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ORIGINAL ARTICLE

Dynamic changes in glycosylation and glycan composition of serum FSH and LH during natural ovarian stimulation

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Abstract

Background. Glycosylation and glycan composition are of fundamental importance for the biological properties of FSH and LH. The aim of this study was to determine the glycosylation, sialylation, and sulfonation of serum FSH and LH throughout the normal menstrual cycle.

Methods. Serum samples were collected from 79 healthy women with regular menstrual cycles. The mean numbers of anionic monosaccharide (AMS), sialic acid (SA), and sulfonated N-acetylgalactosamine (SU) residues per FSH and LH molecule were estimated for all sera with methods based on electrophoreses, neuraminidase treatments, and fluoroimmunoassays of the gonadotrophins.

Results. Di-glycosylated glycoforms (FSHdi, LHdi) were detected in serum in addition to tetra-glycosylated FSH (FSHtetra) and tri-glycosylated LH (LHtri). FSHdi exhibited two peaks: one on day 5 to 7 and one, more pronounced, at midcycle. FSHtetra plateaued at a high concentration from day 5 to 15, without a midcycle peak. There were lower concentrations of LHdi than LHtri, except at midcycle when the opposite occurred. The mean numbers of SA and SU residues per molecule of FSH and LH in serum showed four different patterns during the cycle, all with highly significant ($P < 0.0001$) differences between levels at different phases of the cycle. The pattern of SA residues on FSH was 'M'-shaped, and that of SU on LH 'V'-shaped.

Conclusion. Serum FSH and LH governing the natural ovarian stimulation process exhibited dynamic changes of glycosylation and glycan composition. This new information on the FSH and LH molecular structures may lead to more successful mono-ovulatory treatment regimens for ovulation induction in anovulatory women.

Key words: *di-glycosylated FSH, di-glycosylated LH, glycoforms, isoforms, ovulation induction, sialic acid, sulfonated N-acetylgalactosamine*

Introduction

Human gonadotrophin preparations have been used for the induction of ovulation in anovulatory women during more than five decades. Successful treatments were first described by Gemzell et al. in 1958 using a crude human pituitary follicle-stimulating hormone (FSH) preparation (1). Large pools of pituitaries had been collected at autopsy in hospitals, mainly from elderly men and women. A few years later, Donini et al. reported a method for the extraction of gonadotrophins from human menopausal urine, and these extracts, called human menopausal gonadotrophins (hMG), were soon introduced for ovulation induction (2). Two decades later, highly purified FSH isolated from menopausal urine was marketed for ovulation induction (3). In 1992, recombinant human FSH preparations were introduced for the same purpose (4). These treatments have been highly successful but also associated with a risk for ovarian hyperstimulation and multifetal pregnancies. Although the frequencies of these severe complications have been reduced by using the serum oestradiol levels to monitor the treatments (5-7), later in conjunction with ultrasound and by the use of lower dosages of gonadotrophins, the risk is still substantial (8,9). A principle of step-down protocols has been developed in order to

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mimic the natural ovarian stimulation process more closely (10). After an initial higher dose of FSH, a lower dose of FSH is administered. This is thought to lead to atresia of most of the developing follicles.

Used for ovulation induction, human pituitary- and urinary-derived preparations from elderly individuals are not physiological. We have shown that the molecular carbohydrate structures of serum FSH and lutenizing hormone (LH) in menopausal women and in men are different from those in young women (11,12). Also, recombinant FSH preparations (13) have a different carbohydrate structure compared with FSH of young women. Mono-ovulation is the aim in the treatment of anovulatory women. It has continuously been a desire to try to mimic the natural ovarian stimulation process more closely to achieve this goal. One prerequisite is then a thorough knowledge about the glycosylation and glycan compositions of serum FSH and LH during the normal menstrual cycle.

Like other glycoproteins FSH and LH exhibit a very large heterogeneity. The two hormones are synthesized in the pituitary, secreted and circulate in blood as individual spectra of large numbers of isoforms (12,14). The FSH and LH isoforms isolated from pools of human pituitaries differ in their N-glycan compositions (15-18). In addition, FSH has been reported to exist in human pituitary and urinary preparations as two major glycoforms designated tetra-glycosylated and di-glycosylated hFSH (19,20). The former glycoform was decorated with two glycans on both the α-subunit and the FSH β-subunit and the latter glycoform with glycans only on the α-subunit.

The isoforms can be separated by electrophoresis due to variation in their contents of two terminal anionic monosaccharide (AMS) residues: sialic acid (SA) and sulfonated N-acetylgalactosamine (SU). These terminal AMS residues are of physiological significance as they are decisive for the half-lives of FSH and LH in human circulation (21,22). Both LH and FSH are secreted in a pulsatile manner, and the compositions of the gonadotrophin isoforms in circulation continuously change after each pulse. A consequence of this is that the pituitary effect on the ovary is exerted by continuous qualitative as well as quantitative changes of FSH and LH in the circulation. The aim of the present study was to determine the degree of glycosylation, sialylation, and sulfonation of serum FSH and LH molecules throughout the normal menstrual cycle.

Subjects and methods

Subjects

As part of the training in clinical chemistry for medical students at Uppsala University Hospital, blood samples were taken from students on an ambulatory basis in the morning during the period 2000 to 2011. All female students who agreed to participate in the research project gave a written consent on analytes accepted to be included and signed a health declaration with information about diseases, medications, alcohol intake, and recent physical activity. Information was given about menstrual bleedings (first day of last menstruation, menstrual cycle length, and regularity over the last year) and hormonal contraceptives. The study was approved by the local Ethics Committee.

One serum sample was selected from each of 79 female students, median age 25, range 21–40 y, on the criteria that they were apparently healthy, had regular menstrual cycles with a length of 28 ± 2 days, did not use any hormonal contraception, and did not have the common genetic variant of LH. They all had serum concentrations of FSH and LH within the reference limits for the day of menstrual cycle. The cycle day was adjusted to a 28-day menstrual cycle. Information about the first day of next cycle was confirmed for samples taken on cycle days 26–28. No serum samples were obtained for cycle days 2 and 4.

