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Effect of sialylation and complexity of FSH oligosaccharides on inhibin production by granulosa cells

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Abstract

Granulosa cell (GC) inhibin A and B production is regulated by FSH and gonadal factors. This gonadotrophin is released as a mixture of glycoforms, which induce different biological responses *in vivo* and *in vitro*. Our aim was to determine the effect of recombinant human FSH (rhFSH) glycosylation variants on inhibin A and B production by rat GCs. Preparative isoelectro focusing was used to isolate more acidic/sialylated (pH <4.00) and less acidic/sialylated (pH >5.00) rhFSH charge analogues. Concanavalin A was used to isolate unbound and firmly bound rhFSH glycoforms on the basis of their oligosaccharide complexity. GCs, obtained from oestrogen-primed immature rats, were cultured with either native rhFSH or its glycosylation variants. Inhibin A and B were determined using specific ELISAs. Results were expressed as mean $\pm$ S.E.M. Under basal conditions, inhibin A was the predominant dimer produced (inhibin A: 673 ± 55; inhibin B: 80 ± 4 pg/ml). More acidic/sialylated charge analogues stimulated inhibin B production when compared to inhibin A at all doses studied; by contrast, less acidic/sialylated charge analogues stimulated inhibin A production and elicited no effect on inhibin B. Glycoforms bearing complex oligosaccharides showed a potent stimulatory effect on inhibin B when compared to inhibin A production (i.e. dose 1 ng/ml: 4.9 ± 0.5 vs 0.9 ± 0.1-fold stimulation, $P<0.001$). Glycoforms bearing hybrid-type oligosaccharides favoured inhibin A production (i.e. dose 4 ng/ml 2.9 ± 0.1 vs 1.6 ± 0.1-fold stimulation, $P<0.05$). These results show that the sialylation degree as well as the complexity of oligosaccharides present in the rhFSH molecule may be considered additional factors that differentially regulate dimeric inhibin production by rat GCs.

Reproduction (2013) **145** 127–135

Introduction

Inhibin A and B are the relevant forms of circulating dimeric inhibins in human and rodents; changes in their plasma concentration during the menstrual/oestrous cycle have been described. In women, inhibin B levels are maximal in the mid-follicular phase and fall before ovulation, remaining low during the luteal phase, whereas inhibin A remains low during the early- and mid-follicular phase and predominates in the luteal phase of the menstrual cycle (Groome *et al.* 1994, 1996, Sehested *et al.* 2000). In female rats, inhibin B serum levels peak early in the oestrous cycle, whereas circulating inhibin A increases gradually until just before the primary gonadotrophin surge (Woodruff *et al.* 1996, Arai *et al.* 2002). The regulation of dimeric inhibin production is complex; FSH, growth factors and the stage of follicle development contribute to the differential regulation of inhibin A and B. We have previously shown that rat granulosa cells (GCs) are able to secrete dimeric inhibins; this production, as well as the ratio between inhibin A and B, is regulated by FSH, oestrogen and growth factors. Inhibin A seems to be more sensitive to FSH stimulation during the later stages of follicular growth, whereas inhibin B seems to reflect the action of the members of the TGF-β superfamily in preantral follicles (Lanuza *et al.* 1999).

FSH is secreted in multiple glycosylation variants, which differ from each other in their sialic acid content and inner carbohydrate structure (Ulloa-Aguirre *et al.* 1999). Specific patterns of FSH glycoforms are associated with the degree of ovarian activity. We have previously shown that a characteristic feature of serum FSH in the follicular phase of young women, whose inhibin B levels are within the normal range, is the predominance of an acidic mix of FSH glycosylation variants, bearing highly branched oligosaccharides. By contrast, during the perimenopause and lactational amenorrhoea, two conditions characterized by diminished ovarian activity in the presence of normal immunoreactive FSH circulating levels, there is a

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-12-0228

Online version via www.reproduction-online.org

decrease in the relative proportion of glycosylation variants bearing highly branched oligosaccharides and appear in circulation those bearing incomplete, hybrid type carbohydrate chains. These changes in the microheterogeneity of circulating FSH were associated with a significant decrease in inhibin B serum levels (Velasquez *et al.* 2006, Loreti *et al.* 2009).

In rodents, FSH sialylation varies according to the day and time of the oestrous cycle (Ulloa-Aguirre *et al.* 1988). Furthermore, it has been shown that the structure of oligosaccharides present in the FSH molecule induces a differential response in rat GCs in terms of oestradiol production (Ulloa-Aguirre *et al.* 1995). The pattern of follicular development and oocyte quality in mice are also strongly influenced by rhFSH sialylation (Nayudu *et al.* 2002).

It remains to be established whether the structure of the oligosaccharides present in the FSH molecule may affect other aspects of the endocrine activity in GCs. The aim of this study was to determine the effect of recombinant human FSH (rhFSH) sialylation and carbohydrate complexity on inhibin A and B GC production.

