Oestrogens regulate pituitary $\alpha 2,3$ -sialyltransferase messenger ribonucleic acid levels in the female rat

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ABSTRACT

Follicle-stimulating hormone (FSH) is synthesized by the anterior pituitary gland in multiple molecular forms. Increased acidic/sialylated FSH charge isoforms are associated with conditions characterized by a low oestrogen output. In the present study, we analysed the dynamics of the changes in mRNA levels of the enzyme Gal β 1,3[4]GlcNAc $\alpha 2,3$ -sialyltransferase (2,3-STase) (one of the enzymes that incorporate sialic acid residues into the FSH molecule) in intact and ovariectomized rats. The anterior pituitaries of 4-day regularly cyclic adult female Wistar rats were obtained at 1000 h on the days of pro-oestrus (P), oestrus (O), dioestrus 1 (D1) and dioestrus 2 (D2), at 0200 h, 1400 h, 1800 h and 2200 h on D1, at 1800 h on day of O and at 1000 h after 7, 14, 21, 28 and 45 days of oophorectomy performed on the morning of P. Total RNA was isolated from each gland and the 2,3-STase levels were measured by Northern blot hybridization analysis employing a 346-base pair cDNA probe encoding for a non-conserved amino acid sequence of the catalytic domain of the enzyme. Maximal levels of the enzyme mRNA were detected at 1000 h on D1; thereafter, they progressively decreased by 60% during the ensuing 24 h, reaching the lowest concentration values (26% of the maximally observed level on D1) at 1000 h on day of P and remaining unchanged during

the morning of O. Administration of the potent oestradiol receptor antagonist ICI 182,780 at 1000 h on D1 completely reverted the time-dependent decrease in 2,3-STase mRNA levels observed during the afternoon of D1, whereas oestradiol benzoate administered at 1000 h on day of O significantly reduced the enzyme mRNA levels (to 21% of the levels detected in vehicle-treated controls). In ovariectomized rats, the $\alpha 2,3$ -STase mRNA progressively increased from day 21 to day 45 post castration. Administration of oestradiol benzoate on day 28 after oophorectomy significantly reduced the 2,3-STase mRNA levels (to 36% of the levels detected in vehicle-injected controls); ICI 182,780 partially counteracted this oestradiol-mediated effect. The dynamics of these changes in 2,3-STase mRNA levels partially correlated with changes in the relative abundance of the FSH charge isoforms separated by preparative chromatofocusing of anterior pituitary extracts, particularly in glands obtained during the morning of P and O. These data demonstrate for the first time that pituitary 2,3-STase is a hormonally-regulated enzyme and that the changes in transcription and/or stability of its mRNA may be involved, in part, in the post-translational processing of the FSH molecule during certain physiological conditions. *Journal of Molecular Endocrinology* (1999) **23**, 153–165

INTRODUCTION

Follicle-stimulating hormone (FSH), one of the signals produced by the anterior pituitary (AP)

gland, is involved in the regulation of several essential reproductive processes occurring at the gonadal level. This gonadotrophin is a heterodimer composed of two glycosylated subunits, α and β

