Truncated equine LH β and asparagine⁵⁶-deglycosylated equine LH α combine to produce a potent FSH antagonist

V Y Butnev¹, V Singh², V T Nguyen^{1,3} and G R Bousfield¹

¹Department of Biological Sciences, Wichita State University, Wichita, Kansas 67260-0026, USA

²Hormone Biochemistry Laboratory, North-Eastern Hill University, Shillong, India

³Sedgwick County Coroner-Medical Examiner, Regional Forensic Science Center, 1109 N. Minneapolis, Wichita, Kansas 67214-3129, USA

(Requests for offprints should be addressed to G R Bousfield, Department of Biological Sciences, Box 26, Wichita State University, 1845 Fairmount, Wichita, Kansas 67260-0026, USA; Email: george.bousfield@wichita.edu)

Abstract

Hybrid hormone preparations were prepared by combining intact and Asn⁵⁶-deglycosylated (N⁵⁶dg) equine (e) LH or FSH α subunit preparations with truncated, des(121-149)eLH β (eLH β t), immunopurified, intact eLH β or equine chorionic gonadotropin β (eCG β) preparations, and eFSH β . The LH receptor-binding potencies of N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eFSH α :eLH β t hybrids were equivalent to that of eLH; however, both N⁵⁶dg- α preparations were only 3-4% as active as eLH in the rat testis Leydig cell bioassay. In the granulosa cell FSH bioassay, eLH α :eLH β t stimulated progesterone synthesis and induced aromatase activity, while N⁵⁶dg-eLH α :eLH β t was completely inactive at doses up to 5 μ g. N⁵⁶dg-eLH α :eLH β t inhibited progesterone production and aromatase induction elicited by 0.3 ng eFSH or 2 ng

human (h) FSH. The inhibitory activities of N⁵⁶dg-eLH α :eLH β and N⁵⁶dg-eCG α :eLH β t were only 10% that of N⁵⁶dg-eLH α :eLH β t. N⁵⁶dg-eLH α :eCG β did not inhibit progesterone synthesis stimulated by eFSH at all and appeared to further stimulate aromatase induction at the highest dose tested. Preincubation of N⁵⁶dg-eLH α :eLH β and N⁵⁶dg-eLH α :eLH β t for 72 h at 37 °C resulted in no loss of FSH receptor-binding activity. Preincubation resulted in 50% loss of receptor-binding activity by the eFSH preparation due to subunit dissociation, while 88% of N⁵⁶dg-eLH α :eFSH β activity was lost following 72 h, 37 °C preincubation. While α Asn⁵⁶ oligosaccharide had no effect on eLH β hybrid stability, it did contribute to the stability of the eFSH heterodimer.

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Introduction

The gonadotropic hormones, equine luteinizing hormone (eLH) and equine follicle-stimulating hormone (eFSH), consist of glycosylated α - and β -subunits (Ward *et al.* 1991). The α -subunit carbohydrate attached to Asn⁵⁶ is essential for *in vitro* signal transduction activity, modulates receptor-binding potency, and may contribute to heterodimer stability (Matzuk *et al.* 1989, Bishop *et al.* 1994, Flack *et al.* 1994, Heikoop *et al.* 1998). The Asn⁸² oligosaccharide plays an important role in facilitating α subunit folding (Strickland *et al.* 1985, Thijssen-van Zuylen *et al.* 1998), stabilizes the α subunit (van Zuylen *et al.* 1997) and, in some instances, modulates receptor-binding activity (Butnev *et al.* 1998). The β -subunit carbohydrates play a less important, but measurable role in signal transduction (Liu *et al.* 1984, Matzuk *et al.* 1989). The α Asn^{52/56} oligosaccharide masks the secondary role of β Asn¹³ requiring that the former be eliminated before the impact of the latter can be demonstrated (Matzuk *et al.* 1989). The β subunit oligosaccharides exert their primary influence on hormone activity by determining the circu-

latory half-life of glycoprotein hormones (Rosa *et al.* 1984, Bishop *et al.* 1995), which is an important factor determining *in vivo* biological activity (Thotakura *et al.* 1991).

Deglycosylated (dg) LH and FSH bind their receptors with a higher affinity than the native hormones. However, the biological activity of deglycosylated hormones is much lower in assays that measure a target cell response, such as cAMP accumulation or steroidogenesis (Sairam 1980, Chen *et al.* 1982, Manjunath & Sairam 1982, Manjunath *et al.* 1982, Sairam 1982, Sairam & Manjunath 1982b, Keutmann *et al.* 1983, Sairam 1983, Liu *et al.* 1984, Calvo *et al.* 1986). Deglycosylation of LH and FSH was once considered a promising approach for development of gonadotropin antagonists. While chemically deglycosylated human chorionic gonadotropin (hCG) preparations interfered with reproductive function in rodents *in vivo* (Kato & Sairam 1983, Kato *et al.* 1983, Sairam *et al.* 1986), they acted as weak agonists in two primate models and no antagonist activity was detected (Patton *et al.* 1988, Liu *et al.* 1989). Recombinant hFSH and hCG glycosylation mutants proved unstable (Bishop *et al.* 1994, Flack *et al.*

1994, Heikoop *et al.* 1998), while recombinant hCG/FSH chimera glycosylation mutants retained residual agonist properties (Trout *et al.* 1999).