FSH and LH serum concentration

To exclude individuals with the common variant form of LH, the presence of such forms was first analysed as described (23).The concentrations of LH and FSH in serum samples were measured using time-resolved sandwich fluoroimmunoassays (Delfia, PerkinElmer-Wallac Oy, Turku, Finland), as previously described (24). Gonadotrophin values were expressed in IU/L using the International Standards for pituitary LH (80/552) and FSH (94/632) as reference standards. The detection limits were 0.02 IU/L, and the interassay coefficient of variation (CV) was less than 3% for both hormones.

AMS residues per molecule

The number of AMS residues per molecule was determined by analyses of all serum samples with an electrophoresis technique using a 0.10% agarose suspension in veronal buffer at pH 8.7 (12,25). After electrophoreses FSH and LH activities were measured in 200 μL of the fractions eluted. The area of gonadotrophin activity was resolved into peaks at the positions for different numbers of AMS residues per molecule; for FSH varying from 4 to 10 residues and for LH from 1 to 6 residues per molecule.

Low- and high-glycosylated forms of FSH and LH

The FSH molecule can be decorated with a maximum of four N-glycans and the LH molecule with a

maximum of three. Bousfield et al. reported that human FSH exists in the pituitary and urine also as a di-glycosylated form with no glycans on the β-subunit (19,20). Estimations of di-glycosylated FSH (FSHdi), tetra-glycosylated FSH (FSHtetra), di-glycosylated LH (LHdi), and tri-glycosylated (LHtri) were made on the basis of the number of AMS residues per molecule. Isoforms of serum FSH with 7–10 AMS residues per molecule were pooled as variants of FSHtetra and those with 4–6 AMS residues per molecule as variants of FSHdi, with proportional adjustments for the overlapping between the di- and tetra-glycoforms. Isoforms of serum LH with 4–6 AMS per molecule were pooled as variants of LHtri and those with 1–2 AMS per molecule as variants of LHdi. Fractions of isoforms of LH with 3 AMS per molecule were added to the high- and low-glycosylated pools in proportion to the concentrations of isoforms in these pools. Low- and high-glycosylated isoforms of both hormones differed in sizes and could be separated by gel filtration on Sephadex G-100 (unpublished observations). There were no isoforms with zero AMS per LH molecule and only an average of 0.88% with one AMS per LH molecule, which excluded the likelihood for the presence of mono-glycosylated glycoforms of LH.

SA and SU residues per molecule

Determinations included analyses of all serum samples both before (see above) and after neuraminidase treatment with an electrophoresis technique (12,25). The area of gonadotrophin activity after the neuraminidase treatment was resolved into peaks at the positions for different numbers of SU residues per molecule. The average numbers of SA and SU residues per gonadotrophin molecule in each serum sample were estimated as previously described (12). The method is based upon previous observations (15-18) that negatively charged terminal SA and SU residues on the N-glycans determine the variation of the electric charge of human FSH and LH.

Statistical analyses

In order to illustrate the time-related dynamic changes during the cycle, three-day mean values ± SEM with one-day step-wise movements throughout the cycle were calculated for different data. The calculation started on day 26 in the previous cycle and the two data-free days 2 and 4 were excluded. Data of days 26, 27, and 28 were pooled as the first three-day mean value and of days 27, 28, and 1 as the second mean value, etc. The distributions of data were analysed with D'Agostino and Pearson omnibus (when more than seven observations) and Kolmogorov–Smirnov normality tests. When three-day mean values are illustrated in the figures, the ± SEM are omitted for means of groups that did not pass a normality test (9 out of 416 three-day mean values; 2.2%). Data for serum concentrations of low- and high-glycosylated gonadotrophin forms, numbers of SA and SU residues per molecule, and ratios between SA and SU residues were compared for different periods of the cycle using two-tailed Student's t test. These data passed normality tests. Gonadotrophin serum concentrations and ratios between number of SA and SU residues per molecule were log transformed before statistical analyses and plotted in the figures as geometric mean ± SEM values. A P value less than 0.05 was considered significantly different.

Results

FSH and LH serum concentrations

There was a rise of serum FSH during the early follicular phase, from day 27 in the previous cycle to day 5 (Figure 1, left panel). The midcycle LH and FSH surges coincided and occurred around day 14. Before that the serum LH concentration increased gradually from day 8 to day 12. During the same period the FSH concentration remained at a slightly decreased plateau level. The midcycle surges of FSH and LH were followed by rapid decreases of the serum concentrations of both gonadotrophins.

Numbers of AMS residues per FSH and LH molecule

The mean number of AMS residues, which is the sum of SA and SU residues, during the menstrual cycle was on FSH 6.85 ± 0.022 and on LH 3.13 ± 0.018, and the mean difference 3.71 ± 0.015. There was a highly statistically significant correlation ($r = 0.74$; $P < 10^{-14}$; $n = 79$) between AMS residues on FSH and LH. Assessments of three-day mean numbers of AMS residues per FSH and LH molecule in serum during the menstrual cycle showed that the shapes of the two curves were similar from day 8 to day 25 with a nadir on day 14 and a zenith on day 21 (Figure 1, right panel).

