DEBATE—continued

Is there a physiological role for gonadotrophin oligosaccharide heterogeneity in humans?

III. Luteinizing hormone heterogeneity: a medical physiologist’s perspective

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Clinical evidence for a physiological impact of luteinizing hormone (LH) isoforms includes their unequal in-vitro bioactivity and altered in-vivo LH kinetics. For example, alkaline LH isospecies emerge in an oestrogen-rich milieu, and show greater bioactivity in vitro along with more rapid metabolic removal in vivo. More acidic LH isotypes are predominant in eugonadal men with end-stage renal failure and in postmenopausal women. The relevance of changes in charge distribution in puberty to sexual maturation is not clear. Molecular LH variants may be associated with decreased testis size and reduced linear growth in boys, menstrual irregularity and/or subfertility in women, and possibly protect against polycystic ovarian syndrome (PCOS). This article summarises the provisional physiological implications of LH isotypes based on current evidence.

Key words: bioassays/hormone physiology/LH isoforms/oligosaccharide heterogeneity

Introduction

Glycoprotein hormones display extensive post-translational polymorphism and a spectrum of in-vivo and in-vitro bioactivities, which vary with endocrine status (Dufau et al., 1976a,b; Reiter et al., 1978,1987; Lucky et al., 1979, 1980a; Rajalakshmi et al., 1979; Robertson et al., 1979; Lobo et al., 1984; Warner et al., 1985; Spratt et al., 1986; Dufau and Veldhuis, 1987; Veldhuis et al., 1989; Talbot et al., 1990; Fauser et al., 1991, 1992; Schaefer et al., 1991,1995; Imse et al., 1992; Matikainen et al., 1994; Mitchell et al., 1994, 1995; Huhtaniemi et al., 1996; Nagamani et al., 1999). Structural analysis indicates that follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyrotropin are synthesized and secreted as an array of molecular variants differing by their type and degree of carbohydrate additions. LH isoforms differ principally in the complexity and magnitude of their post-translational glycosylation, sialylation and sulphation modifications (Sairam and Fleschner, 1981; Rosa et al., 1984; Sardanons et al., 1987; Smith et al., 1990; Fiete et al., 1991). The resultant biochemical differences allow molecular separation by charge (Tsuruhara et al., 1972). Such properties also control in-vitro LH bioactivity, which can vary by as much as 10-fold and the in-vivo retention and tissue actions of LH, which can vary by 30-fold (Tsuruhara et al., 1972a,b; Dufau et al., 1976a,b; Wehmann and Nisula, 1979; Baenziger and Green, 1988; Bishop et al., 1995; Burgon et al., 1996). The synthesis of selected glycoforms of LH appears to be under multifactorial endocrine control, wherein gonadotrophin-releasing hormone (GnRH), oestradiol and testosterone play important regulatory roles (Dufau et al., 1977; Chen et al., 1982; Weise et al., 1983; Dufau and Veldhuis, 1987; Veldhuis et al., 1989; Clarke et al., 1990).

The most common method for determining LH bioactivity is in-vitro bioassay of plasma or pituitary extracts, based on testosterone secretion by mouse or rat interstitial (Leydig) cells (Dufau et al., 1976b; Lucky et al., 1979; Robertson et al., 1979; Marut et al., 1981; Huhtaniemi et al., 1996). By comparing LH concentrations obtained by biological and immunological [such as radioimmunoassay (RIA), immunoradiometric (IRM) or immunofluorometric assay (IFMA)] techniques in a given sample, one can also estimate the apparent bioactive to immunoreactive (B/I) LH ratio. This derived parameter has been used as an indirect index of the relative biopotency of LH, at least in relation to any one immunological estimate. The latter is an important consideration, inasmuch as large non-uniformities can exist among immunoassay technologies (Chappel, 1990; Jaaokola et al., 1990; Demers, 1991; Phillips and Wide, 1994; Clark et al., 1997). Subject to these constraints, and within any given validated assay system, a changing B/I ratio should mirror variations in the relative in-vitro biopotency of LH.