We have employed truncated, des(121–149)eLH β (eLH β t) preparations to prepare hybrid hormone preparations that enabled us to study the effects of hormone-specific changes in α Asn⁵⁶ oligosaccharide structure on biological activity (Gotschall & Bousfield 1996, Butnev *et al.* 1998). These derivatives were employed to solve two technical problems: elimination of non-dissociable eLH dimer and separation of hybrid heterodimers from non-associated subunits (Bousfield *et al.* 1989). When N⁵⁶dg-eLH α :eLH β t was tested in an *in vitro* LH bioassay, only 3% biological activity remained; however, when tested in a rat granulosa cell assay, no detectable FSH activity was observed. Elimination of the α Asn⁵⁶ oligosaccharide had no effect on dimer stability, consistent with a recent report involving recombinant eLH/CG (Galet *et al.* 2000). Substitution of intact eLH β , eCG β , or N⁵⁶dg-eCG α produced derivatives with substantially reduced antagonistic activity. These derivatives may be useful in determining how oligosaccharides contribute to the activation of the FSH receptor.

Materials and Methods

Hormone and subunit preparations

Purified hFSH (AFP7298A) and ovine (o) FSH (NIDDK-oFSH-20) preparations were obtained from the National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA and Dr A F Parlow. Purification and characterization of eFSH, eLH and eCG were described earlier (Butnev *et al.* 1996). Dissociation of the purified hormones in 6 M guanidine, separation of eLH or eCG α - and β -subunits by Sephacryl S-200 gel-filtration, and separation of eFSH α and eFSH β by QAE-Sephadex chromatography were carried out as reported previously (Moore & Ward 1980, Bousfield & Ward 1984, Butnev *et al.* 1996). Homogeneity of the preparations was confirmed by automated solid phase Edman degradation using a MilliGen (Bedford, MA, USA) model 6600 ProSequencer (Gotschall & Bousfield 1996). The truncated eLH β derivative, des(121–149)eLH β (eLH β t), was prepared by mild acid hydrolysis of eLH β followed by Sephacryl S-200 chromatography, as previously reported (Bousfield *et al.* 1989).

α -Subunit Asn⁵⁶ deglycosylation with peptide-N-glycanase

Selective Asn⁵⁶ deglycosylation of eLH α , eFSH α , or eCG α was performed according to the protocol described recently (Gotschall & Bousfield 1996). Peptide-N-glycosidase digestion was carried out on 1 mg samples of eLH α , eFSH α or eCG α dissolved in 500 μ l NH₄HCO₃,

pH 8.6, using 5 mU PNGase (Glyco, Novato, CA, USA). After incubation for 24 h at 37 °C, oligosaccharides were separated from the samples by Centricon P-10 ultrafiltration. N⁵⁶dg- α subunit preparations were recovered from the retentate fraction by evaporation in a Savant (Holbrook, NY, USA) SpeedVac.

Immunopurification of β subunit preparations

Protein G-purified monoclonal antibody ECG01 (Bidart *et al.* 1989) was conjugated to a 1-ml Amersham Pharmacia Biotech (Piscataway, NJ, USA) HiTrap-NHS column following the protocol provided by the manufacturer. A 6-mg sample of eLH β was dissolved in 1 ml 20 mM sodium phosphate buffer, pH 7.0, and subjected to four rounds of immunoaffinity chromatography. Column fractions were concentrated and desalted in Centricon P10 ultrafiltration devices and protein recovered by evaporation in a Savant SpeedVac. We recovered 2.8 mg immunopurified eLH β from the fourth unbound fraction, which represented 75% of the theoretical yield. Elimination of the α subunit immunoactivity was achieved following the fourth round of immunopurification as indicated by a solution phase radioimmunoassay using the monoclonal antibody ECG01 and ¹²⁵I-eLH tracer. The sequential purification of the immunopurified beta subunit preparation and the presence of eLH α and β subunits in the bound fractions were confirmed by automated Edman degradation using an Applied Biosystems (Foster City, CA, USA) model 492 Procise sequencer. Two preparations of immunopurified eLH β and eCG β employed in this study were subjected to four cycles of immunopurification and the final products characterized by SDS-PAGE, automated Edman degradation, and solution phase RIA to confirm their purity and integrity.

Hybrid hormone preparation

The retentate fractions containing the N⁵⁶dg- α preparations were lyophilized, dissolved in 500 μ l 0.5 M Tris-acetate buffer, pH 7.0, and combined with 1 mg eLH β t, immunopurified eLH β , immunopurified eCG β , or eFSH β . Following 24-h incubation at 37 °C, the preparations were placed in a P-10 Centricon, the Tris-acetate buffer replaced with 0.126 M ammonium bicarbonate buffer by ultrafiltration, and protein recovered by lyophilization. The dried protein was dissolved in 200 μ l 0.126 M NH₄HCO₃ and purified by Superdex 75 column chromatography (Butnev *et al.* 1998).

