The additional N-glycosylation site of the equine LH/CG receptor is not responsible for the limited cyclic AMP pathway activation by equine chorionic gonadotropin relative to luteinizing hormone

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SUMMARY
In order to investigate the role of the unique seventh N23-glycosylation site of the equine LH/CG receptor (eLHCG) in the cAMP pathway activation, COS-7 cells were transiently transfected with either the wild-type or the mutant eLHCGN23Q cDNA and challenged with porcine LH and eCG for cAMP production. We showed that the N23-glycosylation site of the eLHCG is not required for the functional coupling of the receptor with the cAMP pathway and is not responsible for the limited potency of eCG relative to pLH to activate this receptor. Reproductive Biology 2011 2: 157-164.

Key words: equine CG, LH/CG receptor, N-glycosylation, carbohydrate

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INTRODUCTION
Equine luteinizing hormone (eLH) and equine choriogonadotropin (eCG) share the same polypeptide structure as a single gene encodes for their α-subunits and another gene encodes for their β-subunits [2]. However, they differ in their N- and O-saccharide chains because they are synthesized in different cell types: pituitary gonadotropes for eLH and chorionic girdle cells in the mare placenta for eCG [6, 10]. Furthermore, eCG binds to the equine LH/CG receptor (eLHCGR) with only one tenth or less the affinity of eLH [9, 11, 14], whereas eCG and eLH exhibit similar affinities toward the porcine LHR [9].

As a first step to better comprehend the structure-function relationship of the eLHCGR toward its ligands, we previously cloned and sequenced this receptor [12]. The protein sequence of the eLHCGR was found to be highly homologous (> 88%) with other mammalian LHRs [12]. Interestingly, the eLHCGR displayed an additional N-glycosylation site (N23-L24-S25) in the N-terminal extremity of the 335-amino acid extracellular domain of the eLHCGR i.e. one more than the six conserved N-glycosylation sites in other mammalian LHR binding domains [1]. The binding of both eCG and eLH to the recombinant eLHCGR led to the activation of the adenylate cyclase/cAMP pathway [12], which is the main intracellular signalling pathway in mammalian LHCGRs [1]. However, the stimulating activity of eCG toward the recombinant eLHCGR was 7.1 times lower than that of eLH, as assessed by the cAMP release from transfected COS-7 cells [12]. This study was thus undertaken to investigate the possible role of the specific N-glycosylation site of eLHCGR in this differential activation of the cAMP pathway through the use of mutated recombinant eLHCGR at position N23.

