EXPERIMENTAL STUDY

Human chorionic gonadotropin with C-elongated α -subunit retains full receptor binding and partial agonist activity

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Abstract

Objective: To test whether extension of the C-terminus of human chorionic gonadotropin (hCG) α -subunit ($h\alpha$) alters the bioactivity of the recombined $\alpha\beta$ heterodimer.

Design: The stop codon of $h\alpha$ was mutated to produce a 24 amino acid extension.

Methods: The extended $h\alpha$ (α_{+24}) was co-expressed with hCG β in COS-7 cells and the receptor binding and *in vivo* bioactivity of the secreted hormone was compared with its wild-type counterpart.

Results: This extension did not impair the binding of hCG to rat LH/CG receptors and provoked a sixfold reduction in its stimulatory activity of testosterone secretion in rat Leydig cells.

Conclusions: The extension of α by itself does not lead to inhibition of the $\alpha\beta$ heterodimer to LH receptors but the structure of the extension appears to play an important role. It is thus possible that one-chain hCG chimeras with the β N-terminus fused to the α C-terminus might be active.

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Introduction

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family which also includes luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyrotropin (TSH). These hormones are heterodimeric and consist of two different non-covalently linked glycoprotein subunits: a common α -subunit and a hormone-specific β -subunit. Only the $\alpha\beta$ dimers, but not the dissociated subunits, exhibit high affinity towards the receptors and the specificity of binding is directed by the β -subunit. Nevertheless, the common α -subunit has been shown to play a crucial role in both high affinity binding (1) and signal transduction (2). In this respect, it has been shown by site-directed mutagenesis that the C-terminal residues of the α -subunit of hCG (Tyr⁸⁸-Tyr-His-Lys-Ser⁹²) are involved in its bioactivity. Indeed, truncations of this portion (3, 4) as well as many specific mutations of its individual residues (3–6) lead to reduced stimulatory activity in spite of unchanged binding activity.

In the present work, we produced a mutant hCG with an α -subunit elongated by 24 residues beyond the terminal Ser⁹² residue through mutation of the stop codon in order to evaluate (1) the possible role of the terminal carboxyl group of the α -subunit in hCG binding and signal transduction and (2) the functional consequences of a carboxy extension of the α -subunit in the scope of producing an active $\text{NH}_2\alpha\text{-}\beta\text{COOH}$ single-chain hCG.

Materials and methods

Plasmid constructs

The cDNAs encoding the wild-type hCG α -subunit and the elongated α -subunit (α_{+24}) were kindly given by Dr J J Rémy (INRA, Jouy-en-Josas, France). The α_{+24} cDNA lacks the stop codon of the wild-type α cDNA and this leads to translation of the 3'UTR region down to the next stop codon. The cDNA sequence of the construct was determined on applied model (373A) nucleotide sequencer and the deduced amino acid sequence of the C-terminal extension in $h\alpha_{+24}$ was Lys-Val-Glu-Gly-Ser-Ser-Ile-Pro-Thr-Ser-Ser-Ala-Pro-Ala-Gly-Arg-Gly-Arg-Asp-Ser-Arg-Gly-Ser-Leu. The wild-type hCG β cDNA in the pM² vector was a gift from Dr I Boime (Washington University, St Louis, MO, USA). All cDNAs were inserted at the unique HindIII site in the pCDM8 (InVitrogen) mammalian expression vector as previously described (7). All constructs were amplified in MC1061/P3 bacteria (InVitrogen, Groningen, The Netherlands) and purified using the maxiprep kit (Qiagen, Couteboeuf, France).

Transient expression of recombinant hormones in COS-7 cells

The COS-7 cells (ATCC-CRL 1651) maintained at 37°C under 5% CO₂ in humidified atmosphere in Dulbecco's modified Eagle's medium (7) were co-transfected with

Figure 1 Left: radioreceptor assay of pituitary pLH (▲), urinary hCG (□), recombinant h α .hCG β (◇) and recombinant h α_{+24} .hCG β (◆) using rat testicular membrane fraction and 125 I-oLH. Right: stimulation of testosterone secretion in rat Leydig cells by the same hormones (same symbols). Concentrations of hormones are based on their immunoactivity measured by EIA. The bars show s.d.

