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Original Article

Signal transduction of C-terminal phosphorylation sites for equine follicle stimulating hormone receptor (eFSHR)

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ABSTRACT Equine follicle stimulating hormone receptor (eFSHR) has a large extracellular domain and an intracellular domain containing approximately 10 phosphorylation sites within the G protein-coupled receptor. This study was conducted to analyze the function of phosphorylation sties at the eFSHR C-terminal region. We constructed a mutant of eFSHR, in which the C-terminal cytoplasmic tail was truncated at residue 641 (eFSHR-t641). This removed 10 potential phosphorylation sites from the C-terminal region of the intracellular loop. The eFSHR-wild type (eFSHR-wt) and eFSHR-t641 cDNAs were subcloned into the pCMV-ARMS1-PK2 expression vector. These plasmids were transfected into PathHunter CHO-K1 Parental cells expressing β-arrestin 2 enzyme acceptor fusion protein and analyzed for agonist-induced cAMP response. The cAMP response in cells expressing eFSHR-t641 was lower than the response in cells expressing eFSHR-wt. EC_{50} values of eFSHR-wt and eFSHR-t641 were 1079 ng/mL and 1834 ng/mL, respectively. eFSHR-t641 was approximately 0.58-fold compared with that of eFSHR-wt. The maximal response in eFSHR-wt and eFSHR-t641 was 24.7 nM and 16.7 nM, respectively. The Rmax value of phosphorylation sites in eFSHR-t641 was also decreased to approximately 68.4% of that in eFSHR-wt. The collective data implicate that the phosphorylation sites in the eFSHR C-terminal region have a pivotal role in signal transduction in PathHunter CHO-K1 cells, and indicate that β-arrestin is involved in coupling the activated receptors to the internalization system.

Keywords: eFSHR, PathHunter CHO-K1 Parental cells, phosphorylation sites, signal transduction

INTRODUCTION

The family of glycoprotein hormone receptors including follicle stimulating hormone receptor (FSHR) and lutropin chorionic gonadotropin hormone receptor (LH/CGR) belongs to the superfamily of G protein-coupled receptors (GPCR) that couple extracellular agonists to intracellular effector molecules through the reactions of heterotrimeric G proteins and arrestins (Schlador and Nathanson, 1997; Pierce et al., 2002; Wan et al., 2018). These heterotrimeric G proteins are broadly divided into four families (Gs, Gi/o, Gq/11, and G12/13) based on their structural and functional similarities (Martemyanov and Garcia-Marcos, 2018). Dysfunction of GPCR contributes to some

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of the most prevalent human diseases that are treated using 34% of all drugs approved by the Food and Drug Administration (Wu et al., 2019).

GPCR internalization is one of the specific processes triggered by agonist stimulation, and follows desensitization of the signaling function of receptors (Min et al., 1998). Protein phosphorylation sites in various signaling pathway are very important in regulating cell proliferation. The C-terminal region of eFSHR harbors 10 potential phosphorylation sites on serine and threonine residues. In the rat FSHR (rFSHR), the impaired agonist-stimulated signal transduction in the phosphorylation mutants D389N and Y530P can be rescued by overexpression of G protein-coupled receptor kinase 2 (arrestin 3). However, this increased phosphorylation only rescues the internalization in the D389N mutant. Agonist-induced activation and phosphorylation of rFSHR are not essential for internalization (Nakamura et al., 1998). However, the rFSHR-t635 mutant, which is truncated at residue 635 and contains only one of potential phosphorylation sites in the C-terminal tail, responds with increases in the accumulation of cAMP and inositol phosphate (Hipkin et al., 1995). The rat LHR (rLHR)-5S/T→A mutant, in which five phosphorylation sites in the C-terminal region are mutated to alanine, is drastically reduced in the accumulation of cAMP induced by human chorionic gonadotropin (hCG), and the rate of internalization is slower than that in cells expressing the wild type rLHR (rLHR-wt) (Wang et al., 1997). Residues 632-653 (rLHR-t628 and rLHR-t631) in the C-terminal tail of rLHR are involved in phorbol-12-myristate-13-acetate (PMA)- and hCG-induced desensitization and hCG-induced down-regulation (Sánchez-Yagüe et al., 1992; Wang et al., 1996). The collective prior evidence indicates that the C-terminal cytoplasmic tail of rLHR is important in preventing phosphorylation and retarding or preventing the hCG- or PMA-induced uncoupling. However, rFSHR mutant is not impeded in phosphorylation or uncoupling (Ascoli, 1996).

