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BRIEF REPORT

Serum Insulin-Like Factor 3 Levels during Puberty in Healthy Boys and Boys with Klinefelter Syndrome

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Context: Levels of the Leydig cell-specific hormone insulin-like factor 3 (INSL3) are incompletely characterized in boys during pubertal development.

Objective: The objective of the study was to characterize changes in INSL3 levels during spontaneous puberty in healthy boys, boys with aromatase inhibitor-induced hypergonadotropic hyperandrogenism, and boys with Leydig cell dysfunction.

Design: This was a prospective clinical study.

Setting: The study was conducted at a university hospital pediatric endocrinology outpatient clinic.

Patients: Patients included 30 healthy boys with idiopathic short stature (ISS) aged 9.0–14.5 yr and 14 boys with Klinefelter syndrome (KS) aged 10–13.9 yr.

Intervention: In ISS boys, intervention included aromatase inhibitor letrozole or placebo for 24 months.

Main Outcome Measures: Serum INSL3 levels in relation to bone age, Tanner pubertal stages, and LH and testosterone levels were measured.

Results: Onset of puberty was associated with a significant increase in INSL3 levels from 0.06 ± 0.01 ng/ml at Tanner G1 to 0.32 ± 0.16 ng/ml at G2 ($P < 0.0001$). Adult INSL3 levels (≥0.55 ng/ml) were attained at bone age 13–14 yr. ISS boys with letrozole-induced hypergonadotropic hyperandrogenism had, after 12 months of therapy, higher INSL3 levels than did placebo treated (0.85 ± 0.54 *vs.* 0.26 ± 0.17 ng/ml, $P < 0.01$). In KS boys during spontaneous puberty, after an initial increase similar to that in healthy boys, INSL3 concentrations leveled off despite hyperstimulation by LH. Positive correlations occurred between serum INSL3 and LH and between INSL3 and testosterone levels in all three groups ($P < 0.0001$).

Conclusions: In boys, the Leydig cell-specific hormone INSL3 may serve as a new marker for onset and progression of puberty. Pubertal increase in INSL3 levels seems to depend on LH. In KS subjects, INSL3 concentrations indicate Leydig cell dysfunction from midpuberty onward. **(*J Clin Endocrinol Metab* 91: 4705–4708, 2006)**

INSULIN-LIKE FACTOR 3 (INSL3), a member of the relaxin-insulin family, is a peptide hormone secreted by prenatal and fully differentiated adult Leydig cells (1). It is only weakly expressed in immature prepubertal Leydig cells and Leydig cells that have become hypertrophic or dedifferentiated (1, 2). INSL3 is essential for the transabdominal part of testis descent (1), but its biological significance in adults has remained unclear.

Recently serum INSL3 levels have been measured in normal men and men with different testicular pathologies (3, 4). These studies have shown that circulating INSL3 is entirely of testicular origin and that the concentrations of this hormone seem to reflect the functional status of the Leydig cells (3, 4). For example, adult patients with Klinefelter syndrome (KS) have significantly below normal INSL3 concentrations (3, 4). Production of both INSL3 and testosterone (T) is related to LH (3, 4), but recent data strongly suggest that the production of these hormones is regulated differently because INSL3 secretion is probably dependent on the long-term trophic effect of LH on Leydig cell differentiation (4).

The aim of this study was to characterize the changes in INSL3 levels during normal puberty by means of a newly developed time-resolved fluorescence immunoassay (4). Furthermore, we also studied the role of gonadotropins in the regulation of INSL3 secretion during puberty in two different models: aromatase inhibitor-treated boys with drug-induced hypergonadotropic hyperandrogenism and boys with KS who developed hypergonadotropism due to Leydig cell dysfunction.

Subjects and Methods

Healthy boys

Thirty healthy boys aged 9.0–14.5 yr were enrolled in a study on idiopathic short stature (ISS) at the outpatient clinic for pediatric endocrinology of the Hospital for Children and Adolescents, Helsinki University Central Hospital. Our selection of patients and study protocol have been described in detail previously (5). These boys were randomized to receive either the aromatase inhibitor letrozole (Lz; Femar, Novartis AG, Basel, Switzerland) at a dose of 2.5 mg or placebo (Pl) orally once daily for 2 yr. During the treatment, the boys were examined every 6 months and again 12 months after the cessation of treatment.

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Abbreviations: INSL3, Insulin-like factor 3; ISS, idiopathic short stature; KS, Klinefelter syndrome; Lz, letrozole; Pl, placebo; T, testosterone.