FSH and LH assays

An institutional Animal Care and Use Committee approved all animal procedures. Receptor-binding assays were performed using 25 mg rat testis homogenate tissue/tube and 2.5 ng ¹²⁵I-eFSH or ¹²⁵I-hCG tracer. The

chloramine-T technique was used for iodination, producing specific activities of 30–50 $\mu\text{Ci}/\mu\text{g}$. Duplicate assay tubes were incubated for 2 h at 37 °C in a shaking water bath, then centrifuged, the supernatant aspirated, and the pellet counted in a Packard (Meriden, CT, USA) Cobra II gamma counter. The counting efficiency was >74%. LH *in vitro* biological activity was determined in a rat testis Leydig cell bioassay (Bousfield *et al.* 2001). Testosterone released into the culture medium following 3-h incubation at 37 °C was determined by radioimmunoassay. FSH biological activity was determined by incubating hormone samples in 16-mm wells of 24-well tissue culture plates containing 300 000 granulosa cells obtained from diethylstilbestrol (DES)-primed immature rat ovaries (Butnev *et al.* 1998). The progesterone content of the medium was measured after 72 h and the amount of estradiol converted from testosterone present in the medium was determined after 96 h. All steroid RIAs employed antisera provided by Dr Gordon Niswender (Colorado State University, Fort Collins, CO, USA) (Korenman *et al.* 1974).

Statistical analyses of dose–response curves in Figs 1, 2, and 5 were conducted using Allfit (DeLean *et al.* 1978). Each FSH inhibition curve in Figs 3 and 4 was analyzed by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

FSH stability studies

Three samples of the N⁵⁶dg-eLH α :eLH β preparation were diluted to the ng/ml concentrations required for receptor-binding assay. One set was incubated for 72 h at 4 °C, while the other two were incubated at 37 °C for 24 and 72 h. Following the pre-incubation, each set of samples was tested in a rat testis FSH radioligand assay (RLA). A similar experimental design was employed for the N⁵⁶dg-eLH α :eFSH β hybrid preparation. Samples of intact eFSH and N⁵⁶dg-eLH α :eFSH β were diluted to ng/ml concentrations, incubated at 37 °C for 72 h or 24 and 72 h respectively, and FSH receptor-binding activities compared. In order to determine the cause of the loss of eFSH activity, a 5-mg sample of intact eFSH was incubated in 20 ml RLA buffer for 72 h at 37 °C and subjected to Sephacryl S-200 chromatography. A second 5-mg sample of this eFSH preparation was subsequently applied to the S-200 column without preincubation. Samples of lyophilized protein recovered from both chromatograms were analyzed by FSH radioligand assay, SDS-PAGE, Western blotting, and automated Edman degradation using an Applied Biosystems model 492 Procise sequencer.

Results

We prepared two hybrid preparations by combining partially deglycosylated eLH α (N⁵⁶dg-eLH α) and partially

Figure 1 LH activity of eLH β hybrids. (A) LH receptor-binding activity of N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eFSH α :eLH β t hybrids in rat testis radioligand assay using ¹²⁵I-hCG tracer. (B) Steroidogenic potency of the same hybrids in a rat testis Leydig cell bioassay. Note the N⁵⁶dg-eLH α :eLH β t hybrids require more hormone to stimulate steroidogenesis, yet stimulate a much lower maximum steroid accumulation than intact eLH.

deglycosylated eFSH α (N⁵⁶dg-eFSH α) preparations with an eLH β preparation and tested them in an LH radioligand assay (Fig. 1A). N⁵⁶dg-eLH α :eLH β t, N⁵⁶dg-eFSH α :eLH β t, and eLH exhibited equivalent receptor-binding activity. At the same time, when tested in a rat Leydig cell LH bioassay, both N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eFSH α :eLH β t preparations exhibited significantly ($P < 0.05$) reduced activity amounting to only 2–3% the steroidogenic activity of intact eLH (Fig. 1B). Maximum steroid accumulation obtained with the Asn⁵⁶-deglycosylated hybrids was only 58% that obtained with intact eLH.

Intact eFSH, eLH, and two hybrid preparations, N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eLH α :eFSH β , were tested in a rat testis FSH radioligand assay (Fig. 2A). The eLH preparation was 12% as active as eFSH while both hybrid preparations exhibited a level of activity intermediate between those of eFSH and eLH. The FSH biological activities of eFSH and the hybrid preparations, N⁵⁶dg-eLH α :eLH β t, N⁵⁶dg-eLH α :eFSH β , and eLH α :eLH β , were

Figure 2 FSH activity of eFSH β and eLH β t hybrids. (A) FSH receptor-binding activity of intact eLH, eFSH, $\text{N}^{56}\text{dg-eLH}\alpha\text{:eFSH}\beta$ hybrid, and $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ hybrid preparations in rat testis radioligand assay using ^{125}I -eFSH tracer. (B) Steroidogenic potency of eFSH, $\text{N}^{56}\text{dg-eLH}\alpha\text{:eFSH}\beta$ hybrid, eLH $\alpha\text{:eLH}\beta\text{t}$ hybrid, and $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ hybrid preparations in a DES-primed, rat ovary granulosa cell bioassay. (C) Induction of aromatase as indicated by conversion of testosterone, provided in the medium, to estradiol. Note that while $\text{N}^{56}\text{dg-eLH}\alpha\text{:eFSH}\beta$ shows partial agonist activity, $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ shows none (the apparent rise in estradiol in panel C was not significant, $P>0.05$).

