Endocr (2007) 31:18–26
DOI 10.1007/s12020-007-0009-8

ORIGINAL PAPER

In vitro biological-to-immunological ratio of serum gonadotropins throughout male puberty in children with insulin-dependent diabetes mellitus

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Published online: 10 May 2007

Abstract Information on the impact of prolonged deficient glycemic control in the quality of the gonadotropin signal delivered by the pituitary gland during puberty in children with insulin-dependent diabetes mellitus (IDDM) is scarce. In the present study, we examined the impact of deficient glycemic control on bioactive LH and FSH concentrations and their corresponding biological-to-immunological (B:I) ratio in boys with poorly controlled, but systemically uncomplicated IDDM. Dual control groups comprising patients with well-controlled IDDM and healthy boys of comparable age and body mass index were included for appropriate comparisons within and between each pubertal stage. Patients with poorly controlled and well-controlled IDDM exhibited serum bioactive FSH levels and B:I FSH ratio similar to those showed by the healthy control group. In contrast, in early and mid-pubertal boys with poorly controlled IDDM bioactive LH levels were normal, but its B:I LH relationship was significantly ($P < 0.05$) decreased. This attenuation in the quality of the LH signal did not affect total serum T concentrations, and apparently, progression of puberty. Long-standing uncontrolled diabetes and the consequent metabolic disturbances and/or complications may aggravate the reproductive axis dysfunction and eventually provoke pubertal arrest.

Keywords Puberty · Diabetes Mellitus · LH · FSH · Bioactivity

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Introduction

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are members of the glycoprotein hormone family that also includes thyrotropin and chorionic gonadotropin. These glycoproteins consist of a common α-subunit and a unique β-subunit, which confers receptor specificity [1–4]. Gonadotropins do not exist as a single post-translational molecular structure, but rather as an array of isoforms that are hormonally regulated [5]. In LH and FSH, carbohydrates play a major role in structure and function since they influence not only intracellular subunit folding and secretion of the heterodimer, but also its circulatory survival, receptor binding, and capacity to evoke signal transduction at the receptor level [5, 6]. The number and relative abundance of these isoforms will depend on the endocrine status of the donor at the time of collection of the tissue or sample [5–10].

The onset of puberty is associated with striking hormonal changes that include a progressive increase in the circulating concentrations of gonadotropins and sex steroids, resulting from the re-awakening of the GnRH

pulse generator [11]. During normal puberty, both immunoactive and bioactive LH and FSH concentrations increase gradually, being more evident at the initial stages [12–14]. In both experimental animals and humans, it has been documented that the increase in bioactive gonadotropin levels is associated with biochemical modifications in the oligosaccharide structure of gonadotropins, which in turn leads to changes in the circulating gonadotropin isoform mixture and its in vitro biological-to-immunological (B:I) ratio [5, 15–17]. The recognition in experimental animals and in humans that GnRH and sex steroid hormones modify the charge isoform distribution of gonadotropins, suggests that progressive exposure of the pituitary to these factors is responsible for the changes in gonadotropin glycosylation and for the efficiency of the gonadotropin signal to initiate and/or maintain gonadal function during puberty.

Children suffering from chronic diseases, generally present delayed onset of puberty or pubertal arrest with reduced growth spurt; the basis for these abnormalities are multifactorial [18–20]. In patients with insulin-dependent, type 1 diabetes mellitus (IDDM) the function of the reproductive axis is altered in a variable extent; in fact, this metabolic disorder may delay the onset of sexual maturation in children [21–27]. Recently, López-Alvarenga and colleagues [28], documented that the hypothalamic-gonadotrope axis is severely compromised in young men with poorly controlled, but systemically uncomplicated IDDM and that, paradoxically, the reduced gonadotropin secretion is compensated by an increase in circulation of gonadotropins enriched in biological activity, which are apparently sufficient to temporarily maintain normal levels of sex steroids. To date, there is no information on the impact of prolonged deficient glycemic control in immunoactive and bioactive serum gonadotropin levels during puberty in children with IDDM, or whether, if present, the altered gonadotropic stimulus may potentially affect serum testosterone levels and the onset or progression of puberty.

In the present study, we examined the impact that deficient glycemic control has on the quality of gonadotropins present in the circulation of pubertal IDDM boys, measured by in vitro bioassays. Patient selection was restricted to boys with poorly controlled, but systemically uncomplicated IDDM (IDDM-U) in whom their pubertal stage at the time of the study was accordant with their chronological age. In addition, dual control groups comprising patients with well-controlled IDDM (IDDM-C) and healthy boys of comparable age and body mass index were included for appropriate comparisons within and between each pubertal stage.

Results

Table 1 summarizes basal serum immunoreactive gonadotropins and testosterone (T) levels in the groups studied. In healthy boys, baseline serum gonadotropin and T levels increased progressively throughout puberty. The first significant increase in all three hormones was observed early in puberty and maximal values were detected at Tanner stages (Ts)-3 (T levels), -4 (FSH levels), or -5 (LH levels). In IDDM patients, maximal T levels were attained at Ts-4 (IDDM-C group) or Ts-5 (IDDM-U group), whereas serum gonadotropin levels peaked at Ts-2 (FSH) and Ts-3 or -4 (LH). In each group, there was a considerably large inter-individual variations in hormone concentrations despite its fairly homogeneous composition.