(Pierce & Parsons 1981, Gharib *et al.* 1990). Each subunit of FSH exhibits two N-linked glycosylation sites located at positions Asn52 or 56 and Asn78 or 82 in FSH α and Asn6, 7 or 13 and Asn23, 24 or 30 in FSH β (Fiddes & Goodman 1979, Pierce & Parsons 1981, Esch *et al.* 1986, Watkins *et al.* 1987, Gharib *et al.* 1989). Glycosylation and processing of incorporated oligosaccharides involve a complex biosynthetic pathway which, after initiation in the rough endoplasmic reticulum, continues through the Golgi apparatus until the mature gonadotrophin is transported to secretory granules (Baenziger & Green 1988). As in other related glycoprotein hormones (luteinizing hormone (LH), chorionic gonadotrophin and thyrotrophin (TSH)), oligosaccharide structures on FSH are highly variable and play a key role in determining the biological properties of the hormone (Wilson *et al.* 1990, Ulloa-Aguirre *et al.* 1995, Ulloa-Aguirre & Timossi 1998). In FSH, most oligosaccharide chains are dibranched structures with either both ends terminating in a negatively charged group (GalNAc-SO₄⁻ or Gal-sialic acid) or one branch terminating in a negatively charged residue and the other in noncharged mannose. Dibranched oligosaccharides, with and without a bisecting GlcNAc moiety linked to the β -linked core mannose residues, and tribranched oligosaccharides of different types, but containing terminal sialic acid residues are also found (Green & Baenziger 1988, Stockell Hartree & Renwich 1992). Variations in the structure and distribution of sialylated and, to a lesser extent, sulphated Asn-linked oligosaccharides constitute the main chemical basis for FSH isoform formation and the extensive charge heterogeneity exhibited by this particular gonadotrophin (Ulloa-Aguirre *et al.* 1995).

Multiple FSH charge isoforms have been isolated from AP extracts, serum and urine of several animal species including man (Chappel *et al.* 1983, Ulloa-Aguirre *et al.* 1986, 1988, 1992a, 1995, Simoni *et al.* 1994, Stanton *et al.* 1996). The more acidic/sialylated variants exhibit longer plasma half-life but lower receptor binding activity and *in vitro* biological potency than the less acidic counterparts (Ulloa-Aguirre *et al.* 1984, 1992a, Cerpa-Poljak *et al.* 1993, Zambrano *et al.* 1996). The distribution of the intrapituitary and secreted FSH charge isoforms changes during specific physiological conditions (Padmanabhan *et al.* 1988, Wide 1989, Wide & Bakos 1993, Ulloa-Aguirre *et al.* 1995, Zambrano *et al.* 1995), thus indicating that FSH heterogeneity is a hormonally regulated phenomenon. In terms of endocrine regulation, there is growing evidence suggesting that hypothalamic and gonadal inputs (gonadotrophin-

releasing hormone (GnRH) and/or sex steroids) may be involved in modulating the molecular nature of the hormone and therefore its biological attributes (Ulloa-Aguirre *et al.* 1995). For example, it has been shown that the release of less sialylated, short-lived FSH glycoforms is increased in conditions in which the AP is exposed to high levels of oestrogens such as the periovulatory phase of the menstrual cycle or after long-term oestradiol administration to hypogonadal subjects (Galle *et al.* 1983, Padmanabhan *et al.* 1988, Wide & Bakos 1993, Wide & Naessén 1994, Zambrano *et al.* 1995). However, the molecular mechanisms responsible for the changes in sialylation of the FSH molecule during these and other physiological states remain unclear. Since the oestrous cycle represents an ordered sequence of changing hormonal events during which the pituitary gland is cyclically exposed to variable amounts of gonadal and hypothalamic hormones, we tested the hypothesis that the mRNA levels of rat Gal β 1,3GlcNAc α 2,3-sialyltransferase (2,3-STase) (the enzyme that forms the NeuAca2,3Gal β 1,3GlcNAc sequence typically found to terminate complex type N-linked oligosaccharide chains such as those present in FSH (Weinstein *et al.* 1982, Baenziger & Green 1988, Kleen & Berger 1993)) changes during the oestrous cycle and that oestrogens play a key role in the regulation of this enzyme mRNA levels.

MATERIALS AND METHODS

Adult Wistar female rats were maintained in groups of 5 animals under light-controlled conditions (lights on from 0500 to 1900 h). The body weights of the rats were between 250 and 300 g. Daily vaginal smears were obtained between 0900 h and 1100 h, and no animal was used until it had shown at least three consecutive 4-day oestrous cycles. Rats were decapitated at 1000 h on the days of pro-oestrus (P), oestrus (O), dioestrus 1 (D1) and dioestrus 2 (D2), at 0200 h, 1400 h, 1800 h and 2200 h on D1, at 1800 h on the day of O and at 1000 h after 7, 14, 21, 28 and 45 days of oophorectomy performed on the morning of P. The anterior pituitaries were dissected free from the posterior pituitaries, rapidly removed and immediately frozen at -70 °C until extraction of total cytoplasmic RNA or soluble proteins.