varied widely in a DES-primed granulosa cell assay (Fig. 2B and C). Two hybrid preparations, $\text{N}^{56}\text{dg-eLH}\alpha\text{:eFSH}\beta$ and eLH $\alpha\text{:eLH}\beta\text{t}$, had very low, but detectable FSH activity (0.4% and 2.9% that of eFSH respectively). The partially deglycosylated hybrid, $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$, did not exhibit any steroidogenic activity at

Figure 3 FSH antagonist activity of $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ in rat granulosa cell assay. (A) Inhibition of progesterone synthesis (solid boxes) or conversion of testosterone to estradiol (open boxes) stimulated by a 300 pg dose of eFSH with a $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ preparation. Responses significantly lower than the response to eFSH alone are indicated (* $P<0.05$). (B) Inhibition of a 2000 pg dose of hFSH by the same $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ preparation. Dose-dependent responses to increasing amounts of eFSH or hFSH were confirmed in each assay (data not shown). Responses significantly lower than response to eFSH alone are indicated (* $P<0.05$).

doses up to 5 μg . The absence of FSH activity in the $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ preparation was observed in several different granulosa cell assays (data not shown) and contrasted with its high FSH receptor-binding activity.

We next examined the FSH antagonist properties of $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$. In several experiments, granulosa cells were incubated in the presence of 0.3 ng eFSH, which stimulated 60% of the maximum steroidogenic response to this hormone preparation (Fig. 2B). Increasing doses of $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ added to another set of wells containing 0.3 ng eFSH provided a dose-dependent inhibition of granulosa cell steroidogenic activity (Fig. 3A). At the highest dose tested, only basal levels of progesterone were measured, confirming the absence of FSH agonist activity in this hormone preparation. The order of addition of eFSH and $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$ did not matter, and delayed addition of $\text{N}^{56}\text{dg-eLH}\alpha\text{:eLH}\beta\text{t}$, 24 or 48 h

after exposure to eFSH, produced partial inhibition of FSH-stimulated steroidogenesis (data not shown). In a separate experiment, $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$ antagonized the activity of a 2 ng dose of the clinically significant human hormone, hFSH, in a dose-dependent manner (Fig. 3B). The 21.5 ng dose of $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$ reduced the hFSH-stimulated steroid production by 95%.

Several other αAsn^{56} -deglycosylated derivatives were prepared and tested for FSH antagonist activity. In order to prepare derivatives incorporating eLH β or eCG β , we first eliminated intact hormone contaminating these preparations by several passes over an eLH α -specific, ECG01 monoclonal antibody column. Following the first pass, 47% eLH immunoactivity remained in the unbound fraction containing eLH β . After the second pass, this was reduced to 7%, and we were unable to determine the amount remaining after the third cycle because displacement of the eLH tracer was too low. Automated Edman degradation of 200 pmol samples of the unbound fractions confirmed that these consisted of eLH β and decreasing amounts of intact eLH. The 6.3% eLH α content in the first unbound fraction, based on a phenylthiohydantoin >PhNCS-Phe to >PhNCS-Ser ratio of 0.063, was almost three times the level of eLH contamination indicated by radioligand assay (Bousfield & Ward 1984). This was reduced to 1.6% in the final immunopurified eLH β preparation. Analysis of the bound fractions revealed the presence of both eLH α and β subunits, as the >PhNCS-Phe to >PhNCS-Ser ratio ranged from 1.2 to 1.3. The eLH β and eCG β preparations actually used to prepare the hybrids shown below were characterized after 4–5 cycles of immunoaffinity purification. Equine LH immunoactivity was undetectable, SDS-PAGE confirmed that the preparations were intact, and automated Edman degradation indicated 1.7% and 0.7% α subunit contamination for eLH β and eCG β respectively. The immunopurified eLH β and eCG β preparations were combined with $N^{56}\text{dg-eLH}\alpha$ and purified by Superdex 75 gel filtration chromatography. The third derivative consisted of $N^{56}\text{dg-eCG}\alpha$ combined with eLH β and the heterodimer was purified in the same manner. Both α and β subunits associated with the heterodimer fractions were intact as indicated by SDS-PAGE analysis (data not shown).

The abilities of hybrid preparations, possessing intact β subunits, to inhibit 0.3 ng eFSH in the rat granulosa cell bioassay were compared with that of $N^{56}\text{dg-eCG}\alpha:\text{eLH}\beta$ (Fig. 4). $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$ exhibited a 10-fold reduction in FSH antagonistic activity. $N^{56}\text{dg-eLH}\alpha:\text{eCG}\beta$ did not exhibit any inhibitory activity when progesterone synthesis was used as the endpoint (Fig. 4A), while the highest dose of the potential antagonist actually stimulated estrogen accumulation significantly above baseline ($P < 0.05$) (Fig. 4B). The $N^{56}\text{dg-eCG}\alpha:\text{eLH}\beta$ preparation also exhibited a 10-fold reduction in FSH antagonist activity as compared with $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$, despite the absence of the β subunit C-terminal extension.