Serum from each subject was tested for LH and FSH bioactivity employing heterologous in vitro bioassay systems. Incubation of human embryonic kidney-293 (HEK-293) cells expressing the recombinant LH or FSH receptor (R) with increasing amounts of standard or unknown samples, induced significant and parallel dose-dependent cAMP accumulation (Fig. 1). In all groups, bioactive LH levels dramatically increased during Ts-2 and Ts-3 and then decreased to levels intermediate between those detected at Ts-1 and Ts-2 (Table 1). In contrast, bioactive FSH levels reached maximal values at Ts-2 or Ts-3 and remained constant during latter stages. Comparisons among the resulting relative B:I gonadotropin ratios across puberty revealed that the B:I LH ratio was higher in normal and IDDM subjects at Ts-2 and Ts-3 than in prepubertal children (Fig. 2A). However, this increase in relative B:I LH ratio was less pronounced in IDDM-U patients than in controls; in fact, median values of this ratio in Ts-2 and Ts-3 IDDM-U patients were significantly ($P < 0.05$) lower than in IDDM-C (at Ts-3) and normal controls (at both Ts-2 and Ts-3). An abrupt decline in the B:I LH ratio was observed with progression of puberty in all groups, with no significant differences among the groups at Ts-1, -4, and -5. In all groups, the B:I FSH ratio rose during early to mid-puberty; this increase, however, was less marked than that observed for LH, particularly in patients with IDDM (Fig. 2B). There were no significant differences in realtive B:I FSH ratio among IDDM patients and controls at the same pubertal stage.

Discussion

Several studies in experimentally induced and spontaneously occurring diabetes, have demonstrated that uncontrolled diabetes may potentially alter the function of the reproductive axis [29–34]. Men with uncontrolled IDDM

Table 1 Serum testosterone (T), immunoreactive (i) and bioactive (b) LH, and FSH levels (median and range) in healthy control boys and patients with controlled and uncontrolled IDDM

	Tanner stage 1			Tanner stage 2			Tanner stage 3			Tanner stage 4			Tanner stage 5		
	Subject group			Subject group			Subject group			Subject group			Subject group		
	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U
T (nmol/l)	$0.17^{A/a}$ (0.07–6.86)	$0.16^{A/a}$ (0.08–2.9)	$0.24^{A/a}$ (0.02–1.35)	$13.1^{B/a}$ (7.2–17.6)	$18.7^{B,C/a,b}$ (11.2–33.7)	$25.7^{B,C/b}$ (11.0–36.9)	$20.7^{C/a}$ (8.86–21.8)	$18.2^{B/a}$ (9.81–25.30)	$20.4^{B/a}$ (9.19–35.9)	$19.3^{C/a}$ (12.9–43.3)	$23.0^{C/a}$ (13.5–27.8)	$24.0^{B/a}$ (16.7–28.7)	$26.4^{C/a}$ (15.3–38.8)	$25.1^{C/a}$ (18.1–41.4)	$29.3^{C/a}$ (22.4–38.8)
iLH (IU/l)	$7.5^{A/a}$ (4.67–16.67)	$7.2^{A/a}$ (5.54–8.45)	$5.7^{A/a}$ (4.38–17.33)	$11.2^{A,B/a}$ (6.88–14.15)	$11.7^{B/a}$ (9.03–14.85)	$10.2^{B/a}$ (7.61–16.62)	$11.8^{A,B/a}$ (8.67–19.15)	$9.0^{C/a}$ (6.8–13.89)	$13.0^{B/a}$ (5.27–32.00)	$10.4^{A,B/a}$ (7.04–36.08)	$9.2^{C/a}$ (7.48–11.61)	$9.9^{B/a}$ (7.07–41.12)	$12.5^{B/a}$ (8.38–16.83)	$12.1^{B,C/a}$ (6.85–24.22)	$12.5^{B/a}$ (8.28–28.39)
bLH (IU/l)	$4.2^{A/a}$ (1.23–8.11)	$2.3^{A/a}$ (1.39–4.60)	$2.9^{A/a}$ (1.12–7.16)	$19.7^{B/a}$ (9.90–30.52)	$18.9^{B/a}$ (18.05–24.96)	$16.5^{B,C/a}$ (5.65–23.72)	$35.5^{B/a}$ (18.61–41.75)	$17.3^{B/a}$ (9.70–44.40)	$19.3^{B/a}$ (8.34–35.08)	$11.3^{B,C/a}$ (4.79–55.73)	$4.9^{C/a}$ (3.03–25.09)	$6.5^{B,C/a}$ (3.12–35.77)	$8.5^{C/a}$ (2.88–14.46)	$9.4^{C/a}$ (2.93–20.51)	$7.6^{C/a}$ (2.87–18.55)
iFSH (IU/l)	$2.3^{A/a}$ (1.16–7.10)	$1.8^{A/a}$ (0.25–3.43)	$2.7^{A/a}$ (0.13–7.86)	$4.4^{A/a}$ (1.32–6.63)	$8.6^{B/b}$ (3.16–14.23)	$4.9^{A/a,b}$ (3.60–15.16)	$4.5^{A/a}$ (2.05–9.81)	$3.8^{C/a}$ (1.83–8.71)	$3.7^{A/a}$ (2.97–6.81)	$5.8^{A/a}$ (3.51–7.06)	$4.7^{B,C/A}$ (1.70–8.63)	$2.9^{A/a}$ (1.65–8.90)	$4.0^{A/a}$ (1.77–5.46)	$4.1^{B,C/a}$ (2.26–10.45)	$4.8^{A/a}$ (3.45–7.95)
bFSH (IU/l)	$0.40^{A/a}$ (0.07–0.55)	$0.41^{A/a}$ (0.24–1.85)	$0.34^{A/a}$ (0.17–0.81)	$1.3^{B/a}$ (0.37–7.75)	$1.9^{B/a}$ (0.75–5.61)	$1.6^{B/a}$ (0.33–10.05)	$3.5^{C/a}$ (2.56–5.27)	$2.1^{B/b}$ (1.41–3.67)	$2.4^{B/a,b}$ (2.07–5.22)	$3.5^{B,C/a}$ (0.89–6.53)	$1.3^{B/a}$ (0.81–3.29)	$1.0^{B/a}$ (0.61–2.57)	$1.9^{B/a}$ (0.95–2.27)	$2.6^{B/a}$ (0.69–5.66)	$2.4^{B/a}$ (0.70–4.89)