RNA extraction and Northern blot hybridization analysis

Total RNA from rat brain and anterior pituitary glands was isolated by the single-step acid

FIGURE 1. (Top panel) Model for the domain structure and putative topology of the $\alpha 2,3$ -STase in the Golgi membrane (TMD, transmembrane domain; branched structures, putative glycosylation sites). (Middle panel) Representation of the enzyme according to the model shown on top. Straight lines indicate the untranslated parts of the mRNA/cDNA and the differently filled boxes represent the sequences coding for the corresponding domains shown in the top figure; numbers indicate the length of the different regions (bp, base pairs; aa, amino acids). (Bottom panel) Sequence of the 2,3-STase cDNA synthesized and employed in the present study; filled boxes show the limits of the cDNA sequence.

guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi 1987) using the TRIZol reagent (Gibco, Gaithersburg, MD, USA) and following the instructions supplied by the manufacturer. For Northern blot hybridization analysis, total RNA ($\sim 15 \mu\text{g}$) was fractionated on 1.0% denaturing agarose gel and transferred to Zeta Probe GT membranes (BioRad, Hercules, CA, USA). After UV crosslinking and prehybridization, the membranes were hybridized during 16–24 h at 65 °C with the 2,3-STase cDNA probe radiolabelled with ^{32}P using a random primer labelling kit (Gibco). The filters were then washed at high stringency and autoradiographed on X-OMAT-AR film (Eastman Kodak). Each blot was stripped and rehybridized with a rat cyclophilin (Cyc) cDNA probe, prepared by random priming and used as a

control to compare the amounts of RNA loaded and transferred. Semiquantitative analysis of specific 2,3-STase mRNA was performed by densitometric scanning of the autoradiograms employing an Eagle Eye II video imaging system (Stratagene, La Jolla, CA, USA). Results are expressed as the 2,3-STase/Cyc mRNAs relative optical density (O.D.) ratios.

Synthesis and amplification of rat $\alpha 2,3$ -STase cDNA

Like other glycosyltransferases bearing type II membrane topology, the 2,3-STase has a short cytoplasmic domain, a hydrophobic signal-anchor sequence, a luminal stem region not required for catalytic activity and a large luminal catalytic domain (Kleen & Berger 1993) (Fig. 1). For the

purpose of this study, we synthesized a cDNA probe of 346 bp encoding for a non-conserved amino acid sequence (residues 236 to 350) of the catalytic domain of the enzyme located 10 amino acid residues far from the carboxyl-end of the so-called sialylmotif region (residues 178–225) (Wen *et al.* 1992, Datta & Paulson 1995). The oligonucleotide primers employed for the construction and amplification of cDNA by reverse transcription-polymerase chain reaction (RT-PCR) were chosen based on the 2,3-STase cDNA sequence reported by Wen *et al.* (1992); sequences of the corresponding sense and antisense oligonucleotides are shown in Fig. 1. Reverse transcription of poly(A)⁺ RNA extracted from female rat brain (which expresses 2,3-STase mRNA at relatively high levels (Wen *et al.* 1992, Kitagawa & Paulson 1994)) was performed employing the GeneAmp RNA PCR reagent kit (Perkin Elmer, Foster City, CA, USA) following the instructions of the manufacturer. After completion of the reverse transcription, PCR was initiated by adding 80 μ l of a mix containing 2.5 units AmpliTaq DNA polymerase (Perkin Elmer), 15 mM of the sense and antisense oligonucleotides, 2 mM MgCl₂ and 8 μ l 10 $\times$ PCR buffer II (Perkin Elmer). The temperature was raised to 95 °C for 105 s and the amplification was carried out by cycling 35 times, 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, and ended with a final extension step of 7 min at 72 °C. The 346 bp cDNA fragment was isolated by agarose gel electrophoresis followed by purification in polyester plug spin inserts (Glenn & Glenn 1994) and ethanol precipitation, and then subcloned into the pCR-Script SK (+) cloning vector (Stratagene). The sequence of the cDNA fragment was confirmed by dideoxynucleotide sequencing (Sanger *et al.* 1977). Northern blots of total RNA from different tissues probed with the 2,3-STase cDNA_{704–1050} showed probe hybridization to a mRNA of ~ 2.5 kb only in those tissues previously reported to express this particular enzyme (Wen *et al.* 1992). The relative amounts of $\alpha 2,3$ -STase mRNA detected in pituitary glands from castrated or D1 rats employing this particular cDNA probe represented $\sim 39\%$ of those present in other rat tissues that express this particular mRNA at high levels (i.e. lung, heart, brain, kidney).