Figure 4 FSH antagonist activities of eLH/CG derivatives.

(A) Inhibition of progesterone synthesis stimulated by a 300 pg dose of eFSH by $N^{56}\text{dg-eLH}\alpha$ hybrids, as indicated. (B) Inhibition of testosterone conversion to estradiol by the same cells. The asterisks indicate significant ($P < 0.05$) reduction in progesterone and estradiol present in the medium as compared with eFSH alone. The presence of the eLH β C-terminal peptide (CTP) or eCG α Asn⁸² oligosaccharide reduced the antagonist activity, while the eCG β CTP eliminated it.

Because granulosa cell assay incubations lasted 72–96 h, the reduced antagonistic activities could have resulted from decreased stability (Matzuk *et al.* 1989, Heikoop *et al.* 1998). Therefore, we tested the thermal stability of two representative hybrid preparations, $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$ and $N^{56}\text{dg-eLH}\alpha:\text{eFSH}\beta$. Preincubation of $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta$ at 37 °C had no effect on the receptor-binding activity of this preparation (Fig. 5A). In contrast, the $N^{56}\text{dg-eLH}\alpha:\text{eFSH}\beta$ preparation lost 88% of its activity by 24 h, although it exhibited the same activity after a further 48 h at 37 °C (Fig. 5B). The intact eFSH preparation lost half its receptor binding activity after preincubation at 37 °C for 72 h. In a separate experiment, 72-h preincubation of $N^{56}\text{dg-eLH}\alpha:\text{eLH}\beta\text{t}$ had no significant effect on its FSH receptor-binding activity and no dissociation into subunits was detectable by gel filtration (data not shown).

In order to determine if the loss of FSH receptor-binding activity on the part of eFSH resulted from subunit

Figure 5 Thermal stability of eFSH, N^{56} dg-eLH α :eLH β , and N^{56} dg-eLH α :eFSH β . (A) Serially diluted samples of N^{56} dg-eLH α :eLH β were incubated at 4 °C or 37 °C for 24 to 72 h, as indicated. Duplicate aliquots of each dilution were tested in a rat testis FSH radioligand assay. There was no significant difference between the dose-response curves for any of the N^{56} dg-eLH α :eLH β preparations ($P>0.05$). (B) Serially diluted samples of eFSH and N^{56} dg-eLH α :eFSH β incubated at 4 °C or 37 °C for 24 to 72 h, as indicated. Duplicate aliquots of each dilution were tested in a rat testis FSH radioligand assay. Note that intact eFSH as well as N^{56} dg-eLH α :eFSH β lost activity during the 37 °C preincubation.

dissociation or proteolysis, we pre-incubated a 4.9 mg sample of eFSH at 37 °C for 72 h and separated the dimer and subunit fractions by Sephacryl S-200 gel filtration chromatography (data not shown). Only 2.2 mg lyophilized protein were recovered from the dimer fraction, representing 45% of the amount applied to the column and consistent with a 50% loss of receptor-binding activity. S-200 chromatography of a 5.0 mg sample of the same eFSH preparation without preincubation resulted in a single peak from which 4.1 mg eFSH were recovered. Extensive analysis consisting of SDS-PAGE, Western blotting, and automated Edman degradation indicated that the dissociated subunits were intact. No evidence for proteolytic fragments resulting from the 37 °C preincubation was obtained. Thus, the loss of eFSH receptor binding resulted from subunit dissociation.

Discussion

The eLH derivative, N^{56} dg-eLH α :eLH β t, is a useful FSH antagonist because it binds FSH receptor, but does not exhibit any detectable FSH activity. While a 5 μ g dose of this hormone derivative failed to elicit a significant response from cultured rat granulosa cells, a 20 ng dose completely inhibited the response of these cells to stimulation by 0.3 ng eFSH or 2 ng hFSH. In contrast, FSH derivatives, such as N^{56} dg-eLH α :eFSH β , chemically deglycosylated (dg)-oFSH and dg-hFSH, (Sairam & Manjunath 1982b, Calvo *et al.* 1986), recombinant hFSH α -glycosylation mutants and hFSH β -glycosylation mutants (Bishop *et al.* 1994, Flack *et al.* 1994) behaved as partial agonists. Several factors combined to completely eliminate the FSH biological activity of N^{56} dg-eLH α :eLH β t. First, eLH and eCG were shown to be relatively poor FSH agonists when their effects were mediated exclusively through the FSH receptor (Liu *et al.* 1985, Butnev *et al.* 1998). Secondly, the absence of α Asn 56 oligosaccharide had no detectable effect on eLH β hybrid stability, but still reduced receptor activation. Thirdly, elimination or modification of β subunit oligosaccharides prevented them from partially compensating for the absence of α Asn 56 carbohydrate by promoting receptor aggregation. And fourthly, the small size of eLH α Asn 82 oligosaccharide also prevented it partially compensating for the absence of α Asn 56 carbohydrate.

