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Signal Transduction of Equine Follicle-Stimulating Hormone Receptor (eFSHR) by rec-eelFSHβ/α, Natural Porcine FSH, and Natural Human FSH

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ABSTRACT

In this study, we analyzed signal transduction by equine follicle-stimulating hormone receptor (eFSHR) on stimulation with recombinant eelFSHβ/α (rec-eelFSHβ/α), natural porcine FSH (pFSH), and natural human FSH (hFSH). cAMP stimulation in CHO-K1 cells expressing eFSHR was determined upon exposure to different doses (0-1450 ng/mL) of these hormones. The EC_{50} value of rec-eelFSHβ/α was 53.35 ng/mL. The Rmax values of rec-eelFSHβ/α and pFSH were 28.12 and 2.88 ng/mL, respectively. The activity of rec-eelFSHβ/α was much higher than that of natural pFSH. However, signal transduction in CHO PathHunter Parental cells expressing eFSHR was not enhanced by stimulation with natural hFSH. Thus, rec-eelFSHβ/α was completely active in cells expressing eFSHR. However, natural hFSH did not invoke a signal response in cells expressing eFSHR. Particularly, natural pFSH was weakly active in the same cells. These results showed that eelFSHβ/α has potent activity in cells expressing eFSHR. Thus, rec-eelFSHβ/α may efficiently bind to eFSHR, where as natural hFSH does not bind to eFSHR.

(Key words : eFSHR, rec-eelFSHβ/α, natural pFSH, natural hFSH, cAMP stimulation)

INTRODUCTION

Members of the glycoprotein family, including chorionic gonadotropin (CG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), comprise two non-covalently linked α- and β-subunits (Park *et al*., 2010, 2017). Japanese eel is one of the most important fish species aquacultured in East Asian countries, including Japan and Korea (Kagawa *et al*., 1998). In fish, as in other vertebrates, reproductive activity is regulated by the hypothalamus-pituitary-gonad axis and 2 types of gonadotropin hormone (GTH: FSH and LH) secreted from the pituitary gland (Swanson *et al*., 2003). In recently, rec-eel FSH and LH were widely reported to exhibit biological activity in reproductive endocrinology studies (Kobayashi *et al*., 2010; Kazeto *et al*., 2008; Ohta *et al*., 2007). Eel gonadal development can be induced by administering gonadotropin reagents such as salmon pituitary extract (Kim *et al*., 2007; Kazeto *et al*., 2008; Kobayashi *et al*., 2010).

In eel, rec-FSH and rec-LH produced in CHO-K1 cells provide more effective results than conventional salmon pituitary extract or hCG injections for inducing oogenesis and spermatogenesis (Kazeto *et al*., 2014). A large quantity of high-quality milt was transported at 18～24 h after injection from the testes to the sperm duct for storage following treatment with rec-eelLH (500 mg/kg BW), and administration of rLH as the final shot for males should be given at 6 h before maturation-inducing steroid injection to females to effectively induce spontaneous spawning (Ohta *et al*., 2017).

Deglycosylation performed using PNase F drastically reduced the protein molecular weight to 7-9 kDa in rec-eelFSHβ/α and rec-eelLHβ/α (Kim *et al*., 2016a). We recently reported that specific monoclonal antibodies for rec-eelFSHβ/α were produced and we developed an emzyme-linked immunosorbent assay system to analyze rec-eelFSHβ/α and rec-eelLHβ/α (Kim *et al*., 2016a). rec-eel proteins produced from CHO cells were measured to determine the percentage of germinal vesicle breakdown *in vitro* (Kim *et al*., 2016b). We also reported that glycoprotein hormones (equine CG, eFSH, hFSH, hEPO, and hTPO) in CHO-K1 cells were produced and found that these hormones exhibited bio-

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logical activity both *in vitro* and *in vivo* (Jeoung *et al.*, 2010; Lee *et al.*, 2017; Min *et al.*, 2004; Park *et al.*, 2009, 2017).

In the present study, we constructed a mammalian expression vector containing eelFSHR. The expression vectors were transfected into CHO-K1 cells and produced rec-eelFSHβ/α. We then assessed the signal transduction activity of equine FSHR (eFSHR) upon exposure to rec-eelFSHβ/α, natural porcine FSH, and natural human FSH.