MATERIALS AND METHODS
The cDNA encoding for the eLHCGR was previously cloned in pCDNA3 [12]. A mutant for the supplementary N-glycosylation site has been obtained by site-directed mutagenesis of the N23 into a Q (Quick-change™ site directed Mutagenesis kit, Stratagene, Cambridge, UK) with the eLHCGR in PBluescript KS as template. The sequences of the primers used to obtain the mutation were as follows: 5’- GGC CCG CGG AGG CAA CTC TCC CGA CTA-3’
(forward) and 5’-TAG TCG GGA GAG **TTG** CCT CCG CGG GCC-3’ (reverse), where the underlined nucleotides introduced a Gln instead of an Asn, abolishing the possibility of the addition of a N-glycan. Cycling parameters were 30 s at 95°C then 16 cycles as followed: 30 s at 95°C, 1 min at 55°C and 3 min at 68°C. The mutant eLHCGRN23Q was introduced in pCDNA via Xba1 sites, amplified, purified using the Qiagen maxiprep plasmid kit (Coger, Paris, France) then sequenced. Monkey kidney COS-7 cells were maintained at 37°C in a humidified 5% CO₂ incubator in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes. COS-7 cells were transfected at 65% of confluency in 10 cm diameter Petri dishes with 12 µg pCDNA-construct containing either the wild-type or the mutant eLHCGR, using a calcium phosphate precipitation procedure as previously described [5]. Crude membrane preparations were prepared from transfected cells as previously described [12] and stored at −80°C before used as binding fractions. The protein concentration of COS-7 binding fractions was determined by the method of Bradford [4]. Radio-receptor assays were performed on COS-7 binding fractions pooled from at least three transfections according to a method previously used in our laboratory [11, 12]. Equine LH (National Hormone and Peptide Program, lot no. AFP5130A) was radioactively labelled with ¹²⁵I-Na (Amersham Pharmacia Biotech) using Iodo-Gen (Pierce, Rockford, IL). Crude COS-7 membrane preparations (15 µg proteins/tube) were incubated with ¹²⁵I-eLH (55 pM) and increasing amounts of unlabeled eLH, pLH, eCG (10⁻¹ to 10⁴ ng/ml) or eFSH (10 to 10⁴ ng/ml). Hormones used were all purified in our laboratory and their relative activities were as follows: eLH FL525, 0.8 × eLH-A; pLH DKL-D110, 3.0 × NIH-LH-P1; eCG FL652, 5 000 IU/mg; eFSH FL149, 0.3 × eFSH-A, where eLH-A and eFSH-A are standard preparations previously described [9]. Non-specific binding was determined in the presence of 100 IU hCG (Chorulon, Intervet, Boxmeer, Netherlands). The data were analyzed with the GraphPad PRISM2.01 software package (San Diego, CA) using the nonlinear “one-site competition” curve-fitting procedure in order to estimate the concentration of hormone required to inhibit ¹²⁵I-eLH binding by 50% (IC₅₀). Cyclic AMP assays were performed on transfected cells as previously described [12]. The
agonist concentrations inducing half-maximal stimulation of cAMP production (EC_{50}) were calculated using the GraphPad PRISM2.01 software package. Data are presented as means±SEM. Differences in potencies toward the eLHCGR between eLH (MW 34 000), pLH (MW 25 000) and eCG (MW 44 000) are expressed in fold of changes on a molecular basis. For example, in order to compare EC_{50} between pLH and eCG, the ratio (EC_{50}^{pLH}*44 000)/(EC_{50}^{eCG}*25 000) has been calculated. The Student t-test was used to compare the mean values of EC_{50} between wild-type and mutant eLHCGRs. Differences were considered statistically significant at p<0.05.

RESULTS AND DISCUSSION

In a first step, as purified pLH was available in larger quantities than eLH in the laboratory, we tested pLH as an alternative hormone to eLH to study the activation of the recombinant eLHCGR: on a molar basis, pLH (IC_{50} 24±3 ng/ml) and eLH (IC_{50} 59±4 ng/ml) bound to the recombinant eLHCGR with 15.0-fold and 8.3-fold greater potency, respectively, than eCG (IC_{50} 634±31 ng/ml; n=3 experiments; fig. 1). Thus, eCG displayed lower binding activity toward its receptor compared with pituitary LHs, whatever the LH origin, showing that it is the binding potency of eCG that is specifically limited. The ratio of eCG binding affinity to eLH (12.0%) or to pLH (6.7%) activity was not very different from the eCG/eLH binding activity ratio previously described in luteal tissue from cyclic (2-4%; [6]) or pregnant (2.5-3.9%; [8]) mares. The difference in binding activity between pituitary LHs and eCG could be linked to differences in size and structure of the N- and O-glycan chains linked to their polypeptidic α and β chains. Indeed, eCG, with a carbohydrate content of 41%, is the most glycosylated glycoprotein hormone among mammals [3, 6] and its glycan chains are mainly terminated by sialic acids whereas glycan chains of all LHs are mainly sulphated [13].