In-vitro biopotency may diverge several-fold from in-vivo LH biopotency, especially when LH’s metabolic clearance is altered by the post-translational addition of key residues, such as sulphate, sialic acid and/or galactosamine (Dufau and Veldhuis, 1987; Veldhuis et al., 1989; Burgon et al., 1996). Few studies have simultaneously appraised in-vitro
and in-vivo bioactivities of human LH as a function of variable isoform subtypes. One study utilized in-vitro Leydig cell responsiveness and in-vivo testosterone secretion in the rodent to compare the luteotropic effects of a wide gradation of human LH isotypes differing prominently by sialic acid and/or sulphate charge (Sardanons et al., 1987; Burgom et al., 1996). The degree of sialylation and sulphation strongly determined the whole-animal LH half-life, and thus influence in-vivo bioactivity more remarkably than in-vitro biopotency. For evident ethical reasons, analogous homologous-species comparisons have not been achievable using human Leydig cells in vitro and purified pituitary or plasma human LH preparations for injection in humans in vivo. However, such studies in the hypophysectomized sheep revealed a relatively small range of in-vivo LH biopotencies, at least in this particular ruminant species. Larger kinetic and potency differences emerged in the rat (Dufau et al., 1976b; Sairam and Fleschner, 1981; Clarke et al., 1990). The recent report of a human recombinant LH-receptor-based in-vitro bioassay of LH-stimulated alpha inhibin promoter-driven luciferase expression in HEK 293 cells and of a human granulosa-luteal cell in-vitro assay model could make homologous in-vitro bioassay of human LH more practicable (LH assay) (Huhtaniemi et al., 1993).

Envisioning the eventual availability of biosynthetically pure human LH isomers, we further suggest that a novel strategy for in-vivo homologous bioassay of human LH kinetics and actions could consist of GnRH antagonist-induced down-regulation of endogenous LH secretion followed by selective LH isoform infusion. One could then directly monitor LH elimination on the one hand and testosterone output on the other. This paradigm should clarify more expressly the relative in-vivo biopotencies of various human LH isotypes acting on the human gonad. Such studies would need to appraise the impact of LH isoforms on both acute and prolonged gonadal steroidogenesis in both sexes. Later analyses should also evaluate differential effects of FSH isotypes on gametogenesis in men and women.

Based on the above technical caveats, the following review of the physiological implications of LH isotypes must be viewed as provisional. Indeed, definitive physiological experiments in our view remain necessary to clarify several issues in this arena. Here, we will focus briefly on the following selected contexts of evident LH-isoform regulation in the human: (i) the adult male, (ii) the female menstrual cycle, (iii) the menopause, (iv) polycystic ovary syndrome (PCOS), (v) puberty, and (vi) in relation to molecular variants of LH polypeptide structure.

**LH isoforms in the normal adult male**

The B/I ratio of plasma LH is higher in men than women (Dufau et al., 1976a; Dufau and Veldhuis, 1987; Veldhuis et al., 1987b; Urban et al., 1988a,b,c; Veldhuis et al., 1989a,b), although serum immunoreactive LH concentrations are comparable (Dufau et al., 1976a). Bioactive LH tends to be secreted in high-amplitude pulses in young men and women, albeit at lower amplitudes in the female in the earlier follicular phase (Dufau et al., 1983; Veldhuis et al., 1984; Veldhuis and Dufau, 1993). The plasma B/I ratio of LH may rise towards the end of an endogenous LH secretory pulse (Dufau et al., 1976a, 1983; Veldhuis et al., 1987), although contradictory results arise in some assays (Spratt et al., 1986; Huhtaniemi et al., 1992). These non-uniformities might reflect different isoform specificities of the immunoreassays or in-vitro bioassays used (Rajalakshmi et al., 1979; Demers, 1991; Pettersson and Soderholm, 1991). This consideration could be tested by applying different assays to a library of plasma samples collected in different conditions of health and disease. For example, one study reported that low levels of apparent LH bioactivity remained in LH-depleted plasma (‘higher bioassay’ blank) (Spratt et al., 1986), whereas other analyses showed no detectable in-vitro LH bioactivity in LH-deficient human serum, but low levels of nonspecific polyclonal LH immunoreactivity (Veldhuis et al., 1987b, 1989a, 1992, 1994; Urban et al., 1988, 1990, 1991; Pavlou et al., 1990). Monoclonal-based epitope-specific sandwich-type immunoradiometric (IRM) and immunofluorometric (IFA) procedures along with homologous in-vitro human LH-receptor dependent bioassays will likely help clarify this technical issue.