$N = 7$ subjects per group and per Tanner stage

Different superscript letters indicate the existence of significant ($P < 0.05$) differences between (capital letters) pubertal stages and within (small letters) each stage

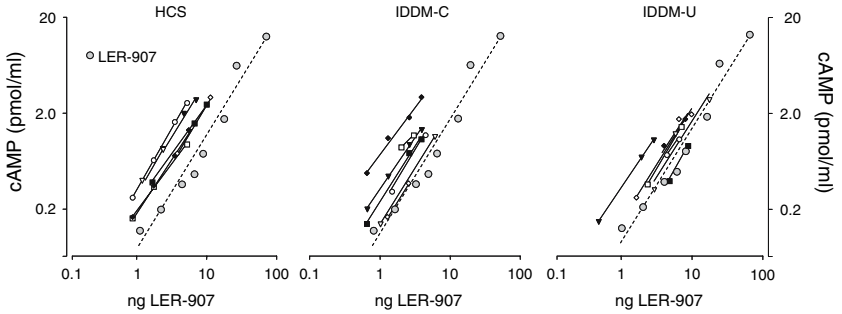

Fig. 1 Impact of increasing concentrations of bioactive LH in serum samples from healthy boys (HCS), IDDM-C, and IDDM-U patients in Tanner stage-3 to elicit cAMP production by HEK-293 cells transfected with the full-length recombinant rat LH receptor. Each symbol represents a single subject. Responses were shifted to the right in the IDDM-U group

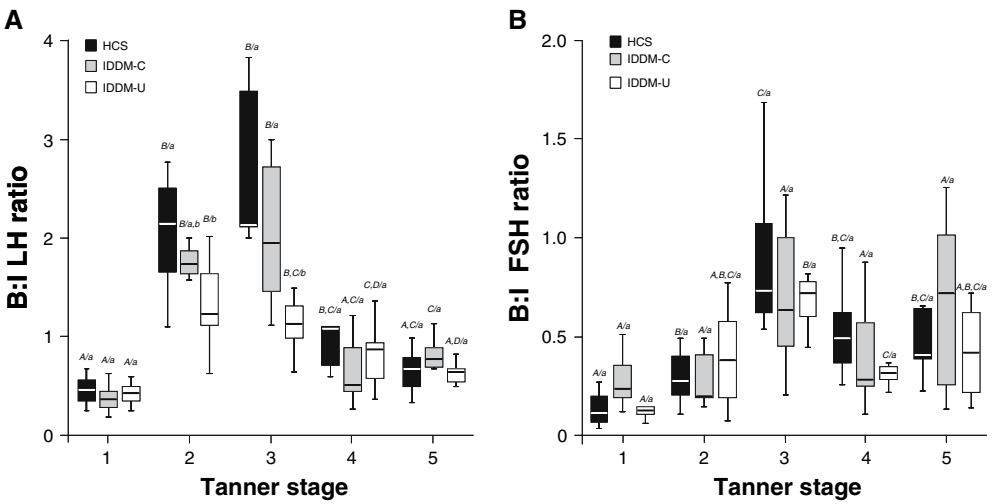

Fig. 2 Biological-to-immunological (B:I) LH (A) and FSH (B) ratio in healthy pre-pubertal and pubertal boys and patients with IDDM. Box plots represent the median (horizontal lines), quartiles (boxes), and extreme values for a single group within clusters defined by Tanner stage. The box length is the interquartile range (75%–25%). Different letters above bars indicate the existence of significant ($P < 0.05$) differences between (capital letters) pubertal stages and within (small letters) each stage. $N = 7$ subjects per group and per Tanner stage

present normal or low serum T and LH concentrations, and exhibit variable gonadotropin responses to exogenous GnRH [22, 27]. Reproductive impairment observed in men with diabetes could be the result of interference by the disease on the hypothalamo–pituitary–testicular axis at multiple levels [28]. This impairment could be due to changes in the net biological potency of both gonadotropins in response to multiple physiopathological processes, including severity and duration of hypoinsulinism and/or hyperglycemia, as well as presence of secondary metabolic disturbances (such as ketosis) that may accompany and potentially aggravate the effects of insulin deprivation on the hypothalamus, the pituitary and/or the testes [27, 33, 35].

The present cross-sectional study, attempted to analyze the effects of deficient diabetic control during male puberty on immunoactive and bioactive serum gonadotropin concentrations and their biological-to-immunological potency relationship, an index known to be strictly influenced by changes in the carbohydrate structure of the gonadotropin molecule [5]. In fact, measurement of the B:I gonadotropin ratio has been shown to be an useful parameter to detect changes in the mixture of circulating isoforms that occur in several conditions, including puberty [16, 17, 36], the menstrual cycle [7, 37–39], and senescence [40, 41], which are characterized by striking changes in sex steroid hormones and GnRH output.

