Truncated equine LHβ and asparagine^{56}-deglycosylated equine LHα combine to produce a potent FSH antagonist

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Abstract

Hybrid hormone preparations were prepared by combining intact and Asn^{56}-deglycosylated (N^{56}dg) equine (e) LH or FSH α subunit preparations with truncated, des(121–149)eLHβ (eLHβt), immunopurified, intact eLHβ or equine chorionic gonadotropin β (eCGβ) preparations, and eFSHβ. The LH receptor-binding potencies of N^{56}dg-eLHα:eLHβt and N^{56}dg-eFSHα:eLHβt hybrids were equivalent to that of eLH; however, both N^{56}dg-α preparations were only 3–4% as active as eLH in the rat testis Leydig cell bioassay. In the granulosa cell FSH bioassay, eLHα:eLHβt stimulated progesterone synthesis and induced aromatase activity, while N^{56}dg-eLHα:eLHβt was completely inactive at doses up to 5 μg. N^{56}dg-eLHα:eLHβt inhibited progesterone production and aromatase induction elicited by 0·3 ng eFSH or 2 ng human (h) FSH. The inhibitory activities of N^{56}dg-eLHα:eLHβ and N^{56}dg-eCGα:eLHβt were only 10% that of N^{56}dg-eLHα:eLHβt. N^{56}dg-eLHα:eCGβ did not inhibit progesterone synthesis stimulated by eFSH at all and appeared to further stimulate aromatase induction at the highest dose tested. Preincubation of N^{56}dg-eLHα:eLHβ and N^{56}dg-eLHα:eLHβt 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 N^{56}dg-eLHα:eFSHβ activity was lost following 72 h, 37 °C preincubation. While αAsn^{56} oligosaccharide had no effect on eLHβ 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 Asn^{56} 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 Asn^{82} 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 αAsn$^{52/56}$ oligosaccharide masks the secondary role of βAsn^{13} 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 circulatory 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 1982*b*, 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.*

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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)eLHβ (eLHβt) preparations to prepare hybrid hormone preparations that enabled us to study the effects of hormone-specific changes in αAsn^{56} 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 N^{56}dg-eLHα:eLHβt 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 αAsn^{56} oligosaccharide had no effect on dimer stability, consistent with a recent report involving recombinant eLH/CG (Galet *et al.* 2000). Substitution of intact eLHβ, eCGβ, or N^{56}dg-eCGα 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 eFSHα and eFSHβ 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 eLHβ derivative, des(121–149)eLHβ (eLHβt), was prepared by mild acid hydrolysis of eLHβ followed by Sephacryl S-200 chromatography, as previously reported (Bousfield *et al.* 1989).

α-Subunit Asn^{56} deglycosylation with peptide-N-glycanase

Selective Asn^{56} deglycosylation of eLHα, eFSHα, or eCGα was performed according to the protocol described recently (Gotschall & Bousfield 1996). Peptide-N-glycosidase digestion was carried out on 1 mg samples of eLHα, eFSHα or eCGα dissolved in 500 µl NH_4HCO_3, 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. N^{56}dg-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 eLHβ 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 eLHβ 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 ^{125}I-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 eLHβ and eCGβ 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 N^{56}dg-α preparations were lyophilized, dissolved in 500 µl 0·5 M Tris–acetate buffer, pH 7·0, and combined with 1 mg eLHβt, immunopurified eLHβ, immunopurified eCGβ, or eFSHβ. 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 µl 0·126 M NH_4HCO_3 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 ^{125}I-eFSH or ^{125}I-hCG tracer. The

chloramine-T technique was used for iodination, producing specific activities of 30–50 μCi/μg. 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 N^{56}dg-eLHα:eLHβ 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 N^{56}dg-eLHα:eFSHβ hybrid preparation. Samples of intact eFSH and N^{56}dg-eLHα:eFSHβ 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 eLHα (N^{56}dg-eLHα) and partially deglycosylated eFSHα (N^{56}dg-eFSHα) preparations with an eLHβt preparation and tested them in an LH radioligand assay (Fig. 1A). N^{56}dg-eLHα:eLHβt, N^{56}dg-eFSHα:eLHβt, and eLH exhibited equivalent receptor-binding activity. At the same time, when tested in a rat Leydig cell LH bioassay, both N^{56}dg-eLHα:eLHβt and N^{56}dg-eFSHα:eLHβt 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 Asn^{56}-deglycosylated hybrids was only 58% that obtained with intact eLH.