The overall orientation of hCG and FSH bound to their respective receptors appears to be similar (Pantel *et al.* 1993, Robert 1995); however, some of the details of receptor activation differ between the two hormones (Simoni *et al.* 1997). The high-affinity binding site for FSH resides in the large extracellular domain that constitutes the N-terminal half of the FSH receptor molecule (Davis *et al.* 1995, Ji & Ji 1995), while receptor activation by intact FSH preparations appears to involve extracellular loop one of the C-terminal transmembrane domain (Ji & Ji 1995). The receptor-binding site for FSH consists of the β subunit seatbelt loop and the α subunit C-terminus (Arnold *et al.* 1998). The common α subunit C-terminus in hCG has been implicated in binding a low-affinity binding site associated with the LH receptor transmembrane domain that is involved in receptor activation (Ji & Ji 1991) and includes Asp 397 located in extracellular loop one (Ji & Ji 1993). However, while the FSH α subunit C-terminus appears to participate in FSH receptor activation, it is also involved in high affinity receptor binding (Zeng *et al.* 1995, Arnold *et al.* 1998). Studies involving the LH receptor have also implicated α Asn 52 carbohydrate with conformation changes induced in the extracellular domain (Moyle *et al.* 1995), which are transmitted to the transmembrane domain through interactions with its extracellular loops (Remy *et al.* 1993). FSH receptors isolated from target tissues emerged as oligomeric assemblies (Dattatreyaumurthy *et al.* 1990), while agonist binding

has been shown to stimulate LH receptor self-association (Roess *et al.* 2000), a phenomenon suggested to be carbohydrate-dependent (Anderson *et al.* 1979). The current conception of G-protein coupled receptors is that they alternate between active and inactive states (Simoni *et al.* 1997); therefore, the three factors involved with receptor activation, extracellular domain binding/conformational change, exoloop one binding, and receptor self-association, may independently contribute to cellular activation by shifting the equilibrium to favor the activated state, with all three required for maximum signal transduction. Since N⁵⁶dg-eLH α :eLH β t binds but does not activate the FSH receptor, all three activation factors must be compromised.

We proposed that the greater inhibitory effect of α Asn⁵⁶ oligosaccharide size on the FSH receptor-binding activities of eLH α -, eFSH α -, and eCG α -eLH β t hybrids than on those of the corresponding eFSH β hybrids indicated a different orientation of the eLH β t hybrids in the FSH receptor extracellular domain from that observed with FSH (Butnev *et al.* 1998). As a result, eLH and eLH β t hybrids displayed a level of FSH biological activity that was only 2–7% that of their FSH receptor-binding potencies, instead of the 71-fold amplification observed for eFSH (Butnev *et al.* 1998). The altered orientation of eLH/CG derivatives in the FSH receptor provided only partial activation of the receptor, possibly because the α C-terminus failed to interact appropriately with extracellular loop one of the receptor transmembrane domain.

The virtually unamplified response of rat Leydig cells to N⁵⁶dg-eLH α :eLH β t stimulation (Bousfield *et al.* 2001) suggested that LH receptor-N⁵⁶dg-eLH α :eLH β t complexes could only activate a single G protein (perhaps preformed LH receptor-Gs complexes) rather than activating several G proteins before desensitization blocked the process. The specific structure of the α Asn⁵⁶ or α Asn⁵² oligosaccharide did not appear to be important for its critical role in linking receptor binding to cellular activation. The major Asn⁵⁶ oligosaccharides of eLH were largely high mannose (G R Bousfield, V Singh, and J Glushka, unpublished data) while those of eCG α were biantennary, extended by lactosamine repeats, and terminated with sialic acid (Butnev *et al.* 1998). These structural differences accounted for a 7.5-fold greater LH receptor-binding activity for eLH α :eLH β t than eCG α :eLH β t; however, they produced only a 0.3-fold difference in LH biological activity in the rat Leydig cell bioassay because of a higher than expected level of biological activity on the part of the eCG α :eLH β t hybrid (Bousfield *et al.* 2001). Likewise, the twofold greater FSH receptor-binding activity of eLH α :eFSH β than eCG α :eFSH β was reduced to an insignificant 0.2-fold difference in FSH activity in the rat granulosa cell bioassay (Butnev *et al.* 1998). The apparent absence of specific structural requirements on the part of hCG α Asn⁵² oligosaccharides prompted Moyle *et al.* (1995) to propose that LH receptor activation involved displacement of the tips of the putative

horseshoe-shaped extracellular domain by this oligosaccharide. Provided it was greater than a minimum size, cellular activation accompanied receptor binding. If carbohydrate was too small or absent, only partial cellular activation ensued, perhaps because interactions between transmembrane loops and the hCG α subunit C-terminus were weakened by inappropriate conformation of the hCG-occupied receptor. As mentioned above, the biological activities of eLH β t hybrid hormone preparations with larger α Asn⁵⁶ oligosaccharides were greater than predicted by their lower receptor binding affinities. Addition of the second branch (absent from most hCG α Asn⁵² oligosaccharides (Weisshaar *et al.* 1991)) to these oligosaccharides appeared to provoke a more robust response by the target cell. Studies with recombinant hCG α Asn⁵² glycosylation mutants suggested a completely nonspecific role for the α Asn⁵² oligosaccharide, heterodimer stabilization. The loss of biological activity was interpreted as loss of functional heterodimer (Heikoop *et al.* 1998).

