Internalization of Rat FSH and LH/CG Receptors by rec-eCG in CHO-K1 Cells

Jong-Ju Park[1], Hun-Ki Seong[1], Jeong-Soo Kim[1], Byambaragchaa Munkhzaya[1],

Myung-Hwa Kang[2] and †Kwan-Sik Min[1]

*[1]Animal Biotechnology, Graduate School of Future Convergence Technology, Department of Animal Life Science,
Institute of Genetic Engineering, Hankyong National University, Ansung 17579, Korea*
[2]Department of Food Science and Nutrition, Hoseo University, Asan 31499, Korea

ABSTRACT : Equine chorionic gonadotropin (eCG) is a unique molecule that elicits the response characteristics of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in other species. Previous studies from this laboratory had demonstrated that recombinant eCG (rec-eCG) from Chinese hamster ovary (CHO-K1) cells exhibited both FSH- and LH-like activity in rat granulosa and Leydig cells. In this study, we analyzed receptor internalization through rec-eCGs, wild type eCG (eCGβ/α) and mutant eCG (eCGβ/α Δ 56) with an N-linked oligosaccharide at Asn^{56} of the α-subunit. Both the rec-eCGs were obtained from CHO-K1 cells. The agonist activation of receptors was analyzed by measuring stimulation time and concentrations of rec-eCGs. Internalization values in the stably selected rat follicle-stimulating hormone receptor (rFSHR) and rat luteinizing/chorionic gonadotropin receptor (rLH/CGR) were highest at 50 min after stimulation with 10 ng of rec-eCGβ/α. The dose-dependent response was highest when 10 ng of rec-eCGβ/α was used. The deglycosylated eCGβ/α Δ 56 mutant did not enhance the agonist-stimulated internalization. We concluded that the state of activation of rFSHR and rLH/CGR could be modulated through agonist-stimulated internalization. Our results suggested that the eLH/CGRs are mostly internalized within 60 min by agonist-stimulation by rec-eCG. We also suggested that the lack of responsiveness of the deglycosylated eCGβ/α Δ 56 was likely because the site of glycosylation played a pivotal role in agonist-stimulated internalization in cells expressing rFSHR and rLH/CGR.

Key words : rec-eCGβ/α, Deglycosylated mutants, Internalization, rFSHR, rLH/CGR

INTRODUCTION

Equine chorionic gonadotropin (eCG) is a unique member of the gonadotropin family, as it can perform both LH- and FSH-like activity in non-equid species as well (Apparailly and Combarnous, 1994; Chopineau et al., 2001). Members of the glycoprotein family, which includes chorionic gonadotropin (CG), luteinizing hormone (LH), folli-cle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), comprise two non-covalently linked α- and β-subunits (Min et al., 2004; Park et al., 2010). The β-subunits of eCG and eLH have identical primary structures (Min et al., 1994). Thus, eCG could be an ideal model for studying structure/function relationships of gonadotropins, as it exhibits the properties of both its pituitary and placental counterparts (Guillou and Combarnous, 1983; Galet et

al., 2009).

In mares, eCG is secreted from binucleate trophoblastic cells, called endometrial cups, in maternal plasma during the first half of equine gestation. These cells detach from the chorionic girdle of the conceptus between days 37–120 of pregnancy (Boeta and Zarco, 2012; Flores-Flores et al., 2014; Conley, 2016). Progesterone has not been detected in the second half of gestation in mares (Conley, 2016). The presence of eCG during pregnancy may have a luteogenic effect, leading to the formation of supplementary CL after 35 days of pregnancy as a result of the ovulation and/or luteinization of dominant follicles at different times (Allen, 2001a). Previous studies on biological activity of rec-eCG, rec-eCGβ/α also had higher *in vitro* bioactivity than the eLH (Min et al., 1996). Deletion of carboxy-terminal peptides (CTP) from the dimeric eCG induced a 50% decrease in the secretion of the truncated hormone, compared to the wild type (Galet et al., 2000). The protein was truncated downstream of residue 109, which significantly decreased its secretion. Thus, the CTP upstream of the residues of the eCGβ-subunit at the 'seat-belt' region were also involved in the folding and secretion of gonadotropins (Galet et al., 2009).

In our previous study, we had produced rec-eCG from CHO-K1 cells, which were shown to exhibit dual LH- and FSH-like activity in rat Leydig and granulosa cells (Min et al., 1996, 2004), which expressed rLH/CGR and rFSHR (Park et al., 2009, 2010; Jeoung et al., 2010). A deletion downstream of amino acid 87 of the eCGα-subunit resulted in it not being secreted into the supernant of the medium in both the transiently and stably transfected cells (Jeoung et al., 2010). A mutant with a deletion downstream of amino acid 95 of the eCGα-subunit showed no LH-like or FSH-like activities, indicating that the C-terminal region of the eCGα-subunit plays a pivotal role in the activity of cells expressing the rFSHR and rLH/CGR. We had also reported that the amino acid sequence at 94–96 of eCGβ-subunit appeared to be very important for signal transduc-

tion of the FSHR (Park et al., 2010).