Results

The isolation of rhFSH glycosylation variants according to their sialylation degree and oligosaccharide complexity was carried out as depicted in Fig. 1.

Distribution profile of rhFSH charge analogues

The relative proportion of rhFSH charge analogues recovered in 0.29 pH units, after preparative isoelectric focusing (IEF), is shown in Fig. 2A. The gonadotrophin was detected in 12 fractions of the gradient, within a pH range of 3.20–5.90. A predominant proportion (35.9±4.8%, $P<0.001$) of rhFSH charge analogues focused at a 4.40–4.69 pH interval. A lower proportion of the hormone was isolated either below pH 4.10 (22.8±8.3%) or above pH 5.00 (17.0±1.7%). In order to obtain a more acidic/sialylated charge analogues mix (rhFSH-AC), fractions from pH 2.56 to 4.00 were combined and concentrated; fractions at pH >5.00 were combined to obtain a less acidic/sialylated preparation (rhFSH-BA). These two rhFSH preparations were selected to explore the effect of their sialylation degree on inhibin production, based on the previously observed variations in the relative proportion of circulating gonadotrophin isolated at these two pH intervals that were associated with changes in ovarian activity in normal women (Velasquez *et al.* 2006, Loreti *et al.* 2009).

Distribution profile of native rhFSH glycoforms after concanavalin A chromatography

In order to isolate rhFSH glycosylation variants on the basis of its carbohydrate complexity, concanavalin A (ConA) chromatography was used. The three groups of glycoforms disclosed by lectin were detected in native rhFSH: unbound glycoforms bearing complex, triantennary and bisecting oligosaccharides (rhFSH-UB); weakly bound glycoforms bearing biantennary and truncated oligosaccharides (rhFSH-WB), and firmly bound glycoforms bearing high mannose and hybrid-type oligosaccharides (rhFSH-FB). A clear predominance of glycoforms bearing high mannose and hybrid-type oligosaccharides was observed (70.7±4.9% vs 18.3 ±4.7% and 12.3±3.5%, rhFSH-FB vs rhFSH-WB and rhFSH-UB respectively, $P<0.001$) (Fig. 2B). To confirm this finding, rhFSH-FB glycoforms were re-analysed in a

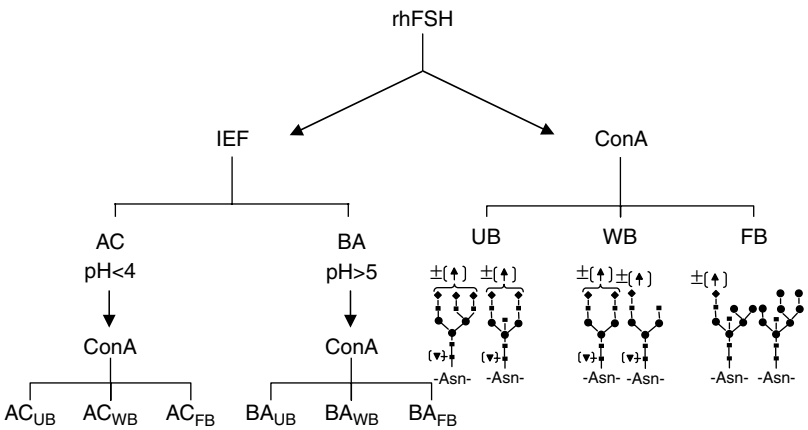

Figure 1 General outline of the analytical procedure followed to isolate and characterize rhFSH glycosylation variants according to their charge (IEF, preparative isoelectric focusing) and oligosaccharide complexity (ConA, concanavalin A chromatography). AC: more acidic charge analogues isolated at pH <4; BA: less sialylated charge analogues isolated at pH >5. Oligosaccharide structures of rhFSH glycoforms isolated after ConA chromatography are shown: UB, unbound, triantennary and bisecting oligosaccharides; WB, weakly bound, biantennary and truncated oligosaccharides; and FB, firmly bound, high mannose and hybrid-type oligosaccharides. $AC_{UB,WB,FB}$, unbound, weakly and firmly bound more acidic charge analogues; $BA_{UB,WB,FB}$, unbound, weakly and firmly bound less sialylated charge analogues. Asn, asparagine; closed square, *N*-acetylglucosamine; closed circle, mannose; closed diamond, galactose; closed down triangle, fucose; uparrow, sialic acid; ± with or without sialic acid.