Chromatofocusing of anterior pituitary extracts

AP glands were individually homogenized at 4 °C with ten strokes of a Teflon pestle in 100 μ l chromatofocusing eluent buffer (1:8 dilution of Polybuffer 74 (Pharmacia Fine Chemicals,

Piscataway, NJ, USA) in deionized water, pH 4.0) containing the protease inhibitor phenylmethylsulphonylfluoride (0.2 mg/ml; Sigma, St Louis, MO, USA). The AP homogenate was centrifuged at 1000 *g* for 30 min at 4 °C, and the supernatant fraction (AP extract) removed and stored frozen at -20 °C. Groups of four AP extracts were separately chromatofocused in 30 $\times$ 1 cm columns of PBE-94 (Pharmacia) following a chromatofocusing procedure previously described (Ulloa-Aguirre *et al.* 1992a). After measuring the pH of the eluent fractions (110 to 130 2-ml fractions/column), they were stored frozen at -20 °C until the day of radioimmunoassay (RIA) of FSH. A total of three or four AP extracts per animal group were pooled and chromatofocused separately in triplicate columns. Recoveries of FSH by this method were $76 \pm 4\%$ of the total amount originally applied to the column.

RIA of FSH and oestradiol

Purified rat FSH-I-7 (NIADDK, Bethesda, MD, USA) was iodinated by the Chloramine T method. The RIA of FSH was performed using the rat RIA kit provided by the NIADDK as previously reported (Ulloa-Aguirre *et al.* 1988); the reference preparation employed was rat FSH-RP-2 (NIADDK). To avoid interassay variations, all samples from a single column were included in the same batch. Intra-assay variability for this RIA system was 7% and the sensitivity was 0.1 ng/ml. The RIA of 17 β -oestradiol was performed employing a solid phase commercial reagent kit (Diagnostic Products Corporation, Los Angeles, CA, USA). All samples were analysed in a single RIA run; the intra-assay variability was 3% and the sensitivity was 4 pg/ml.

Statistical analysis

One-way analysis of variance and the unpaired *t*-test were used to analyse differences between groups.

RESULTS

Adult cyclic rats were killed at the times shown in Fig. 2 and total pituitary RNA was analysed for the presence of 2,3-STase mRNA. As shown in the figure, the mRNA of the enzyme changed according to the day and time of the oestrous cycle. The highest levels were recorded on the morning of D1, whereas the lowest were on the morning of the days of P and O. A progressive increase in the

FIGURE 2. Representative experiment showing the changes in anterior pituitary 2,3-STase mRNA levels throughout the rat oestrous cycle (means $\pm$ s.d. of 3 to 5 animals per group). Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group. Also shown are representative Northern blots of total RNA from anterior pituitary glands probed with radiolabelled 2,3-STase and cyclophilin cDNA probes. Similar results were found in three additional experiments.

enzyme mRNA levels was observed at 1800 h on the day of O and at 0200 h on D1 (not shown). With castration on the morning of the day of P, the AP $\alpha 2,3$ -STase mRNA levels remained at the values found on day of P until day 21 post castration, when the enzyme mRNA levels showed a progressive increase reaching maximal values after day 28 post-oophorectomy (Fig. 3).