Along with the FSHR, LH/CGR is a member of the rhodopsin-like subfamily of G-protein-coupled receptors (GPCRs), which has been shown to mediate the internalization of its agonist (Segaloff and Ascoli, 1993; Ascoli et al., 2002; Flores-Flores et al., 2014). The internalized receptors are either degraded in lysosomes (Ghinea et al., 1992) or undergo resensitization and reinsertion into the plasma membrane (Ascoli and Segaloff, 1987; Lazari et al., 1998). Thus, the majority of internalized GPCRs are recycled back to the plasma membrane, instead of being routed to the lysosomes. Thus, internalization is thought to play an important role in receptor resensitization, rather than desensitization (Krupnick and Benovic, 1998; Lefkowitz, 1998).

We also reported on the function of the deglycosylated rec-eCGs produced from CHO-K1 cells (Min et al., 1996, 2004). However, we did not revealed the specific factors for the reduced biological activity in receptor cells. To better define the involvement of the biological activity of the rec-eCG stimulated cAMP synthesis, we have now analyzed the internalization of the rFSHR and rLH/CGR by deglycosylated rec-eCG. In the present study, we constructed a mammalian expression vector of rFSHR and rLH/CGR. The expressing vectors transfected to CHO-K1 cell and assessed the internalization of rFSHR and LHR by the bound rec-eCGβ/α.

MATERIALS AND METHODS

1. Materials

The expression vector pcDNA3 was purchased from Invirogen (CA, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Endonucleases were Boehringer Mannheim (MA, USA) and Takara (Osaka, Japan). Polymerase chain reaction (PCR) reagents were from Takara (Japan). Ham's F-12, CHO-S-SFM II, Geneticin, Lipofectamine 2000 and fetal

bovine serum (FBS) were from Gibco BRL (MD, USA). The QIAprep-Spin plasmid kit was purchased from QI-AGEN Inc. (Hilden, Germany). PMSG enzyme-linked immunosorbent assay (ELISA) kit was from DRG (USA). The pCORON 1000 SP VSV-G tag expression vector, cAMP kit and CypHer 5-labeled anti-VSVG were from GE Healthcare Life Science (Buckinghamshire, UK). The oligonucleotides were synthesized by Green Gene Bio (Seoul, Korea). Fetal bovine serum was from Hyclone laboratories (Utah, USA). Centriplus Centrifugal Filter Devices were purchased from Amicon Bio separations (MA, USA). All other reagents used were from Sigma-Aldrich (USA) and Wako Pure Chemicals (Osaka, Japan).

2. Construction of the tethered eCGβ/α and eCGβ/α⊿56

cDNAs encoding the tethered eCGβ/α were inserted in the pcDNA3 mammalian expression vector and used as templates to construct the mutants, in which Asn^{56} (AAC) at the glycosylated site of the α-subunit was substituted for Gln (CAG), as previously reported (Min et al., 2004). The epitope, *myc-tag* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu), inserted into the tethered eCGβ/α and mutant (eCGβ/αΔ56) between the first and second amino acids in the β-subunit of the mature protein. Site-directed mutagenesis was performed using the three primers method in a single-step PCR (Min et al., 2004). The schematic diagrams of rec-eCGβ/αWT and eCGβ/αΔ56 are shown in Fig. 1. These fragments were digested with *EcoR* I and *Sal* I and ligated into the eukaryotic expression vector, pcDNA3. The plasmids were then purified and sequenced in both directions by automated DNA sequencing to ensure that the correct mutations had been introduced (designated as pcDNA3-eCGβ/α; β/αΔ56).

3. Cell culture and functional expression

Cultured CHO-K1 cells were transfected into expression

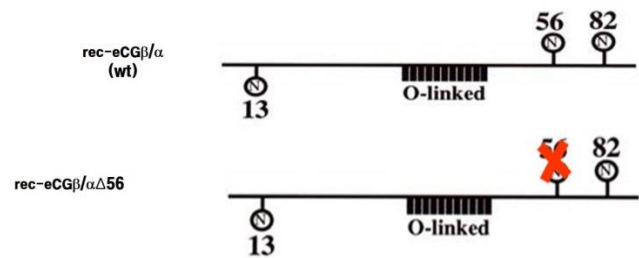**Fig. 1. Schematic diagram of rec-eCGβ/α and receCGβ/αΔ56.** The eCG cDNA was ligated into the pcDNA3 mammalian expression vector. Wild type (eCGβ/α) and eCGβ/αΔ56 (deglycosylated at Asn^{56} in the α-subunit replaced with Gln) were constructed.

vectors using the liposome transfection method, as previously described (Min et al., 2004). The transfected cells were cultured for 48 h in a serum-free medium (CHO-S-SFM-II) and then harvested and centrifuged at 15,000 rpm for 10 min. The supernatant was collected and stored at −20℃ until the assay. Six to 8 pools of stably transfected cells were selected by incubating the cells in a growth medium [Ham's F12 medium with 10% FCS, penicillin (100 IU/mL), streptomycin (100 μg/mL) and glutamine (2 mM) supplemented with G418 (800 μg/mL)] for 2–3 weeks after transfection, as previously reported method (Min et al., 2004). The culture medium was centrifuged at 15,000 rpm for 10 min to remove cell debris. The supernatant was collected and concentrated in an Amicon Stirred cell concentrator and stored at −20℃ until the assay.