Employing a heterologous in vitro bioassay system and a polyclonal antibody-based immunoassay with sufficient sensitivity to detect relatively low-serum gonadotropin concentrations, we found that in normal boys and IDDM patients serum immunoreactive and bioactive gonadotropin concentrations rose gradually during early and mid-puberty. However, the increment in bioactive LH and FSH concentrations exceeded their corresponding immunoreactive levels, leading consequently to a marked increase in the calculated B:I ratio during these pubertal stages. This observation agrees with previous studies showing that both plasma bioactive LH concentrations and the calculated B:I LH ratio increase across male puberty [42, 43], but not with studies employing monoclonal antibody-based immunoassay procedures [44, 45], thus emphasizing the potential effect of differential glycosylation on the reactivity of

glycoproteins toward certain antibodies [46–49]. The observed changes in relative in vitro bioactivity and B:I ratio of gonadotropins across puberty, may result from modifications in the carbohydrate structure of gonadotropins as documented by studies showing that both serum LH and FSH charge isoform distribution changes during early normal puberty in males [16, 17], due presumably to progressive exposure to sex steroid hormones and/or GnRH [38, 41, 50–58].

In contrast to the pattern observed in normal controls and IDDM-C patients, boys at Ts-2 and Ts-3 with uncontrolled IDDM exhibited significantly lower relative B:I LH ratios. Interestingly, it is during these pubertal stages when both the reactivation of the hypothalamic–pituitary–gonadal axis and pubertal growth spurt occur [59–61]. This decrease in net relative in vitro biopotency in IDDM-U could explain, in part, the previously noticed delayed and/or slow progression in sexual development and growth showed by chronically uncontrolled IDDM children [62–64]. The fact that the IDDM-U patients studied herein did not exhibit either pubertal delay, growth arrest, or decreased serum T levels may be due to the relatively short period of glycemic lack of control and/or the absence of acute complications such as ketosis. The factor(s) responsible for the altered B:I LH ratio in poorly controlled IDDM is unknown. Nevertheless, changes in this relationship could reflect abnormal post-translational processing of the LH molecule. In fact, glycosylation and sulfation of terminal oligosaccharide residues control the in vivo metabolic clearance and the in vitro biopotency of human LH [5, 6, 65]. In this scenario, the decreased in vitro B:I ratio in a setting of normal serum bioactive LH levels in uncontrolled IDDM pubertal boys, could result from the retention in the circulation of immunoreactive LH species with decreased potency at the target cell level. Further, a sustained exposure to a glucose-enriched endogenous milieu may promote non-enzymatic glycation of LH molecules, rendering isoforms with reduced functional properties, as previously observed for glycated insulin species in type 2 diabetes [66].

Our finding of decreased relative B:I LH ratio in poorly controlled IDDM contrasts with a previous study in which in vitro bioassay of LH revealed elevated values in young adult men with uncontrolled diabetes mellitus [28]. The precise basis for this latter contrast is not known. Plausible explanations include (1) differences in age and thus in the maturity of mechanisms controlling gonadotropin synthesis, glycosylation, and secretion; (2) use of different sampling protocols (single vs. pooled samples) and/or (3) differences in the in vitro bioassays employed (heterologous vs. homologous recombinant receptor bioassay systems). Whatever the reason for these apparent discrepancies, the overall data indicate that the B:I LH ratio and thus the glycosylation of the gonadotropin molecule, may be susceptible to alterations by relatively prolonged hypoinsulinism.

In summary, the B:I LH relationship is altered in early and mid-pubertal boys with poorly controlled, but systemically uncomplicated IDDM. This attenuation in the net biopotency of the LH signal does not affect total serum T concentrations and apparently, progression of puberty, which may be explained by the absence of systemic complications, preserved LH-independent compensatory mechanisms and/or residual insulin activity. Long-standing uncontrolled diabetes and the consequent metabolic disturbances and/or complications may alter the function of the reproductive axis and eventually provoke pubertal arrest.

Subjects and methods

Subjects

A total of 105 prepubertal [Tanner stage (Ts) -1), pubertal (Ts-2 to -4) and postpubertal (Ts-5) male subjects, classified on the basis of testicular volume [volume = $\pi/6$ (largest diameter × smallest diameter^2)] [67] agreed to participate in the study. Informed written consent from each patient and/or his parents was obtained. The study protocol was approved by the Institute's Human Research Committee.

Three different groups of subjects were included. The primary cohort comprised 35 IDDM subjects, aged 11–16 years with poor (but nonketotic) glycemic control (IDDM-U group), defined as glycated hemoglobin A1c levels ≥10% over at least the preceding 3 months (Table 2). Daily insulin doses (administered in a 1 or 2 injection of intermediate insulin/day) ranged from 1.03 to 1.69 U/Kg. A disease-specific control group included 35, 9–17 years old IDDM patients, with optimal or suboptimal metabolic control (IDDM-C group), defined as glycated hemoglobin A1c levels <8% over at least the preceding 3 months. Daily insulin requirements for this group ranged from 0.84 U/Kg to 1.55 U/Kg. None of the IDDM patients were taking medication that could potentially alter serum gonadotropin levels and none had clinical or biochemical evidence of proliferative retinopathy, renal or thyroid disease. A healthy control cohort (HCS group) included 33 normal boys or young adult men, aged 11–23 years with normal glycated hemoglobin concentrations. Subjects in this group were selected among boys attending our endocrine outpatient clinic for non-pathological conditions (i.e., constitutional short stature) or relatives of others patients.Insulin-dependent diabetes mellitus patients were included only when two HbA1C concentration values,

Table 2 Age, HbA1c, years since diagnosis, testicular volume, and body mass index (BMI) (mean ± SD) in healthy control boys and patients with controlled and uncontrolled IDDM

