bone age ($r = 0.527$; $P = 0.0016$;

Table II). Inhibin B concentrations increased between stage I and II puberty, reached a peak in stage III puberty, and decreased thereafter. The correlation with bone age was not significant ($r = 0.172$). Activin A concentrations did not differ significantly with pubertal stage, although the trend in concentrations at each pubertal stage mirrored those of inhibin B (Figure 1).

Follistatin concentrations

Total follistatin concentrations had an apparent decrease between pubertal stages I to IV and stage V puberty (Figure 1), exhibiting a significant decrease with advancing bone age ($r = –0.634$; $P = 0.0001$; Table II). Free follistatin concentrations were near the limit of assay detection in all subjects (Figure 1). When determined by an ELISA specific for follistatin 288, follistatin concentrations exhibited a peak in stage III puberty and declined significantly thereafter. Analysis of the relationship between follistatin 288 concentration and bone age showed that $r = –0.321$ (not significant).

Correlation of gonadal peptides with FSH and oestradiol

FSH concentrations demonstrated a positive correlation with increasing concentrations of oestradiol and LH (Figure 1, Table II). There was a positive correlation between inhibin B concentration and FSH concentration, but no significant correlations between inhibin A, activin A, or follistatin and FSH concentrations (Table II). There were positive correlations between oestradiol concentration and inhibin A and B concentrations, but not with activin A concentration (Table II). Total follistatin concentration had a significant negative correlation with oestradiol concentration, but there was no correlation between oestradiol concentration and follistatin 288 concentration (Table II).

Discussion

In this study, changes in activin A, inhibin A and B, and follistatin concentrations during pubertal development in girls were examined, and changes in gonadal peptide concentrations were correlated to the developmental marker of bone age and with FSH and oestradiol concentrations. The results indicate that the concentrations of two negative regulators of FSH secretion inhibin A and total follistatin change in opposite directions during progression of puberty. Inhibin concentrations increased while follistatin concentrations decreased with pubertal progression in girls. Total concentrations of the positive regulator of FSH, activin A, remain unchanged during pubertal progression. The decline in follistatin, a binding neutralizer of activin, in the face of relatively constant total activin A concentrations suggests that the bioavailable activin (free activin A) increases with pubertal progression, such that activin could contribute to the increase in FSH that occurs during puberty.

Changes in inhibins in this study agree in general with those observed in earlier studies. In both boys and girls, inhibin concentrations are lowest during pubertal stage I (Andersson *et al*., 1997). However, the mid-pubertal peak in inhibin B that was noted in the girls in the current study has not been observed in boys or in a previously reported longitudinal study in girls (Crofton *et al.*, 1997). It is difficult to make any meaningful comparisons between the current and the published report (Crofton *et al.*, 1997), as there were four girls in stage III puberty available for inclusion in the current study, while only one girl had a sample obtained at stage III puberty in the study of Crofton *et al.* Further, earlier studies (Andersson *et al.*, 1997; Crofton *et al.*, 1997) utilized single samples in their design, while the current study design incorporated an integrated measure over a 10 h period at night when gonadotrophin secretion is most active in pubertal children (Penny *et al.*, 1977; Foster *et al.*, 1989; Cemeroglu *et al.*, 1996; Kletter *et al.*, 1997). It is also of interest that in an earlier study (Boepple *et al*., 1996), inhibin B concentrations found in girls with precocious puberty were similar to those in adult women, and inhibin B concentration declined when gonadotrophin secretion was down-regulated with GnRH agonist therapy. Since inhibin B is found in granulosa cells of developing follicles (Roberts *et al*., 1993), the general increase in numbers of developing follicles during mid puberty may contribute to the increased concentrations of inhibin B observed in the mid-pubertal girls of the current investigation. Studies relating inhibin B concentrations to changes in follicular size and oestradiol and progesterone concentrations in the pubertal transition may shed additional light on how gonadal peptide concentrations change with ovarian development.

As both inhibin A and B suppress FSH secretion (Ying, 1987), the sum total of inhibin A and inhibin B should provide an index of total peripheral inhibin input. Estimation of total inhibin by summing inhibin A and inhibin B concentrations suggests that total inhibin concentrations increase through mid puberty and decline thereafter. The directionality of inhibin changes is dictated by the higher circulating concentrations of inhibin B. Until it is established that these two immunoassays predict the protein mass of inhibin A and B correctly, and it is demonstrated that the relative biological potency of these two isomers in suppressing human FSH secretion is similar, this estimate should be viewed with caution.