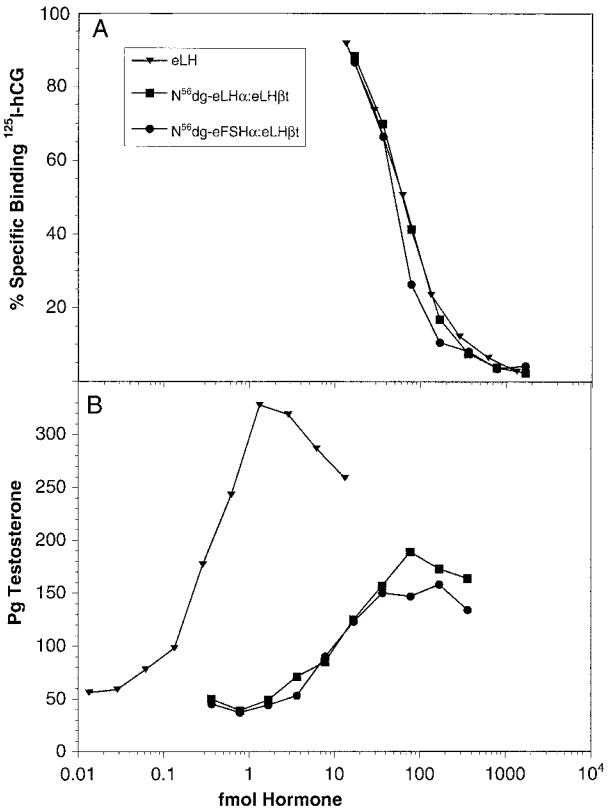

Figure 1 LH activity of eLHβt hybrids. (A) LH receptor-binding activity of N^{56}dg-eLHα:eLHβt and N^{56}dg-eFSHα:eLHβt hybrids in rat testis radioligand assay using ^{125}I-hCG tracer. (B) Steroidogenic potency of the same hybrids in a rat testis Leydig cell bioassay. Note the N^{56}dg-eLHα:eLHβt hybrids require more hormone to stimulate steroidogenesis, yet stimulate a much lower maximum steroid accumulation than intact eLH.

Intact eFSH, eLH, and two hybrid preparations, N^{56}dg-eLHα:eLHβt and N^{56}dg-eLHα:eFSHβ, 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, N^{56}dg-eLHα:eLHβt, N^{56}dg-eLHα:eFSHβ, and eLHα:eLHβ,