2 μ g of one vector encoding a h α -subunit (α or α_{+24}) and 2 μ g of the vector encoding the wild-type hCG β -subunit. This was performed at 65% confluency in 6 cm Petri dishes using the calcium phosphate precipitation procedure (7). Control cells were transfected with the empty pCDM8 expression vector. The supernatants were collected and kept frozen at -20°C until assays.

Natural hormones

Partially purified urinary hCG (1000 IU/vial) was purchased from Intervet (Oss, Holland) and its immunoactivity was checked in the specific EIA described below against the WHO international reference preparation. The concentrations of hCG were calculated on the basis of 15 000 IU/mg for the pure hormone. Porcine LH (pLH CY1354; $2.0 \times \text{NIH LH S1}$) and ovine LH (oLH CY1086; $3.5 \times \text{NIH LH S1}$) were purified in our laboratory. The radioiodination of oLH was performed using the chloramine T oxidation method (8).

Hormone assays

Quantification of expression was performed by an enzyme immunoassay (EIA) specific for the hCG $\alpha\beta$ heterodimer with a polyclonal antibody raised in the rabbit against hCG (M P Dubois, Nouzilly) using a previously published protocol (9). In this assay, the cross-reactions of the free h α - and hCG β -subunits are 0.1 and 0.3% respectively relative to hCG, showing that the antibody is heterodimer-specific. In addition, the $\alpha\beta$ and $\alpha_{+24}\beta$ heterodimers exhibit parallel competition curves, indicating that the +24 extension at the C-terminus of h α probably does not affect the conformation of the heterodimer. The binding activity of the recombinant dimers was measured by a radioreceptor assay using rat testicular membranes and radioiodinated oLH (^{125}I -oLH) and their *in vitro* bioactivity was measured by stimulation of testosterone secretion in rat Leydig cells (10).

Results

Figure 1 shows the dose-response curves of inhibition of ^{125}I -oLH binding to rat testicular membranes (left panel) and testosterone secretion in rat Leydig cells (right panel) by the $\alpha\beta$ and $\alpha_{+24}\beta$ dimers as compared with highly purified urinary hCG and pituitary pLH. Table 1 shows the calculated potencies of the four molecules in the two assays and the ratios between the two assays. The recombinant hCG $\alpha_{+24}\beta$ dimer exhibits unchanged binding activity to the LH receptor compared with the $\alpha\beta$ dimer. Its potency in the stimulation of testosterone secretion is sixfold less than that of wild-type recombinant hCG ($\alpha\beta$). The recombinant wild-type hCG ($\alpha\beta$) exhibits a tenfold higher activity than urinary hCG in the two assays. We previously observed the same difference between recombinant and natural hormones for equine LH, equine CG and porcine LH when the former were produced in either CHO, COS-7 or 293 cells (N Martinat, F Guillou & Y Combarous unpublished data). The $\alpha_{+24}\beta$ dimer, like pLH, had no antagonistic activity on the secretion of testosterone stimulated by hCG in rat Leydig cells (not shown).

Discussion

In the present work, we have shown that the elongation of the α -subunit of hCG by 24 amino acid residues does not impair its binding to rat LH receptors. This shows

Table 1 Potency of the natural and recombinant hormones in the radioreceptor assay and stimulation of testosterone secretion by rat Leydig cells.