In a second step, we tested the stimulating activity of pLH on COS-7 cells transiently transfected with the wild-type recombinant eLHCGR. On a molar basis, pLH (EC_{50} 287±90 ng/ml) stimulated cAMP production from transfected COS-7 cells 4.8 times better than eCG (EC_{50} 245 ±497 ng/ml; n=4 experiments; fig. 2).
**Figure 1.** Competition curves of $^{125}$I-eLH binding to membrane preparations from COS-7 cells transiently transfected with the wild-type recombinant eLHCGR construct. Cell membranes were incubated in triplicate with a constant amount of $^{125}$I-eLH and increasing concentrations of unlabelled hormones. Data are presented as mean±SEM of triplicate observations from a single experiment. The experiment was repeated three times and a representative assay is shown. eCG: equine choriogonadotropin; eLH: equine luteinizing hormone; pLH: porcine luteinizing hormone; eFSH: equine follicle stimulating hormone.

**Figure 2.** Effect of various gonadotropins on media cAMP concentrations in COS-7 cells transiently transfected with the wild-type recombinant eLHCGR construct. Cos-7 cells were cultured for 1 h in the presence of increasing doses of pLH, eCG or eFSH. Data are presented as mean±SEM of triplicate observations from a single experiment. The experiment was repeated four times and a representative assay is shown. eCG: equine choriogonadotropin; pLH: porcine luteinizing hormone; eFSH: equine follicle stimulating hormone.
This result is close to the value obtained previously in the laboratory with eLH: on a molar basis, eLH stimulated cAMP release from transfected COS-7 cells 7.1 times greater than eCG [12]. So the binding potency of eCG toward the eLHCGR was 10-15 times less than pituitary LH but this binding resulted in comparatively higher activation of the receptor. The roles of eCG glycan chains in the greater coupling of intracellular responses after eCG binding to the eLHCGR remains to be explored.

As the comparison between eLHCGR with all other LHRs indicated a supplementary potential N-glycosylation site at position N23, we then tested the involvement of this site in the differential activation of eCG versus pLH by testing cAMP pathway activation on COS-7 cells transfected with mutated eLHCGR\textsuperscript{N23Q}. Stimulation of transfected cells with increasing concentrations of pLH and eCG resulted in dose-dependent increases in cAMP production whereas eFSH did not stimulate cAMP production (fig. 3).

![Figure 3](image)

**Figure 3.** Effect of various gonadotropins on media cAMP concentrations in COS-7 cells transiently transfected with the mutated recombinant eLHCGR\textsuperscript{N23Q} construct. Cos-7 cells were cultured for 1 h in the presence of increasing doses of pLH, eCG or eFSH. The data are expressed as the mean±SEM of triplicate observations from a single experiment. The experiment was repeated four times and a representative assay is shown. eCG: equine choriogonadotropin; pLH: porcine luteinizing hormone; eFSH: equine follicle stimulating hormone.
On a molar basis, pLH (EC\textsubscript{50} 403±90 ng/ml) stimulated cAMP production 4.8 times greater than eCG (EC\textsubscript{50} 3 428±526 ng/ml; n=4 experiments). These EC\textsubscript{50} values were not statistically different from the ones obtained on cells transfected with the wild-type eLHCG. Therefore, the N23-glycosylation site of the eLHCG was not required for the functional coupling of the occupied receptor with the cAMP pathway and was not implicated in the difference in cAMP production between eCG and the pituitary pLH. Consistent with these results, the rat LHR devoid of all its six N-linked carbohydrates was able to mediate the hCG-stimulated cAMP release from transfected cells with similar efficiency as the glycosylated receptor \cite{8}. This finding does not exclude the possibility of there being a role for the additional N-linked carbohydrate of the eLHCG in the intracellular receptor folding and trafficking. For instance, the first three glycosylation sites in the rat LHR have been shown to have a role in facilitating the processing of the receptor precursor to the mature cell surface form \cite{7}. In conclusion, the study shows that the additional glycosylation site at N23 of the eLHCG extracellular domain is not responsible for the specific limitation of eCG to activate the adenylate cyclase/cAMP pathway relative to porcine LH.

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