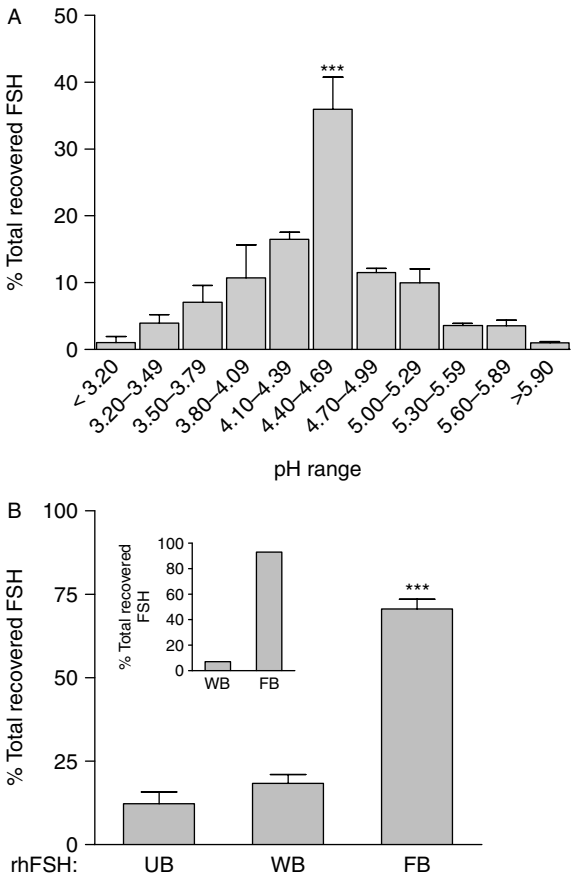

Figure 2 Distribution profile of rhFSH charge analogues after IEF, isolated within pH intervals of 0.29 units in a pH 3–10 gradient (A). Distribution profile of rhFSH glycosylation variants isolated by concanavalin A chromatography (B): unbound (rhFSH-UB: triantennary and bisecting oligosaccharides); weakly bound (rhFSH-WB: biantennary and truncated oligosaccharides) and firmly bound (rhFSH-FB: high mannose and hybrid-type oligosaccharides). Inset: Re-chromatography of rhFSH-FB fraction. Results are expressed as mean ± S.E.M. percentage of total recovered rhFSH of triplicate incubations of three independent experiments. ***$P<0.001$, proportion of rhFSH isolated at a pH interval of 4.40–4.69 vs those isolated at pH intervals above and below these values (A). ***$P<0.001$, rhFSH-FB proportion vs rhFSH-UB and rhFSH-WB proportions (B).

new ConA column; 90% of the hormone was again firmly bound to the lectin.

To explore the effect of rhFSH carbohydrate complexity on inhibin production by GCs, glycoforms bearing complex oligosaccharides and those bearing high mannose and hybrid-type oligosaccharides, with marked differences in the complexity of their oligosaccharides, were selected. In previous studies, we found that it was the relative proportion of these two types of glycosylation variants that were more affected by changes in ovarian cyclicity and ageing in normal women. FSH glycoforms, bearing triantennary and bisecting oligosaccharides, circulate in the follicular phase of the normal menstrual cycle, when a fully functional gonad is present; however, they are absent in postmenopausal women (Creus *et al.* 1996, Velasquez *et al.* 2006). During the perimenopause, there is a relative decrease in circulating FSH glycoforms bearing triantennary and bisecting oligosaccharides, concomitantly with the appearance of a small proportion of glycosylation variants bearing high mannose and hybrid-type oligosaccharides (Loreti *et al.* 2009).

Distribution profile of more and less sialylated rhFSH after ConA chromatography

The more acidic/sialylated and the less sialylated rhFSH charge analogues (rhFSH-AC and rhFSH-BA) were concentrated and individually applied to ConA columns in order to characterize the glycosylation variants on the basis of their oligosaccharide complexity (Fig. 3A and B). A clear predominance of rhFSH glycoforms bearing complex oligosaccharides was observed when the more acidic/sialylated rhFSH charge analogue preparation was analysed (rhFSH-AC_{UB}: 39.5 ± 5.5% and rhFSH-AC_{WB}: 45.5 ± 7.5% of total recovered rhFSH); a lower proportion of glycoforms bearing high mannose and hybrid-type oligosaccharides was found (rhFSH-AC_{FB}15.0 ± 2.0, $P<0.01$).

rhFSH glycoforms bearing high mannose and hybrid-type oligosaccharides (rhFSH-BA_{FB}) were the more abundant glycosylation variants found in the less acidic preparation (83.8 ± 7.7% vs 13.0 ± 6.1% and 3.3 ± 1.8%, rhFSH-BA_{FB} vs rhFSH-BA_{WB} and rhFSH-BA_{UB} respectively, $P<0.001$).