The crystal structure of hCG suggested a potential role for α Asn⁵² oligosaccharide in stabilizing the heterodimer as the seatbelt loop embraced α L2 in such a manner that Asn⁵² oligosaccharide must slip through the seatbelt loop to permit physical dissociation of the subunits. Once the subunits separated, the steric barrier provided by the branched oligosaccharide rendered the dissociation irreversible at circulating and experimental hormone concentrations, because the seatbelt loop disulfide bond did not open under non-reducing conditions (Xing *et al.* 2001), while it has been reported that triantennary oligosaccharides attached to free hCG α Asn⁵² prevented dimer formation (Blithe 1990). Although there have been no reports that oligosaccharide branching stabilized the heterodimer, only 50% of hFSH was actually dissociated into subunits by 8 M urea (Parlow & Shome 1974, Rathnam & Saxena 1975). The destabilizing effect accompanying removal of α Asn⁵² carbohydrate from recombinant hCG is curious. Chemically deglycosylated-oFSH and dg-hCG were reportedly more stable to heat denaturation at 100 °C than their intact counterparts (Sairam & Manjunath 1982a, Sairam 1983), while α Asn⁵² hCG-glycosylation mutants were reportedly unstable at lower temperatures (Matzuk *et al.* 1989, Heikoop *et al.* 1998). The crystal structure of dg-urinary hCG suggested hydrogen bonds between the GlcNAc(β 1–4)GlcNAc disaccharide remnant and hCG β side chains (Lapthorn *et al.* 1994); however, a nuclear magnetic resonance spectroscopy (NMR) study of intact recombinant hCG provided no evidence for hydrogen bonds between the biantennary oligosaccharide attached to α Asn⁵² and the hCG β subunit (Weller *et al.* 1996). The crystal structure of recombinant hFSH, bearing intact, high mannose oligosaccharides also indicated hydrogen bonds between hFSH β and the α Asn⁵² oligosaccharide (Fox *et al.* 2001). There is a report of a significant loss of hCG biological activity associated with an α Asn⁵²-glycosylation mutant

following a 4-h preincubation at 37 °C that was not observed with a similar glycosylation mutant stabilized with an intra-subunit disulfide bond (Heikoop *et al.* 1998). Subunit dissociation was inferred from the loss of biological activity and attributed to the absence of carbohydrate rather than to mutations eliminating the N-glycosylation sequence.

Hormone instability was not a factor in the present study, as no significant loss of receptor-binding activity was observed following 72-h preincubation of N⁵⁶dg-eLH α :eLH β or N⁵⁶dg-eLH α :eLH β t at 37 °C. The stability of the latter was confirmed by gel filtration. These results were consistent with an earlier study showing that eLH was stable for up to 72 h at 25 °C, even at pH 3 (Bousfield & Ward 1986), and with a recent report that recombinant eLH/CG remained functional even when the seatbelt disulfide bond between β subunit Cys residues 26 and 110 was eliminated by mutagenesis (Galet *et al.* 2000). While α Asn⁵⁶ oligosaccharides (largely biantennary) certainly contributed to the stability of eFSH, their presence could not prevent the slow dissociation of that heterodimer. Dissociation of eFSH into subunits was corroborated by gel filtration, while the absence of proteolysis, another potential explanation for loss of receptor-binding activity (Ward *et al.* 1986, Bousfield & Ward 1988, 1994), was established by SDS-PAGE, Western blot analysis, and automated Edman degradation. Thus, loss of eFSH receptor-binding activity was caused by subunit dissociation, not by proteolytic damage, a commonly cited reason for FSH inactivation (Papkoff *et al.* 1967, Parlow & Shome 1974). There seems to be no clear-cut relationship between heterodimer stability and biological activity. While thermal stability studies may be necessary to determine if alterations in carbohydrate destabilize glycoprotein hormone derivatives, they fail to provide predictive information, as the least stable preparation, N⁵⁶dg-eLH α :eFSH β , exhibited greater FSH activity than the much more stable N⁵⁶dg-eLH α :eLH β t.

Although chemical deglycosylation studies consistently showed that removal of carbohydrate from both α and β subunits was required for maximum loss of biological activity (Sairam 1980, Kalyan & Bahl 1983, Liu *et al.* 1984), the classic N-glycosylation site mutagenesis studies of recombinant hCG (Matzuk *et al.* 1989) demonstrated a critical role for α Asn⁵² oligosaccharide. When carbohydrate was present at this site, elimination of any of the three other N-linked sites in hCG had no detectable effect on biological activity. Once α Asn⁵² carbohydrate was eliminated, then β Asn¹³, but not β Asn³⁰, oligosaccharide was shown to play a secondary role in activating target cells. Elimination of both α Asn⁵² and β Asn¹³ glycosylation sites reduced biological activity to the level observed for the quadruple glycosylation mutant. Additional evidence for a secondary effect of β Asn¹³ oligosaccharide was provided by chemical deglycosylation studies with oLH, which possesses only the β Asn¹³ oligosaccharide (Liu *et al.*

1984). Because β Asn¹³ oligosaccharides extend outward from the putative receptor-hormone interface, they could participate in carbohydrate-mediated receptor association.