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Boys with KS

Fourteen nonmosaic 47,XXY boys aged 10.0–13.9 yr were followed up at the same university clinic every 4–6 months. Some of the resultant clinical and hormonal data have already appeared (6, 7). None of the subjects were or had been on androgen therapy.

The parents of each boy gave their informed consent for participation in these studies, which had been approved by the research ethics committee of the Hospital District of Helsinki and Uusimaa.

Clinical follow-up

In both groups, at each visit, stage of puberty was evaluated according to Tanner (8), and venous blood samples were drawn for biochemical measurements. Bone ages assessed by the method of Greulich and Pyle (9) rather than chronological ages were used because the majority of the ISS boys had delayed bone age.

Biochemical measurements

INSL3 serum concentrations were analyzed by a semicompetitive time-resolved fluorescence immunoassay specific for human INSL3. This assay has a detection limit of 0.05 ng/ml, and intra- and interassays coefficients of variations of 8.0 and 11.3%. For further details, see Bay *et al.* (4). Serum LH, T, and inhibin B levels determinations were by methods previously described (5–7).

Statistical analyses

Values, unless otherwise stated, are expressed as mean ± SD. To reduce frequency bias, means were calculated to obtain for each subject one value for each age or puberty stage interval. Comparisons between the Pl-treated ISS and KS groups and within these groups were by unpaired two-tailed Student's *t* test. Differences between the Lz- and Pl-treated ISS boys were analyzed by repeated-measures ANOVA. Relations between INSL3 and other hormones were tested by linear regression analysis. $P < 0.05$ was considered statistically significant.

Results

INSL3 levels during puberty in healthy boys with ISS

Onset of puberty was associated with a marked increase in INSL3 levels (from Tanner pubertal stage 1 to 2) that occurred concomitantly with significant increases in T and LH levels (Table 1). Significant increases in serum INSL3 and LH levels took place between bone ages 10–11 yr and 11–12 yr, and adult INSL3 concentrations 0.55 ng/ml or greater (4) were reached at bone age 13–14 yr (Table 1).

INSL3 levels in healthy boys with ISS during hyperandrogenism induced with an aromatase inhibitor

The nine boys who entered puberty within 18 months from the start of Lz therapy showed a marked increase in serum INSL3 levels from 0.21 ± 0.19 ng/ml (month 0) to 1.15 ± 0.55 ng/ml (24 months), whereas the eight Pl-treated ISS boys, who entered puberty during follow-up, showed an increase from 0.08 ± 0.06 ng/ml (month 0) to 0.53 ± 0.14 ng/ml (24 months) (Fig. 1). One year after cessation of Lz therapy, a slight decrease in INSL3 levels was noted to 1.00 ± 0.36 ng/ml (Fig. 1).

INSL3 levels in boys with KS

In KS boys in comparison with healthy boys, no significant difference in INSL3 levels emerged in assessment according to bone age or Tanner pubertal stages (Table 1). In the KS boys, the increase in serum INSL3 was significant between bone ages 11–12 and 12–13 yr and from Tanner pubertal stage P1 to P2 (Table 1). Adult INSL3 levels (≥0.55 ng/ml) (4) were reached at bone age 12–13 yr. Thereafter INSL3 and T concentrations leveled off despite increasing LH levels (Table 1).

After this study, two of the KS boys were started on T substitution therapy. After 6 months on T (Sustanon 250; Organon, Oss, The Netherlands), 1 mg/kg every fourth week, their serum INSL3 decreased from 0.68 to 0.11 ng/ml and from 0.49 to 0.04 ng/ml. At the same time, their serum LH levels decreased from 21.3 to 4.1 IU/liter and from 17.0 to 1.4 IU/liter.

TABLE 1. Serum concentrations of INSL3, T, and LH in 30 healthy boys with ISS at baseline, in 14 Pl-treated ISS boys during follow-up, and in 14 boys with KS according to bone age and Tanner pubertal stage