Effect of native rhFSH on inhibin A and B production

Rat GCs, cultured under basal conditions, predominantly produced inhibin A (inhibin A, 673 ± 55; inhibin B, 80 ± 4 pg/ml); the INHA/INHB ratio was 8.4 ± 0.3. To assess the effect of rhFSH on the production of both inhibins, GCs were cultured with increasing doses of gonadotrophin (0.5–16 ng/ml). As shown in Table 1, rhFSH favoured inhibin A production; the lowest dose used (0.5 ng/ml) was able to significantly stimulate the production of this dimer ($P<0.01$ when compared to basal). The lowest dose of rhFSH that was effective to induce an increase in inhibin B secretion was 4 ng/ml ($P<0.05$ when compared to basal).

Effect of rhFSH sialylation on inhibin A and B production

When rat GCs were stimulated with more and less acidic/sialylated rhFSH charge analogues, there was a differential effect on inhibin production depending on the charge analogues mix to which the cells were exposed (Fig. 4). More acidic/sialylated charge analogues isolated at pH below 4 (rhFSH-AC) stimulated inhibin B production (3.6- to 13.7-fold over basal,

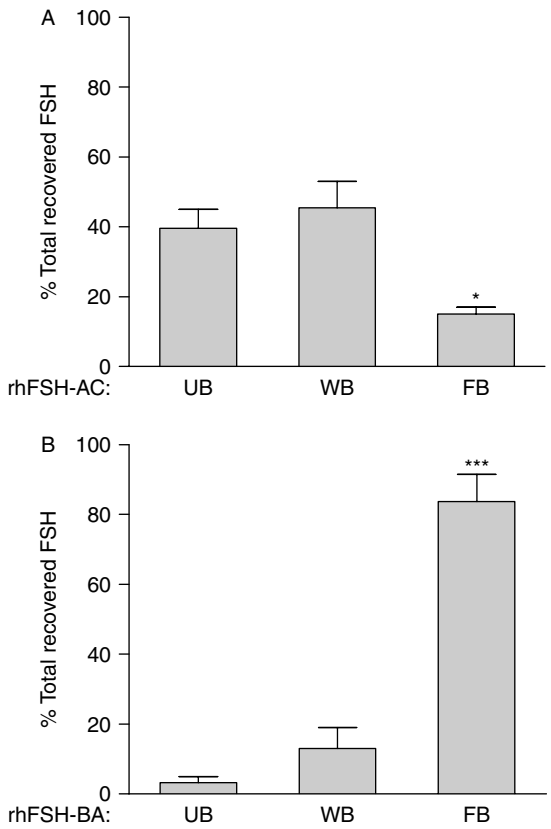

Figure 3 Distribution profile of rhFSH charge analogues after concanavalin A chromatography. rhFSH-AC: more acidic charge analogues isolated at pH <4 (A); rhFSH-BA: less sialylated charge analogues isolated at pH >5 (B). rhFSH-AC_{UB}, unbound, triantennary and bisecting oligosaccharides; rhFSH-AC_{WB}, weakly bound, biantennary and truncated oligosaccharides and rhFSH-AC_{FB}, firmly bound, high mannose and hybrid-type oligosaccharides. Results are expressed as mean±S.E.M. percentage of total recovered rhFSH of triplicate incubations of three independent experiments. *$P<0.05$; ***$P<0.001$, FB proportion vs UB and WB proportions.

$P<0.01$) over a dose range of 1–16 ng/ml. Only minor changes were observed in inhibin A production, even at the highest doses (8 and 16 ng/ml) used in the culture: 1.3- and 2.9-fold stimulation over basal respectively. By contrast, the cells exposed at the less acidic/sialylated counterparts isolated at pH above 5 (rhFSH-BA) responded with a marked increment of inhibin A secretion (from 4.9- to 7.6-fold stimulation over basal) at a dose range of 2–16 ng/ml; no effect was observed on inhibin B production at any of the doses studied.

Effect of rhFSH glycan branching

The complexity of rhFSH oligosaccharides also affected dimeric inhibin production (Fig. 5). Both rhFSH glycosylation variants, rhFSH-UB and rhFSH-FB, stimulated the production of inhibin A and B; however, those bearing triantennary and bisecting oligosaccharides (rhFSH-UB) clearly favoured inhibin B production. A significant increment was observed at a low dose, 1 ng/ml: 4.9± 0.5-fold stimulation over basal ($P<0.05$); maximal stimulation was reached at the dose of 2 ng/ml (6.9± 0.4-fold stimulation over basal). Under these experimental conditions, inhibin A was not affected. Higher doses of rhFSH-UB glycoforms were needed to significantly increase inhibin A production: 4–16 ng/ml (1.9- to 7.5-fold stimulation over basal, $P<0.01$) (Fig. 5A).

rhFSH glycoforms bearing high mannose and hybrid-type oligosaccharides (rhFSH-FB fraction) stimulated inhibin A production over a range of doses between 4 and 16 ng/ml (2.9- to 9.0-fold stimulation over basal, $P<0.001$). Minor changes were observed in inhibin B production at the highest doses used in the culture (Fig. 5B).