Based on the results of the time course study, the effects of oestradiol administration on the pituitary $\alpha 2,3$ -STase mRNA levels were then tested. For this purpose, animals were subcutaneously injected with either vehicle (corn oil) or 10 μ g oestradiol benzoate (OB) on the morning of the day of O and total AP RNA was thereafter obtained at 1000 h on the presumptive D1. In a separate experiment, D1 animals received at 1000 h 2 mg/kg body weight (b.w.) of the potent oestrogen receptor antagonist ICI 182,780 (Wakeling *et al.* 1991) and its effects on 2,3-STase mRNA levels were then examined eight and twenty-four hours after its administration. As

shown in Fig. 4, administration of OB resulted in a significant decrease in the 2,3-STase mRNA to levels similar to those detected during the morning of O. In contrast, administration of a single dose of ICI 182,780 at 1000 h on D1 completely reversed the naturally occurring, time-dependent decrease in 2,3-STase mRNA levels observed at 1800 h on D1 and partially (by $30 \pm 5\%$) that occurring at 1000 h on D2 (Fig. 5). Castrated rats also received one dose of 10 μ g OB or vehicle on day 28 after oophorectomy and the levels of 2,3-STase mRNA were analysed 24 h and 48 h after OB administration. As shown in Fig. 6, oestradiol administration to female castrates significantly reduced the levels of the enzyme mRNA, although to a lesser extent than in OB-treated intact animals (to $56.4 \pm 5.7\%$ and $36.8 \pm 7.8\%$ of the levels detected 24 h and 48 h after OB administration in vehicle-injected castrated controls respectively). In this animal group, administration of ICI 182,780 and oestradiol benzoate, in tandem, reversed by 36% the effects of

FIGURE 3. Changes in anterior pituitary 2,3-STase mRNA levels before and after castration on the morning of the day of P (means $\pm$ s.d.). Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group. Also shown are representative Northern blots of total RNA from anterior pituitary glands probed with radiolabelled 2,3-STase and cyclophilin cDNA probes. The results are representative of 3 independent experiments.

OB on 2,3-STase mRNA levels observed at 48 h (Fig. 7).

Chromatofocusing fractionation of AP extracts revealed a marked increase in the relative abundance of FSH charge isoforms with elution pH values ≥ 5.0 at the time of highest serum oestradiol concentrations and lowest 2,3-STase mRNA levels, i.e. on the morning of P (Fig. 8). These less acidic FSH isoforms remained relatively increased during the day of O in a setting of low enzyme mRNA and relatively decreased serum oestradiol levels. Finally, on the morning of D2, there was a dissociation between the enzyme mRNA and serum oestradiol levels and the charge distribution of AP FSH, with the highest values for the most acidic isoforms in a setting of moderately increased serum oestradiol and decreased levels of the 2,3-STase mRNA (Fig. 8). In castrated rats, the pH distribution pattern of AP FSH was similar to that exhibited by intact animals killed at 1000 h on D1, with $>80\%$ of total FSH recovered in elution pH values <5.0 ; a

modest and not significant increase in less acidic isoforms (elution pH values ≥ 5.0) was detected exclusively on day 28 post-oophorectomy (not shown).