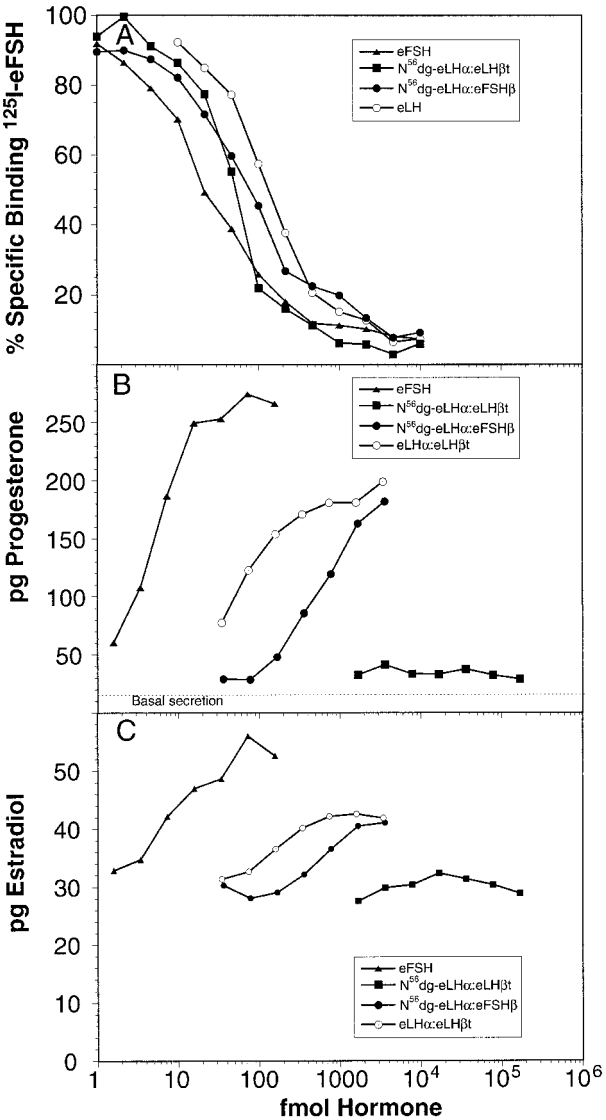

Figure 2 FSH activity of eFSHβ and eLHβt hybrids. (A) FSH receptor-binding activity of intact eLH, eFSH, N^{56}dg-eLHα:eFSHβ hybrid, and N^{56}dg-eLHα:eLHβt hybrid preparations in rat testis radioligand assay using ^{125}I-eFSH tracer. (B) Steroidogenic potency of eFSH, N^{56}dg-eLHα:eFSHβ hybrid, eLHα:eLHβt hybrid, and N^{56}dg-eLHα:eLHβt 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 N^{56}dg-eLHα:eFSHβ shows partial agonist activity, N^{56}dg-eLHα:eLHβt shows none (the apparent rise in estradiol in panel C was not significant, $P>0{\cdot}05$).

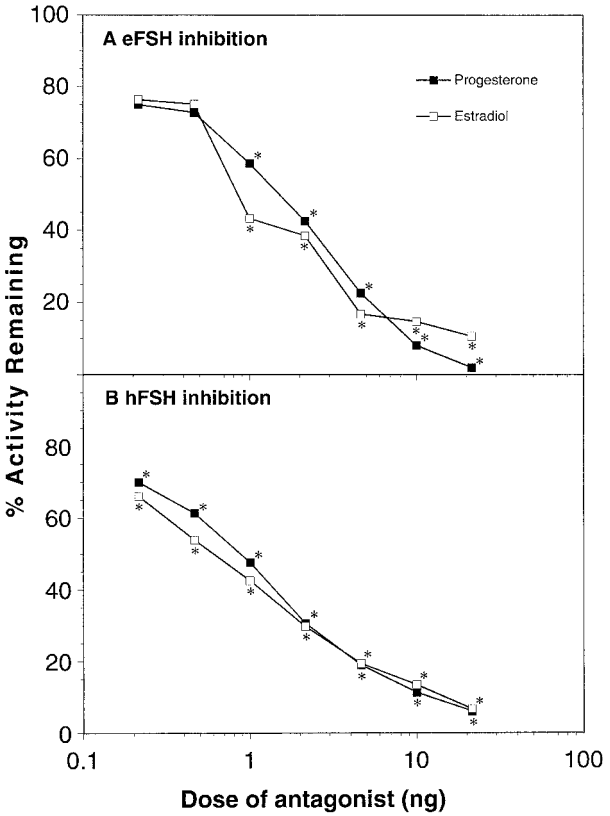

Figure 3 FSH antagonist activity of N^{56}dg-eLHα:eLHβt in rat granulosa cell assay. (A) Inhibition of progesterone synthesis (solid boxes) or conversion of testosterone to estradiol (open boxes) stimulated by a 300 pg dose of eFSH with a N^{56}dg-eLHα:eLHβt preparation. Responses significantly lower than the response to eFSH alone are indicated (*$P<0{\cdot}05$). (B) Inhibition of a 2000 pg dose of hFSH by the same N^{56}dg-eLHα:eLHβt 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 (*$P<0{\cdot}05$).