Correlation data between hormone concentrations do not imply a cause-and-effect relationship, but are of interest in determining whether a relationship may be present. Hence, the correlation coefficients between the gonadal peptides and FSH and oestradiol concentrations were determined in the subject population of this study. Previously, it was reported that both inhibin A and B concentrations exhibit positive correlations with oestradiol and FSH concentrations (Crofton *et al.*, 1997). In the current study, only inhibin B and not inhibin A concentrations exhibited a significant correlation with FSH. The positive correlation between inhibin B and FSH suggests that the increase in FSH may actually drive the increase in inhibin B and—at least during puberty—inhibin B may not be negatively regulating FSH secretion. Such a relationship is also seen with oestradiol and gonadotrophin secretion.

FSH is believed to increase oestradiol secretion during puberty, and the profound negative feedback of oestradiol on gonadotrophin secretion seen in prepubertal children is lost during puberty. This appears to be due to a change in neurotransmitter control of GnRH secretion which results in

resistance of the hypothalamus to negative feedback. As puberty progresses, the pituitary gonadotroph assumes increasing importance over the hypothalamus as the site of sex steroid negative feedback (Kletter *et al*., 1992; Cemeroglu *et al*., 1998). This change of feedback appears to permit the increase of gonadotrophin secretion seen at puberty. If gonadal peptides exhibit their primary endocrine effects at the level of the pituitary, negative feedback may not be possible until gonadotrophin secretion from the pituitary is well established, as in late puberty. As inhibin A and B concentrations both increase significantly with the increase in oestradiol concentrations, and inhibin B concentrations increase significantly with FSH concentrations, these data suggest that inhibin concentration may serve as a developmental marker of ovarian follicle development or increase in follicle number during puberty.

The cross-sectional data in this study suggest that activin concentrations do not change significantly with advancing puberty. Activin assays are relatively new (Knight *et al.*, 1996), and hence this is the first report on changes in activin concentrations during the onset of puberty. Surprisingly, total follistatin concentrations declined steadily with advancing bone age, and follistatin 288 concentrations (determined by a separate ELISA) were also at their lowest values in late pubertal girls and adult women. Follistatin has not been shown to vary during pubertal maturation in other studies (Kettel *et al*., 1996), but this may be due to differences in assays for this peptide and how the assay recognizes the different variants of follistatin. Free follistatin concentrations were at or near the assay sensitivity in all of the subjects in this study, indicating that virtually all of the circulating follistatin is activin-bound. These results also corroborate the earlier finding that almost all of the free follistatin appears to be activin-bound in the circulation of adult cycling women (McConnell *et al*., 1998). As activin A concentrations appear to be constant, the decline in follistatin concentrations during late puberty suggests that more activin A may be available to regulate FSH secretion in late puberty. Animal studies have shown that activin at concentrations as low as 0.01 nmol/l can stimulate FSH secretion (Bilezikjian *et al*., 1998). In the absence of measures of activin B and AB, which are also produced by the ovary, the total activin drive cannot be estimated.

Since the ovary is increasing in size with advancing puberty, the finding that total follistatin concentration declined during pubertal maturation was unexpected. Follistatin 288 concentrations also exhibited a decline in late puberty, but the correlation with bone age was not as strong. A number of studies have suggested that the ovary is not a significant source of follistatin entering into the circulating pool (Klein *et al*., 1993; Khoury *et al*., 1995). Therefore the observation that follistatin concentrations declined during pubertal maturation may reflect changes in the paracrine regulatory loop involving activin and follistatin in the pituitary (Phillips and de Kretser, 1998). This notion is also consistent with the significant inverse correlation between total follistatin concentrations and oestradiol concentration in the present study, and the observation that the pituitary is a net secretor of follistatin into the circulation (Phillips and de Kretser, 1998). The fact that total follistatin concentration has a significant inverse correlation with oestradiol concentration suggests that increasing oestradiol concentrations with advancing puberty might be responsible for suppressing follistatin, but additional studies will be needed to demonstrate a cause-and-effect relationship.

The data in this study, coupled with other recent findings, indicate that inhibin concentrations vary dynamically with pubertal maturation. Changes in inhibins during pubertal maturation seem to be a reflection of ovarian maturation rather than an indicator of FSH feedback regulation. Activin A concentrations do not vary significantly with pubertal maturation, but the decline in follistatin concentrations with increasing pubertal maturation suggests that more activin could be available to increase pituitary FSH release as the ovary develops. Analysis of dynamic changes of all gonadal peptides will be needed to understand whether and how these peptides function in the complex regulation of gonadotrophin secretion.

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