4. Quantification of rec-eCG proteins

The tethered eCG proteins secreted by the transient transfected CHO-K1 cells and the stable cell lines into serum-free media were collected, and rec-eCG was quantified using the PMSG ELISA (enzyme-linked immunosorbent assay) kit, according to the supplier's protocol (DRG, USA). Briefly, the collected medium was dispensed into wells coated with a monoclonal antibody against unique antigenic site on the eCG molecule. The wells were incubated for 60 min at RT and 100 μL of conjugate was

then added into each well. The substrate solution (100 μL) was also added, and the mixture was then incubated for 30 min at RT. Finally, 50 μL stop solution was added to stop the enzymatic reaction. The optical density (OD) of the product solution was read at 450 nm with a micro titer plate reader.

5. Selection of cell lines expressing rLH/CGR and rFSHR

The cDNAs for rLH/CGR and FSHR were cloned and expressed in CHO-K1 cell lines, as previously described (Min et al., 1998; Min, 1999). The receptor cDNAs were subcloned into the eukaryotic expression vector, pCORON 1000 SP VSV-G tag (GE Healthcare Life Science, Buckinghamshire, UK), for transfection. Stably transfected cell lines were selected by G418 treatment, as previously described (Min et al., 2004, Seo et al., 2015). Briefly, cloned cells were cultured in standard 96-well microplates at concentrations of 10^5–10^6 cells/mL and incubated overnight. Cholera toxin was added and the mixture was incubated for 30 min. Finally, the cyclic AMP kit was used according to the supplier's protocol, and the OD was measured at 630 nm with a microplate reader. The establishment and properties of a clonal cell line expressing *vsvg*-rLH/CGR-wt [designated as *vsvg*-rLH/CGR (wt-9)] and one expressing *vsvg*-rFSH-wt [designated *vsvg*-rFSHR (wt-7)] were studied based on cAMP responsiveness to an agonist, cholera toxin, as previously reported (Park et al., 2009).

6. Internalization assays of rLH/CG and rFSH receptors by rec-eCGs

A novel assay technology, which relies on the intrinsic ability of cell surface receptors to internalize into acidic endosomal vesicles upon agonist stimulation, was recently reported (Lowe et al., 2015). This technology uses a novel, fluorogenic, pH-sensitive cyanine dye, CypHer 5. It is therefore ideal for reporting the movement of receptors from the cell surface into the acidic internal endosomes. The rLH/CGR cells and FSHR cells were seeded into a 96-well tissue culture view plate (Perkin Elmer; 6005710, precoated with poly-D-lysine) at a density of 2×10^3 cells/cm^2 in a complete Nutrient Mix F-12 Ham's medium with G418 (700 μg/mL). The cells were incubated at 37℃ overnight until the cells were 20–40% confluent. The supernatant was removed and the cells gently washed twice with Kreb's Ringer Buffer (KRB) (120 mM sodium chloride, 25 mM HEPES, 4.8 mM potassium chloride, 1.2 mM potassium dihydrogen orthophosphate, 1.2 mM magnesium sulphate heptahydrate, and 1.3 mM calcium chloride) at pH 7.4. The culture medium was replaced with 70 μL/well of KRB. KRB with CypHer5-labeled antibodies (2-5 μg/mL), 1μM Hoechst 33342, and 1% FBS was then added to the plate. After incubating at RT for 10 min, 30 μL of KRB containing the agonist (1, 3, 6, 10, and 20 ng/mL of rec-eCG and 500 ng/mL of cholera toxin) was added to the plate. The mixture was incubated at RT for 60 min, and 75 μL/well of ice-cold 8% paraformaldehyde was then added. After incubating on ice at 4℃ for 20 min, the supernatant was removed and the cells were gently washed twice with PBS. Finally, 100 μL/well of PBS was added. The Cy5-labeled receptors and the Hoechst 33342 were visualized with the In Cell Analyzer 3000 device (GE Healthcare Life Science, Buckinghamshire, UK).

RESULTS

1. Stable expression rec-eCGβ/α and rec-eCGβ/α⊿56

The eCGβ/α and rec-eCGβ/αΔ56 vectors were transfected into CHO-K1 cells and the cells stably expressing genes were then selected with G418 treatment. The rec-eCGs secreted by the stably transfected cells into the serum-free medium were isolated and concentrated. The rec-eCGs were quantified using ELISA. The quantity of rec-eCG

obtained from stably transfected cells was about 180-240 mIU/mL. The size of the rec-eCGβ/α protein was found to be approximately 40-45 kDa (data now shown), which was consistent with previous studies (Park et al., 2009, 2010; Jeoung et al., 2010). However, the size of the rec-eCG secreted by the mutant with the deglycosylated Asn[56] in the α-subunit was decreased approximately 4-5 kDa.