Serum concentrations of FSHdi, FSHtetra, LHdi, and LHtri

The time pattern of the serum FSHdi concentration was characterized by a steep rise from day 27 to day 3–6 and then a dip to a decreased concentration lasting from day 7 to day 11 followed by a pronounced midcycle peak (Figure 2, left panel; Table I). After the

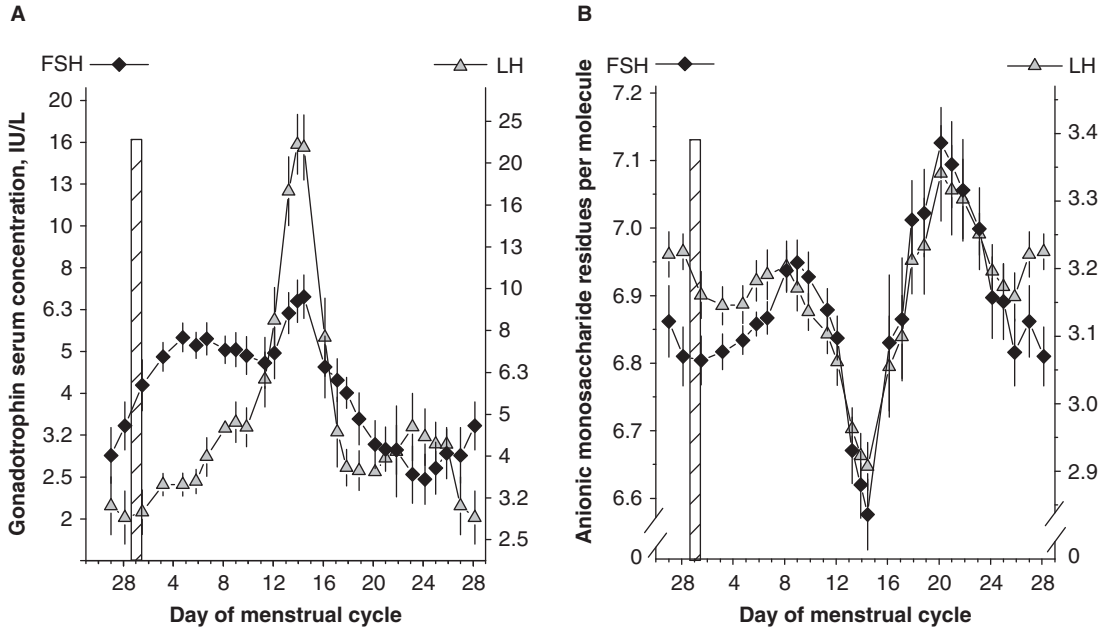

Figure 1. A: Concentrations of FSH and LH in serum of 79 women with a normal menstrual cycle, plotted as geometric mean ± SEM values. B: The number of anionic monosaccharide (AMS) residues per molecule of FSH and LH in the same serum samples, plotted as three-day mean ± SEM values. Data in this figure and in Figures 2,3,4,5 have been plotted as three-day moving mean values starting during the last days of the previous cycle and with the first day of the cycle indicated by a vertical hatched bar.

midcycle peak there was a rapid decrease to the lowest level on day 17–19. The FSHtetra concentration increased to a high plateau level lasting from day 3 to day 15 followed by a slow decrease without any sign of a midcycle peak. Both the LHdi and LHtri concentrations showed a pronounced mid-cycle peak (Figure 2, right panel; Table I). The concentrations of LHdi showed a larger variation

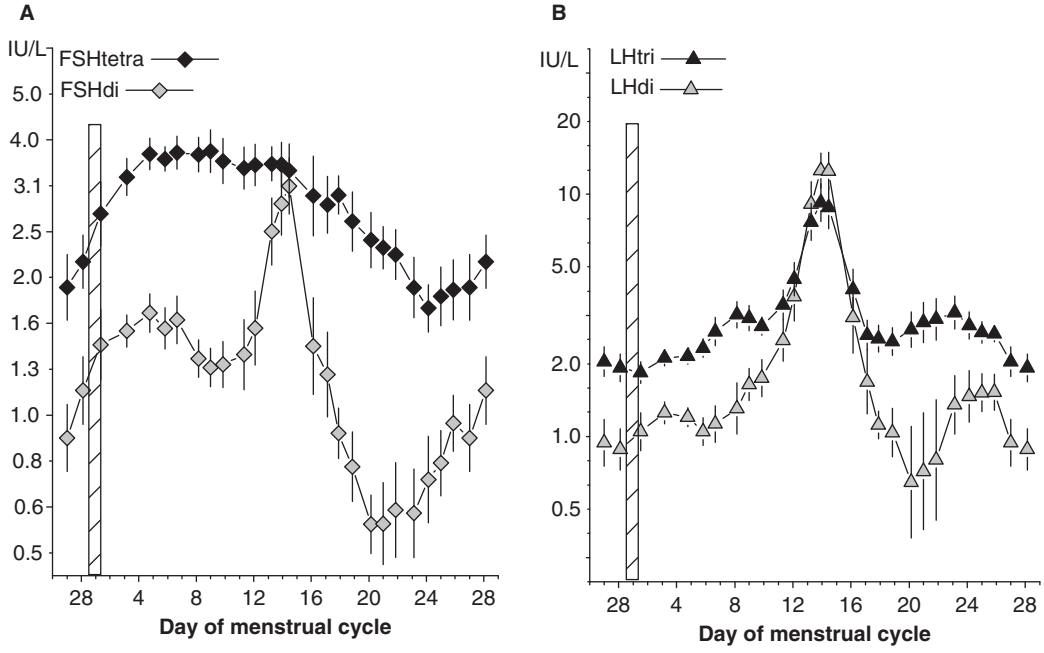

Figure 2. Concentrations of FSHdi and FSHtetra, A, and LHdi and LHtri, B. Geometric scales. See also legend to Figure 1.

Table I. Serum concentration of FSHdi, FSHtetra, LHdi, and LHtri at different periods of the normal menstrual cycle. Geometric mean values and their 95% limits are shown.