DISCUSSION

In the present study, we found that the mRNA levels of the 2,3-STase, one of the enzymes that add sialic acid to FSH, varied significantly according to the time and day of the rat oestrous cycle and that the dynamics of these changes in the enzyme mRNA levels were temporally associated with variations in serum oestradiol concentrations. Specifically, during the preovulatory days (D2 and P days) we observed a progressive decrease in 2,3-STase mRNA levels in the face of increasing serum oestradiol concentrations, reaching its lowest values immediately before and after maximal pituitary exposure to this sex steroid, i.e. in the

FIGURE 4. Changes in anterior pituitary 2,3-STase mRNA after administration of 10 μ g oestradiol benzoate (OB) or vehicle (Vh) on the morning of the day of oestrus (grey bars on the right) (means $\pm$ s.d. of three separate experiments). The dynamics of the changes in pituitary 2,3-STase mRNA throughout the cycle in untreated animals is also shown for comparison (black bars on the left). Results are expressed as percentage change considering the normalized O.D. in the oestrous group as 100%. Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group. Representative Northern blots of total RNA from anterior pituitary glands probed with radiolabelled 2,3-STase and cyclophilin cDNA probes are also shown.

morning of P and O respectively; conversely, a significant rise in enzyme mRNA levels occurred at the time of lowest oestradiol output, thus suggesting that changes in oestrogen levels may influence the rate of transcription and/or stability of this glycosyltransferase mRNA. This possibility was in fact experimentally documented by the studies showing that administration of OB to both intact and castrated female rats blocked the naturally occurring increase in $\alpha 2,3$ -STase mRNA imposed by the low oestrogenic milieu, whereas the opposite effect was observed when the pure anti-oestrogen ICI 182,780 was injected to block the effects of endogenous or exogenous oestrogens. Apparently, the suppressive effects of oestradiol on the enzyme mRNA levels may extend throughout several hours

FIGURE 5. 2,3-STase mRNA levels in intact rats exposed during 8 h and 24 h to either vehicle (control) or the anti-oestrogen ICI 182,780 (2 mg/kg b.w.) administered subcutaneously at 1000 h on D1 (means $\pm$ s.d.). Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group. Representative Northern blots of total RNA from anterior pituitary glands probed with radiolabelled 2,3-STase and cyclophilin cDNA probes are also shown. The results are representative of 3 separate experiments.

or even days after maximal oestradiol exposure as disclosed by the persistently low 2,3-STase mRNA levels during the morning of O and after gonadal removal on the day of P. Nevertheless, the finding that the $\alpha 2,3$ -STase mRNA levels remained persistently low at least for 14 days following oophorectomy, and that the response to both oestradiol benzoate and ICI 182,780 in the gonadectomized group was considerably attenuated as compared with those exhibited by the intact animals, suggests that other extrapituitary factors may be additionally involved in the control of this enzyme mRNA levels (see below).

We and others have shown that sialic acid plays a key role in determining both the charge isoform distribution and the biological activity of FSH and other glycoprotein hormones (Reichert 1971, Ulloa-Aguirre *et al.* 1984, Chappel *et al.* 1982, 1984, Wide 1989, Schaaf *et al.* 1997). Incubation of rat

FIGURE 6. Changes in anterior pituitary 2,3-STase mRNA levels after 24 h and 48 h of oestradiol benzoate administration (OB; 10 μ g) on day 28 post-oophorectomy (means $\pm$ s.d. of 3 independent experiments). Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group. Representative Northern blots of total RNA from anterior pituitary glands probed with radiolabelled 2,3-STase and cyclophilin cDNA probes are also shown.