2. Isolation of CHO-K1 cell lines stably expressing rFSHR and rLH/CGR

The mammalian expression vectors for rFSHR and rLH/CGR were transfected into CHO-K1 cells. The 10 clones were then isolated by G418 treatment. The cAMP level was determined to evaluate the response for agonist stimulation. The most stimulated cells were rFSHR-7 and rLH/CGR-9, as shown in Fig. 2. The maximal cAMP responses were 2,100 pmol/mL and 3,500 pmol/mL in the rFSHR and rLH/CGR, respectively.

3. Internalization assay results of receptors by rec-eCGs in time- and concentration-dependent manner

The effects of rec-eCGβ/α on the internalization of rFSHR and rLH/CGR in CHO-K1 cells were determined in a time-dependent manner (0–70 min). As shown in Fig. 3A,
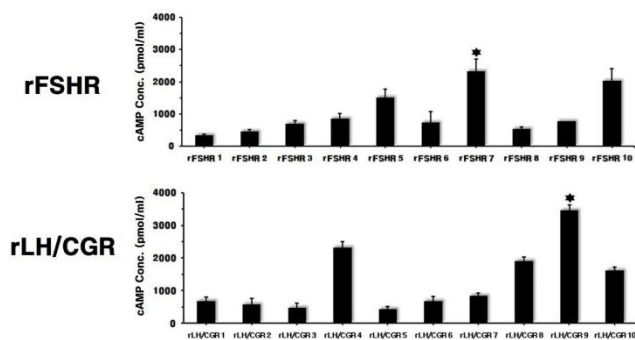
rFSHR rLH/CGR were most internalized at 50 min after incubating rec-eCGβ/α (10 ng/mL). We also measured the effect of rec-eCGβ/α on the internalization by receptors in a concentration-dependent manner (Fig. 3B), and found that the internalization increased proportional to the concentration of rec-eCGβ/α. The granularity values for both the receptors were found to be 800–900. The results of the agonist activation experiment also suggested that both the receptors were internalized in endosomes. Therefore, we used Cy5-labeled receptors to detect the localization after 60 min of incubation with rec-eCGβ/α (Fig. 4). Next, we analyzed the differences between the effects of rec-eCGβ/α and rec-eCGβ/αΔ56 on stably expressed these receptors (rFSHR-7 and rLH/CGR-9). The results of this analysis showed that the rec-eCGβ/αΔ56 in cells expressing rFSHR and rLH/CGR was completely flat; no agonist-stimulated internalization was induced in either cell type by eCGβ/α
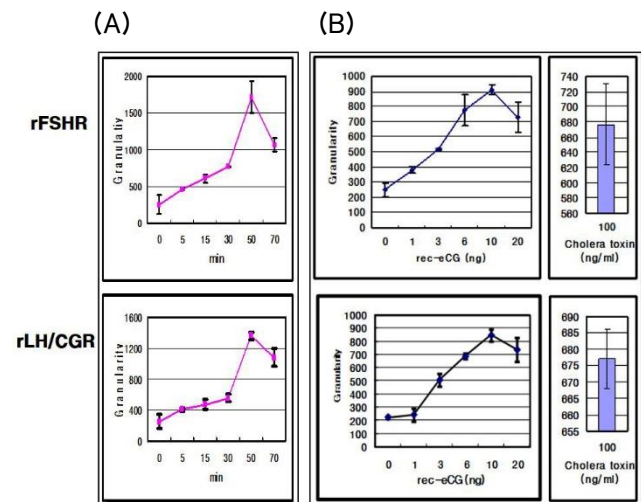

Fig. 3. **Internalization results for cells expressing rFSHR and rLH/CGR.** (A) Internalization results for time-dependent (0–70 min) at a fixed rec-eCGβ/α concentration (10 ng/mL) in rFSHR and rLH/CGR. (B) Internalization results for agonist-dependent (1–20 ng/mL). The rLH/CGR cells (wt-9) and FSHR cells (wt-7) cells were seeded at a density of 2×10^3 cells. The results were visualized using the In Cell Analyzer 3,000 device and the granularity of the receptors internalized into the endosomes was calculated.