varied widely in a DES-primed granulosa cell assay (Fig. 2B and C). Two hybrid preparations, N^{56}dg-eLHα:eFSHβ and eLHα:eLHβt, had very low, but detectable FSH activity (0·4% and 2·9% that of eFSH respectively). The partially deglycosylated hybrid, N^{56}dg-eLHα:eLHβt, did not exhibit any steroidogenic activity at doses up to 5 μg. The absence of FSH activity in the N^{56}dg-eLHα:eLHβt 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 N^{56}dg-eLHα:eLHβt. 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 N^{56}dg-eLHα:eLHβt 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 N^{56}dg-eLHα:eLHβt did not matter, and delayed addition of N^{56}dg-eLHα:eLHβt, 24 or 48 h

after exposure to eFSH, produced partial inhibition of FSH-stimulated steroidogenesis (data not shown). In a separate experiment, N^{56}dg-eLHα:eLHβt 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}dg-eLHα:eLHβt 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 eLHβ or eCGβ, we first eliminated intact hormone contaminating these preparations by several passes over an eLHα-specific, ECG01 monoclonal antibody column. Following the first pass, 47% eLH immunoactivity remained in the unbound fraction containing eLHβ. 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 eLHβ and decreasing amounts of intact eLH. The 6·3% eLHα 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 eLHβ 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 eLHβ and eCGβ 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 eLHβ and eCGβ respectively. The immunopurified eLHβ and eCGβ preparations were combined with N^{56}dg-eLHα and purified by Superdex 75 gel filtration chromatography. The third derivative consisted of N^{56}dg-eCGα combined with eLHβt 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}dg-eCGα:eLHβt (Fig. 4). N^{56}dg-eLHα:eLHβ exhibited a 10-fold reduction in FSH antagonistic activity. N^{56}dg-eLHα:eCGβ 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}dg-eCGα:eLHβt preparation also exhibited a 10-fold reduction in FSH antagonist activity as compared with N^{56}dg-eLHα:eLHβt, despite the absence of the β subunit C-terminal extension.

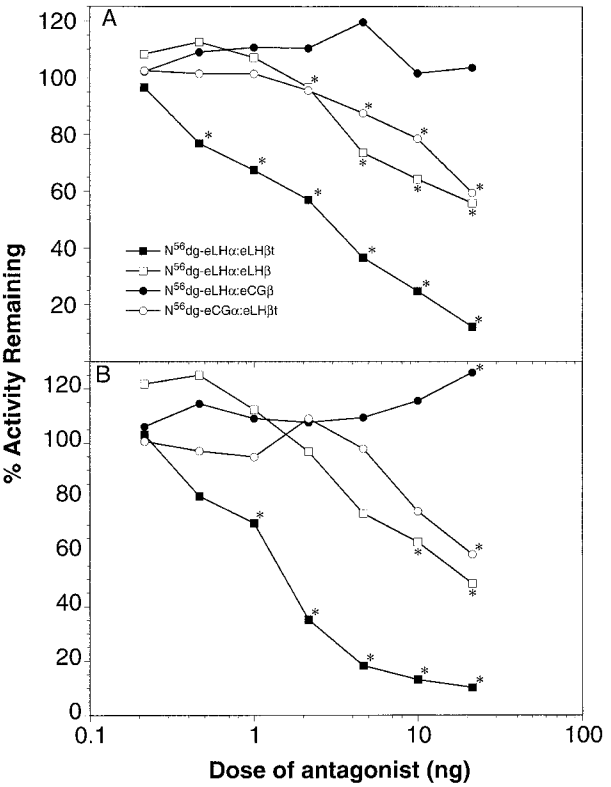

Figure 4 FSH antagonist activities of eLH/CG derivatives. (A) Inhibition of progesterone synthesis stimulated by a 300 pg dose of eFSH by N^{56}dg-α hybrids, as indicated. (B) Inhibition of testosterone conversion to estradiol by the same cells. The asterisks indicate significant ($P<0·05$) reduction in progesterone and estradiol present in the medium as compared with eFSH alone. The presence of the eLHβ C-terminal peptide (CTP) or eCGα Asn^{82} oligosaccharide reduced the antagonist activity, while the eCGβ 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}dg-eLHα:eLHβ and N^{56}dg-eLHα:eFSHβ. Preincubation of N^{56}dg-eLHα:eLHβ at 37 °C had no effect on the receptor-binding activity of this preparation (Fig. 5A). In contrast, the N^{56}dg-eLHα:eFSHβ 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}dg-eLHα:eLHβt 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