Quantitative changes in plasma LH B/I ratios do not necessarily denote unequal secretion of bio-enriched versus bio-depleted isoforms of LH, but could also reflect their unequal rates of in-vivo metabolic interconversion or removal (Ashwell and Harford, 1982; Dufau and Veldhuis, 1987; Veldhuis et al., 1987a,b, 1989a,b; Pavlou et al., 1990; Burgom et al., 1996). In this regard, women show a rise in the LH B/I ratio postmenopaually (Dufau et al., 1976a; Reader et al., 1983; Davis et al., 1987; Urban et al., 1988a,b,c, 1990; Kolp et al., 1992), whereas ratios decrease or remain unchanged in ageing men (Marrama et al., 1984; Warner et al., 1985; Urban et al., 1988; Mitchell et al., 1995). In one study, mean plasma concentrations of bioactive LH and the calculated half-life of endogenous LH were similar in healthy older and young men (Urban et al., 1988a). However, in tamoxifen-treated older men, the amplitude (but not the frequency) of bioactive LH pulses failed to rise equivalently, suggesting an impaired reserve capacity for maximal bioactive LH secretion (Urban et al., 1988a; Veldhuis et al., 1989a). An age-related distinction in bioactive LH secretory responsiveness was also evident in response to short-term antiandrogen administration (Veldhuis et al., 1989a, 1992, 1994).

Men with chronic renal failure treated with hemodialysis manifest elevated serum immunoreactive LH (and α subunit) concentrations, but reduced B/I ratios and impaired testosterone output (Talbot et al., 1990). In addition, the distribution of plasma LH isoforms in uremic patients includes more basic moieties. Conversely, acidic LH species in azotemic men correlated with higher B/I LH ratios and normal serum testosterone concentrations (Mitchell et al., 1994). This relationship would coincide with the predictably longer in-vivo half-life of highly sialylated (more acidic) LH, and hence its extended availability in the circulation to stimulate gonadal steroidogenesis (Veldhuis et al., 1989a).

Taken together, the foregoing clinical pathophysiological data suggest that differential LH isoform production may play...
a role in men during ageing and influence gonadal function in end-stage renal failure. However, the precise significance of LH heterogeneity in the normal young adult male remains to be determined (Urban et al., 1988a,b).

**LH isoforms during the female menstrual cycle**

Plasma bioactivity in healthy women varies within the menstrual cycle, and rises dramatically after the menopause (Dufau et al., 1983; Veldhuis et al., 1984). It has been reported that LH B/I ratios decline in the luteal phase of the menstrual cycle compared with those in the early follicular phase and at mid-cycle (Suginami et al., 1982). Veldhuis and co-workers used frequent blood sampling to identify the highest plasma LH B/I ratios in the late follicular phase (Veldhuis et al., 1984). The latter finding is in keeping with preovulatory studies in the female Rhesus monkey (Marut et al., 1981), and the report of Wide and Bakos (Wide and Bakos, 1993) demonstrating the emergence of more basic LH isoforms at midcycle with elevated in-vitro LH biopotency. Thus, preferentially more alkaline LH products with higher in-vitro B/I ratios tend to be predominant in young oestrogen-enriched women. In contrast, acidic (long-lived) isoforms of LH tend to circulate in postmenopausal individuals (Veldhuis et al., 1983, 1989a).

No apparent changes were reported in LH B/I ratios in response to bolus i.v. GnRH stimulation (using conventional RIA or IFMA) during the early follicular phase of the menstrual cycle in healthy women (Ding and Huhtaniemi, 1991). During the late follicular phase, the LH B/I ratio increased according to a conventional RIA, but remained unchanged when estimated by IFMA. Opiate-receptor antagonist administration in healthy normally cycling women induced an equivalent elevation in bioactive and immunoreactive LH concentrations with a PCOS is the most common reproductive endocrinopathy of normally cycling women induced an equivalent elevation in bioactive and immunoreactive LH concentrations with a PCOS is the most common reproductive endocrinopathy of

**LH isoforms in the menopause**

In postmenopausal women, ovarian steroidogenesis and gametogenesis become quiescent, resulting in increased gonadotrophin secretion due to withdrawal of negative feedback (Evans et al., 1992). The plasma LH B/I ratio also rises postmenopausally compared with that of fertile young women and men (Dufau et al., 1976a, 1983). In addition, LH isoforms in the pituitary gland and plasma become more acidic (Reader et al., 1983; Wide, 1985; Wide and Naessen, 1994). Treatment of postmenopausal women with oestradiol suppresses LH bioactivity and causes a shift toward more basic isoforms (Veldhuis et al., 1989a; Urban et al., 1991; Wide and Naessen, 1994). Progestogens may also alter gonadotrophin charge, but in a temporally biphasic manner (Wide et al., 1996). Acute progestogen exposure in oestradiol-supplemented postmenopausal women tends to oppose the ability of oestradiol to maintain more alkaline LH isoforms, so that acidic LH species predominate. With longer-term progestogen co-administration, circulating gonadotrophin isoforms recover more basic properties. These time-dependent actions of progestogens in the oestrogen-primed milieu might reflect in part the androgenic properties of these agents, changes in the pattern of GnRH release from the hypothalamus, and/or direct pituitary actions.