and monkey AP extracts and recombinant human FSH with the enzyme neuraminidase progressively decreased the most acidic components of the hormone while concomitantly increasing the proportion of less acidic forms until only FSH isoforms with elution pH values of 6.4 or greater were detected (Chappel *et al.* 1984, Ulloa-Aguirre *et al.* 1984, Galway *et al.* 1990). Studies with neuraminidase-treated human sera and pituitary extracts also demonstrated that removal of sialic acid residues induces a shift in the charge of FSH isoforms towards less acidic isomers, thus suggesting that some of the charge variations detected in different physiological conditions are mainly due to differences in the terminal sugar content of FSH (Wide 1982, 1985, 1989). In the present study, chromatofocusing fractionation of AP extracts allowed the identification of significant shifts in FSH charge isoform distribution throughout the cycle, with a significant increase in the relative

abundance of the less acidic FSH isoforms during the morning of the day of P and to a lesser extent during O, times at which low 2,3-STase mRNA levels were detected. However, such a close inverse relationship between the abundance of less acidic isoforms and enzyme mRNA levels was practically absent during the following days of the cycle, particularly on D2, as well as after 7 and 14 days of castration, conditions during which the highest relative abundance of more acidic isoforms was detected in a setting of significantly decreased 2,3-STase mRNA levels. The existence of a partial rather than a complete correlation between this enzyme mRNA levels and the changes in FSH charge isoform distribution may be due to the following: (1) the concomitant participation of other sialyltransferases (e.g. $\alpha 2,6$ -STase) or glycosyltransferases (which may in turn be regulated by one or several extrapituitary factors) in FSH processing, including sialylation and/or sulphation (Baenziger &

FIGURE 7. The effects of oestradiol benzoate (OB) or ICI 182,780 (ICI) plus OB on 2,3-STase mRNA levels in oophorectomized rats (means $\pm$ S.D. of three separate experiments). Groups of four rats were castrated on the morning of P. On day 28 after oophorectomy, animals were divided into three treatment groups. The control group received vehicle at all times; the OB group was treated with vehicle at 1000 h, OB (10 μ g subcutaneously) at 1100 h and again with vehicle at 1000 h on the next day. Finally, the ICI 182,780 group received 2 mg/kg b.w. of the anti-oestrogen at 1000 h, followed by OB at 1100 h and again ICI 182,780 at 1000 h on the next day. All animals were killed by decapitation 48 h after the start of the experiment and the pituitary glands were processed for 2,3-STase mRNA measurement as described in Materials and Methods. Results are expressed as percentage change considering the normalized O.D. in the control group as 100%. Different letters above the bars indicate the existence of significant ($P < 0.05$) differences between each group.

Green 1988, Darmesh & Baenziger 1993, Helton & Magner 1994a,b, Kitagawa & Paulson 1994, Rafferty *et al.* 1995); (2) to variations in substrate availability, which may eventually allow for effective sialylation in conditions characterized by low enzyme levels and reduced FSH production, such as those present during both dioestrous days (Smith *et al.* 1975, Ortolano *et al.* 1988, present study); (3) to oestrogen-independent variations in the half-life of the enzyme, and/or (4) to the inability of our FSH RIA system to allow for a clear distinction between increased production of more acidic/sialylated isoforms and decreased immunogenicity of epitopes present in certain FSH glycoforms bearing less complex oligosaccharide structures

and whose relative abundance may be increased at a given time (Dahl & Stone 1992, Stanton *et al.* 1996).

