Differential effects of the charge variants of human follicle-stimulating hormone

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Abstract

FSH is synthesized and secreted by the anterior pituitary gland in multiple molecular forms; the release of these isoforms depends on the endocrine status of the donor at the time of sample collection. In the present study, we analysed the possibility that the FSH charge isoforms may exert differential effects at the target cell. Seven FSH isoform mixes were isolated from pooled anterior pituitary glycoprotein extracts by high resolution chromatofocusing, followed by affinity chromatography, which removed nearly 90% of the LH that co-eluted with the FSH isoforms during chromatofocusing. The isoforms (isoform I, pH >7.10; II, pH range 6.60–6.20; III, pH 5.47–5.10; IV, pH 5.03–4.60; V, pH 4.76–4.12; VI, pH 4.05–3.82 and VII, pH <3.80) were then tested for their capacity to stimulate cAMP release, androgen aromatization and tissue-type plasminogen activator (tPA) enzyme activity and cytochrome P450 aromatase, tPA and inhibin/afii9825-subunit mRNA production by rat granulosa cells in culture. cAMP and oestradiol production were determined by RIA, tPA enzyme activity by SDS-PAGE and zymography and all mRNAs by northern blot hybridization analysis and semiquantitative RT-PCR. All isoforms, with the exception of isoform I, stimulated synthesis and release of cAMP, oestrogen and tPA enzyme activity in a dose-dependent manner; the potency of the less acidic isoforms (pH 6.60–4.60) was greater than that exhibited by the more acidic/sialylated analogs (pH 4.76 to <3.80; potencies II >III >IV >V >VII >VI). A similar trend was observed in terms of cytochrome P450 aromatase and tPA mRNA production. In contrast, when FSH-stimulated production of α-inhibin mRNA was analysed, isoforms V–VII were significantly more potent (two- to threefold) than the less acidic/sialylated counterparts (II–IV). In contrast to isoforms II–VII (which behaved as FSH agonists), isoform I (elution pH >7.10) completely blocked P450 aromatase and tPA mRNA expression, without altering that of a constitutively expressed gene (glyceraldehyde-3-phosphate dehydrogenase). These results show for the first time that the naturally occurring human FSH isoforms may exhibit differential or even unique effects at the target cell level.

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Introduction

The pituitary gonadotrophin follicle-stimulating hormone (FSH) regulates the development and maintenance of several basic reproductive processes, including gametogenesis, follicular maturation and ovulation (Chappel et al. 1983). This gonadotrophin is a member of a glycoprotein hormone family that includes luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and choriogonadotrophin and consists of a common α-subunit and a unique β-subunit (Pierce et al. 1971, Boothby et al. 1981, Pierce & Parsons 1981). Each subunit has two asparagine-linked (N-linked) oligosaccharides, attached at positions 52 and 78 on the α-subunit and at positions 7 and 24 on the β-subunit of human FSH (Baenzinger & Green 1988). These play a significant role in determining the plasma
half-life, specific interaction with the target-cell receptor and capability of the hormone to activate one or more intracellular signal transduction pathways (Morell et al. 1971, Sairam & Bhargavi 1985, Sairam 1989, Flack et al. 1994h, Valove et al. 1994, Arey et al. 1997). In all glycoprotein hormones, the oligosaccharides are highly variable (Baenzinger & Green 1988). Recent evidence indicates that, regardless of sex, this variability is strongly influenced by the endocrine milieu of the donor at the time of tissue or sample collection (Padmanabhan et al. 1988, Wide & Bakos 1993, Zambrano et al. 1995, Anobile et al. 1998). Variations in the oligosaccharide structures on these glycoprotein hormones constitute the main biochemical basis for isoform formation and the large array of molecular forms found within the pituitary gland and in the circulation (Ulloa-Aguirre et al. 1995).

FSH isoforms may be separated on the basis of their charge, determined by the structure and distribution of sialylated N-linked oligosaccharide structures (Baenzinger & Green 1988, Ulloa-Aguirre et al. 1995). Although in both the pituitary gland and serum the relative proportion of highly acidic/sialylated isoforms predominates over that of the less sialylated forms, the latter variants substantially increase during the periovulatory period (Padmanabhan et al. 1988, Wide & Bakos 1993, Zambrano et al. 1995, Anobile et al. 1998). As a consequence of their structural differences, FSH isoforms differ in their capability to bind to target-cell receptors, survive in the circulation and evoke biological responses (Ulloa-Aguirre et al. 1988a, 1992, Yding Andersen et al. 1999, Zambrano et al. 1999). Highly acidic/sialylated isoforms have considerably longer plasma half-lives but more modest and/or slower capacities to elicit cellular responses in vitro and in vivo (Wide 1986, Ulloa-Aguirre et al. 1992, Timossi et al. 1998b, Vitt et al. 1998, Barrios de Tomasi et al. 1999, Yding Andersen et al. 1999). Although studies in a variety of species and in humans have clearly demonstrated heterogeneity of FSH (and other glycoprotein hormones), the functional significance of such a variety of isoforms for a single hormone remains unclear. In this regard, we have previously proposed (Ulloa-Aguirre et al. 1995) that at least three criteria must be met to assign physiological and clinical significance to FSH heterogeneity: isoforms identified in the anterior pituitary gland must be secreted into the circulation and reach the target cell, circulating FSH isoforms must be differentially regulated by the endocrine milieu, and changes in the distribution of circulating isoforms during different physiological conditions must be of sufficient magnitude to alter the net potency of the hormone or, alternatively, the biological actions rather than potencies must differ among the various isoforms. Although abundant evidence exists from several laboratories in support of the first two criteria (Padmanabhan et al. 1988, Wide & Bakos 1993, Zambrano et al. 1995, Phillips et al. 1997, Anobile et al. 1998), evidence supporting the third is somewhat circumstantial (Zambrano et al. 1995, Timossi et al. 1998a). Limited studies comparing chemically derived ovine FSH isoform mixes have shown that deglycosylated FSH is less potent than the native FSH variant in stimulating cAMP and oestrogen production, but more potent in provoking α-inhibin production (Beitinz & Padmanabhan 1991, Ulloa-Aguirre et al. 1995). Similar studies using natural variants testing the potential differential functions have not been undertaken.