	Tanner stage 1			Tanner stage 2			Tanner stage 3			Tanner stage 4			Tanner stage 5		
	Subject group			Subject group			Subject group			Subject group			Subject group		
	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U	HCS	IDDM-C	IDDM-U
Age (years)	10.7 (±1.1)^{a}	8.8 (±1.1)^{a}	10.7 (±1.1)^{a}	12.3 (±0.6)^{a}	13.3 (±1.1) ^{a}	14.7 (±0.3)^{b}	14.5 (±0.9)^{a}	13.9 (±1.8)^{a}	15.7 (±1.4)^{a}	16.3 (±0.9)^{a}	15.0 (±0.7)^{a}	14.7 (±1.1)^{a}	22.9 (±1.5)^{a}	16.9 (±3.9)^{a,b}	16.2 (±1.1)^{b}
HbA1c (%)	5.1 (±0.3)^{a}	6.6 (±0.9)^{b}	11.2 (±0.3)^{c}	5.4 (±0.3)^{a}	7.5 (±0.8)^{b}	13.0 (±1.8)^{c}	5.7 (±0.4)^{a}	7.6 (±0.6)^{b}	10.9 (±2.4)^{c}	5.6 (±0.2)^{a}	5.8 (±0.7)^{a}	11.3 (±0.5)^{b}	5.5 (±0.2)^{a}	7.4 (±0.9)^{b}	11.5 (±1.4)^{c}
Years since diagnosis	–	4.0 (±2.5)^{a}	4.9 (±1.8)^{a}	–	2.4 (±2.3)^{a}	8.2 (±3.5)^{a}	–	1.1 (±4.5)^{a}	11.2 (±3.1)^{b}	–	3.0 (±1.3)^{a}	5.0 (±2.9)^{a}	–	7.9 (±5.2)^{a}	4.2 (±2.3)^{a}
TV[&] (cm^3)	3.0 (±0.6)$^{A/a}$	0.9 (±0.1)$^{A/b}$	1.1 (±0.2)$^{A/b}$	6.0 (±0.6)$^{B/a}$	5.7 (±1.0)$^{B/a}$	6.7 (±0.7)$^{B/a}$	10.2 (±0.4)$^{C/a}$	10.0 (±0.6)$^{C/a}$	10.3 (±0.6)$^{C/a}$	12.7 (±0.7)$^{D/a}$	12.3 (±0.4)$^{D/a}$	13.3 (±0.5)$^{D/a}$	20.5 (±1.5)$^{E/a}$	18.9 (±2.0)$^{E/a}$	18.5 (±1.3)$^{E/a}$
BMI (Kg/m^2)	18.1 (±2.6)^{a}	16.7 (±1.2)^{a}	17.6 (±2.3)^{a}	19.2 (±1.5)^{a}	20.9 (±1.4)^{a}	19.6 (±1.1)^{a}	20.2 (±0.9)^{a}	18.7 (±1.4)^{a}	20.0 ±1.8^{a}	23.0 (±2.2)^{a}	20.6 (±1.7)^{a}	19.6 (±2.6)^{a}	24.2 (±0.5)^{a}	24.0 (±1.8)^{a}	23.2 (±2.5)^{a}

$N = 7$ subjects per group and per Tanner stage

[&] Testicular volume

Different superscript letters indicate the existence of significant ($P < 0.05$) differences between (capital letters) pubertal stages and within (small letters) each stage

measured in samples obtained in two consecutive visits to our outpatient clinic, were above or below the limits pre-established in the study design (≥10% for the IDDM-U group and <8% for the IDDM-C group). No IDDM patients with glycemia <3.9 mmol/l or > 16.8 mmol/l on the day of study were included.

Blood samples (7 ml each) were collected from an antecubital vein, after an overnight fast. Samples were allowed to clot at room temperature for 30 min, and then were centrifuged at 1,000 × *g* for 10 min. Aliquots from each individual serum sample were stored frozen at –20°C until later assay.

Methods

In vitro bioassay of human LH and FSH

In vitro LH and FSH bioassays and RIA were applied separately to single serum samples. These in vitro bioassays monitor cAMP production by human embryonic kidney-293 (HEK-293) cells stably transfected with either the full-length rat LH receptor or the rat FSHR. The recombinant LH and FSH receptors cloned into the pcDNA3.1/V5-His-TOPO-TA expression vector were generated as described elsewhere [68]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA), pH 7.3, supplemented with 2.5% (LHR bioassay system) or 5% (FSHR-based assay) fetal calf serum (Gibco, BRL), 2 mmol/l L-glutamine (Sigma Chemicals Co, St Louis, MO, EUA), 100 mg/ml geneticin (Life Technologies), 50 IU (LH bioassay) or 100 IU (FSH bioassay)/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co, St. Louis, MO, USA), and grown in 162-cm^2 flasks (Costar, Cambridge, MA, USA). Confluent cells were scraped and plated in 24-well culture plates (Costar) for 24 h at 37°C in 5% CO_2. Cells (5×10^4 cells/culture dish) were cultured for 24 h, and then washed with unsupplemented DMEM and exposed to increasing doses of either each sample or the LER-907 reference standard (National Hormone and Peptide Program, Torrance, CA) in the presence of 0.125 (LH bioassay) or 0.2 (FSH bioassay) mmol/l 1-methyl-3-isobutyl-xanthine (Sigma) dissolved in 400 μl of DMEM supplemented with fetal calf serum, L-glutamine and antibiotics for 24 h at 37°C. Samples (unknowns and standards) were diluted with serum from women treated with oral contraceptives that contained LH and FSH immuno- and bioactivities not distinguishable from the zero dose, such that the concentration (vol/vol) of serum in each sample did not exceed 10% (LH bioassay) or 20% (FSH bioassay). To avoid the interference of serum factors in the FSH bioassay, serum samples were heated at 56°C for 10 min [69]. After incubation, the media and cells were boiled at 95°C for 3 min and stored frozen at –20°C until the cAMP assay. The minimal detectable responses of the assays were 0.075/well mIU LER-907/tube for LH bioactivity and 0.25 mIU/well for FSH bioactivity. Each sample was assayed in duplicate incubations and at 2–3 different doses (12.5–75 μl per sample) to ascertain for parallelism with the standard curve. Between- and within-assay coefficients of variation at the 50% effective dose were <18% and <10% for the LHR bioassay system and <17% and <10% for the FSHR-based bioassay, respectively.