In summary, LH bioactivity rises markedly in the ovariprival postmenopausal woman, and exhibits increased isoform acidity due to greater post-translational sialylation and/or sulphation. Oestrogen reverses these changes. The putatively more short-lived alkaline LH isoforms induced by oestrogen replacement might thus contribute to effective suppression of plasma LH concentrations by oestrogen in postmenopausal women by accelerating LH removal (Veldhuis et al., 1987a). How the route of oestrogen delivery might alter these responses is not established. Moreover, how oestrogen-induced changes in endogenous LH isoforms might impact LH-dependent ovarian stromal-cell androgen secretion in the post-climacteric female is not known.

**LH isoforms in PCOS**

PCOS is the most common reproductive endocrinopathy of young women (Carmina and Lobo, 1999). PCOS is marked by pubertal onset and a constellation of metabolic features (hyperinsulinism, dyslipidemia), variable obesity, amenorrhea, hyperandrogenism, hirsutism and reduced fertility. Garcia-Rudaz and co-workers reported that adolescent girls with PCOS exhibit augmented LH secretion due to an increase in immunofluorometric and deconvolution-estimated LH secretory burst mass and frequency, as well as greater basal LH release. Ropelato and co-workers (Ropelato et al., 1999) further identified relatively elevated in-vitro LH bioactivity and a preponderance of more basic LH isoforms in pubertal patients with PCOS. The estimated half-life of endogenous LH was reduced commensurately, consistent with presumptively more rapid in-vivo metabolic removal of alkaline LH isoforms. The finding of elevated in-vitro LH bioactivity in adolescents with PCOS corroborates earlier studies using other bioassays in adults (Lobo et al., 1983; Ding and Huhtaniemi, 1991; Fauser et al., 1991; Imse et al., 1992). A greater proportion of basic LH isoforms in girls with PCOS also confirms data from Ding and Huhtaniemi in adults with PCOS (Ding and Huhtaniemi, 1991). Consequently, highly biopotent basic LH isoforms may drive excessive ovarian androgen secretion in both pubertal and adult PCOS patients. However, the inferentially shorter half-life of more basic LH isoforms...
Physiological role for gonadotrophin heterogeneity

The heterogeneity of gonadotrophin isoforms is appraised by chromatofocusing procedures, which quantify charge variations among molecules (Weise et al., 1983). Thereby, Wide and co-workers identified a shift toward more acidic isoforms of LH (and FSH) during the later stages of puberty in a small number of children with precocious puberty (Wide et al., 1996). Less conspicuous qualitative changes emerged during female puberty. However, early normal male puberty was marked by a dramatic shift towards more acidic isoforms of both gonadotrophins (Phillips et al., 1997). This sex difference in isoform estimates may allow for more ready detection of the initiation of puberty in boys. However, the underlying mechanisms regulating the changes in gonadotrophin charge distribution in puberty are unclear, and their precise in-vivo relevance (if any) to sexual maturation in girls and boys is unknown.

Haavisto and co-workers (Haavisto et al., 1990) reported that greater basal and GnRH-stimulated bioactive and immuno-reactive LH in children with precocious puberty (Wide et al., 1996). Earlier RIA studies often identify a 2–3 fold only (Lucky et al., 1993). However, another report did not concur with this inference in adolescent girls (Reiter et al., 1999). We thus infer that in-vitro estimates of plasma LH bioactivity are elevated in girls and women with PCOS, reflecting the presence of more basic LH isotypes. Whether the molecular heterogeneity of circulating LH isospecies is due to relative hyperoestrogenism and/or hyperinsulinism in PCOS is not known. However, we speculate that the inferentially more rapid in-vivo clearance of basic LH molecules in PCOS offers some protection against the degree of LH elevation, which would otherwise exacerbate hyperandrogenism further.

LH isoforms in puberty

During human sexual development, serum concentrations of immunoreactive LH increase 30 to 100-fold from prepuberty to adulthood as measured by highly sensitive IFMA (Apter et al., 1989; Wu et al., 1996). Earlier RIA’s often identify a rise of 2–3 fold only (Lucky et al., 1980b; Reiter et al., 1982). Plasma bioactive LH concentrations also increase remarkably across puberty. In some studies, the calculated B/I ratio rises concurrently in boys or in both sexes (Reiter et al., 1978, 1987; Kasa-Vubu et al., 1993; Kletter et al., 1993). However, another report did not concur with this inference in adolescent girls. The latter disparity likely mirrors the relatively poor sensitivity of earlier LH RIA’s in quantitating low prepubertal and early pubertal LH secretion accurately. Overestimating low LH concentrations by immunoassay would obscure a changing B/I ratio. Indeed, more recent analyses using a 30-fold more sensitive IFMA along with an enhanced in-vitro LH bioassay reported no change in B/I LH ratios in human puberty (Huhtaniemi et al., 1996). The latter study also unveiled large inter-individual variations in B/I ratios even in a fairly homogeneous group of pubertal boys. On the other hand, the LH B/I ratio remained relatively constant within individual subjects during longitudinal follow-up (Huhtaniemi et al., 1996).