In human FSHR (hFSHR), most of the hFSHR complex that is internalized by hFSH is recycled back to the plasma membrane. Truncation of the hFSHR-t678 re-routes a substantial portion of the internalized complex to lysosomal degradation in KK-1 mouse granulosa cells (Krishnamurthy et al., 2003). The more pronounced loss of receptors observed in KK-1 cells expressing hFSHR-t678 was accompanied by a more pronounced loss of cAMP responsiveness (Bhaskaran and Ascoli, 2005). The loss of surface receptor was approximately 50% and approximately 75% in cells expressing hLHR-wt and hLHR-t682 (Bhaskaran and Ascoli, 2005). The majority of the hLHR-t682 complex with hCG is instead routed to a lysosomal degradation pathway (Kishi et al., 2001; Hirakawa et al., 2003; Galet et al., 2004). Recently, we also reported that the half maximal response concentration (EC_{50}) value of eel FSHR-t614, in which all the potential phosphorylation sites in the C-terminal cytoplasmic tail were deleted, was approximately 60.4% that of eelFSHR-wt in CHO-K1 cells. The maximal response of eelFSHR-t614 was also drastically lower than that of eelFSHR-wt (Kim et al., 2018). We also reported that the constitutively activated eelFSHR-D540G mutant displayed remarkably increased response (13.1 to 19.6-fold) when compared to the wild type receptor (Kim et al., 2019), suggesting that cAMP production was greatly increased in the absence of eelFSH stimulation. The findings indicate that these cells are involved in the signal transduction of glycoprotein hormone receptors. The C-terminal region of hFSHR and eFSHR has a potential 10 phosphorylation sites and highly conserved leucine motif, which is important for trafficking of the mature receptor from ER to the cell surface (Duvernay et al., 2004). The C-tail of FSHR plays an indispensable role in palmitoylation, receptor phosphorylation, interaction with beta arrestin proteins, and internalization of FSH-FSHR complex (Banerjee and Mahale, 2015). Thus, we suggest that C-terminal region in eFSHR needs to be determined the function of signal transduction.

To characterize the signal transduction through eFSHR and to assess the specificity of the β-arrestin function, we examined the effects of both eFSHR-wt and eFSHR-t641 on the agonist-stimulated cAMP responsiveness. The results demonstrate that phosphorylation sites in the C-terminal region of eFSHR significantly affected the signal transduction through the G protein stimuli. eFSHR was also associated with a potential role for β-arrestin expression in PathHunter CHO-K1 cells.

MATERIALS AND METHODS

Materials

The oligonucleotides used in this study were synthesized by Genotech (Daejeon, Korea). The pCMV-ARMS1-PK2 mammalian expression vector, Freestyle MAX reagent,

antibiotics, purified hFSH, and AssayComplete medium were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The PathHunter CHO-K1 Parental cell line expressing β-arrestin 2 was purchased from DiscoveRx (San Diego, CA, USA). The restriction enzyme and DNA ligation reagent were purchased from Takara Bio (Shiga, Japan). Fetal bovine serum was from Hyclone Laboratories (Logan, UT, USA). The cAMP Dynamic 2 immunoassay kit was purchased from Cisbio (Codolet, France). All other reagents used were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA).

Construction of eFSHR-wt and eFSHR-t641 expression vectors

eFSHR cDNA was cloned using cDNA of equine testis and ovary, as previously reported (Park et al., 2009, 2010). The polymerase chain reaction (PCR) fragments were ligated into pcDNA3 mammalian expression vector. For the PathHunter CHO-K1 Parental cells, eFSHR cDNA digested using Nhe1 and Sac1 was cloned into the same enzyme sites as the pCMV-ARMS1-PK2 expression vector (designated pcAMV-ARMS1-PK2-eFSHR-wt) as previously described (Lee et al., 2017). As shown in Fig. 1, the truncated mutant in which the potential phosphorylation sites had been deleted, was constructed using PCR by deletion after amino acid residue 640 (designated pcAMV-ARMS1-PK2-eFSHR-t641). There is no stop codon in the C-terminal region of Sac1 enzyme site. The direction was confirmed through restriction mapping and verified by sequencing the entire open reading frame.