Cycle days		Serum concentration, IU/L geometric		Comparison with previous group *P* value[a]
mean; range	Number of women	mean	95% limits	
FSHdi				
27.0; 26–28	8	0.89	0.59–1.33	
4.0; 3–5	8	1.54	1.18–2.01	< 0.05
6.0; 6	5	1.90	1.17–3.10	ns
9.0; 7–11	11	1.30	1.09–1.55	< 0.05
13.9; 13–15	14	2.90	2.06–4.07	< 0.001
18.8; 17–19	8	0.91	0.68–1.24	< 0.0001
22.1; 20–24	12	0.57	0.41–0.78	< 0.05
26.4; 25–28	11	0.96	0.69–1.33	< 0.05
FSHtetra				
27.0; 26–28	8	1.90	1.29–2.80	
4.0; 3–5	8	3.56	2.72–4.66	< 0.01
13.9; 13–15	14	3.52	2.76–4.48	ns
22.7; 20–25	15	1.97	1.63–2.38	< 0.001
LHdi				
28.6; 26–1	11	1.04	0.70–1.53	
11.3; 10–12	9	2.49	1.58–3.90	< 0.01
13.9; 13–15	14	12.5	8.75–17.9	< 0.01
20.6; 19–22	9	0.79	0.28–2.23	< 0.001
LHtri				
1.4; 26–5	19	2.10	1.66–2.66	
13.9; 13–15	14	9.24	6.27–13.6	< 0.0001
18.9; 17–21	13	2.55	1.99–3.27	< 0.0001

[a]Two-tailed Student's *t* test.

than that of LHtri during the cycle. The LHdi concentrations were lower than those of LHtri during the follicular and luteal phases of the cycle and higher at midcycle.

FSHdi and LHdi in serum expressed as a percentage of total gonadotrophin concentrations

The three-day mean values of serum LHdi varied from 23.2% to 58.4% and those of FSHdi from 19.7% to 48.3% (Figure 3). The mean value for the entire cycle period was 36.6% for LHdi and 32.0% for FSHdi. There was a similar time pattern of fluctuations in FSHdi and LHdi percentages over the menstrual cycle. The percentage of FSHdi was significantly ($r = 0.69$; $P < 10^{-11}$) correlated with that of LHdi. Moreover, percentages of FSHdi and LHdi were significantly correlated to the log concentration of LH (for FSH: $r = 0.62$; $P < 10^{-8}$; for LH: $r = 0.65$; $P < 10^{-10}$). Finally, the percentage of FSHdi was significantly ($r = 0.38$; $P < 0.001$) correlated to the log concentration of FSH.

Number of AMS per glycan on FSH and LH

The number of AMS per glycan on the FSH molecules was significantly ($P < 0.0001$) higher at the midcycle phase, with a mean value ± SEM of 2.16 ± 0.030 ($n = 14$), than at the follicular phase, 2.02 ± 0.008 ($n = 32$), or at the luteal phase, 2.01 ± 0.013 ($n = 33$). The mean number of AMS per glycan on the LH molecule during the menstrual cycle was 1.20 ± 0.002 ($n = 79$) without any significant variation between different phases of the cycle.

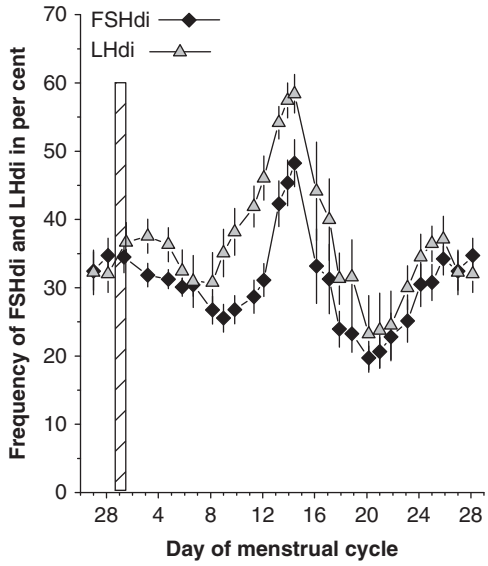

Figure 3. Concentrations of FSHdi and LHdi, expressed as per cent of total concentrations of FSH and LH. See also legend to Figure 1.

Numbers of SA and SU residues per FSH and LH molecule

The time-related change for the number of SA residues on FSH during the cycle was 'M'-shaped with the lowest levels at midcycle and at day 28 (Figure 4, left panel; Table II). The three-day mean number of SA residues varied from 6.28 to 6.73 per FSH molecule. The highest numbers were found at mid-follicular phase, day 7–11, and at mid-luteal phase with a plateau from day 17 to day 21.

As regards numbers of SU residues per FSH molecule during the cycle, there was a fast continuous decrease for 9 days, from cycle day 27 in the previous cycle to a nadir on day 8 followed by a slow increase for 17 days to a maximum on day 27 (Figure 4, left panel; Table II). The number of SU residues was low, and the three-day mean values varied from 0.23 to 0.56 per FSH molecule. There was a highly significant correlation between the percentages of FSHdi and the average number of SA residues per molecule ($r = -0.87$; $P < 10^{-24}$) and no significant correlation with the number of SU residues ($r = 0.03$; $P = 0.78$).

The time pattern of numbers of SA residues on LH was characterized by a low plateau level from late luteal phase into early follicular phase and then an increase from the first days of the cycle to a peak on day 12 (Figure 4, right panel; Table II). After this peak there was a decrease to day 15 and then a plateau level to day 21, followed by a decrease to the low plateau level during late luteal and early follicular phase.