Fig. 2. **Selection of cells expressing rFSHR and rLH/CGR from stably transfected CHO-K1 cells.** Cloned cells were cultured at concentrations of 10^5-10^6 cells/mL. Cholera toxin (100 ng/mL) was added, incubated, and cyclic AMP was analyzed using a cAMP kit.
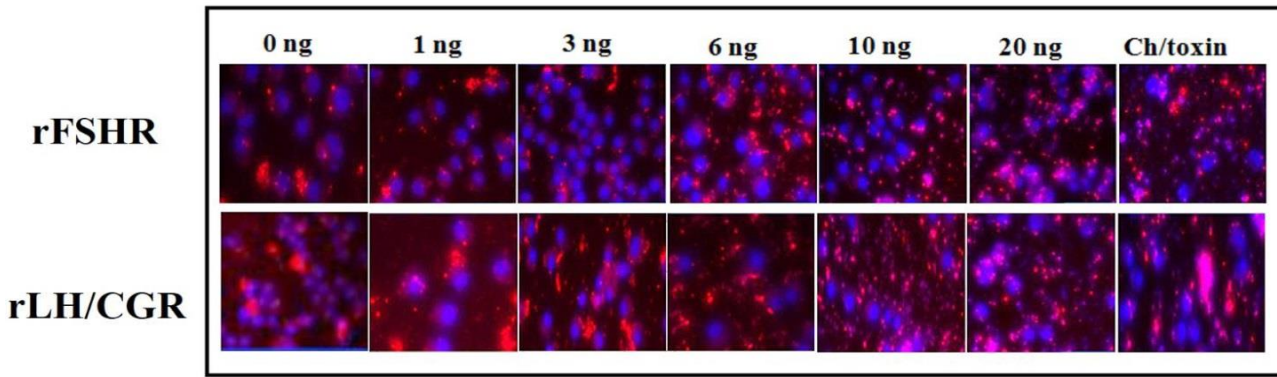
Fig. 4. Visualization of internalization results with In Cell Analyzer 3000 device. The cells were labeled with CypHer5 antibody and the agonist (rec-eCGβ/α) was added, as shown in Fig. 3. The Cy5-labeled receptors and the Hoechst 33342 were visualized using the In Cell Analyzer 3,000 device.
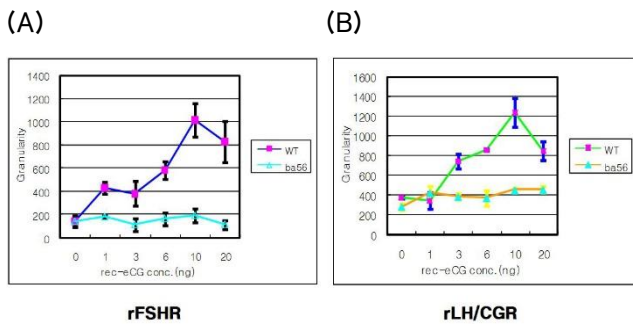
(A)

(B)

rFSHR

rLH/CGR

Fig. 5. Internalization results of rec-eCGβ/α and rec-eCGβ/αΔ56 using cells expressing rFSHR and rLH/CGR. (A) rFSHR cells. (B) rLH/CGR cells. Both rec-eCGβ/α and rec-eCGβ/αΔ56 were induced by stably transfected rFSHR and rLH/CGR cells. Internalization assays were analyzed in a rec-eCGβ/α-dose-dependent manner, as described in Fig. 3.

Δ 56 (Fig. 5A,B). Together, these data suggested that rec-eCGβ/α induced receptor internalization in cells expressing rFSHR and rLH/CGR. However, the mutant with the eCGβ/α Δ 56 did not induce this agonist stimulated internalization. Thus, the glycosylation site (Asn[56]) in the eCGα-subunit played a pivotal role in receptor internalization.

DISCUSSIONS

The results of the present study showed that rec-eCGβ/α induced the internalization of rFSHR and rLH/CGR in CHO-
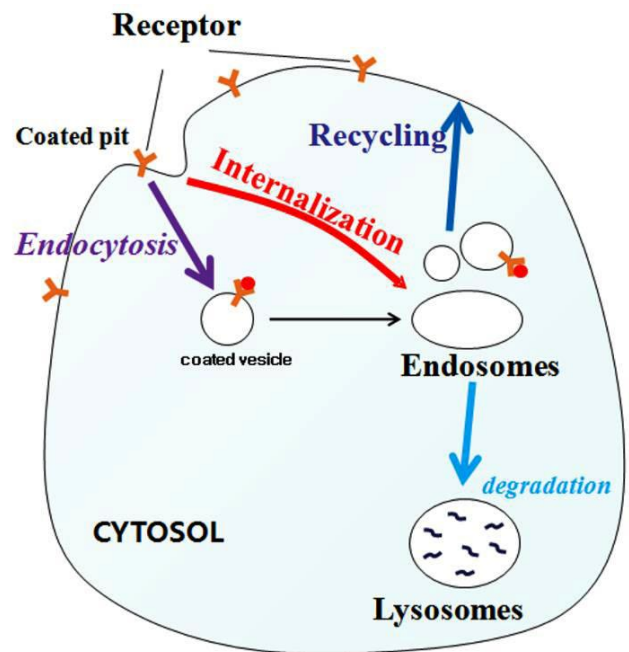

Fig. 6. Pathways of internalization, recycling, degradation and endocytosis of G protein-coupled receptors. When receptor cells are exposed to a agonist, the responsiveness wanes with time, in spite of the continuous presence of the agonist. It called to as desensitization that occur at the level of the hormone receptor, post-receptor steps. There are two categories of regulatory events. Uncoupling is defined as a change in the functional properties of a constant number of receptors. The other one is down-regulation defining as a reduction in the density of cell surface receptors (Hipkin et al., 1993).

K1 cell lines that expressed those receptors in a time- and concentration- dependent manner. We also showed that deglycosylation at Asn[56] of the α-subunit of rec-eCGβ/α inhibited this agonist-induced internalization in both cell types.