In the present study we explored the possibility that naturally occurring, pituitary-derived FSH isoforms may exert differential effects at the target cell level. To investigate this issue, we analysed the effects of seven anterior pituitary FSH charge isoform mixes on granulosa cell function, monitoring the FSH-induced generation of two different end products (oestradiol-17β) and tissue-type plasminogen activator (tPA) with their corresponding mRNAs, in addition to expression of α-inhibin mRNA, which is also under the control of this gonadotrophin.

### Material and Methods

#### Pituitary extracts

Adult human pituitaries from victims of accidental death were collected at autopsies performed no later than 24 h postmortem. No selection was made for age and sex and the bodies were kept at 8 °C within 3–4 h after death. The anterior pituitary glands were stored frozen at −70 °C until extracts were prepared. Total anterior pituitary glycoprotein extracts from two different batches of pooled pituitaries were obtained by the method of Jones et al. (1979). Extracts were mixed, separated in several aliquots and kept frozen at −70 °C until required for chromatofocusing. The study was approved by the human and animal research ethics committees of the Institute at which the work was conducted.

#### Preparative chromatofocusing of pituitary glycoprotein extracts and LH immunoextraction

High-resolution chromatofocusing of FSH contained in the two pooled batches of pituitary glycoprotein extracts was performed as described previously (Zambrano et al. 1996, Timossi et al. 1998a). After RIA determination of LH and FSH contained in 5–15 µl aliquots of each fraction collected from three chromatofocusing separations, fractions containing the greatest concentrations of immunoreactive FSH (within a pH gradient of 7.10 to 3.8) and those recovered at both ends of the pH window (elution pH values >7.10 and <3.8) were pooled as shown in Fig. 1. The pools were transferred to dialysis membrane tubes (molecular mass cut-off 12 000–14 000 Da), dialysed for 24 h at 4 °C against deionized water and then against 0.01M ammonium carbonate, pH 7.5. The dialysed...
solution was dried by lyophilization. Powders containing each isoform pool (I–VII in Fig. 1) were then redissolved in 0·1 M ammonium bicarbonate pH 7·4 and transferred to 1 × 10 cm columns of monoclonal antiLH-IgG (antiLH-BG7; Cuba–Mexico World Health Organization RIA Reagents Program, Mexico DF, Mexico) immobilized in Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ, USA) in order to remove the LH that co-eluted with the isoforms during the chromatofocusing separation. This procedure removed >90% of the immunoreactive LH present in each original concentrate as determined by RIA. Each isoform concentrate was finally redissolved in McCoy’s 5A modified medium (Gibco Brl, Gaithersburg, MD, USA) and stored frozen at −70 °C until required for use. Both prolactin and growth hormone were undetectable in the isoform pools used in the experiments when analysed at final dilutions by highly specific RIAs.

Granulosa cell culture and in vitro bioassays of FSH

Granulosa cells were collected by follicular puncture of ovaries from 21-day-old Wistar rats implanted for 4 days with diethylstilboestrol (Sigma Chemical Co., St Louis, MO, USA)-containing silastic capsules and cultured in 16 mm 24 well plates at a density of 1·0–1·5 × 10⁵ cells/ml in McCoy’s 5A medium supplemented with 2 mM t-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) in 5% CO₂ at 37 °C and 80% humidity. After 24 h the cells were washed with unsupplemented medium and then incubated in the presence or absence of the various FSH isoforms or standards added to 500 µl fresh McCoy’s medium supplemented with t-glutamine, antibiotics, 0·125 mM methyl-isobutyl-xantine (MIX) (Sigma) and 10⁻⁶ M androstenedione (Sigma). At the end of the culture period (48 h for oestrogen and cAMP production, 72 h for induction of tPA enzyme activity, 24 h for cytochrome P450 aromatase (P450_aro) and α-inhibin mRNA production and 12 h for tPA mRNA expression), media were collected and either stored frozen at −70 °C until quantitation of oestrogen and cAMP or dried by vacuum centrifugation and immediately processed for determination of tPA enzyme activity. Cells were scraped with a rubber policeman and the content of wells belonging to the same incubation group pooled and processed for total RNA isolation as described below.