Total (intra-plus extracellular) cAMP was measured by RIA after acetylation of samples, as previously described [70]. The sensitivity of the assay was 4 fmol/tube and the between- and within-assay coefficients of variation ranged were <11% and <7%. Mean bioactive LH and FSH was calculated by interpolation of at least two different concentration doses assayed. Data are expressed as the mean relative B:I activity ratio (i.e., the biopotency of LH or FSH exhibited by the unknown samples in the in vitro bioassays expressed as a function of total (immunoreactive) hormone present in the circulation), calculated after conversion of the results to IU/L of the 2nd IRP-HMG). Considered as a functional parameter, changes in in vitro B:I ratio mirror variations in the relative in vitro biopotency of a given sample at the target cell level [9, 71]. As previously reported [5, 9, 71], changes in this ratio is a reliable indicator of the occurrence of structural modifications in the carbohydrate structure of the gonadotropin molecule.

LH, FSH, and testosterone immunoassays

Aliquots (12.5–100 μl) of samples from each subject were analyzed for immunoreactive LH and FSH content by previously reported [72, 73] radioimmunoassay (RIA) procedures. Briefly, the RIA of LH was performed employing ^{125}I-labeled LH-I3 as the tracer (specific activity 70–90 μCi/μg protein), the reference LH preparation LER-907 as the standard, and the antiserum for human LH-3 at a final dilution of 1:800,000 [19]. Cross-reactivity of this antiserum with highly purified FSH, growth hormone, and prolactin is <0.2%. The sensitivity of the assay was 0.70 IU/l as expressed in terms of the Second International Reference Preparation of human menopausal gonadotropins [(2nd IRP-HMG); 1 mg LER 907 = 277 IU]. The FSH RIA was performed employing ^{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 at a final dilution of 1:250,000 as the antiserum [73]. This antiserum exhibits less than 0.1% cross-reactivity with highly purified human LH and prolactin, and undetectable reactivity with free α-subunit and growth hormone. The sensitivity of the assay was 0.24 mIU/tube (2nd IRP-HMG;

1 mg LER-907 = 53 IU)]. All LH and FSH RIA reagents were generously provided by the National Hormone and Peptide Program. In both RIAs, the within- and between-assay coefficients of variation were determined using multiple replicates (3 replicates per dose) of a serum pool collected from postmenopausal women, assayed at dose levels that displaced the corresponding tracers from the antibody at 10–20%, 45–60%, and 75–90% total binding; these coefficients ranged from 4–6% to 6–15%, respectively.

Serum testosterone levels were determined in duplicate by time-resolved immunofluorometric assay (IFMA) (Delfia, PerkinElmer, Mexico) [74, 75]. The sensitivity of the assay was 0.3 nmol/l. To avoid interassay variations, samples from subjects in same pubertal stage were analyzed in the same assay run. The within- and between-assay coefficients of variation were <5.0% and <7.5%, respectively.

Statistical analysis

Given the relatively small sample size in each pubertal stage (7 subjects *per* Tanner stage) and that the different measurements did not fit a normal distribution (according to the Kolmogorov–Smirnov test for normality), differences between groups were calculated employing non-parametric statistics (Kruskall–Wallis one-way analysis of variance and the Mann–Whitney test for post-hoc contrasts). Probabilities <0.05 were considered statistically significant.

Acknowledgments This study was supported by grant IMSS-2002/189 from the FOFOI-Instituto Mexicano del Seguro Social, México (to Daniela Söderlund.). Alfredo Ulloa-Aguirre is recipient of a Research Career Development Award from the Fundación IMSS, México.