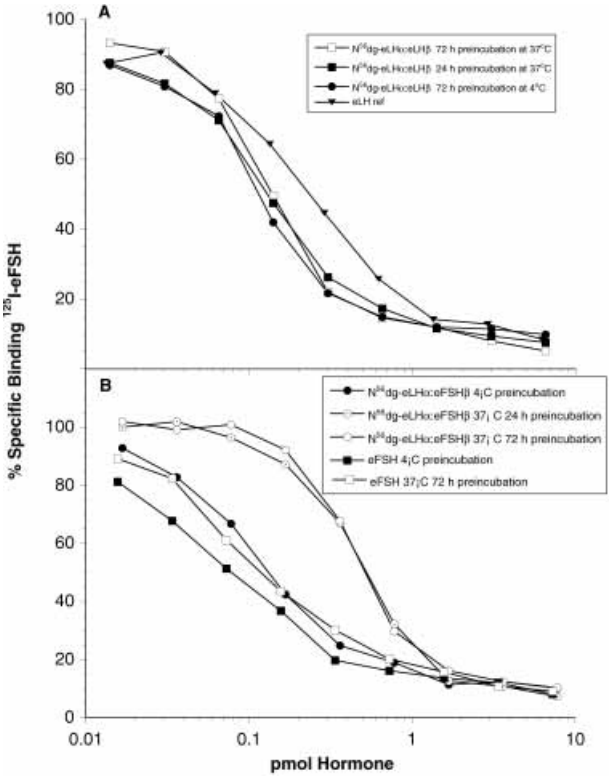

Figure 5 Thermal stability of eFSH, N^{56}dg-eLHα:eLHβ, and N^{56}dg-eLHα:eFSHβ. (A) Serially diluted samples of N^{56}dg-eLHα:eLHβ were incubated at 4 °C or 37 °C for 24 to 72 h, as indicated. Duplicate aliquots of each dilution were tested in a rat testis FSH radioligand assay. There was no significant difference between the dose–response curves for any of the N^{56}dg-eLHα:eLHβ preparations (P>0·05). (B) Serially diluted samples of eFSH and N^{56}dg-eLHα:eFSHβ 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-eLHα:eFSHβ 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-eLHα:eLHβt, is a useful FSH antagonist because it binds FSH receptor, but does not exhibit any detectable FSH activity. While a 5 μg 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-eLHα:eFSHβ, chemically deglycosylated (dg)-oFSH and dg-hFSH, (Sairam & Manjunath 1982*b*, Calvo *et al.* 1986), recombinant hFSHα-glycosylation mutants and hFSHβ-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-eLHα:eLHβt. 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 αAsn^{56} oligosaccharide had no detectable effect on eLHβ hybrid stability, but still reduced receptor activation. Thirdly, elimination or modification of β subunit oligosaccharides prevented them from partially compensating for the absence of αAsn^{56} carbohydrate by promoting receptor aggregation. And fourthly, the small size of eLHα Asn^{82} oligosaccharide also prevented it partially compensating for the absence of αAsn^{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 αAsn^{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 (Dattatreyamurty *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 N^{56}dg-eLHα:eLHβt binds but does not activate the FSH receptor, all three activation factors must be compromised.