Discussion

The differential actions of FSH glycosylation variants on GC function and follicular development have been previously reported (Ulloa-Aguirre *et al.* 1995, Vitt *et al.* 1998). The results obtained in this study show, for the first time, that this differential action of FSH glycosylation on GCs also affects inhibin production.

rhFSH was used to isolate glycosylation variants, based on their carbohydrate sialylation and complexity, considering that this preparation is extremely pure and has no LH contamination (Loumaye *et al.* 1995). The results obtained herein confirmed previous reports describing the isolation of rhFSH charge analogues within a pH range similar to that found in this study; the predominant proportion of the gonadotrophin was also isolated at a similar pH interval (Horsman *et al.* 2000). Nevertheless, the proportion of more acidic/sialylated charge analogues (isolated below pH 4.4) was clearly lower in the rhFSH preparation when compared with that physiologically secreted during the follicular phase of the normal menstrual cycle (Zambrano *et al.* 1995, Velasquez *et al.* 2006, Loreti *et al.* 2009). When the carbohydrate complexity of glycosylation variants was analysed, a clear predominance of glycoforms bearing incomplete carbohydrates was found in

Table 1 Effect of native rhFSH on inhibin A and B production by rat granulosa cells.

rhFSH (ng/ml)	**0**	**0.5**	**1**	**2**	**4**	**8**	**16**
INHA (pg/ml)	673±55	1117±86†	1142±54†	1397±151‡	1854±185‡	2368±200‡	4159±69‡
INHB (pg/ml)	80±4	82±7	100±13	123±15	129±5*	174±17‡	338±27‡

Rat granulosa cells were cultured for 72 h with or without increasing concentrations of native recombinant human FSH (rhFSH; expressed in terms of hFSH-2 standard, NIDDKNHPP). Inhibins A (INHA) and B (INHB) were determined in the conditioned media. Results are expressed as mean±S.E.M. of triplicate incubations of three independent experiments. *$P<0.05$; $^{\dagger}P<0.01$; $^{\ddagger}P<0.001$ compared with respective control.

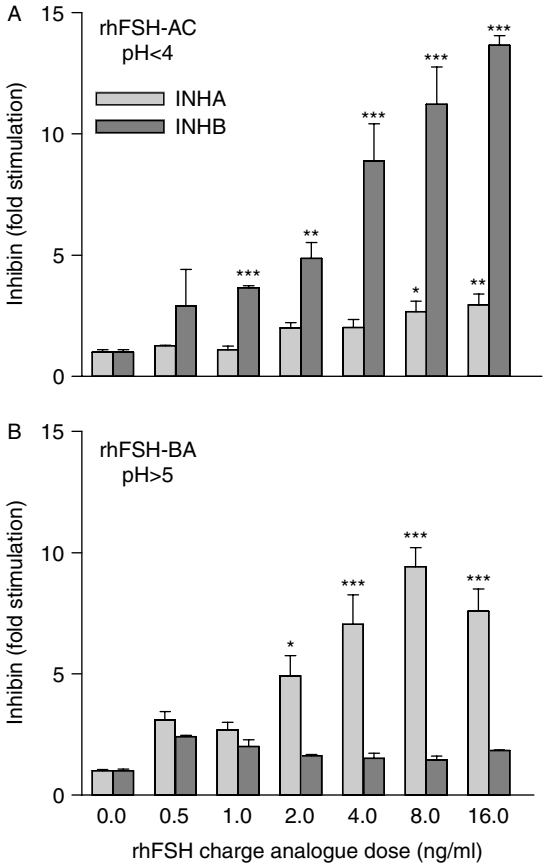

Figure 4 Effect of sialylation degree of rhFSH on inhibin A (INHA) and inhibin B (INHB) production by rat granulosa cells (GCs). GCs were cultured with or without increasing concentrations of more acidic charge analogues isolated at pH <4, rhFSH-AC (A), and less acidic charge analogues isolated at pH >5, rhFSH-BA (B). The rhFSH dose is expressed in terms of hFSH-2 standard (NIDDKNHPP). Inhibin A and B were determined in the conditioned media after 72 h of culture. Results are presented as fold stimulation over respective basal production. Values are mean±S.E.M. of triplicate incubations of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with respective control.

this preparation. These results were not unexpected considering the source of production of this hormone, the CHO cells. This non-human cell line does not express β-galactoside α2,6-sialyltransferase and *N*-acetylglucosaminyltransferase III. Therefore, only α2,3-linked sialic acid residues are added to rhFSH carbohydrate chains and there is no possibility to add bisecting GlcNAc residues to generate oligosaccharide complex structures (Hård *et al.* 1990, Xu *et al.* 2011). Interestingly, the physiologically produced FSH in the human pituitary contains not only α2,3-linked but also α2,6-linked sialic acid residues (Green & Baenziger 1988), and glycoforms bearing complex oligosaccharides are predominant in human pituitary extracts (Creus *et al.* 2001). It is worth noting that the differences in the characteristic of the carbohydrates present in the FSH molecule, currently used to stimulate follicular development and ovulation, may have a relevant impact on GC endocrine function.