Previous studies have demonstrated that rec-eCGβ/α was expressed in the baculovirus-Sf9 insect cell system, either as a single-chain with the C-terminal of the β-subunit fused to the N-terminus of the α-subunit or as noncovalently linked heterodimers with or without polyhistidine tags at various locations (Legardinier et al., 2008). Heterodimeric rec-eCG exhibited the same thermal stability as natural pituitary LH, and its advantages over the single-chain eCG included higher secretion, higher *in vitro* bioactivity, and lower risk of immunogenicity (Legardinier et al., 2008). From the labeling and pulse-chase experiments, the secretion of the eCGβ-subunit from CHO-K1 cells was inefficient (medium recovery of 16–25%) and slow ($t_{1/2}$>6.5 h). This secretion of eCGβ-subunit resembled that of hLHβ-subunit, rather than that of the hCGβ-subunit (Cohen et al., 2015). In a previous study, rec-eCG was expressed in commercially available Mimic[TM] insect cells that were stably transformed into Sf9 cells expressing five mammalian genes that encoded glycosyltransferases involved in the synthesis of complex-type monosialylated N-glycans. the rec-eCG produced from these Minic[TM] insect cells showed mammalian-like nonsialyl complex-type N-glycosylation (Legardinier et al., 2005). Natural eCG contains complex-type N-glycans terminating with both Siaα2,3Gal and Siaα2,6Gal. In previous studies, we had reported rec-eCG production in CHO-K1 cells (Min et al., 2004; Park et al., 2009, 2010; Jeoung et al., 2010). But, the specific glycosylation site is very important for the secretion of rec-eCG in CHO-K1 cells (in preparation). Our results accord to reports that rec-eCG was exhibited biologically activity (Min et al., 1996, 2004; Park et al., 2009, 2010). Taken together, these results indicated that rec-eCG produced from mammalian cells could be useful for improving the development of dominant and pre-ovulation follicles in non-equids.

We also stably transfected CHO-K1 cells with rFSHR and rLH/CGR cDNAs. In previous studies, rec-eCGβ/α was shown to have dual activity of both rFSHR and rLH/CGR in not-equids, but only LH-like activity in equid species (Park et al., 2009; 2010). However, there have been no reports on the internalization of rec-eCGs in rFSHR and rLH/CGR. Our results demonstrated that receptors of rFSH and rLH/CG were internalized in a time- and concentration-dependent manner by rec-eCGs. However, when deglycosylated at the Asn[56] of α-subunit, rec-eCG did not induce the internalization of those receptors. Our results support the data from a previous study that showed that the internalization and degradation of chemically deglycosylated hCG (dghCG) was slower than that of hCG in MA-10 cells, and that this effect was not due to its inability to stimulate cAMP production (Hoelscher et al., 1991). In fact, dghCG was not capable of uncoupling the LH/CGR in Leydig tumor cells (Rebois and Fishman, 1983). In contrast to hCG, addition of dghCG to ^{32}P-labeled 293L cells resulted in little or no increase in the phosphorylation of LH/CG receptors (Hipkin et al., 1993). Based on the data presented, we suggested that the rate of the mutant rec-eCG, which was deglycosylated at Asn[56] of the α-subunit, was not increased because of the conformation of the hormone-receptor complex. A previous study suggested that four serine residues in the C-terminal tail of rLH/CGR had distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization (Lazari et al., 1998). A constitutive active mutant of hLH/CGR, hLH/CGR-L457R, was not routed to the lysosomes, but was recycled to the cell; hormone degradation was barely detectable (Galet and Ascoli, 2006). Similarly, the removal or simultaneous mutation of two residues (Cys[699] and Leu[683]) rerouted internalized hCG to a degradation pathway and internalized hLHR to the lysosomes (Galet et al., 2004). Replacement of all of the Ser/Thr residues in the carboxyl-terminal tail and truncation of the domain containing these

residues completely inhibited vasoactive intestinal poly-peptide (VIP)-stimulated phosphorylation and receptor internalization (Langlet et al., 2005). This GPCR internalization has been shown to be agonist-specific and G protein regulated kinase (GRK)-dependent (Hossain et al., 2009). This type of feedback regulation is conventional, as it requires the activation of classic G proteins (Kohout and Lefkowitz, 2003; Lodowski et al., 2003) as shown in Fig. 6. In the presented study, we showed that deglycosylated rec-eCG did not induce agonist-stimulated internalization. Therefore, we suggested that the glycosylation site in rec-eCG played a pivotal role in the internalization of rFSHR and rLH/CGR. The established stable cells expressing rFSHR and rLH/CGR could be utilize agonist-stimulated receptor internalization when stimulated with rec-eCG mutants that were deglycosylated at other sites. Further studies are required to conclusively determine whether internalization is indeed responsible for the uncoupling of rFSHR and rLH/CGR. These activity will be analyze the oocyte ovulation *in vivo* using mice.

REFERENCES

Allen WR (2001a) Luteal deficiency and embryo mortality in the mare. Reprod Domest Anim 36:121-131.