Figure 1 The pattern of pH distribution of pituitary FSH after chromatofocusing of an anterior pituitary glycoprotein extract. The fractions pooled to obtain the various FSH isoform concentrates (I–VII) are denoted by the brackets. The arrow indicates the addition of 1 M NaCl to the chromatofocusing column.

Radioimmunoassays

RIA of LH and FSH Purified human FSH (human FSH-I1, National Institute of Diabetes, Digestive and Kidney Disease, National Hormone and Pituitary Program (NHPP), Torrance, CA, USA) was iodinated by the lactoperoxidase–glucose oxidase method (Bex & Corbin 1981). After separation of the protein-bound and free iodine–125 by Sephadex G-100 (Pharmacia) column chromatography, ¹²⁵I-labelled FSH was further purified by concanavalin A chromatography (Pharmacia) as described by Dufau et al. (1972). The RIA was performed using reagents provided by the NHPP. The reference preparation LER-907 (which contains almost all FSH charge isoforms detected in crude pituitary extracts (Chappel et al. 1986)) was used to construct the standard curve and anti-human FSH-6 was used at a final dilution of 1 : 250 000. This antiserum exhibits less than 0·1% cross-reactivity with highly purified human LH and undetectable reactivity with highly purified free α-subunit. In this RIA system, all isoforms displaced ¹²⁵I-labelled FSH from the antibody in a parallel fashion when tested at seven to 10 different dilutions; in fact, simultaneous curve fitting of the dose–response curves revealed no significant differences among the slopes generated by FSH present in the LER-907 standard and the several isoform pools fractionated by chromatofocusing (Zambrano et al. 1996). FSH concentrations were expressed in terms of the FSH-I1 standard unless indicated otherwise. The LH RIA was performed using ¹²⁵I-labelled LH (LH-II) as the tracer, anti-human LH-2 as antiserum and LER-907 as the curve standard (NHPP). For both RIAs, the inter- and intra-assay coefficients of variation were <13% and <8% respectively.

RIA of oestradiol-17β The amount of oestrogens released into the medium was determined using an antiserum against oestradiol-17β kindly provided by the Cuba–Mexico World Health Organization RIA Reagents Program at a final dilution 1 : 210 000. Cross-reactivity of this antiserum was <0.02% with cortisol, progesterone and testosterone, 0·8% with oestradiol and 22% with oestrone. Results are expressed as total oestrogens released from granulosa cells in culture. All samples from a single

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experiment were assayed in the same RIA. The intra-assay coefficient of variation was less than 4%, and the sensitivity was 6·3 pg/tube.

**RIA of cAMP** The cAMP RIA was performed using 2-O-monomuccinyl cAMP tyrosylmethyl ester (Sigma) radiolabelled by the chloramine T method and a cAMP antiserum (anti-cAMP C-1) prepared in one of our laboratories (P M C). This antiserum showed <0·1% cross-reactivity with cGMP, 2',3'-cAMP, 5'-cAMP, 3'-cAMP, ADP, GDP, ATP, CTP and MIX. After incubation at 4 °C for 24 h, antibody-bound and free cAMP were separated by ethanolic precipitation followed by centrifugation at 3000 r.p.m. for 15 min at 4 °C. The sensitivity of the assay was 4 fmol/tube. All samples from a single experiment were analysed in the same assay. The intra-assay coefficient of variation was <6%.

**Detection of tPA enzyme activity**

Detection of tPA enzyme activity was assessed by zymography using the method of Heussen & Dowdle (1980) optimized for samples from culture medium (Timossi et al. 1998a). Semiquantitative estimation of tPA-mediated protease activity detected in pooled vacuum-centrifugation-dried media was performed by densitometric scanning using a digital image analyser (Eagle-Eye II, Stratagene, La Jolla, CA, USA). As the urokinase-type plasminogen activator (uPA) enzyme activity was not altered by FSH treatment, relative amounts of tPA enzyme activity could be standardized with the corresponding uPA-dependent protease activity in the same gel. Results are expressed as the tPA : uPA relative OD ratio—the ratio of the lytic area corresponding to tPA relative to that stimulated by uPA.