It has been demonstrated that differences in FSH sialylation affect GC as well as follicular functions. In particular, a differential regulation induced by pituitary FSH charge analogues on inhibin α-subunit expression has been reported using a similar experimental model (Timossi *et al.* 2000). Our results showed that the sialylation degree of rhFSH is involved in the differential secretion of a specific inhibin dimer. It was clear that more sialylated rhFSH favoured inhibin B production; the less sialylated counterparts were not able to stimulate inhibin B but exerted a marked stimulatory effect on inhibin A. This particular effect exerted by the rhFSH degree of sialylation on the GC may contribute to the characteristic pattern of dimeric inhibin secretion profile

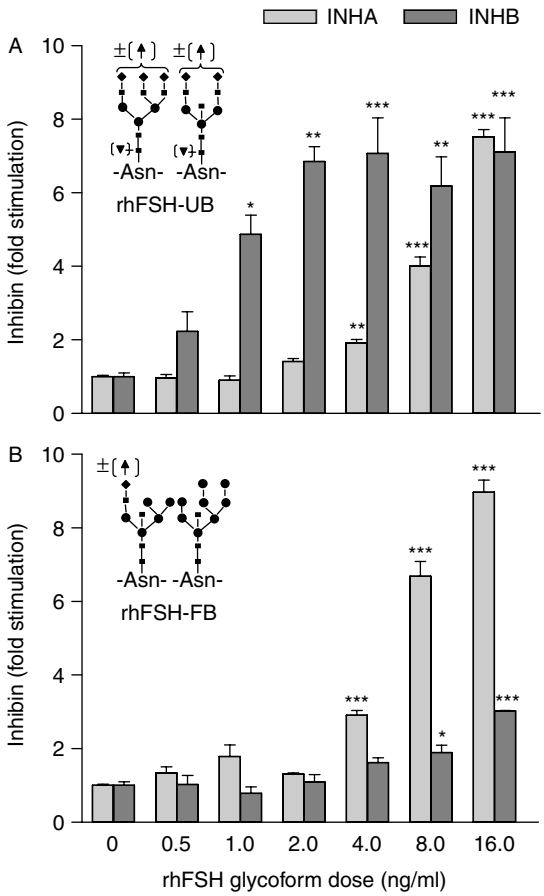

Figure 5 Effect of rhFSH glycan branching on inhibin A (INHA) and inhibin B (INHB) production by rat granulosa cells (GCs). GCs were cultured with or without increasing concentrations of rhFSH glycoforms bearing triantennary and bisecting oligosaccharides (rhFSH-UB; A) and glycoforms bearing high mannose and hybrid-type oligosaccharides (rhFSH-FB; B). The rhFSH dose is expressed in terms of hFSH-2 standard (NIDDKNHPP). Inhibin A and B were determined in the conditioned media after 72 h of culture. Results are presented as fold stimulation over respective basal production. Values are mean± S.E.M. of triplicate incubations of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with respective control.

observed across the menstrual cycle. FSH sialic acid content varies across the menstrual cycle (Padmanabhan *et al*. 1988, Zambrano *et al*. 1995); concomitantly with the presence of more acidic FSH, raising serum inhibin B concentrations are observed in the early follicular phase of the menstrual cycle, whereas the less acidic counterparts found in the late follicular phase are associated with the appearance of inhibin A in circulation.

In a previous study, we showed that not only the degree of sialylation but also the complexity of the FSH carbohydrate influences the biological response in the Sertoli cell in terms of oestradiol production (Creus *et al*. 2001). Dimeric production in GCs was also affected by the oligosaccharide structure of rhFSH; however, it is worth noting that the differential effect induced by oligosaccharide complexity was not identical to that exerted by the degree of hormone sialylation. rhFSH glycosylation variants bearing complex oligosaccharides (rhFSH-UB) were able to stimulate inhibin A and B. Nevertheless, GCs showed a high sensitivity to respond to these glycosylation variants producing, predominantly, inhibin B. The differential effect of oligosaccharide complexity on inhibin production disappeared when cells were exposed to high doses of the hormone.

The analysis of the oligosaccharide complexity in the more and less sialylated rhFSH charge analogues revealed the predominance of oligosaccharides that have completed the addition of all sugar residues in the rhFSH-AC preparation and the presence of a very high proportion of incomplete, high mannose/hybrid-type oligosaccharides in the rhFSH-BA preparation.