Allen WR (2001b) Fetomaternal interactions and influences during equine pregnancy. Reproduction 121:513-527.

Apparailly F, Combarnous Y (1994) Role of sialic acid residues in the *in vitro* superactivity of human chori-ogonadotropin (hCG) in rat Leydig cells. Biochim Biophys Acta 1224:559-569.

Ascoli M, Fanelli F, Segaloff DL (2002) The lutropin/chorio-gonadotropin receptor, a 2002 perspective. Endocr Rev 23:141-174.

Ascoli M, Segaloff DL (1989) On the structure of the lute-inizing hormone/chorionic gonadotropin receptor. Endocr Rev 10:27-44.

Boeta M, Zarco QL (2005) Progesterone and equine chori-onic gonadotropin concentrations around the time of pregnancy loss in mares impregnated by donkeys or stallions. J Equine Vet Sci 25:531-538.

Boeta M, Zarco QL (2012) Luteogenic and luteotropic effects of eCG during pregnancy in the mare. Anim Reprod Sci 130:57-62.

Chopineau M, Martinat N, Galet C, Guillou F, Combarnous Y (2001) β-subunit 102–104 residues are crucial to confer FSH activity to equine LH/CG but are not sufficient to confer FSH activity to human CG. J Endocrinol 169: 55-63.

Cohen L, Bousfield GR, Ben-Menahem D (2015) The recombinant equine LHβ subunit combines divergent intracellular traits human LHβ and CGβ subunits. Theriogenology 83:1469-1476.

Combarnous Y, Guillo F, Martinat N (1984) Comparison of *in vitro* follicle-stimulating hormone (FSH) activity of equine gonadotropins (luteinizing hormone, FSH, and chorionic goandotropin) in male and female rats. Endocrinology 115:821-1827.

Conley AJ (2016) Review of the reproductive endocrinology of the pregnant and parturient mare. Theriogenology 86:355-365.

Daels PF, Albrecht BA, Mohammed HO (1998) Equine chorionic gonadotropin regulate luteal steroidgenesis in pregnant mares. Biol Reprod 59:1062-1068.

Flores-Flores G, Velazquez-Canton E, Boeta M, Zarco L (2014) Luteoprotective role of equine chorionic gonadotropin (eCG) during pregnancy in the mare. Reprod Dom Anim 49:420-426.

Freick M, Passarge O, Weber J (2017) Lack of effects of an equine chorionic gonadotropin (eCG) administration between days 9 and 15 postpartum on reproductive performance in a Holstein dairy herd. Reprod Domest Anim (inpress), doi: 10.1111/rda.12928

Galet C, Ascoli M (2006) A constitutively active mutant of the human lutropin receptor (hLHR-L457R) escapes lysosomal targeting and degradation. Mol Endocrinol

20:2931-2945.

Galet C, Guillou F, Foulon-Gauze F, Combarnous Y, Chopineau M (2009) The β104-109 sequence is essential for the secretion of correctly folded single-chain ba horse LH/CG and for its activity. J Endocrinol 203: 167-174.

Galet C, Hirakwa T, Ascoli M (2004) The postendocytotic trafficking of the human lutropin receptor is mediated by a transferable motif consisting of the C-terminal cysteine and an upstream leucine. Mol Endocrinol 20: 2931-2945.

Galet C, Menck L, Bourhis C, Chopineau M, Griec LG, Perrin A, Magallon T, Attal J, Viglietta C, Houdebine LM, Guillou F (2000) Expression of a single βα chain protein of equine LH/CG in milk of transgenic rabbits and its biological activity. Mol Cell Endocrinol 174:31-40.

Garcia-Ispierto I, Lopez-Helguera I, Martino A, Lopez-Gatius F (2012) Reproductive performance of anoestrous high-producing dairy cows improved by adding equine chorionic gonadotrophin to a progesterone-based oestrous synchronizing protocol. Reprod Domest Anim 47:752-758.

Ghinea N, Vuhai MT, Groyer-Picard MT, Houllier A, Schoevaert D, Milgrom E (1992) Pathways of internalization of the hCG/LH receptor: immunoelectron microscopic studies in Leydig cells and tranfected L cells. J Cell Biol 118: 1347-1358.

Guillou F, Combarnous Y (1983) Purification of equine gonadotropins and comparative study of their acid-dissociation and receptor-binding specificity. Biochim Biophys Acta 755:229-236.

Hipkin RW, Sanchez-Yague J, Ascoli M (1993) Agonist-induced phosphorylation of the luteinizing hormone/chorionic gonadotropin receptor expressed in a stably transfected cell line. Mol Endocrinol 7:823-832.

Hoelscher SR, Sairam MR, Ascoli M (1991) The slow rate of internalization of deglycosylated human chorionic gonadotropin is not due to its inability to stimulate cyclic adenosine monophosphate accumulation. Endocrinology 128: 2837-2843.

Holtan DW, Squires EL, Lapin DR, Ginther OJ (1979) Effect of ovariectomy on pregnancy in mares. J Reprod Fertil Supple 27:457-463.