	ISS boys			KS boys		
	INSL3 (ng/ml)	T (ng/dl)	LH (IU/liter)	INSL3 (ng/ml)	T (ng/dl)	LH (IU/liter)
Bone age (yr)						
10–11	0.11 ± 0.12 (10)	34.7 ± 54.5 (10)	0.6 ± 0.6 (10)	0.00 (1)	25.3 ± 17.3 (4)	0.5 ± 0.3 (4)
11–12	0.30 ± 0.19 (10)*	77.8 ± 85.4 (9)	1.6 ± 0.8 (10)**	0.20 ± 0.24 (8)	34.6 ± 22.3 (7)	1.4 ± 1.7 (9)
12–13	0.46 ± 0.25 (7)	193.9 ± 105.3 (5)*	1.6 ± 0.8 (7)	**0.55 ± 0.30** (6)*	115.2 ± 148.1 (8)	3.9 ± 4.3 (11)
13–14	**0.65 ± 0.14** (3)	395.4 and 424.9 (2)§	3.2 ± 0.8 (3)	0.48 ± 0.40 (6)	152.5 ± 131.5 (7)§	6.5 ± 4.7 (9)
14–15	**0.66 ± 0.18** (3)		3.0 ± 1.5 (3)	0.54 ± 0.27 (6)	156.2 and 277.5 (2)	9.9 ± 6.7 (7)
Pubertal stage G						
1	0.06 ± 0.05 (25)	9.7 ± 6.4 (25)	0.4 ± 1.5 (25)	0.03 ± 0.04 (6)	15.7 ± 8.9 (7)	0.2 ± 0.1 (7)
2	0.32 ± 0.16 (13)****	63.2 ± 33.3 (11)****	1.8 ± 0.9 (13)****	0.28 ± 0.32 (11)	74.8 ± 86.4 (11)	2.5 ± 3.8 (13)
3	0.45 ± 0.15 (7)	215.4 ± 80.0 (6)	1.7 ± 0.8 (7)	0.47 ± 0.12 (6)	140.3 ± 130.2 (8)	5.1 ± 4.7 (11)
4	**0.78 ± 0.22** (6)	436.2 ± 47.5 (3)§	0.7 ± 0.8 (6)§§	**0.59 ± 0.14** (5)	181.9 ± 138.6 (6)§	8.5 ± 4.4 (9)§§
5	**0.56** and **0.81** (2)		4.1 and 4.7 (2)	**0.68 ± 0.17** (4)	253.2 (1)	14.1 ± 6.3 (4)
Pubertal stage P						
1	0.13 ± 0.14 (29)	32.3 ± 47.1 (29)	0.7 ± 0.6 (29)	0.11 ± 0.14 (9)	48.5 ± 73.1 (8)	1.1 ± 2.0 (9)
2	0.37 ± 0.16 (6)***	112.9 ± 47.1 (4)**	1.7 ± 0.8 (6)**	0.45 ± 0.41 (5)*	150.9 ± 147.8 (9)	4.1 ± 4.3 (11)
3	**0.83 ± 0.23** (4)	395.4 and 488.3 (2)§§	2.6 ± 0.8 (4)§	**0.59 ± 0.27** (8)	138.1 ± 90.8 (7)§§	8.8 ± 5.5 (10)*§
4	**0.70 ± 0.16** (4)	424.9 (1)	2.9 ± 1.3 (4)	**0.63 ± 0.18** (5)	189.3 (1)	11.4 ± 6.9 (5)

Data are expressed as means ± SD, with the number of patients in *parentheses*. *Bold* indicates INSL3 levels greater than 0.55 ng/dl, 2.5 percentile in healthy adult men (4).

* and § indicate the stages when differences first became significant between stages within the groups or between the groups, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$. §, $P < 0.05$; and §§, $P < 0.01$.

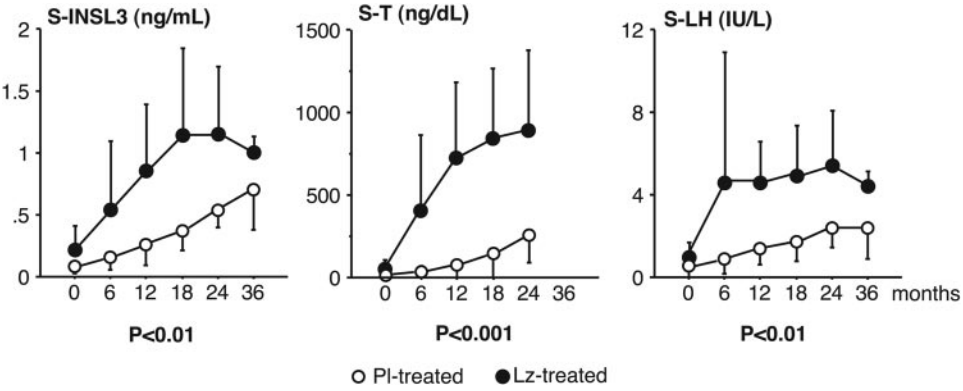

FIG. 1. Serum (S)-INSL3, S-LH, and S-T concentrations (mean ± SD) in healthy boys with ISS treated for 24 months with Pl (n = 8) or Lz (n = 9). *P* values, differences between the groups.