References

1. J.G. Pierce, T.F. Parsons, Annu. Rev. Biochem. **50**, 465 (1981)
2. J.C. Fiddes, K. Talmadge, Recent. Program. Horm. Res. **40**, 43 (1984)
3. L.A. Bishop, T.V. Nguyen, P.R. Schofield, J. Clin. Endocrinol. Metab. **136**, 2635 (1995)
4. A. Ulloa-Aguirre, E. Zambrano, C. Timossi, A. Olivares, A. Quintanar, M. Aguinaga, L. Díaz-Cueto, J.P. Méndez, Arch. Med. Res. **26**(Supl), 219 (1995)
5. A. Ulloa-Aguirre, R. Midgley, I.Z. Beitins, V. Padmanabhan, Endocr. Rev. **16**, 765 (1995)
6. A. Ulloa-Aguirre, A. Maldonado, P. Damián-Matsumura, C. Timossi, Arch. Med. Res. **32**, 520 (2001)
7. E. Zambrano, A. Olivares, J.P. Méndez, L. Guerrero, L. Díaz-Cueto, J.D. Veldhuis, A. Ulloa-Aguirre, J. Clin. Endocrinol. Metab. **80**, 1647 (1995)
8. A. Ulloa-Aguirre, C. Timossi, P. Damián-Matsumura, J.A. Días, Endocrine **11**, 205 (1999)
9. A. Ulloa-Aguirre, C. Timossi, J.P. Méndez, Hum. Reprod. **16**, 599 (2001)
10. J.A. Días, Hum. Reprod. **16**, 825 (2001)
11. T.M. Plant, M.L. Barker-Gibb, Hum. Reprod. Update. **10**, 67 (2004)
12. I.M. Burr, P.C. Sizonenko, S.L. Kaplan, M.M. Grumbach, Pediatr. Res. **4**, 25 (1970)
13. Z. Dickerman, R.J. Barkey, R. Prager-Lewin, F. Gahnem, Z. Laron, Horm. Metab. Res. **21**, 42 (1989)
14. A. Olivares, D. Söderlund, C. Castro-Fernández, T. Zariñán, E. Zambrano, J.P. Méndez, A. Ulloa-Aguirre, Endocrine **23**, 189 (2004)
15. J.Z. Kasa-Vubu, V. Padmanabhan, G.B. Kletter, M.B. Brown, E.O. Reiter, P.C. Sizonenko, I.Z. Beitins, Pediatr. Res. **34**, 829 (1993)
16. D.J. Phillips, L. Wide, J. Clin. Endocrinol. Metab. **79**, 814 (1994)
17. D.J. Phillips, K. Albertsson-Wikland, K. Eriksson, L. Wide, J. Clin. Endocrinol. Metab. **82**, 3103 (1997)
18. S. Valenti, M. Giusti, D. McGuinness, R. Guido, P.G. Mori, G. Giordano, K.D. Dahl, Eur. J. Endocrinol. **133**, 48 (1995)
19. C. Castro-Fernández, A. Olivares, D. Söderlund, J.C. López-Alvarenga, E. Zambrano, J.D. Veldhuis, A. Ulloa-Aguirre, J.P. Méndez, J. Clin. Endocrinol. Metab. **85**, 4603 (2000)
20. T.L. Chambers, C.R. Buchanan, J. Royal. Coll. Phys. London. **34**, 141 (2000)
21. M. Salerno, A. Argenziano, S. Di Maio, N. Gasparini, S. Formicola, G. De Filippo, A. Tenore, Diab. Care. **20**, 721 (1997)
22. J.C. Daubresse, J.C. Meunier, J. Wilmotte, A.S. Luyckx, P.J. Lefebvre, Diab. Metab. **4**, 233 (1978)
23. A. Zeidler, R. Gelfand, J. Draus, J.K. Tauscher, R.T. Chopp, Fertil. Steril. **35**, 653 (1981)
24. A. Zeidler, R. Gelfand, E. Tamagna, R. Marrs, R. Chopp, O. Kletzky, Andrology **14**, 62 (1982)
25. F.T. Murray, H.U. Wyss, R.G. Thomas, M. Spevack, A.G. Glaros, J. Clin. Endocrinol. Metab. **65**, 127 (1987)
26. H. Fushimi, H. Horie, T. Inoue, M. Kameyama, K. Kanao, S. Ishihara, T. Tsujimura, H. Nunotani, T. Minami, Y. Okazaki, Diab. Res. Clin. Pract. **6**(Suppl 2), 297 (1989)
27. M. Valimaki, K. Liewendahl, P. Nikkanen, R. Pelkonen, Scand. J. Clin. Lab. Invest. **51**, 385 (1991)
28. J.C. López-Alvarenga, T. Zariñán, A. Olivares, J. González-Barranco, Veldhuis J.D., A. Ulloa-Aguirre, J. Clin. Endocrinol. Metab. **87**, 5507 (2002)
29. Q. Dong, R.M. Lazarus, L.S. Wong, M. Vellios, D.J. Handelsman, J. Endocrinol. **131**, 49 (1991)
30. S. Sudha, G. Valli, P.M. Julie, J. Arunakaran, P. Govindarajulu, K. Balasubramanian, Exp. Clin. Endocrinol. Diabetes **108**, 14 (2000)
31. T. Tanaka, S. Nagatani, D.C. Bucholtz, S. Ohkura, H. Tsukamura, K. Maeda, D.L. Foster, Biol. Reprod. **62**, 1256 (2000)
32. M. Tanaka, S. Nakaya, T. Kumai, M. Watanabe, N. Matsumoto, S. Kobayashi, Endocr. Res. **27**, 109 (2001)
33. D.C. Bucholtz, A. Chiesa, W.N. Pappano, S. Nagatani, H. Tsukamura, K.I. Maeda, D.L. Foster, Biol. Reprod. **62**, 1248 (2000)
34. P. Kovacs, A.F. Parlow, G.B. Karkanias, Neuroendocrinology **76**, 357 (2002)
35. I. Pitton, G.E. Bestetti, G.L. Rossi, Andrologia **19**, 464 (1987)
36. D.D. Zalesky, H.E. Grotjan, Biol. Reprod. **44**, 1016 (1991)
37. C.J. Anobile, J.A. Talbot, S.J. McCann, V. Padmanabhan, W.R. Robertson, Mol. Hum. Reprod. **4**, 631 (1998)
38. V. Padmanabhan, L.L. Lang, J. Sonstein, R.P. Kelch, I.Z. Beitins, J. Clin. Endocrinol. Metab. **67**, 465 (1988)
39. L. Wide, O. Bakos, J. Clin. Endocrinol. Metab. **76**, 885 (1993)