Hossain M, Rashid M, Bhuiyan MA, Nakamura T, Ozaki M, Nagatomo T (2009) Mutagenesis of important amino acid reveals unconventional homologous internalization of β1-adrenergic receptor. Life Science 85:339-344.

Jeoung YH, Yoon JT, Min KS (2010) Biological functions of the COOH-terminal amino acids of the α-subunit of tethered equine chorionic gonadotropin. Reprod Dev Sci 34:47-53.

Kohout TA, Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. Mol Pharmacology 63:9-18.

Krupnick JG, Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annual Review of Pharmacological Toxicology 38:289-231.

Langlet C, Langer I, Vertongen P, Gaspard N, Vanderwinden JM, Robberecht P (2005) Contribution of the carboxyl terminus of the VPAC1 receptor to agonist-induced receptor phosphorylation, internalization, and recycling. J Biol Chem 280:28034-28043.

Lazari MF, Bertrand JE, Nakamura K, Liu X, Krupnick JG, Benovic JL, Ascoli M (1998) Mutation of individual serine residues in the C-terminal tail of the lutropin/choriogonadotropin receptor reveal distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization. J Biol Chem 273:18316-18324.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and β-arrestins in receptor signalling and desensitization. J Biol Chem 273:18677-18680.

Legardinier S, Poirier JC, Klett D, Combarnous Y, Cahoreau C (2008) Stability and biological activities of

heterodimeric and single-chain equine LH/chorionic goandotropin variants. J Mol Endocrinol 40:185-198.

Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ, Tesmer JJ (2003) Keeping G proteins at bay: A complex between G protein-coupled receptor kinases 2 and G beta-gamma. Science 23:1256-1262.

Lowe JD, Sanderson HS, Cooke AE, Ostovar M, Tsisanova E, Withey SL, Chavkin C, Husbands SM, Kelly E, Henderson G, Bailey CP (2015) Role of G protein-coupled receptor kinases 2 and 3 in μ-opiod receptor desensitization and internalization. Mol Pharmacol 88:347-356.

Min KS (1999) Functional expression of lutropin/choriogonadotropin receptor cDNAs in 293 cells. Reproduction and Development 23:347-352.

Min KS, Hattori N, Aikawa JI, Shiota K, Ogawa T (1996) Site-directed mutagenesis of recombinant equine chorionic gonadotropin/luteinizing hormone: differential role of oligosaccharides in luteinizing hormone- and follicle-stimulating hormone-like activities. Endocrine J 43:585-593.

Min KS, Hiyama T, Seong HH, Hattori N, Tanaka S, Shiota K (2004) Biological activities of tethered equine chorionic gonadotropin (eCG) and its deglycosylated mutants. J Reprod Dev 50:297-304.

Min KS, Liu X, Fabritz J, Jaquette J, Abell AN, Ascoli M (1998) Mutations that induce constitutive activation and mutations that impair signal transduction modulate the basal and/or agonist-stimulated internalization of the lutropin/choriogonadotropin receptor. J Bio Chem 273:34911-34919.

Pacala N, Corin N, Bencsik I, Dronca D, Cean A, Boleman A, Caraba V, Papp S (2010) Stimulation of the reproductive function at cyclic cows by ovsynch and PRID/eCG.

Anim Sci Biotech 43:317-320.

Park JJ, JarGal N, Yoon JT, Min KS (2009) Function of the tethered rec-eCG in rat and equine receptors. Reprod Dev Biol 33:229-236.

Park JJ, JarGal N, Yoon JT, Min KS (2010) β-Subunit 94-96 residues of tethered recombinant equine chorionic gonadogropin are important sites luteinizing hormone and follicle stimulating hormone like activities. Reprod Dev Biol 34:33-40.

Rebois RV, Fishman PH (1983) Deglycosylated human chorionic gonadotropin: an antagonist to desensitization and down-regulation of the gonadotropin receptor-adenylate cyclase system. J Biol Chem 258:12775-12778.

Rensis FD, Lopez-Gatius F (2014) Use of equine chorionic gonadotropin to control reproduction of the dairy cow: a review. Reprod Dom Anim 49:177-182.

Rostami B, Niasari-Naslaji A, Vojgani M, Nikjou D, Amanlou H, Gerami A (2011) Effect of eCG on early resumption of ovarian activity in postpartum dairy cows. Anim Reprod Sci 128:100-106.

Segaloff DL, Ascoli M (1993) The lutropin/ choriogonadotropin receptor; 4 years later. Endocr Rev 14:324-347.

Seo EB, Kang MH, Min KS (2015) Biological activities of tethered recombinant equine chorionic gonadotropin mutants (βGln94Alaα, βIle95Alaα and βLys96Alaα). Academia J Biotechnology 3:122-231.

Wei SC, Gong ZD, Zhao HW, Liang HQ, Lai LJ, Deng YY (2016) Equine chorionic gonadotropin influence on sheep oocyte in vitro maturation, apoptosis, and follicle-stimulating hormone receptor and luteinizing hormone receptor expression. Genet Mol Res 15: doi: 10.4238/gmr15049162.