Correlations of INSL3 levels with testicular volume and serum LH, T, and inhibin B levels

Significant correlations ($|r| = 0.60–0.90$, $P < 0.0001$) appeared in all three patient groups between INSL3 and testicular volume, LH, T, and inhibin B concentrations. No differences in INSL3/testicular volume and INSL3/LH correlations appeared between the Pl- and Lz-treated ISS groups. The Lz-treated had significantly lower INSL3 to T ratios ($P < 0.05$) and furthermore, significantly higher INSL3/inhibin B ratios ($P < 0.001$) than did the Pl treated.

Because testicular volumes in the KS boys never exceeded 4.5 ml, the KS boys displayed higher INSL3 to testicular volume ratios than did ISS boys ($P < 0.0001$). The KS boys displayed low INSL3 levels when the hypergonadotropism with high LH levels occurred; this difference in INSL3 to LH ratios from those of the healthy boys was significant ($P < 0.0001$). In INSL3/T correlations, the differences between these two groups were nonsignificant ($P = 0.07$). Furthermore, the KS boys showed an inverse correlation between INSL3 and inhibin B ($r = -0.63$, $P < 0.0001$) because secretion of inhibin B was very rapidly suppressed after onset of puberty.

Discussion

The present study describing changes in serum INSL3 concentrations in adolescent boys shows that there occurs from prepubertal stages G1 and P1 to pubertal stages G2 and P2 a significant rise in serum INSL3 levels. During progression of puberty, serum INSL3 levels increased in close correlation with testicular growth and LH and T concentrations until adult levels were achieved at Tanner pubertal stages G4 and P3 and ages 13–14 yr. This continuous increase in INSL3 levels during puberty indicates that INSL3 may be used as a Leydig cell-specific marker for onset and progression of puberty.

Studies have suggested that LH regulates INSL3 and T secretion differently. T secretion is more acutely sensitive to LH, whereas INSL3 secretion is more dependent on the trophic effect on Leydig cell differentiation (3, 4, 10). The dependence of INSL3 secretion on LH was verified in this study because, concomitantly with pubertal activation of LH secretion, INSL3 concentrations began to rise. Furthermore, the boys treated with the aromatase inhibitor Lz developed hypergonadotropic hyperandrogenism (5) and had significantly higher INSL3 levels than did Pl-treated boys.

Secretion of the Sertoli cell hormone inhibin B increases significantly at onset of puberty, but thereafter serum levels remain relatively constant (11, 12). Inhibin B concentrations are regulated by FSH, and during puberty an inverse relationship develops between FSH and inhibin B (11, 13). Although both FSH and LH rose significantly during Lz treatment, throughout the treatment period, only INSL3 levels showed a marked increase, not inhibin B levels (5). Thus, contrary to the inverse relationship between FSH and inhibin B, the positive relationship between LH and INSL3 was sustained in healthy boys throughout puberty. Furthermore, in the two KS boys started on T substitution, LH as well as INSL3 levels decreased markedly, verifying the dependence of INSL3 on LH stimulation during puberty. The gene for the INSL3 receptor LGR8 is expressed in the pituitary (14), but neither the present study nor previous studies (3, 4, 10) have shown a negative feedback regulation by INSL3 on LH secretion.

In KS boys, after an initial rise in serum INSL3 concentrations at onset of puberty, a tendency for a leveling off in the concentrations occurred despite stimulation by increasing LH levels. This is consistent with the histological findings in our earlier study (6); at onset of puberty, as the juvenile Leydig cells were transformed to adult type cells, they also gradually became hypertrophic. However, whereas Leydig cell function after onset of puberty remains within the low normal range as indicated by INSL3 and T levels, inhibin B secretion decreases very rapidly (6, 7, 15), reflecting the rapid degeneration of the seminiferous tubules.

Earlier studies have suggested that INSL3 is more sensitive than T to Leydig cell dysfunction (3, 4, 10). Actually we have shown that during puberty, T levels in KS boys did not differ from those in healthy boys during puberty (7). In that study we concluded that KS boys do not require androgen substitution until midpuberty, when other subtle signs of androgen deficiency become evident (7). Our observations in the present study on INSL3 concentrations in these KS boys support that finding.

In conclusion, our study shows that serum INSL3 concentrations may serve as novel markers for onset and normal progression of puberty. Secretion of INSL3 was dependent on LH, and during pubertal development, there existed a strong positive correlation with serum LH and T levels as well as testicular volume. In KS subjects, INSL3 measure-

ments also indicate impaired Leydig cell function from mid-puberty onward.

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