MATERIALS AND METHODS

Materials

The expression vector, pcDNA3, was purchased from Invitrogen (Carlsbad, CA, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Endonucleases and polymerase chain reaction (PCR) reagents were from Takara (Shiga, Japan). Ham's F-12, CHO-S-SFM II, Geneticin, Lipofectamine 2000, and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, USA). Porcine FSH and hFSH were from Sigma-Aldrich Corp. (St. Louis, MO, USA). The QIAprep-Spin plasmid kit was purchased from QIAGEN, Inc. (Hilden, Germany). FreeStyle MAX reagent, FreeStyle CHO expression medium, pCMV-ARMS1-PK2 expression vector, and assay complete medium were purchased from Invitrogen, the Path Hunter CHO-K1 β-arrestin Parental cell line was obtained from DiscoveRx (San Diego, CA, USA) and disposable spinner flasks were from Corning Inc. (Corning, NY, USA). The cAMP Dynamic 2 immunoassay kit was from Cisbio Bioassay (Codolet, France).

The oligonucleotides were synthesized by Green Gene Bio (Seoul, Korea). Centriplus Centrifugal filter devices were purchased from Amicon Bio separations (EMD Millipore, Burlington, MA, USA). All other reagents used were from Sigma-Aldrich and Wako Pure Chemicals (Osaka, Japan).

Construction of rec-eelFSH

cDNA encoding the tethered eelFSHβ/α was inserted in the pcDNA3 mammalian expression vector as previously reported (Park *et al.*, 2016). The schematic diagrams of rec-eelFSHβ/α production are shown in Fig. 1. These fragments were digested with *Eco*RI 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-eelFSHβ/α).

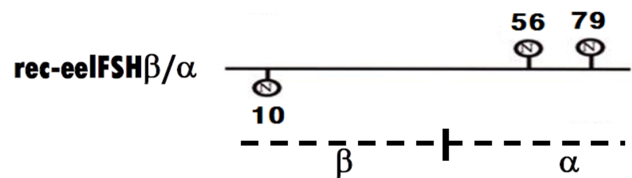

Fig. 1. Schematic diagram of rec-eelFSHβ/α. The eelFSHβ/α cDNA was ligated into the pcDNA3 mammalian expression vector. The glycosylation sites at Asn56 and Asn79 of eelFSHα-subunit was shown as number 56 and 79. The eelFSHβ-subunit has one N-linked glycosylation site at Asn10. N: N-linked oligosaccharide site.

Production and quantification of rec-eelFSHβ/α protein in CHO suspension cells

rec-eelFSHβ/α protein was expressed as described previously (Park *et al.*, 2016) by transfecting the expression vector into CHO-S cells using the FreeStyle MAX reagent transfection method according to the supplier's instructions. Briefly, CHO-S cells were cultured with FreeStyle CHO expression medium at a density of 1×10^7 cells/30 mL for 3 days. One day prior to transfection, the cells were passaged at $5\sim6\times10^5$ cells/mL in CHO expression medium in 125-mL disposable spinner flasks. On the day of transfection, the cell density was approximately $1.2\sim1.5\times10^6$ cells/mL. Next, the plasmid DNA of eelFSHβ/α (160 μg) was mixed gently in 1.2 mL of OptiPRO serum-free medium (SFM), and FreeStyle MAX reagent (160 μL) was also mixed gently in 1.2 mL of OptiPRO SFM. Both medium mixtures were incubated for 5 min at room temperature. The solutions were then mixed and the complex (2.4 mL) was added to each cell suspension flask. For the rec-protein assay, the culture medium was collected on day 7. Finally, the culture media were centrifuged at 100,000 x *g* rpm at 4℃ for 10 min to remove the cell debris.

The supernatant was collected and concentrated by freeze-drying in an Amicon stirred cell concentrator and stored at −20℃ until the assays were performed. rec-protein was analyzed by enzyme-linked immunosorbent assay as previously reported by our lab (Kim *et al.*, 2016).