Carbohydrate-mediated gonadotropin receptor-complex microaggregation was proposed to explain the inhibitory effects of hCG-derived glycopeptides on hCG stimulation of adenylyl cyclase in the absence of receptor-binding inhibition (Calvo & Ryan 1985) and the clusters of 2–3 silver grains observed in high resolution autoradiography of rat corpora lutea after intravenous injection of ¹²⁵I-hCG (Anderson *et al.* 1979). It is an attractive model because more than one oligosaccharide could participate in this mechanism. Conflicting evidence subsequently appeared showing inhibition of hCG binding to LH receptors by several oligosaccharide preparations (Thotakura *et al.* 1990). However, reversal of the inactivation caused by chemical deglycosylation of hCG was reported using high concentrations of antibodies to hCG by two laboratories (Rebois & Fishman 1984, Rebois & Liss 1987, Hattori *et al.* 1988). Although antibody-induced change in conformation of the dg-hCG was proposed as a mechanism for hormone reactivation, wheat germ agglutinin could produce the same result, even though the lectin had no effect by itself (Hattori *et al.* 1989). Moreover, the crystal structure of glycosylated and fully active recombinant hFSH (Fox *et al.* 2001) indicated no significant conformational differences from the crystal structures reported for dg-hCG (Lapthorn *et al.* 1994, Wu *et al.* 1994). Studies involving fluorescence resonance energy transfer measurements on cells expressing LH receptor-green fluorescent protein chimeras have recently provided strong evidence for LH- and hCG-induced LH receptor association (Roess *et al.* 2000). Whether this is mediated by oligosaccharide remains an open question; however, it could explain the reduced FSH antagonist activities of our other partially deglycosylated derivatives. Thus, N⁵⁶dg-eLH α :eLH β exhibited reduced FSH antagonist activity because of either sialic acid terminating the β Asn¹³ oligosaccharide or the 11 O-linked oligosaccharides attached to residues 121–149. The absence of FSH antagonist activity associated with N⁵⁶dg-eLH α :eCG β made it more likely that O-glycosylation rather than N-glycosylation was responsible for the reduced antagonist activity of N⁵⁶dg-eLH α :eLH β because O-linked oligosaccharides attached to eLH β differ substantially from those attached to eCG β (Bousfield *et al.* 1985, Damm *et al.* 1990, Hokke *et al.* 1994, Bousfield *et al.* 2001).

The reduction in FSH antagonist activity associated with N⁵⁶dg-eCG α :eLH β t may also fall into the category of another carbohydrate partially compensating for the absence of the α Asn⁵⁶ oligosaccharide by promoting receptor association. This is an interesting case because it demonstrates that oligosaccharide structure is important. The lowest receptor-binding activities were reported for eCG α :eLH β t, while eFSH α :eLH β t exhibited intermediate activity (Butnev *et al.* 1998). Following

elimination of α Asn⁵⁶ oligosaccharides, N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eFSH α :eLH β t preparations exhibited equivalent receptor-binding activities, but N⁵⁶dg-eCG α :eLH β t still remained less active, as if the α Asn⁸² oligosaccharide interfered with receptor binding despite its distant location and orientation in the molecule (Lapthorn *et al.* 1994). This contrasted with the absence of an effect of hCG α Asn⁷⁸ oligosaccharide on receptor-binding affinity (Matzuk *et al.* 1989), but was consistent with an increase in FSH receptor binding activity when recombinant hFSH α Asn⁷⁸ deglycosylation mutants were examined (Bishop *et al.* 1994, Flack *et al.* 1994), a reflection of the structural differences between hCG and hFSH oligosaccharides. In the present study, reduced FSH antagonist activity was associated with N⁵⁶dg-eCG α :eLH β t either because of the lower receptor-binding activity or because the oligosaccharide conferred partial agonist activity.

Elimination of sialic acid was reported to have no effect on *in vitro* hCG biological activity (Dufau *et al.* 1971) while eliminating *in vivo* biological activity (Morell *et al.* 1971). However, several investigators have reported that sialic acid is essential for the *in vitro* biological activity of hCG (Moyle *et al.* 1975, Amir *et al.* 1987, Amano & Kobata 1993, Reddy *et al.* 1996). The same conflicting data exist for intact and desialylated eCG (Moyle *et al.* 1978, Moore & Ward 1980, Aggarwal & Papkoff 1981). Reduction in the FSH antagonist properties of N⁵⁶dg-eLH α :eLH β t and N⁵⁶dg-eCG α :eLH β t and elimination of the FSH antagonist properties of N⁵⁶dg-eLH α :eCG β were mediated by oligosaccharides largely terminated with sialic acid (Damm *et al.* 1990, Matsui *et al.* 1991, Smith *et al.* 1993, Hokke *et al.* 1994, Matsui *et al.* 1994), although eLH β Asn¹³ oligosaccharides were also terminated with sulfate (Matsui *et al.* 1994). Further studies with these hormone derivatives could resolve the conflicting evidence of the role of sialic acid in the activation of FSH target cells because a complete loss of FSH activity should accompany sialic acid removal, if it is involved.

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