40. S. Creus, E. Pellizzari, S.B. Cigorraga, S. Campo, Clin. Endocrinol. (Oxf). **44**, 181 (1996)
41. L. Wide, T. Naessen, Clin. Endocrinol. (Oxf). **40**, 783 (1994)
42. E.O. Reiter, M.L. Dufau, A.W. Root, K.J. Catt, Pediatr. Res. **12**, 330 (1978)
43. G.B. Kletter, V. Padmanabhan, M.B. Brown, E.O. Reiter, P.C. Sizonenko, I.Z. Beitins, J. Clin. Endocrinol. Metab. **76**, 432 (1993)
44. I. Huhtaniemi, Y.Q. Ding, R. Tahtela, M. Valimaki, J. Clin. Endocrinol. Metab. **75**, 1442 (1992)
45. I.T. Huhtaniemi, A.M. Haavisto, R. Anttila, M.A. Siimes, L. Dunkel, Pediatr. Res. **39**, 180 (1996)
46. M.J. Papandreou, I. Sergi, M. Benkirane, C. Ronin, Mol. Cell. Endocrinol. **73**, 15 (1990)
47. M.J. Papandreou, I. Sergi, G. Medri, C. Labbe-Jullie, J.M. Braun, C. Canonne, C. Ronin, Mol. Cell. Endocrinol. 78, 137 (1991)
48. C. Labbe-Jullie, I. Sergi, C. Canonne, H. Darbon, C. Ronin, Endocrinology **131**, 1999 (1992)
49. M. Zerfaoui, C. Ronin, Eur. J. Clin. Chem. Clin. Biochem. **34**, 749 (1996)
50. S.B. Rulli, S. Creus, E. Pellizzari, S.B. Cigorraga, R.S. Calandra, S. Campo, Neuroendocrinology. **63**, 514 (1996)
51. L.J. Wide, Clin. Endocrinol. Metab. **55**, 682 (1982)
52. L. Wide, T. Naessen, D.J. Phillips, Clin. Endocrinol. (Oxf). **42**, 59 (1995)
53. K.D. Dahl, M.P. Stone, J. Androl. **13**, 11 (1992)
54. A.R. Solano, A. García-Vela, K.J. Catt, M.L. Dufau, Endocrinology **106**, 1941 (1980)
55. J.D. Veldhuis, M.L. Dufau, J. Clin. Invest. **80**, 631 (1987)
56. J.D. Veldhuis, M.L. Johnson, M.L. Dufau, J. Clin. Endocrinol. Metab. **64**, 1275 (1987)
57. P. Damián-Matsumura, V. Zaga, A. Maldonado, C. Sánchez-Hernández, C. Timossi, A. Ulloa-Aguirre, J. Mol. Endocrinol. **23**, 153 (1999)
58. S.M. Dharmesh, J.U. Baenziger, Proc. Natl. Acad. Sci. USA **90**, 11127 (1993)
59. G. Van Vliet, Horm. Res. **36**, 93 (1991)
60. E.O. Retter, M.M. Grumbach, Annu. Rev. Physiol. **44**, 595 (1982)
61. F.C.W. Wu, G.E. Butler, C.J.H. Kelnar, I. Huhtaniemi, J.D. Veldhuis, J. Clin. Endocrinol. Metab. **81**, 1798 (1996)
62. M.V.L. Du Caju, R.P. Rooman, L. Op de Beeck, Pediatr. Res. **38**, 607 (1995)
63. M.L. Ahmed, M.H. Connors, N.M. Drayer, J.S. Jones, D.B. Dunger, Diab. Care. **21**, 831 (1998)
64. R.W. Holl, M. Grabert, E. Heinze, W. Sorgo, K.M. Debatin, Eur. J. Pediatr. **157**, 972 (1998)
65. J.A. Summerfield, J. Vergalla, E.A. Jones, J. Clin. Invest. **69**, 1337 (1982)
66. S.J. Hunter, A.C. Boyd, F.P. O'Harte, A.M. McKillop, M.I. Wiggam, M.H. Mooney, J.T. McCluskey, J.R. Lindsay, C.N. Ennis, R. Gamble, B. Sheridan, C.R. Barnett, H. McNulty, P.M. Bell, P.R. Flatt, Diabetes **52**, 492 (2003)
67. W.A. Marshall, J.M. Tanner, Arch. Dis. Child. **45**, 13 (1970)
68. C. Castro-Fernández, G. Maya-Núñez, J.P. Méndez, Endocrine **25**, 49 (2004)
69. T. Gudermann, H. Brockmann, M. Simoni, J. Gromoll, E. Nieschlag, Endocrinology **135**, 2204 (1994)
70. E. Zambrano, T. Zariñán, A. Olivares, J. Barrios-de-Tomasi, A. Ulloa-Aguirre, Endocrine **10**, 113 (1999)
71. M. Bergendah, J.D. Veldhuis, Hum. Reprod. **16**, 1058 (2001)
72. A. Olivares, M. Cárdenas, C. Timossi, T. Zariñán, V. Díaz-Sánchez, A. Ulloa-Aguirre, Hum. Reprod. **15**, 2285 (2000)
73. T. Zariñán, A. Olivares, D. Söderlund, J.P. Méndez, A. Ulloa-Aguirre, Hum. Reprod. **16**, 1611 (2001)
74. T. Lövgren, I. Hemmilä, K. Pettersson, J.U. Eskola, E. Bertolt, Talanta. **31**, 909 (1984)
75. I. Hemmilä, S. Dakubu, V.M. Mukkala, H. Siitari, T. Lövgren, Annal. Biochem. **137**, 335 (1984)