Transient transfection of eFSHR in CHO-K1 cells and stable transfection of eFSHR in PathHunter CHO-K1 EA-Parental cells

The method for constructing cDNAs encoding eFSHR was previously reported and conducted with some modifications (Lee *et al.*, 2017). Transfection of CHO-K1 cells was performed using the liposome transfection method as previously described (Lee *et al.*, 2017).After combining diluted DNA with Lipofectamine reagent, the mixture was incubated for 20 min. CHO cells were grown to 80∼90% confluence in 6-well plates. CHO

cells were washed with Opti-MEM and the DNA-Lipofectamine complex was added to each well. Fresh growth medium was added at 24 h after transfection. The cells were then subjected to cAMP analysis at 48～72 h after transfection. PathHunter CHO-K1 EA-Parental cells were transiently and stably transfected according to the supplier's protocol. PathHunter CHO-K1 EA-Parental cells were cultured in AssayComplete CHO-K1 culture medium. For stable cell lines, transfected cells were seeded at 500 and 1,000 cells per 100-mm culture dish at 24～48 h after transfection. The cells were cultured in Assay Complete medium containing G418 for 2～3 weeks to select. Approximately 20 clones were recovered and cultured in a 24-well plate. Finally, 5 cell clone lines were isolated and stocked as previously reported (Lee *et al*., 2017).

Evaluation of cAMP level by homogenous time-resolved foster resonance energy transfer (HTRF)

cAMP accumulation in CHO cells and PathHunter CHO-K1 EA-Parental cells was measured using cAMP Dynamics 2 competitive immunoassay kits as described previously (Lee *et al*., 2017). The transfected eFSHR cells were plated at 10,000 cells per well into 384-well plates. Five microliters of compounds in medium buffer were added to each well; next, cAMP-d2 (5 μL) and anti-cAMP-cryptate (5 μL) were added to each well. The plate was read on a compatible HTRF reader. The results were calculated from the 665 nm/620 nm absorption ratio and expressed as Delta F % (cAMP inhibition). The concentrations of standard samples were 0.17～712 nM (final concentration of cAMP per well).

$$\text{Delta F\%} = (\text{Standard or sample ratio} - \text{Sample negative}) \times 100/\text{ratio negative}$$

The cAMP concentrations for Delta F% value were calculated using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA).

Data analysis

Dose-response curves were fitted to a nonlinear regression variable slope equation using GraFit 5.0 (Erithacus Software Limited, Surrey, UK) and GraphPad Prism 6.0 (GraphPad, Inc.). Curves fitted in a single experiment were normalized to the background signals measured in mock-transfected cells (0%). The sum of each curve was calculated from at least three independent experiments.

RESULTS AND DISCUSSION

The effect on cAMP stimulation in CHO-K1 cell lines expressing eFSHR genes was determined to evaluate the activity of rec-eelFSHβ/α, natural porcine FSH, and natural human FSH. Receptor-expressing cells were incubated with various concentrations (0.085～1,500 ng/mL) of ligands. As shown in Fig. 2, Delta F% for rec-eelFSHβ/α (B) and natural porcine FSH (B) gradually decreased in a dose-dependent manner as compared to the standard curve (0.17～712 nM) (A). Here, cAMP production, expressed as Delta F%, was inhibited by activation of transfected eFSHR. However, no response to human FSH treatment was observed (B). Next, these data were calculated by cAMP concentration (nM), as shown in Fig. 3. The cAMP concentration increased in proportion to the rec-eelFSHβ/α concentration. The cAMP level was very low in the porcine FSH treatment group. The EC_{50} value of rec-eelFSHβ/α was 53.35 ng/mL (Table 1). Porcine FSH did not show a very low EC_{50} value in Rmax. Thus, the data suggest that rec-eelFSHβ/α and natural porcine FSH bound to eFSHR and stimulated the receptors. However, porcine FSH was stimulate data low level. The curve was also flat, as shown in Fig. 2B. This suggests that eFSHR does not bind to human FSH. Thus, there were no changes in the cAMP concentration in eFSHR-expressing cells in response to human FSH binding. Therefore, porcine FSH and human FSH are not suitable for promoting equine ovulation and maturation.

The results of the present study showed that rec-eelFSHβ/α induced signal transduction by eFSHR in CHO-K1/PathHunter CHO-K1EA-Parental cell lines expressing receptors in a time- and concentration-dependent manner. However, natural porcine FSH showed little response. Human FSH did not exhibit any response in eFSHR cells.