The pattern for the SU content on LH (Figure 4, right panel; Table II) was 'V'-shaped with a high plateau level during the late luteal phase into the first day of next cycle and a nadir on day 13–15, that was followed by an increase to a high plateau level from day 19 to day 28. There was a highly significant inverse correlation between the percentages of LHdi and the average number of SU

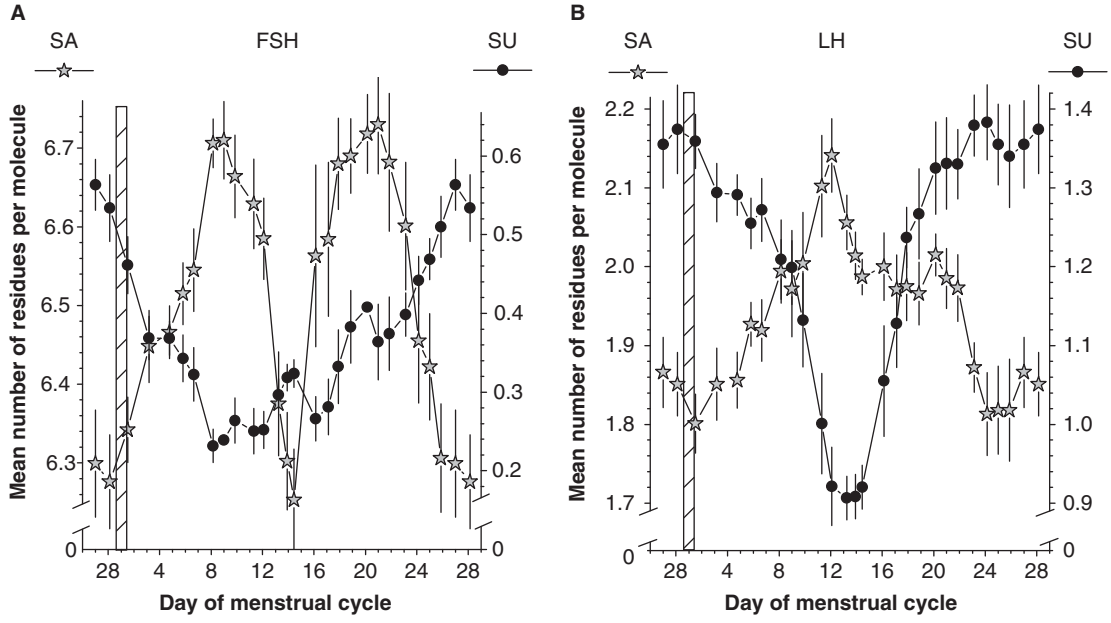

Figure 4. Numbers of sialic acid (SA) and sulfonated N-acetylgalactosamine (SU) residues per molecule of FSH, A, and LH, B. See also legend to Figure 1.

Table II. Number of sialic acid (SA) and sulfonated N-acetylgalactosamine (SU) residues per FSH or LH molecule and ratio of SA/SU residues at different periods of the normal menstrual cycle. Mean values and their 95% limits are shown.

	Cycle days		Number of residues per molecule		
	mean; range	Number of women	mean	95% limits	Comparison with previous group *P* value[a]
FSH					
SA	27.0; 26–28	8	6.30	6.14–6.46	
	9.0; 7–11	11	6.67	6.59–6.75	< 0.0001
	13.9; 13–15	14	6.30	6.17–6.44	< 0.0001
	18.9;17–21	13	6.71	6.62–6.79	< 0.0001
	26.4; 25–28	11	6.29	6.17–6.41	< 0.0001
SU	27.0; 26–28	8	0.56	0.49–0.64	
	8.5; 7–10	9	0.24	0.19–0.29	< 0.0001
	14.2; 14–15	11	0.33	0.29–0.37	< 0.01
	26.4; 25–28	11	0.54	0.47–0.61	< 0.0001
LH					
SA	3.2; 1–5	11	1.85	1.75–1.95	
	12.1; 10–13	10	2.14	2.04–2.25	< 0.001
	18.1; 15–21	17	2.00	1.95–2.05	< 0.01
	25.6; 23–28	16	1.84	1.77–1.91	< 0.001
SU	28.6; 26–1	11	1.34	1.24–1.45	
	13.9; 13–15	14	0.91	0.85–0.97	< 0.0001
	23.8; 19–28	25	1.36	1.30–1.41	< 0.0001
			Geometric		
FSH			mean	95% limits	
SA/SU	27.0; 26–28	8	11.3	9.71–13.2	
	9.9; 7–12	16	28.7	24.2–34.0	< 0.0001
	14.2; 14–15	11	19.1	16.5–22.2	0.001
	25.9; 24–28	14	12.6	11.0–14.3	0.0001
LH					
SA/SU	28.6; 26–1	11	1.39	1.23–1.57	
	12.1; 11–13	10	2.35	2.01–2.74	< 0.0001
	25.9; 24–28	14	1.34	1.20–1.48	< 0.0001

[a]Two-tailed Student's *t* test.

residues per molecule ($r = -0.70$; $P < 10^{-12}$) but no statistically significant correlation with regard to the number of SA residues ($r = 0.03$; $P = 0.78$).

Ratio between numbers of SA and SU residues per FSH and LH molecule

There was an increase of the SA/SU ratio for FSH from day 27 in the previous cycle to a peak on day 7–12 (Figure 5; Table II). After that the SA/SU ratio decreased to day 14 and then varied during the first part of the luteal phase followed by a decrease from day 21 to day 27.

The ratio SA/SU values for LH increased from day 1 to a peak value on day 11–13 (Figure 5; Table II). After this peak the ratios decreased continuously to a low plateau level from day 24 to day 28.