**RNA extraction and northern blot hybridization analysis**

Total RNA from granulosa cells was isolated by the single-step acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987) using the TriZol reagent (Gibco), following the instructions supplied by the manufacturer. Approximately 20 µg total RNA extracted from three individual culture wells were electrophoresed on 1% denaturing agarose gels (Sambrook et al. 1989). Thereafter the RNA was transferred to nylon membranes (Z-probe GT, Bio-Rad), u.v. cross-linked (Strataginker 1800, Stratagene) and prehybridized with 25 mM Na₂HPO₄, 7% SDS–1 mM EDTA, pH 8·0 at 65 °C for 4 h. A specific phosphorus-32- or digoxigenin-labelled cDNA probe for rat tPA (provided by Dr Tor Ny, University of Umeå, Umeå, Sweden), rat P45₀ₐ₉₉ (kindly provided by Dr JoAnne S Richards, Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA), or human α-inhibin (Genentech, San Francisco CA, USA; 80% homology with the rat α-inhibin cDNA sequence (according to Genestream II Networks Servers for Biology, |Align Online Software, Institut de Génétique Humaine, Montpellier, France)) was added to the membrane and hybridized and washed at high stringency and autoradiographed on X-Omat AR films (Eastman Kodak de Mexico, Mexico DF, Mexico) or incubated with an anti-digoxigenin, alkaline phosphate-conjugated antibody and thereafter with the chemiluminescence substrate CDP-STAR (Boehringer Mannheim Bioquimica, Mexico DF, Mexico) and exposed for 3–10 min to X-Omat AR films. Semiquantitative analysis of each specific mRNA was performed by densitometric scanning of the films. Results are expressed as the amount of mRNA relative to that of 18 S rRNA, in OD units.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Specific mRNA production was also assessed by semiquantitative RT-PCR assay. Briefly, 2 µg total RNA sample were reverse-transcribed (PCR Superscript, Gibco) in a volume of 20 µl containing 200 U reverse transcriptase MuLV, a deoxynucleotide mixture (dNTPs) and oligo-dT as transcription primer, for 60 min at 42 °C. The cDNA obtained was subjected to PCR amplification using specific oligonucleotide primers for each cDNA (Table 1). Each reaction contained 2·5 IU Taq polymerase (Perkin Elmer, Foster City, CA, USA), 2 mM primer pairs and 1 mM dNTPs in PCR buffer I (Perkin Elmer). Paired reactions were carried out using 100 ng RNA to detect amplification from contaminating genomic DNA and one additional tube per assay containing no cDNA to detect contamination. Tubes were incubated in a thermal cycler (Thermal Cycler 9600, Perkin Elmer) for 30 cycles as follows: 15 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C for P45₀ₐ₉₉ mRNA; 15 s at 94 °C, 30 s at 54 °C and 45 s at 72 °C for tPA mRNA and glyceraldehyde-3-phosphate dehydrogenase (G3PD). For α-inhibin mRNA, incubations were performed for 15 cycles, 15 s at 94 °C, 30 s at 54 °C, and 75 s at 72 °C. Extension time for all last cycles was 7 min. The PCR products so obtained were then electrophoresed in a 1% agarose gel for 30 min at 50 V and the gels dyed with ethidium bromide and digitalized in an image analyser (Eagle-Eye II, Stratagene). Individual PCR reactions were standardized to that of G3PD and are expressed as relative OD ratios.

**Statistical analysis**

One-way analysis of variance (ANOVA) was used to determine between-group differences in biological to immunological (B/I) FSH activity ratios and cAMP production responses. When differences existed, Bonferroni protected t-tests were used to determine which means were different. Probabilities <0·05 were considered statistically significant.
Results

Separation of anterior pituitary glycoprotein extracts through chromatofocusing columns disclosed the presence of multiple distinct peaks of FSH and LH immunoreactivity with pH values ranging from >7·1 to <3·75 (Fig. 1). Fractions comprising the various major FSH peaks (I–VII in Fig. 1) were processed as described in Materials and Methods and tested for in vitro bioactivity. All isoform concentrates displaced 125I-labelled FSH from the antibody and from cultured granulosa cells in a dose-dependent manner in parallel with the results obtained with LER-907 and highly purified human FSH (not shown).

In preliminary experiments, cultured granulosa cells exposed to human recombinant FSH (rFSH; Org32489, Organon International BV, Oss, Holland) for 6, 12, 24, 48, 60 and 72 h produced progressively more oestrogen, cAMP and tPA enzyme activity, with 48–72 h of exposure to rFSH being most effective for oestrogen production, 48 h for cAMP accumulation and 72 h for tPA enzyme activity (not shown). As previously reported (Timossi et al. 1998a), the capacity of isoform concentrate I to stimulate androgen aromatization and cAMP production and induce tPA enzyme activity was considerably reduced compared with that exhibited by the less acidic, mid-acidic and strongly acidic isoforms (not shown). In contrast, incubation of rat granulosa cells in culture with increasing doses (0·18–3·0 ng) of FSH isoforms II–VII for 48 h, stimulated significant dose-dependent oestrogen (Fig. 2) and cAMP production (not shown). The in vitro biological activity (expressed as the B/I activity ratio) of isoforms II–VII, decreased as the elution pH value of the corresponding isoform declined (Table 2). cAMP production was also significantly (P<0·05) greater when the cells were stimulated by FSH isoforms with elution pH values 6·60 to 4·60 (ED50 values: isoform II = 2·44 ± 0·1; III = 2·48 ± 0·09; IV = 2·48 ± 0·09; mean ± s.d.) than when they were incubated with isoforms with pH values 4·76 to <3·80 (ED50 values: isoform V = 2·70 ± 0·15; VI = 2·89 ± 0·20; VII = 2·78 ± 0·16). Similar effects were observed when induction of tPA enzyme activity by cultured granulosa cells exposed to 2·8 ng (a dose to provoke nearly maximal response to LER–907 or human recombinant FSH in terms of oestrogen production (Fig. 2) or tPA enzyme activity (Timossi et al. 1998a)) of these FSH isoforms for 72 h was measured as the biological end point (Fig. 3).