We previously reported that rec-eCG was efficiently secreted and has a biological activity (Min *et al*., 2004; Park *et al*., 2009, 2010; Jeoung *et al*., 2010). rec-eCGβ/α was shown to activate both rFSHR and rLH/CGR in non- equids, but only LH-like activity was observed in equid species (Park *et al*., 2009; 2010). However, eCG does not bind to FSH receptors in equine follicles (Murphy & Martinuk, 1991; Guillou & Combarnous, 1983) or testis (Moore & Ward, 1980), suggesting that eCG is primarily an LH-like hormone in horses. rec-eCG produced from mammalian cells may be useful for improving the development of dominant and pre-ovulation follicles in non-equids. We also reported that rec-eel FSHβ/α has full bioactivity in eel FSHR (Kim *et al*., 2016a,b).

Based on the data presented, natural porcine FSH and natural human FSH did not increase cAMP responsiveness in cells expressing eFSHR. Thus, we suggest that the activity of FSH differs between mammals and fish. However, it greatly increased the bioactivity between eFSHR and rec-eelFSHβ/α. Thus, equine FSHR and

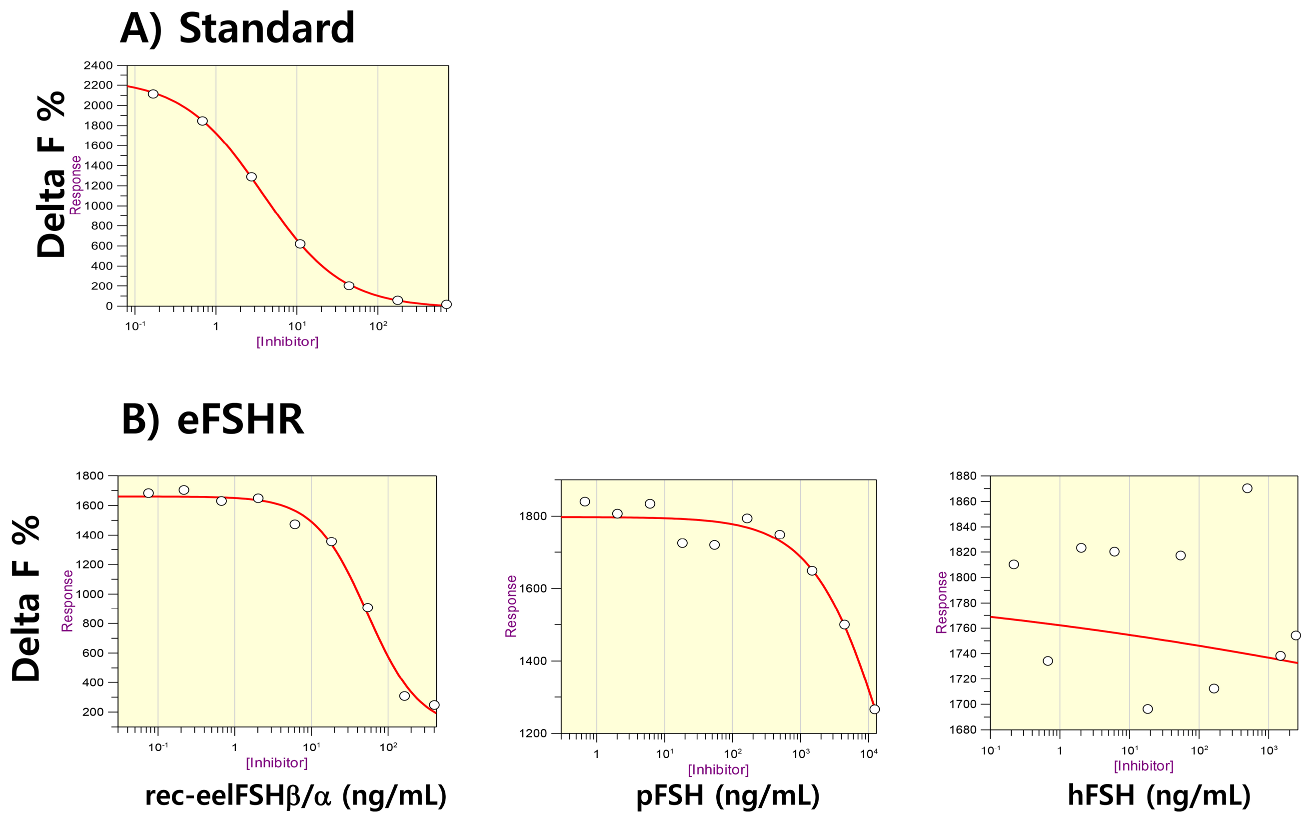

Fig. 2. Dose-dependent inhibition and increase in cAMP accumulation induced by rec-eelFSHβ/α, natural porcine FSH, and natural human FSH in cells transiently and stably expressing eFSHR. CHO-K1 and CHO-K1 EA Parental cells were transfected with eFSHR. The transfection method is described in the Materials and Methods section. The transfected culture media were replaced with new CHO growth medium 24 h after transfection. Cells were subjected to cAMP analysis at 48~72 h after transfection. The standard samples were prepared in concentration ranges of 0.17~712 nM. The plate was