The promoter of the variant LH β gene also is ~50% more active in transfected cell lines than the wild-type gene (Jiang et al., 1999). The foregoing differences in LH bio-synthesis, in-vitro action and in-vivo kinetics in subjects who are homo-or heterozygous for the variant LH allele can be associated epidemiologically with delayed puberty (Raivio et al., 1996), PCOS (Rajkhowa et al., 1995; Tapanainen et al., 1999) and subfertility (Ramanujam et al., 2000). Two studies also reported a higher prevalence of menstrual irregularity in Japanese women homozygous for the variant LH β allele (Furui et al., 1994; Suganuma et al., 1995). Finally, in one analysis, the
above variant LH gene type was associated with relatively greater frailty in healthy older men (van den et al., 1999).

Boys with normal pubertal onset but a variant (homo- or heterozygous) LH genotype have smaller testis volumes, shorter stature, slower linear growth rates, and lower serum IGFBP-3 levels than their peers (Raivio et al., 1996). However, in cohorts of boys with a delayed onset of puberty, the frequency of the variant LH β allele does not differ from that in the reference population, indicating that existence of the variant LH genotype does not generally influence the timing of pubertal GnRH activation (Raivio et al., 1996). Based on these studies in pubertal boys, we infer that the polymorphic LH variant may affect testis growth and the somatotropic GH-IGF-1-BP-3 axis in pubertal boys, but not the age of pubertal onset.

Obese patients with PCOS exhibit a lower frequency of variant LH molecules both in Finland and the Netherlands, with a similar trend in the United States (Tapanainen et al., 1999). If these observations are correct, then the variant LH gene type may in part protect obese women from developing symptomatic PCOS. In contrast, Rajkhowa and co-workers in the UK (Rajkhowa et al., 1995) observed a higher frequency of variant LH isotypes in obese women with PCOS compared with eugonadal controls. Thus, the role of variant LH in PCOS remains to be established.

Selected technical issues
By way of monitoring in-vivo biological activity of LH, an ideal measurement system would require an ensemble estimate of overall luteotropic bioactivity in the circulation as well as an individual representation of relative isoform prevalence. This ideal has not been achieved at present. Indeed, serum contains a variety of potentially nongonadotrophin factors, which could be capable of modulating or directly stimulating gonadal cell activity in vivo as well as potentially in in-vitro systems. Accordingly, various endpoints have been devised predominantly for in-vitro systems to appraise putative LH bioactivity, such as binding assays, the generation of cyclic AMP, and the stimulation of de-novo steroidogenesis. Nonetheless, the challenge remains to identify the optimal target cell for such analyses in vitro, the most informative cell dispersion methodology, duration of incubation, standards and a buffer choices presence of serum, etc. In principle, the perfect in-vitro biological assay of LH activity would achieve a faithful rendition of in-vitro LH drive on the endpoint of interest. Given these challenges, among different assays and even under certain in-vitro assay circumstances, the bio:immuno LH ratio may be unstable, inconsistent or falsely estimate changes in endogenous LH drive. Such limitations presently restrict the diagnostic role of LH bioassay applications in day-to-day clinical studies, making serial comparisons within a single assay preferred and restricting use to primarily a research context. Likewise, the ultimate therapeutic application of suitably validated measurements of biologically active LH will require further development. Indeed, the advent of recombinant human LH protein does not fully obviate these technical issues, since recombinant proteins require specialized post-translational modification to mimic or match endogenous glycosylated gonadotrophin forms. Nonetheless, the broad background experimentally and clinically concerning regulated output of LH bioactivity points to a promise for significant developments in this arena.

Summary
In summary, clinical evidence for a physiological impact of LH isoforms includes their unequal in-vitro bioactivity and altered in-vivo LH kinetics. For example, alkaline LH iso-species emerge in an oestrogen-rich milieu, and show greater bioactivity in-vitro along with more rapid metabolic removal in vivo. More acidic LH isotypes predominant in eugonadal men with end-stage renal failure and in postmenopausal women. Molecular LH variants may be associated with decreased testis size and reduced linear growth in boys, more menstrual irregularity and/or subfertility in women, and possibly protect against PCOS in women.

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