In granulosa cells exposed to rFSH for periods ranging from 6 to 72 h, maximal P450 arom mRNA levels were observed after 24 h of FSH exposure, whereas those

| Table 1 Primer oligonucleotide sequences used to amplify the cDNA sequences as described in Material and Methods |
|---------------------------------|--------------|
| cDNA & | Sequence | Reference |
| Aromatase Sense | CTTGTGGATGGGGATTGGAAG | Rappaport & Smith 1996 |
| Aromatase Antisense | CCACACAGATTTTCGATCTGC | Rappaport & Smith 1996 |
| G3PD Sense | GTAGCCATATTCATGTGCTA | * |
| G3PD Antisense | AAGGCATCCGACGGCAACCA | * |
| Rat α-inhibin Sense | TGTCTCCAGGCGATCCTTCCCCA | * |
| Rat α-inhibin Antisense | AAAGAAGCTGGGAGGGTACAGTC | * |
| tPA Sense | GCATCCAGGGAATTCACTGC | * |
| tPA Antisense | GAATCCAGGGAATTCACTGC | * |

* Sequences obtained from the OLIGO 4.0 software, National Biosciences Inc., Plymouth, MN, USA
corresponding to tPA mRNA showed two peaks of maximal production, the first occurring 12 h and the second 60 h after exposure to rFSH (not shown). The effect of the isoforms on P450arom and tPA mRNA levels is shown in Figs 4–6. Incubation of cultured granulosa cells in the presence of 2·8 ng/well isoforms II–VII or the LER-907 standard for 48 h resulted in the induction of the previously reported (Hickey et al. 1990) three P450arom mRNA transcripts (3·3, 2·6 and 1·9 kb in size) as disclosed by northern blot hybridization analysis (Fig. 4). The same mRNA transcripts were also detected in total RNA extracted from ovarian tissue of 18-day-pregnant rats used as a positive control (Richards 1994). Less acidic isoforms, specifically isoforms II, III and IV (pH 6·6–7·0), exhibited greater capacity to induce P450arom mRNA than their more acidic counterparts (Fig. 4A). Comparable results regarding the potency of isoforms to induce P450arom mRNA were found by semiquantitative RT-PCR (Fig. 4B). Granulosa cells exposed to equivalent amounts of isoforms II–VII during a 12-h period produced tPA mRNA in a fashion similar to that observed for P450arom mRNA, as disclosed by both northern blot hybridization and RT-PCR analysis (Fig. 5).

In a recent study (Timossi et al. 1998a), we showed that FSH isoform I, which binds to the FSH receptor and presumably possesses a low sialic acid content, behaved as an antagonist of FSH action. In the present study, we extended these observations by analysing the effects of isoform I on the FSH-induced expression of both P450arom and tPA mRNA. As shown in Fig. 6, in the presence of the ED50 of rFSH for maximal androgen aromatization and tPA enzyme activity (2·8 ng/culture well), isoform I antagonized the rFSH-induced expression of P450arom and tPA mRNAs without blocking the production of G3PD mRNA, which is constitutively expressed by the granulosa cells.

The last end point measured in this study was induction of α-inhibin mRNA by the pituitary FSH isoforms II–VII (the amount of isoform I available for this particular analysis was insufficient). As shown in Fig. 7A, incubation of granulosa cells for 24 h (time of maximum production of FSH-induced α-inhibin mRNA) in the presence of equivalent amounts of each isoform resulted in the expression of α-inhibin mRNA in a fashion opposite to that observed for P450arom mRNA production; α-inhibin mRNA induction was greater in incubations including the more acidic/sialylated isoforms (particularly isoform VII) than in those in which the less acidic counterparts were

### Table 2

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*S* Significant differences (*P*<0·05) among isoforms (one-way analysis of variance and Bonferroni protected *t* tests).

#### Figure 3

Upper panel: Detection of tPA enzyme activity in conditioned media from granulosa cells exposed to FSH isoforms. The location of tPA (M, ~70 000) and high molecular weight (M, ~50 000) urokinase-type plasminogen activator (uPA) activities are noted. No proteolytic activity with M, ~70 000 or M, ~50 000 was detected when plasminogen was omitted from the polyacrylamide gels (not shown). Lower panel: Relative OD (tPA/uPA OD ratio) of the lytic areas from the zymograph shown above. The results are representative of three independent experiments. C, control incubation in the absence of FSH.