These and other previously reported *in vivo* experiments (Padmanabhan *et al.* 1988, Wide & Naessén 1994) do not clarify unambiguously whether oestradiol modifies 2,3-STase mRNA levels and thus protein sialylation directly on the pituitary gland, on the hypothalamus to regulate GnRH activity, or by a combination of several pathways. Although some studies strongly implicate GnRH in modulating the glycosylation and degree of sialic acid-dependent charge heterogeneity of LH and FSH respectively (Galle *et al.* 1983, Miller *et al.* 1983, Liu *et al.* 1992, 1994, Ulloa-Aguirre *et al.* 1992b), more recently, several *in vivo* studies involving experimental animals and humans have allowed us to distinguish between pituitary and hypothalamic sites of regulation by oestrogens. Using the nutritionally growth-restricted ovariectomized lamb as the experimental model (state of hypogonadotrophism due to central inhibition of GnRH secretion in which the pituitary responsiveness to exogenous GnRH is preserved (Ebling *et al.* 1990)) it has been shown that whereas pulsatile administration of GnRH failed to alter the pattern of charge isoform distribution of intrapituitary and secreted FSH, oestradiol treatment selectively increased the secretion of less acidic/sialylated isoforms (Hassing *et al.* 1993, Lee *et al.* 1998). Likewise, although in normally ovulating women the relative abundance of less acidic FSH isoforms increased dramatically during the periovulatory period (a time of increased oestradiol levels and pituitary GnRH sensitivity) (Padmanabhan *et al.* 1988, Wide & Bakos 1993, Zambrano *et al.* 1995), the changes observed in the distribution of more and less acidic/sialylated serum FSH isoforms were not significantly altered by the consecutive administration of a low and high GnRH dose during each cycle phase (Zambrano *et al.* 1995). Although such studies data do not exclude additional intrapituitary loci of control via autocrine and/or paracrine interactions (whose nature and role, if any, in modifying the post-translational molecular forms of FSH are not known), the overall data strongly indicate that oestrogens play a determinant role in defining the degree of glycosylation of the FSH molecule during certain physiological conditions.

The present results are in agreement with other studies showing that products from target glands under the control of the AP gland are tightly involved in the posttranslational processing of the trophic signal (Miura *et al.* 1989, Gyves *et al.* 1990, Darmesh & Baenziger 1993, Helton & Magner 1994a,b, Persani *et al.* 1998). In fact, it has

FIGURE 8. Temporal relationship between changes in anterior pituitary 2,3-STase (STase) mRNA levels throughout the oestrous cycle (a), the serum oestradiol concentrations (black bars in (b)) and the charge distribution of pituitary FSH at 1000 h on each day of the oestrous cycle (c); data in (c) are presented as the proportion of FSH recovered within elution pH values ≥ 5.0 and < 5.0 normalized as a percentage of total FSH recovered from each single chromatofocusing run ($n=$ three columns per day of cycle). Intrapituitary FSH content was 0.15 ± 0.04 , 0.16 ± 0.02 , 0.06 ± 0.01 and 0.08 ± 0.01 μg FSH-RP-2/gland on the days of P, O, D1 and D2 respectively. For each graph the results represent the means $\pm$ S.D. The pattern of oestradiol levels in serum samples taken at frequent intervals throughout the oestrous cycle as reported by Smith *et al.* (1975) is also shown for comparative purposes (closed circles in (b); means $\pm$ S.E.M.). Horizontal bars represent the dark period.

been demonstrated that activities of some glycosyltransferases ($\alpha 2,6$ -sialyltransferase and GalNAc-transferase) and mannosidases (α -mannosidase-II), may be modulated specifically at the pituitary level by thyroid hormones (Helton & Magner 1994a,b) and oestrogens (Darmesh & Baenziger 1993), thereby potentially affecting sialylation and sulphation of TSH and LH respectively. Thus, regulation of the structure and biological properties of glycoprotein hormones by specific stimuli may represent an additional level of feedback control by which the target cell regulates the duration and intensity of the trophic signal released from the AP gland.

ACKNOWLEDGEMENTS

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACyT, grants 0004P-N9505 and G016M), the Programa Latinoamericano de Capacitación e Investigación en Reproducción Humana (PLACIRH), the Universidad Autónoma Metropolitana-Iztapalapa, and PADEP-UNAM (500307), México DF. The authors are indebted to the NIADDK for the rat FSH RIA material, to Drs Victor Sales and José Luis Velázquez from Zeneca Farma, Mexico, for the ICI 182,780 and to Mr Roberto Chavira for performing the RIA of 17β -oestradiol. Pablo Damián-Matsumura is a postgraduate student from the Facultad de Estudios Profesionales-Zaragoza, Universidad Nacional Autónoma de México, Mexico.

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REVISED MANUSCRIPT RECEIVED 21 April 1999