Discussion

Glycosylation of FSH and LH

Di-glycosylated glycoforms of both FSH and LH, in addition to the FSHtetra and the LHtri forms, were detected in serum throughout the menstrual cycle. These four gonadotrophin forms exhibited different

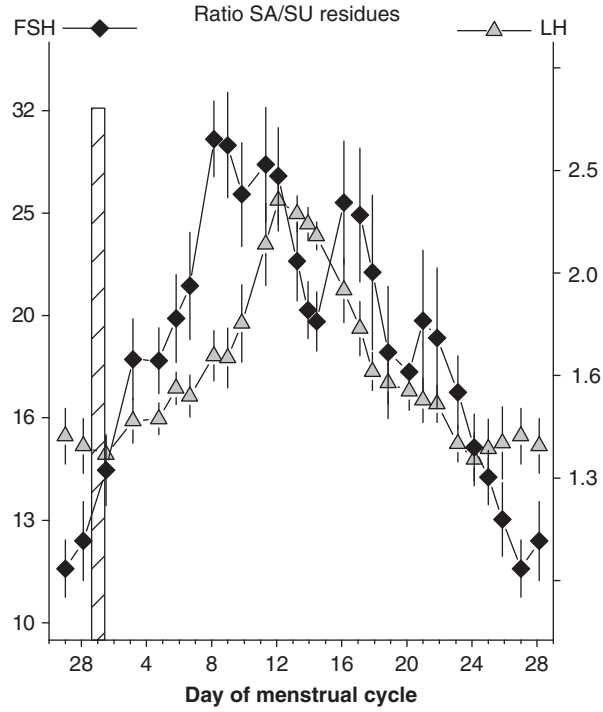

Figure 5. Ratios between sialic acid (SA) and sulfonated N-acetylgalactosamine (SU) residues per FSH and LH molecule. Geometric scales. See also legend to Figure 1.

concentration patterns during the menstrual cycle. FSHdi exhibited two peaks—one peak on day 5–7 and one, more pronounced, at midcycle. FSHtetra had a high plateau concentration from day 5–15, without a midcycle peak. LHdi had lower concentrations than LHtri, except at midcycle when the opposite occurred. In all graphs data have been presented as three-day moving mean values during the menstrual cycle, starting during the last days of the previous cycle. With this continuous presentation of the results, dynamic time-related changes of data were revealed that had been otherwise hidden when more conventional fixed periods of menstrual cycle phases had been used.

N-glycosylation is a post-translational process in which an oligosaccharide is transferred and attached to asparagine on a nascently translated polypeptide (26). The glycosylation of the peptide structures of the subunits of FSH and LH and the following synthesis of the N-glycans to terminal SA and SU residues in the gonadotrophin-producing cells of the anterior pituitary have been schematically shown in Figure 6. N-glycosylation of the common α-subunit and β-subunits of FSH and LH takes place in the rough endoplasmic reticulum (ER) simultaneously in the same compartment. A dolichol (Dol)-linked oligosaccharide is attached to an oligosaccharyltransferase (OST) complex, and the oligosaccharide then becomes transferred to a nascently translated polypeptide.

The glycosylated α-subunits are associated with glycosylated β-subunits of FSH and LH to form FSHtetra and LHtri. Furthermore, an FSHdi glycoform has been demonstrated in pituitary and urinary preparations, and it consists of an α-subunit with two glycans associated with a non-glycosylated FSH β-subunit (19,20). In our study the three-day mean frequency of FSHdi varied in serum from 20% to 48% during the menstrual cycle. These figures are lower than the relative abundance of 60–65% of non-glycosylated β-subunits on FSH reported by Bousfield et al. for immunopurified extracts of some pituitaries from 21–43-year-old women (20). The difference between the pituitary and serum values may be explained by a shorter half-life in the circulation of FSHdi, due to a lower number of sialic acid residues, compared with FSHtetra.

The FSHtetra and FSHdi glycoforms were identified by the number of AMS per molecule. Similarly, both LHtri and LHdi glycoforms were detected in serum. We suggest that, in analogy with FSH, the LHdi consists of an α-subunit with two N-glycans and a non-glycosylated LH β-subunit. It has been shown that the N-glycan at the position Asn30 of the LH β-subunit has a very high abundance of SU residues (17). A lack of this N-glycan on the LH β-subunit is compatible with the finding of a negative correlation between the percentage of di-glycosylated LH forms and the average number of SU residues per LH molecule. The Asn78 glycan on the α-subunit has a low number of SU residues (17), and the Asn52 glycan on the α-subunit has been shown to be important for signal transduction and for cAMP and steroid formation (29). It seems less likely that one of these glycans on the α-subunit is missing on the LHdi glycoform.

The mean number of AMS per glycan on FSH during the follicular and luteal phases of the cycle was 2.02. This number was significantly ($P < 0.0001$) raised to 2.16 at midcycle, when the concentration of FSHdi, with glycosylation on the α-subunit only, was increased compared with that of FSHtetra. A possible explanation is that the glycans on the α-subunits of the FSH molecule are more branched than those on the FSH β-subunits. The mean number of AMS per glycan on the LH molecule during the cycle was 1.20 without any significant variation during the cycle. This suggests that the branching of the glycans on the LH α-subunits is similar to that of the glycan on the LH β-subunits.

How is the formation of the FSHdi and LHdi glycoforms regulated? It was recently suggested by Bousfield and Dias that a possible mechanism for a