Recent studies have shown that pituitary hFSH consists of two classes of molecules: those that possess a non-glycosylated β-subunit and those that possess di-glycosylated β-subunits (Walton *et al*. 2001, Bousfield *et al*. 2007). These differences impact on the β-subunit and FSH molecular weight. It cannot be ruled out that in our preparations, particularly in rhFSH-BA and rhFSH-FB, non-glycosylated β-subunits may be present. However, the differential effects exerted by rhFSH glycosylation variants on inhibin production by GCs cannot account for variations in the gonadotrophin mass added to the cultures. The amount of native rhFSH as well as its glycosylation variants was evaluated by RIA using a polyclonal antibody that is unable to differentiate the rhFSH glycosylation variants according to their molecular weight. All the glycosylation variants isolated from rhFSH showed the same affinity for the antibody when referred to an identical standard: LER 907. This standard was previously used to evaluate immunological FSH activity when the *in vitro* biopotency of different hFSH preparations was assessed (Zambrano *et al*. 1996, Yding Andersen *et al*. 1999).

Based on all these findings, it can be concluded that GCs need the stimulus of an FSH molecule bearing two concomitant characteristics: a heavily sialylated carbohydrate chain and a complex carbohydrate chain in order to produce inhibin B. On the other hand, inhibin A production may not require such a strict molecular feature; it may be stimulated by an FSH molecule bearing different degrees of oligosaccharide complexity, but the presence of an abundant sialic acid content in the oligosaccharide moiety of the gonadotrophin might hinder the production of this dimer.

Further studies will be needed to elucidate the molecular mechanism induced by FSH glycosylation variants to exert this differential regulation on GC inhibin production. Unlike the α- and βA subunits, transcription of the βB-subunit is not regulated by cAMP-responsive elements (Dykema & Mayo 1994) and the canonical Gs/cAMP/PKA pathway is not the unique mechanism leading to FSH biological actions (Gloaguen *et al*. 2011). Therefore, it can be speculated that specific signal transduction pathways could be activated by FSH glycoforms in order to induce not only the expression of inhibin/activin subunits but also other genes involved in the regulation of important regulatory mechanisms of GC function.

Materials and Methods

Reagents

rhFSH was purchased from the National Hormone and Peptide Program of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK)-NIH (Torrance, CA, USA). Diethylstilbestrol (DES) was purchased from Sigma–Aldrich, Inc. DMEM, nutrient Mixture F-12 (Ham), fungizone (250 μg/ml) and gentamicina (10 mg/ml), was obtained from Invitrogen. All other chemicals were of reagent grade from standard commercial sources.

Rat GC isolation and culture

Ovaries were obtained from 21- to 23-day-old immature female Sprague–Dawley rats, injected s.c. with 1 mg DES daily for 3 days. Animals were killed by cervical dislocation, and all experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local Institutional Ethic Committee (IBYME-CONICET). GCs were isolated by follicular puncture, as described previously (Bley *et al*. 1992). Cells were seeded onto plastic 96-well plates (Nunc, Roskilde, Denmark). Initial plating density was 2×10^5 viable cells/cm^2 and cells were maintained at 37 °C with 5% CO_2. After 2 h, medium was changed to remove non-attached cells and replaced with fresh DMEM:F12 (1:1, vol/vol)–bicarbonate (2.2 g/l) supplemented with fungizone, gentamicin, in the absence or presence of native rhFSH and its glycosylation variants (0.5–16 ng/ml) for 72 h.

Isolation of rhFSH glycosylation variants

Glycosylation variants were isolated according to either their charge or their oligosaccharide complexity. Two native rhFSH

ampoules (40 mg LER-907) were applied for each procedure; three independent and consecutive analyses were carried out. The content of each ampoule was dissolved in double-distilled and deionized water (Barnstead NANOPure II, Thermo Scientific, Baltimore, MD, USA) and applied into a preparative IEF cell or into a ConA column.

Preparative IEF

Preparative IEF was used to isolate rhFSH charge analogues according to their sialylation degree using a Rotofor system (Rotofor Preparative Cell, Bio-Rad Laboratories, Inc.) as described previously (Bedecarrás *et al.* 1998, Loreti *et al.* 2009). Focusing was carried out at 12 W constant power (Power Pac 3000 Bio-Rad Laboratories, Inc.) for 4 h, maintaining the chamber refrigerated (Refrigerated Circulator, Forma Scientific, Inc., Marietta, OH, USA). Twenty fractions (2.5 ml each) were harvested and their pH was determined. Each individual fraction was exhaustively dialysed against 1 mol/l NaCl to completely eliminate ampholytes and detergent and I-FSH content was determined by double-antibody RIA. The range of recovered FSH was 70–85%. Fractions from pH 2.56 to 4.00 (rhFSH-AC) and at pH >5.00 (rhFSH-BA) were combined and concentrated using Centriprep-10 membrane (cut-off 10 000; Amicon, Beverly, MA, USA) and stored at −20 °C.