incubated for 30 min after adding rec-eelFSHβ/α (0~1,500 ng/mL). cAMP d2 and anti cAMP-cryptate were added and incubated at RT for 1 h. Inhibition of cAMP accumulation was shown by Delta F%. A) Standard curve, B) Delta F% value was shown by inhibition of cAMP responsiveness (GraFit).

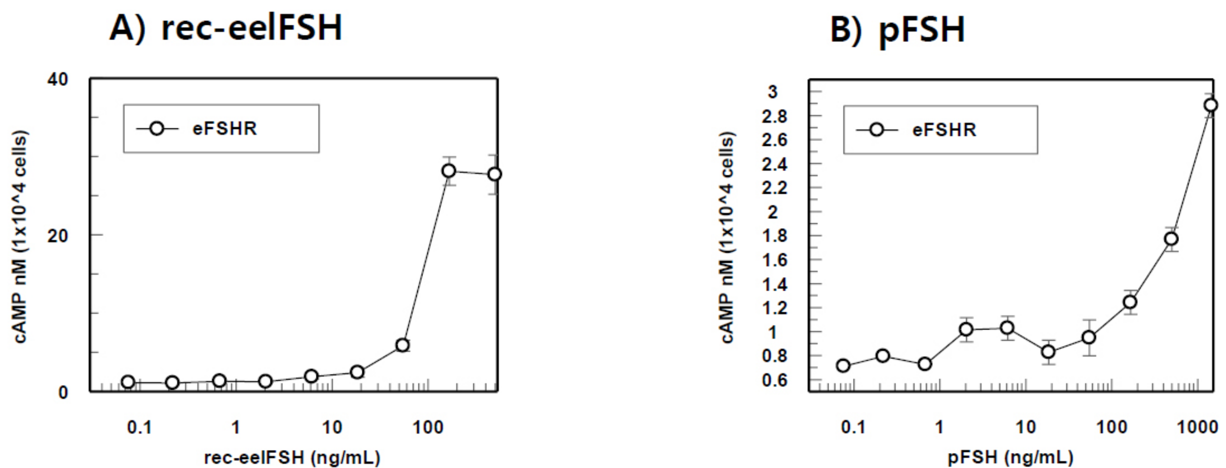

Fig. 3. Dose-dependent increase in cAMP accumulation induced by rec-eelFSHβ/α and natural porcineFSH in stably cells expressing eFSHR. PathHunter CHO-K1 EA Parental cells were transfected with eFSHR. The stably expressing cells were seeded at 10,000 cells per well into 384-well plates. The cAMP concentration was calculated by GraphPad Prism software.

Table 1. Bioactivity of rec-eelFSHβ/α, porcine FSH, and human FSH in CHO-K1 cells expressing equine FSHR

Ligands	cAMP responses		
	Basal ($nM/10^4$ cells)	EC_{50} (ng/mL)	Rmax ($nM/10^4$ cells)
rec-eelFSHβ/α	1.140±0.08	53.35±10.3	28.12±2.74
Porcine FSH	0.711±0.04	-	2.88±0.31
Human FSH	-	-	-

Values are the means of triplicate experiments. The EC_{50} values used to determine the potency were obtained from the concentration-response curves for the *in vitro* bioassays.

eelFSH may function as switches to induce ovulation and maturation. Although eCG did not elicit any activity in cells expressing eelFSHR, eCG increased the biological activity in cells expressing eelLH/CGR (in preparation). In the present study, rec-eelFSH hormone bound to equineFSHR, and rec-eFSHβ/α may have bound to eelFSHR.

Therefore, rec-eelFSH stimulates cells expressing eFSHR. The FSH- and LH-activity of these hormones is slightly different between mammalian and fish species.

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