#### Figure 4

This figure shows the effects of FSH isoforms on the production of P450arom and tPA mRNA. The mRNA levels were assessed by RT-PCR and northern blot hybridization. The results are shown for isoforms II–VII in the presence of 2·8 ng/well, respectively. The mRNA levels were normalized to the levels of G3PD mRNA, which is constitutively expressed by the granulosa cells.
added (Fig. 7A). On the basis of previous studies indicating that oestrogens may inhibit α-inhibin gene expression (Tate et al. 1996, Tekmal et al. 1996), we examined whether the relatively decreased production of α-inhibin mRNA exhibited by the less acidic isoforms may be due to their enhanced ability to stimulate androgen aromatization and oestrogen production. As shown in Fig. 7B, addition of the potent oestradiol receptor antagonist ICI 182,780 or omission of androstenedione (the aromatization substrate) in incubations of cells exposed to FSH isoforms II or VII did not significantly modify the isoform-induced α-inhibin mRNA production by cultured granulosa cells. Finally, to rule out the possibility that the differential effect of the FSH isoforms on α-inhibin mRNA production were actually produced by non-FSH factors that may potentially inhibit or stimulate unspecifically the production of α-inhibin mRNA, cultured granulosa cells were incubated with isoform II or VII in the presence or absence of either rFSH or anti-human FSH-6 (at a titre sufficient to decrease significantly the androgen aromatization provoked by rFSH, without interfering with the incubation conditions (Timossi et al. 1998a)), respectively. As shown in Fig. 8, addition of rFSH to wells containing isoform II increased α-inhibin mRNA production, whereas co-incubation of isoform VII with anti-FSH decreased α-inhibin mRNA to levels slightly greater than those detected in control and isoform II incubations (lanes 1 and 3 in Fig. 8).

Discussion

It is well known that the oligosaccharide residues of glycoprotein hormones determine several of the biological features of the hormone at the target cell level (Ulloa-Aguirre et al. 1995). The main difference among the various isoforms is caused by charge, which in FSH is mainly determined by its content in terminal sialic acid residues (Chappel et al. 1983, Baenzinger & Green 1988, Ulloa-Aguirre et al. 1995), although differences in non-terminal carbohydrates also play an important role in determining the heterogeneous nature of the several members of the glycoprotein hormone family (Ulloa-Aguirre et al. 1988b, Papandreou et al. 1993, Creus et al. 1996). We and others have found that the receptor binding activity of the intrapituitary FSH isoforms varies depending on the charge of the isoform (Stanton et al. 1992, Ulloa-Aguirre et al. 1992, Yding Andersen et al. 1999). Less acidic/sialylated FSH isoforms exhibit lower dissociation constants than the more sialylated counterparts when assessed by

Figure 4  Cytochrome P450<sub>arom</sub> mRNA expression in cells exposed to FSH isoforms II–VII (2·8 ng/culture well) for 48 h. (A) Upper panel: Representative northern blot hybridization analysis of P450<sub>arom</sub> mRNA production in response to FSH exposure. +, Positive control (ovarian tissue from 18-day-pregnant rats); −, negative control (granulosa cells cultured in the absence of FSH); Std, granulosa cells exposed to LER-907. Lower panel: Relative OD (2·6 kb P450<sub>arom</sub> mRNA/18S rRNA OD ratio) of the autoradiograph shown above. (B) Upper and middle panels: Representative RT-PCR assay of expression of P450<sub>arom</sub> and G3PD mRNAs in cells exposed to isoforms II–VII. Std, Molecular mass standards. Lower panel: Relative OD (584 bp reversed-transcribed P450<sub>arom</sub> cDNA/513 bp reverse-transcribed G3PD cDNA OD ratio) of the P450<sub>arom</sub> and G3PD bands shown above. The results are representative of three independent experiments.
heterologous receptor assay systems, which may explain the greater capacity of the former variants to stimulate oestrogen production by cultured granulosa cells (Ulloa-Aguirre et al. 1992, Zambrano et al. 1999, present study). However, the potential exists that, depending upon the particular oligosaccharide structure of the ligand, the same receptor or its membrane-expressed variants may produce a spectrum of cellular responses that are reflected in activation/inhibition of various intracellular signalling pathways located downstream of the receptor, thus raising the interesting possibility for isoform-specific stimulation of different functions (Arey et al. 1997). Although recent studies using glycoforms of recombinant human TSH have shown that some of the isoforms of this glycoprotein hormone may exert differential effects (Schaaf et al. 1997), data for the naturally occurring FSH variants are scarce.

The findings of the present study support the hypothesis that the glycosylation variants of FSH may evoke several cellular functions with distinct potencies. Less acidic isoforms, which presumably possess relatively low amounts of sialic acid residues and greater amounts of high mannose- and hybrid-type oligosaccharides (Creus et al. 1996), exhibited a greater capability to induce expression of cytochrome P450arom mRNA and tPA mRNA and protein than the variants bearing more complex, highly sialylated oligosaccharides. The mechanism(s) subserving these differences in potency among the isoforms is uncertain. Using heterologous assay systems, we have found that the affinity of the less acidic human FSH isoforms for its cognate receptor is greater than that showed by the more acidic analogues, which suggests that differences in receptor affinity may account for the observed variations.