ConA chromatography

ConA chromatography was used to isolate three groups of rhFSH glycosylation variants according to the complexity of their oligosaccharides. The isolation of these three groups of glycoforms was based on the different affinities that the carbohydrate structures have for this particular lectin. Galactosyl residues present in glycoproteins bearing complex type or highly branched oligosaccharides do not interact with the lectin, unbound fraction: UB (Goldstein *et al.* 1965). On the contrary, carbohydrate chains containing two interacting α-mannopyranosyl residue, frequently present in high mannose and hybrid-type oligosaccharides, are strongly retained in the column, firmly bound fraction: FB (Ogata *et al.* 1975). The presence of *N*-acetylglucosamine residues, frequently present in glycoproteins bearing biantennary and truncated carbohydrate chains, considerably weakens binding to the lectin, weakly bound fraction: WB (Goldstein *et al.* 1965). The technique described by Narasimhan *et al.* (1979), modified by Cheng *et al.* (1984) and Creus *et al.* (1996), was employed. Briefly, equilibrium buffer (50 mm Tris–HCl; pH 7.4, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$) was used to elute unbound rhFSH glycoforms (rhFSH-UB); equilibrium buffer containing 10 mM methyl-α-D-glucopyranoside (glucoside) was used to elute the weakly bound rhFSH glycoforms (rhFSH-WB) and equilibrium buffer containing 0.1 M methyl-α-D-mannopyranoside (mannoside) was used to elute the firmly bound rhFSH glycoforms (rhFSH-FB).

Eluates containing rhFSH-UB, -WB and -FB glycoforms were dialysed against 0.01 mol/l NaCl and concentrated (Centriprep membranes, Amicon). The procedure was carried out at 4 °C. The range of recovered rhFSH was 75–90%. The distribution profile of rhFSH glycoforms was obtained by considering each fraction as a proportion of the total rhFSH recovered after ConA chromatography.

Distribution profile of rhFSH charge analogues after ConA chromatography

More acidic/sialylated rhFSH (6.8 mg LER-907) and less sialylated rhFSH (5.4 mg LER-907) charge analogues were individually applied to ConA columns. Figure 1 shows the general outline of the analytical procedure. The elution protocol was identical to the one described in the previous paragraph.

Hormone measurements

The rhFSH content of samples was measured using an in-house double-antibody RIA with reagents provided by NIDDK (Bethesda, MD, USA). The RP LER-907 (1 mg LER-907 = 53 IU Second International Reference Preparation, hMG) was used to construct the standard curve. The polyclonal antibody, anti-hFSH-6, was used as antiserum. Purified hFSH (hFSH-I-SIAFP-1) was iodinated using the chloramine-T method (Greenwood *et al.* 1963). To minimize the effects of interassay variations, as well as to determine the degree of parallelism between the unknown samples and the FSH standards, all glycoform preparations were analysed at multiple dose levels in the same assay run. The intra- and interassay coefficients of variation were <9 and 12% respectively. Simultaneous curve fitting of the dose–response curves obtained in the RIA of the glycoforms revealed no significant differences among the slopes generated by the standard LER-907 and the different rhFSH glycosylation variants, suggesting that the glycoforms were equally recognized by the antibody. rhFSH is expressed in terms of hFSH-2 standard (NIDDKNHPP).

Inhibin A and B levels in the culture media were measured using specific two-site ELISAs (Oxford Bio-Innovation Ltd., Oxon, UK) as described previously (Groome *et al.* 1994, 1996). Recombinant human inhibin A and B (Genentech, San Francisco, CA, USA) were used as standards. The assay sensitivity was 7 pg/ml for inhibin A and 15 pg/ml for inhibin B. Inhibin A had <0.5% cross-reaction in the inhibin B assay whereas inhibin B had <0.1% cross-reaction in the inhibin A assay. The human inhibin A and B assays had been validated and successfully used in the rat (Lanuza *et al.* 1999, Arai *et al.* 2002).

Statistical analysis

Results are expressed as mean ± S.E.M. Data were log transformed before statistical analysis when appropriate; percentage of FSH glycosylation variants was transformed to arcsine $(p)^{1/2}$, where $0<P<1$. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons. The significance was established when the two-tailed P value was <0.05. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of the present research reported.

Funding

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 5479 to S Campo) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 25365 to S Campo).

Acknowledgements

The authors are very grateful to NIDDK-NIH's National Hormone & Peptide Program for supplying rhFSH. S Campo is an established member of CONICET.

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Received 16 June 2012
First decision 6 August 2012
Revised manuscript received 23 October 2012
Accepted 19 November 2012