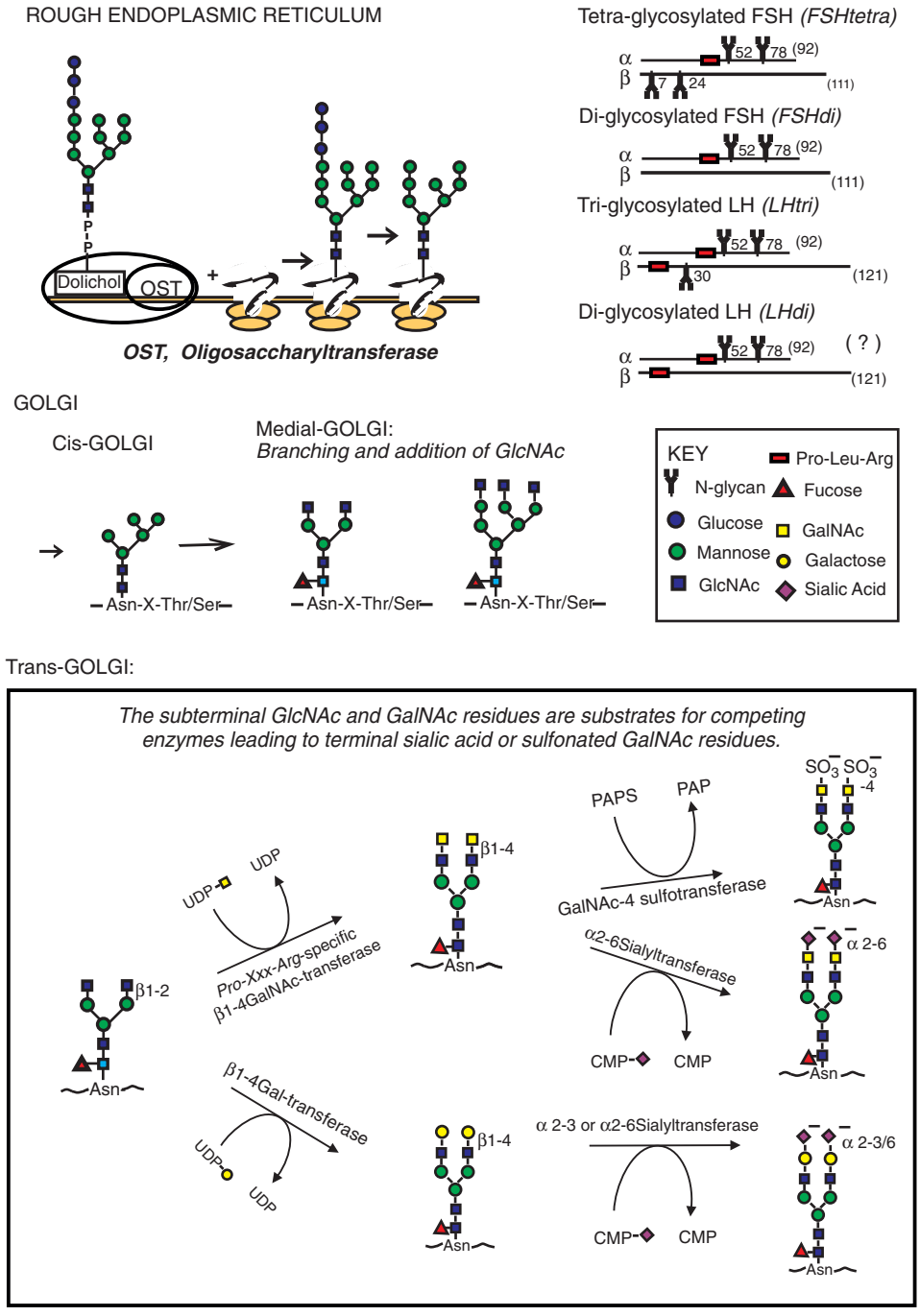

Figure 6. Schematic drawings of the glycosylation of FSH and LH in the rough endoplasmic reticulum and the synthesis of the N-glycans in the Golgi of human anterior pituitary gonadotrophin-producing cells. Symbol nomenclature according to *Essentials of Glycobiology*, 2nd ed. (27). Pathways and design from refs. (16,17,26,28).. (CMP = cytidine monophosphate; PAPS = 3′phosphoadenyl-5′phosphosulfate; UDP = uridine diphosphate).

selective formation of non-glycosylated FSH β-subunits could involve inhibition of OST isoforms specific for FSH β-subunits (30). The results of the present study suggest an alternative explanation. The number of AMS residues on FSH and LH was highly significantly correlated ($r = 0.74$; $P < 10^{-14}$). There was a striking parallelism between the frequencies of FSHdi and LHdi glycoforms expressed in per cent of total (Figure 3), and the correlation between the two di-glycosylated forms was highly significant ($P < 10^{-11}$). The percentages of the FSHdi and LHdi glycoforms were significantly ($P < 10^{-8}$ and $P < 10^{-10}$, respectively)

correlated with the log concentration of LH in serum. These results suggest that the glycosylation of the two hormones may be restricted by similar mechanisms. The availability of the Dol-P and the level of the OST activity have been shown to be able to restrict and regulate the glycosylation process (26). When, during the menstrual cycle, the production rate of the gonadotrophins increases, the available Dol-P or OST activity may not increase in parallel. A higher production rate of non-glycosylated β-subunits of both hormones will then be expected. The common α-subunit is produced in large excess to the β-subunits, and enough of di-glycosylated α-subunits may be synthesized. It seems most likely that oestrogens play a major role for the inhibition of the glycosylation process of both FSH and LH throughout the menstrual cycle.

Glycan composition on FSH and LH

Glycans on FSH are more branched than those on LH. Both tri-antennary and tetra-antennary glycans are produced on FSH in the medial-Golgi (Figure 6). A sub-terminal N-acetylglucosamine (GlcNAc) residue is added in the medial-Golgi to the asparagine-linked oligosaccharide chains and serves in the trans-Golgi as a substrate for two competing enzymes: a β1-4galactosyltransferase starting a pathway to terminal sialic acid, and a β1-4GalNAc-transferase starting a pathway to terminal SU and SA residues (17). The number of terminal SA and SU residues per molecule depends on the pathway-substrate concentration and the enzymatic activities along the two pathways. The β1-4GalNAc-transferase recognizes a Pro-Leu-Arg tripeptide on the LH β-subunit which enhances its activity and leads to more sulfonated residues on LH compared to FSH (28,31,32). The tripeptide motif on the LH β-subunit is not present on the FSH β-subunit, and the α-subunit recognition motif is thought to be masked by the β-subunit of FSH (33). Therefore, for FSH, the activity of the peptide-specific β1-4GalNAc-transferase is low, and the sialylation pathway dominates.