**Figure 5** Tissue-type plasminogen activator mRNA expression in cells exposed to 2·8 ng/culture well FSH isoforms II–VII for 12 h. (A) Upper panel: Representative northern blot hybridization analysis of tPA mRNA expressed by FSH exposure. Middle panel: The corresponding 18S rRNA. Lower panel: Relative OD (tPA mRNA/18S rRNA OD ratio) of the tPA mRNA and 18S rRNA bands shown above. (B) Upper and middle panels: Representative RT-PCR assay of expression of tPA (539 bp) and G3PD (525 bp) mRNAs in cells exposed to isoforms II–VII. The first lane in each gel shows the migration of the molecular mass standards. Lower panel: Relative OD (reverse-transcribed tPA cDNA/reverse-transcribed G3PD cDNA OD ratio) of the tPA and G3PD cDNA bands shown above. Similar results were found in two additional experiments.

**Figure 6** RT-PCR assay of expression of P450arom (upper panel) and tPA (lower panel) mRNAs in cells exposed to 2·8 ng/culture well rFSH, FSH isoform I (FSH-I) or rFSH plus FSH-I. Std, Molecular mass standards; C, control incubations in the absence of FSH; +tPA and +P450arom, positive controls (reversed-transcribed cDNA from total RNA samples previously extracted from cells exposed to rFSH in which the tPA and P450arom mRNAs were identified by northern blot hybridization analysis).
in vitro biopotency between the various isoforms (Zambrano et al. 1999). This possibility is supported by evidence from structural studies on human FSH, which demonstrated high correlation of binding and signal transduction (for reviews see Dias et al. 1998, Ulloa-Aguirre & Timossi 1998). However, using a homologous assay system that presumably expresses a unique human FSH receptor population we recently found that, despite the absence of differences in receptor affinity, less acidic isoforms were more potent activators of the receptor and more effective triggers of Gs-mediated intracellular signal transduction than their more acidic counterparts (Zambrano et al. 1999). Taken together, these data suggest that, in addition to differences in receptor affinity, less acidic/sialylated FSH isoforms may exhibit dominant negative properties via preferential coupling to the Gi-mediated transducing machinery, may account for some of the pleiotropic actions of FSH (Sairam et al. 1996). Although expression of these or other receptor variants in naturally occurring cell systems (Simoni et al. 1999) may potentially contribute to the differences in in vivo bioactivity of the FSH isoforms, more studies are required before a functional role can be assigned to such receptor diversity.

In contrast to the observed effects on P450arom and tPA mRNA expression, the less acidic/sialylated FSH isofoms mixes were less potent than the more acidic analogues as stimulators of /alpha/-inhibin mRNA production. This divergent effect was in fact surprising, particularly considering that all biological end points measured (/alpha/-inhibin mRNA, tPA enzyme activity and androgen aromatization) are FSH-inducible effects believed to be triggered solely by activation of the same Gs–cAMP-dependent signalling pathway (Reichert & Dattatreyamurtty 1989, Pei et al. 1991, Fitzpatrick & Richards 1994, Holmberg et al. 1995). Although the effect is difficult to explain on the basis of distinct ligand–receptor interactions, it is not possible to rule out the possibility of cross-talk between the FSH receptor and the insulin-like growth factor-I receptor.

Figure 7 /alpha/-Inhibin subunit mRNA expression in granulosa cells exposed to FSH isoforms (2.8 ng/culture well) for 24 h. (A) Upper panel: Representative northern blot hybridization analysis of /alpha/-inhibin mRNA production induced by exposure of cells to FSH isoforms II–VII. Middle panel: The corresponding 18S rRNA bands. Lower panel: Relative OD (/alpha/-inhibin mRNA/18S rRNA OD ratio) of the mRNA and 18S rRNA bands shown above. Similar results were found by semiquantitative RT-PCR (not shown). Inset: P450arom mRNA expression in cells exposed to the same isoforms in a parallel experiment. (B) Upper panel: /alpha/-inhibin mRNA expression in cells exposed for 24 h to isoforms II and VII in the presence or absence of androstenedione (A) or the oestrogen receptor antagonist ICI 182,780 (ICI). Lower panel: Relative OD (/alpha/-inhibin mRNA/18S rRNA OD ratio) of the mRNA bands shown above. Results are representative of three independent experiments. C, Incubations in the absence of FSH.
(which is involved in FSH-stimulated α-inhibin expression (Danmei et al. 1998)) systems provoked by glycosylation-dependent diverse FSH signals that may induce and/or stabilize distinct receptor conformations, allowing for preferential or different degrees of activation/inhibition of alternative signals. It is possible also that these divergent responses may be subserved by autocrine mechanisms triggered by the increased concentrations of oestrogen produced in response to the less acidic FSH isoforms, which in turn may amplify some specific responses (P450arom and also, potentially, tPA mRNA expression; Hickey et al. 1988, Fitzpatrick & Richards 1991, 1994) without interfering with the expression of the α-inhibin mRNA, as disclosed by the lack of effects of androgen deprivation and oestrogen receptor blockade on inhibin α-subunit mRNA levels. Alternatively, the attenuated α-inhibin mRNA response to the less acidic FSH isoforms may be mediated by cAMP-dependent autoregulatory mechanisms, such as isoforms of the cAMP-response element modulatory protein, which may act as specific early transcriptional repressors of some genes expressed in granulosa cells, including the inhibin α-subunit gene, the expression of which is regulated by the gonadotrophin-activated G_\text{c} −cAMP signalling pathway (Molina et al. 1993, Mukherjee et al. 1998). Whatever the mode of action through which the FSH isoforms exert this divergent or differential effect, this finding extends those of previous studies using chemically deglycosylated ovine FSH variants (Beitinz & Padmanabhan 1991, Padmanabhan et al. 1993, Ulloa-Aguirre et al. 1995) and strongly suggests that selective changes in FSH glycosylation not only alter the net potency of the hormone but may also elicit divergent responses at the target cell level.