Hormone	Binding (B)	Stimulation S	S/B
pLH	1	1	1
hCG	2.1	111	53
h α .hCG β	26	572	22
h α_{+24} .hCG β	26	94	3.6

that the additional peptide portion does not interfere either with the combination of subunits or with the association of the so-formed dimer with the LH receptor. These data contrast with those of Furuhashi *et al.* (11) who produced an hCG chimera with an additional β carboxyterminal peptide (hCG β -CTP) fused to the C-terminus of the α -subunit. This elongated α -subunit (h α -CTP) recombined efficiently with wild-type hCG β -subunit but the so-formed dimer exhibited very low binding activity to the human LH receptor expressed in the human fetal kidney 293 cell line (<5% relative to wild-type hCG).

Thus, the +24 peptide does not exert any inhibitory activity on hCG binding to rat LH receptors when present at the C-terminus of the α -subunit. This extension is only slightly shorter than the β -CTP (28 amino acid residues) but the latter is known to bear O-saccharide chains on four of its eight serine residues (Ser^{121, 127, 132, 138}) in the natural hCG molecule (12) and was also found to be O-glycosylated when fused to the C-terminus of the h α -subunit (11). Multiple O-glycosylation in segments of proteins impedes the folding of these regions, leading to bulky extended flexible structures (13). It is thus very likely that the bulky O-glycosylated CTP at the C-terminus of the α -subunit hinders the access of the dimer to the receptor. It can thus be hypothesized that the +24 peptide at the same location does not diminish the binding of the dimer to the receptor because of less steric hindrance compared with β CTP. The observed full binding activity of the $\alpha_{+24}\beta$ dimer also demonstrates that the α -carboxyl group of the C-terminal Ser⁹² residue is not crucial for high-affinity binding to rat LH receptors.

However, it cannot be ruled out from the present data using rat LH receptors and those of Furuhashi *et al.* (11) with human receptors that extension of the h α -subunit, with β -CTP or +24 peptide, leads to binding inhibition of hCG to the human receptors but not to the rat receptors. This is in keeping with the observation of Jia *et al.* (14) that the recombinant human LH receptor exhibits a very narrow specificity since it binds only the human gonadotropins hLH and hCG but not the equine, rat or ovine LHs. In contrast, LHs and CGs from all species tested bind to the rat testicular LH receptor *in vitro* (15, 16) but, as far as we know, the binding of non-human gonadotropins to natural human LH receptors from testis or ovary has not been studied; therefore it is impossible to ascertain whether the difference arises from the species origin of the receptors (human vs rat) or from the cells in which they are expressed (L293 vs Leydig).

The potency of the $\alpha_{+24}\beta$ dimer in the stimulation of testosterone secretion in rat Leydig cells is 16% that of the $\alpha\beta$ dimer. This indicates that the presence of an additional sequence at the C-terminus of the α -subunit does interfere with signal transduction but, nevertheless, does not suppress it completely. Previous studies have shown that truncations or mutations in this location also

diminish signal transduction (3–6). Moreover, reciprocal mutagenesis of Lys⁹¹ of the hCG α -subunit and of Asp³⁹⁷ of the exoloop 1 of the LH receptor suggests that these residues interact for receptor activation but not for high-affinity binding (17). It is likely that the additional +24 peptide at the C-terminus of the α -subunit diminishes the flexibility of this region and consequently slows down its adaptation with exoloop 1 of the receptor and subsequent activation of the receptor. It is interesting to point out that in spite of the unfavorable effect of the extension of the α -subunit, the stimulatory activity of the $\alpha_{+24}\beta$ dimer remains higher than that of pLH. After binding, hCG exhibits a much higher transduction efficiency than pLH in rat Leydig cells (15). In order to get a better comprehension of the molecular mechanism of the LH/CG receptor activation, it will be necessary to determine whether the elongation of the α -subunit only affects the 'superactivity' of hCG or also affects the 'normal' activity of pLH. Moreover, the retention of bioactivity in hCG with C-elongated α -subunit opens new ways in the production of active gonadotrope molecules for the control of fertility. In particular, active single-chain gonadotropins with their α - β -subunits fused in this order should be synthesized in addition to the different β - α molecules already described (18–20).

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