Each one of the four pituitary gonadotrophin glycoforms, designated FSHdi, FSHtetra, LHdi, and LHtri, is heterogeneous and present in blood as spectra of isoforms varying in their glycan compositions. The glycan compositions vary with respect to branching and terminal AMS residues which affect the physical-chemical properties of the isoforms. The biological effect of such spectra of isoforms, for example on their half-life in the circulation, will be a resultant of that of the multiple isoforms. The isoforms that we measure in serum in this study are representative for those reaching the ovary at the moment when the sample was harvested. The individual isoforms secreted from the pituitary have different half-lives in circulation. When the pituitary isoforms are secreted into the bloodstream, they are mixed with those remaining from previous pulsatile secretions having the longest half-lives. The composition of the isoforms secreted from the pituitary is thus slightly different from that of the isoforms circulating in blood. For FSH, the terminal SA residues on the glycans prolong survival implying that the composition of isoforms in blood becomes much more anionic than that of those secreted from the pituitary. The disappearance rate of the LH molecules is regulated both by the terminal SA and SU residues on the glycans. LH molecules with two or more terminal SU residues are quickly removed from the human circulation suggesting the presence of a mannose/sulfonated N-acetylgalactosamine-specific receptor in the human liver similar to that in rodents (34,35). A consequence of this is that the composition of LH isoforms in blood is usually slightly less anionic compared with that of LH isoforms analysed in age-matched pituitary extracts of men or women (unpublished observation).

The number of AMS residues per molecule during the menstrual cycle was at minimum at the midcycle for both FSH and LH. This is in agreement with a reported shift to a more basic pH range of FSH and LH isoforms at the midcycle phase compared with follicular and luteal phases of the normal menstrual cycle (36,37). The contents of SA and SU residues per molecule of FSH and LH in serum showed four different patterns during the cycle, all with highly significant ($P < 0.0001$) differences between levels at different phases of the cycle. In a previous study we observed that at the midcycle surge there was a considerable decrease in number of SU and increase of SA residues per LH molecule (12). In contrast to LH, the number of SA residues per serum FSH decreased at the midcycle surge. The present investigation on sera from 79 women with a normal menstrual cycle confirmed the previous results but permitted thorough studies of sulfonation and sialylation also during the follicular and luteal phases. This revealed that there were substantial time-related changes of sialic acid and SO_3-GalNAc residues per LH and FSH molecule in serum within both the follicular and luteal phases. The most impressive changes during these phases of the cycle were those of the SA residues per FSH molecule and of the SU residues per LH molecule. The values presented for FSH are mean values of FSHdi and FSHtetra and for LH of LHdi and LHtri, all four glycoforms existing as a large number of isoforms with different glycan compositions.

Relationship between degree of glycosylation and glycan composition of FSH and LH

There was a highly significant ($P < 10^{-24}$) negative correlation between the percentages of FSHdi and the average number of SA residues per molecule in our study samples. This is in agreement with a low SA content on FSHdi with a non-glycosylated β-subunit (18). As the SA residues are known to prolong the half-life of FSH in the human circulation, the FSHdi glycoforms are expected to have a shorter half-life than the FSHtetra forms. The average number of SA residues on FSH is low at midcycle and at the last days of the cycle, when the ratios of the concentrations of FSHdi to those of FSHtetra are high. An expected biological consequence is a shorter half-life of FSH during these two periods of the cycle and a longer half-life around days 8 and 20 of the cycle when the average number of SA per molecule is high. This is in agreement with shorter plasma half-lives reported for FSH molecules released in response to gonadotrophin-releasing hormone at midcycle compared with those released during follicular and luteal phases (38). A shorter half-life in the circulation was also reported for FSH during GnRH receptor blockade in women at midcycle compared with early follicular phase (21).

There was a highly significant ($P < 10^{-12}$) negative correlation between the percentage of di-glycosylated LH forms and the average number of SU residues per LH molecule. A low content of SU residues on the LHdi is expected to lead to a prolonged half-life for this LH form, as SU residues play a major role for the rapid elimination of LH from circulation. At midcycle, the concentration of LHdi was high, the average SU content on LH was low, and the SA content on LH was high compared with the rest of the cycle. An expected biological consequence of this is a prolonged half-life of LH during the midcycle phase.

It has been reported that the negative feedback effect of oestrogen on gonadotrophin secretion is a direct effect at the pituitary level (39). Administration of 17β-oestradiol and progestogen preparations to postmenopausal women has been shown to change the contents of SA and SU residues per molecule on both FSH and LH in serum (40). This suggested steroid effects on the enzymes in the trans-Golgi of the pituitary gonadotrophin-producing cells. The present demonstration of variations of the serum concentrations of the FSHdi and LHdi during the menstrual cycle opens for an additional possible explanation. The number of glycans and their compositions on the di-glycosylated forms differ from those of the tri- or tetra-glycosylated forms. The changes in serum concentration of the di-glycosylated forms in relation to those of FSHtetra and LHtri, respectively, will contribute to the variations observed for mean molecular contents of SA and SU during the cycle. Simulation experiments were made with different estimations of SA and SU contents on the low- and high-glycosylated forms of FSH and LH in combination with the values obtained in this study for the concentrations of low- and high-glycosylated forms. These simulation experiments resulted in an 'M'-shaped pattern for SA residues per FSH molecule and a 'V'-shaped pattern for SU residues per LH molecule during the cycle, similar to the patterns illustrated in Figure 4. These results indicate that the variation in the degree of glycosylation in ER may have a substantial regulatory effect on the average number of SA and SU residues per serum FSH and LH molecules during the menstrual cycle.

Conclusion

As it is known that the glycosylation and glycan composition of the pituitary gonadotrophins regulate their biological properties, we conclude that the changes observed in this study most likely are important for the fine regulation of the natural ovarian stimulation process. This information may be useful when gonadotrophins are used for the inductions of ovulation in anovulatory women. The use of mixtures of FSHdi and FSHtetra glycoforms with different glycan compositions, perhaps combined also with LHdi and LHtri glycoforms, may lead to more successful mono-ovulatory treatment regimens.

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