We proposed that the greater inhibitory effect of αAsn^{56} oligosaccharide size on the FSH receptor-binding activities of eLHα-, eFSHα-, and eCGα-eLHβt hybrids than on those of the corresponding eFSHβ hybrids indicated a different orientation of the eLHβt hybrids in the FSH receptor extracellular domain from that observed with FSH (Butnev *et al.* 1998). As a result, eLH and eLHβt 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 N^{56}dg-eLHα:eLHβt stimulation (Bousfield *et al.* 2001) suggested that LH receptor–N^{56}dg-eLHα:eLHβt 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 αAsn^{56} or αAsn^{52} oligosaccharide did not appear to be important for its critical role in linking receptor binding to cellular activation. The major Asn^{56} oligosaccharides of eLH were largely high mannose (G R Bousfield, V Singh, and J Glushka, unpublished data) while those of eCGα 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 eLHα:eLHβt than eCGα:eLHβt; 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 eCGα:eLHβt hybrid (Bousfield *et al.* 2001). Likewise, the twofold greater FSH receptor-binding activity of eLHα:eFSHβ than eCGα:eFSHβ 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 αAsn^{52} 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 eLHβt hybrid hormone preparations with larger αAsn^{56} oligosaccharides were greater than predicted by their lower receptor binding affinities. Addition of the second branch (absent from most hCG αAsn^{52} oligosaccharides (Weisshaar *et al.* 1991)) to these oligosaccharides appeared to provoke a more robust response by the target cell. Studies with recombinant hCG αAsn^{52} glycosylation mutants suggested a completely nonspecific role for the αAsn^{52} 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 αAsn^{52} oligosaccharide in stabilizing the heterodimer as the seatbelt loop embraced αL2 in such a manner that Asn^{52} 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 hCGα Asn^{52} 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 αAsn^{52} 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 1982*a*, Sairam 1983), while αAsn^{52} 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(β1–4)GlcNAc disaccharide remnant and hCGβ 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 αAsn^{52} and the hCGβ subunit (Weller *et al.* 1996). The crystal structure of recombinant hFSH, bearing intact, high mannose oligosaccharides also indicated hydrogen bonds between hFSHβ and the αAsn^{52} oligosaccharide (Fox *et al.* 2001). There is a report of a significant loss of hCG biological activity associated with an αAsn^{52}-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 N^{56}dg-eLHα:eLHβ or N^{56}dg-eLHα:eLHβt 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 αAsn^{56} 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, N^{56}dg-eLHα:eFSHβ, exhibited greater FSH activity than the much more stable N^{56}dg-eLHα:eLHβt.

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 αAsn^{52} 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 αAsn^{52} carbohydrate was eliminated, then βAsn^{13}, but not βAsn^{30}, oligosaccharide was shown to play a secondary role in activating target cells. Elimination of both αAsn^{52} and βAsn^{13} glycosylation sites reduced biological activity to the level observed for the quadruple glycosylation mutant. Additional evidence for a secondary effect of βAsn^{13} oligosaccharide was provided by chemical deglycosylation studies with oLH, which possesses only the βAsn^{13} oligosaccharide (Liu *et al.* 1984). Because βAsn^{13} 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 ^{125}I-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, N^{56}dg-eLHα:eLHβ exhibited reduced FSH antagonist activity because of either sialic acid terminating the βAsn^{13} oligosaccharide or the 11 O-linked oligosaccharides attached to residues 121–149. The absence of FSH antagonist activity associated with N^{56}dg-eLHα:eCGβ made it more likely that O-glycosylation rather than N-glycosylation was responsible for the reduced antagonist activity of N^{56}dg-eLHα:eLHβ because O-linked oligosaccharides attached to eLHβ differ substantially from those attached to eCGβ (Bousfield *et al.* 1985, Damm *et al.* 1990, Hokke *et al.* 1994, Bousfield *et al.* 2001).

The reduction in FSH antagonist activity associated with N^{56}dg-eCGα:eLHβt may also fall into the category of another carbohydrate partially compensating for the absence of the αAsn^{56} 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 eCGα:eLHβt, while eFSHα:eLHβt exhibited intermediate activity (Butnev *et al.* 1998). Following

elimination of αAsn^{56} oligosaccharides, N^{56}dg-eLHα:eLHβt and N^{56}dg-eFSHα:eLHβt preparations exhibited equivalent receptor-binding activities, but N^{56}dg-eCGα:eLHβt still remained less active, as if the αAsn^{82} 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 αAsn^{78} oligosaccharide on receptor-binding affinity (Matzuk *et al.* 1989), but was consistent with an increase in FSH receptor binding activity when recombinant hFSH αAsn^{78} 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 N^{56}dg-eCGα:eLHβt 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 N^{56}dg-eLHα:eLHβt and N^{56}dg-eCGα:eLHβt and elimination of the FSH antagonist properties of N^{56}dg-eLHα:eCGβ 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 eLHβ Asn^{13} 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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