We recently reported that the basically charged FSH isoform (isoform I) behaves as an antagonist of FSH action and that its inhibitory effects are specific and probably exerted at steps subsequent to cAMP formation because: (a) in low doses, this particular isoform moderately increased FSH-stimulated cAMP production by cultured granulosa cells; (b) coincubation of the antagonist isoform with dibutyryl cAMP (a potent cAMP analoge) completely inhibited the effects of the analogue on oestrogen and tPA production; (c) the isoform was able to stimulate cAMP production in a human fetal cell line expressing the recombinant human FSH receptor; (d) the inhibitory effects of this isoform on dibutyryl cAMP-provoked oestrogen and tPA production were reversed by the addition of a highly specific antibody directed against human FSH, further demonstrating that the antagonistic effects observed were due to FSH-like molecules (Timossi et al. 1998a). In the present study, we have confirmed and extended this finding by showing that isoform I completely inhibited the FSH-dependent expression of P450arom and tPA mRNAs, without blocking the expression of other non-gonadotrophin regulated mRNAs, such as that of the enzyme G3PD. Therefore the inhibitory effects exhibited by this isoform affected specifically the expression of those genes of which the transcription is regulated by FSH.

It should be noted that, in contrast to other studies in which highly purified preparations were used to test some in vitro actions of the FSH isoforms (reviewed in Stanton et al. 1996), in the present experiments we used isoform mixes that were only partially purified (devoid of LH, growth hormone and prolactin immunoactivities), thus raising the possibility that some of the effects observed may have been provoked by non-FSH molecules. However, several lines of evidence indicate that this was not the case. The affinity of the chromatofocusing-resolved pituitary isoform mixes for the rat and human FSH receptor was similar to that exhibited by highly purified human pituitary and recombinant FSH preparations in the same receptor systems, thus ruling out the possibility of non-specific interference with isoform binding (Zambrano et al. 1999). Highly purified isoforms of human recombinant FSH and urinary FSH also exhibit varying potencies to evoke specific responses, with the less acidic preparations being more potent or effective than the more acidic/sialylated counterparts (Cerpà-Poljak et al. 1993, Flack et al. 1994a, de Leeuw et al. 1996, Timossi et al. 1998b, Vitt et al. 1998, D’Antonio et al. 1999). Conversely, it has been shown that chemically
Deglycosylated ovine FSH variants may evoke differential or divergent effects on androgen aromatization and α-inhibin production by mouse Sertoli cells (Beitinz & Padmanabhan 1991, Padmanabhan et al. 1993, Ulloa-Aguirre et al. 1995). In the present study, exposure of granulosa cells to both isoform II and rFSH resulted in an increased production of α-inhibin mRNA, ruling out the possibility of a contaminating factor as the cause of the decreased capability of isoform II to induce expression of this mRNA. Further, the results of the immunoneutralization experiments performed in both the present and the previously reported study (Timossi et al. 1998a) strongly suggest that the inhibitory action of the basically charged isoform on some FSH-provoked effects, in addition to the potent agonistic effect of the more acidic variant on α-inhibin mRNA expression, were in fact the result of the specific action of FSH molecules rather than of non-specific effects of contaminants present in the preparation. Nevertheless, further studies using highly purified pituitary FSH isoforms, with preserved bioactivity, will undoubtedly strengthen the series of data pointing toward the differential and divergent effects of the FSH isoforms at the target cell level.

In summary, the anterior pituitary gland produces different glycosylated variants of FSH with agonist or antagonist properties. The potential of the isoforms to evoke a specific effect at the target cell level differs depending on the acidic sugar content (and also probably on the content of other carbohydrate residues internal to this terminal sugar (Ulloa-Aguirre et al. 1988b, Papandreou et al. 1993, Creus et al. 1996)) of the isoform. Whereas less acidic FSH isoforms are more potent in stimulating oestrogen production and tPA enzyme activity, the more acidic/sialylated analogues possess a greater potency for inducing synthesis of the α-subunit mRNA of inhibin. These divergent effects may be critical for a more precise regulation of the ovarian response to the gonadotrophic stimulus in vivo, particularly during the periovulatory period during which a significant increase in secretion of less acidic isoforms has consistently been observed (Padmanabhan et al. 1988, Wide & Bakos 1993, Zambrano et al. 1995, Anobile et al. 1998). In contrast, the existence of isoforms with antagonist properties may prevent overstimulation of the growing follicle and in selected abnormal conditions may counteract in excess the physiological gonadotrophic stimulus leading to a hypogonadal state (Dumestic et al. 1993). Further studies are still needed to clarify in more detail the biochemical mechanisms subserving this variety of responses to the FSH signal during the course of regulated follicular growth and maturation.

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