Transient transfection of PathHunter CHO-K1 Parental cells

PathHunter CHO-K1 Parental cells are engineered to stably express the enzyme acceptor-tagged β-arrestin fusion protein. These cells were transiently transfected according to the supplier's protocol as previously described (Lee et al., 2017; Kim et al., 2018). Transfections were performed using the liposome transfection method as previously described (Byambaragchaa et al., 2018). The transfected cells were adjusted for cAMP analysis 48-72 h after transfection.

cAMP assay via homogenous time-resolved foster resonance energy transfer (HTRF)

Measurement of AMP accumulation in PathHunter CHO-K1 Parental cells was performed using cAMP Dynamics 2 competitive immunoassay kits (Cisbio) as described previously (Byambaragchaa et al., 2018). The cAMP response assay uses a cryptate-conjugated anti-cAMP monoclonal antibody and d2-labeled cAMP reagent. The cell transfected with eFSHR-wt and eFSHR-t641 cDNA were added

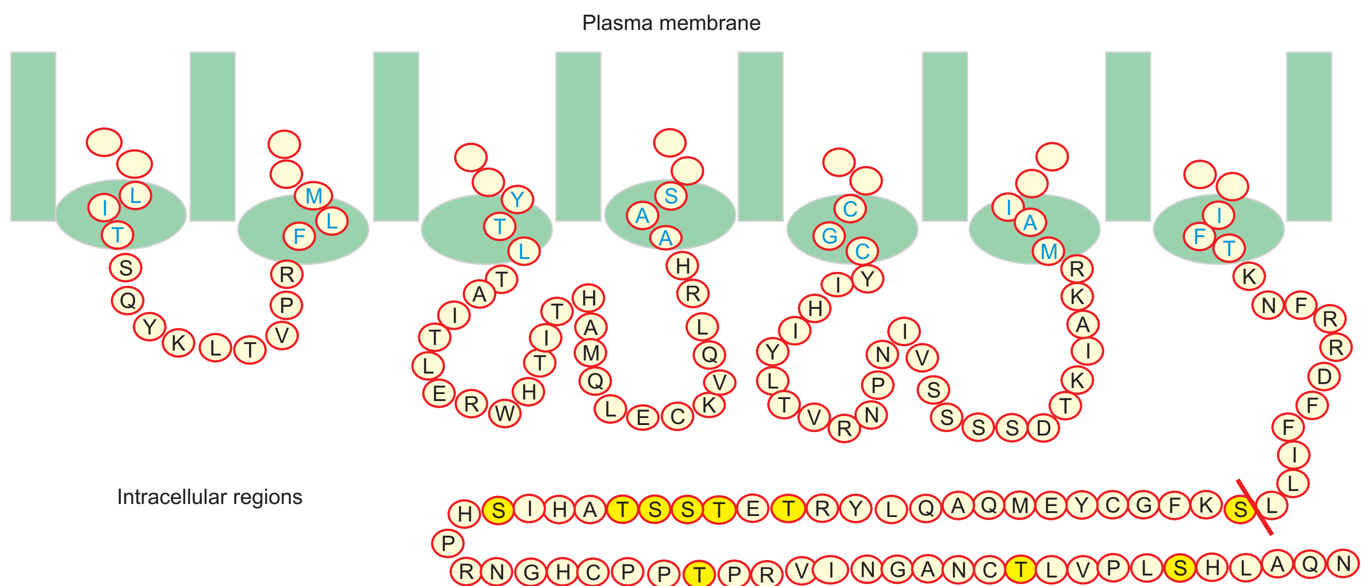

Fig. 1. Intracellular region of equine follicle stimulating hormone receptor (eFSHR). The amino acid sequence of the three cytoplasmic loops and the C-terminal cytoplasmic tail of eFSHR are shown. The 10 potential phosphorylation sites (serine and threonine residues) are S641, T655, T657, S658, S659, T660, S664, T674, T684 and S689. The truncation site (t641) is also shown. The amino acid sequence was amplified with equine ovary and testis cDNA from our laboratory and sequenced as described by Park et al. (2010).

to 384 wells, with 10,000 cells per well. MIX was added to cell dilution buffer in order to prevent cAMP degradation. The standard samples were prepared to cover an average range of 0.17-712 nM. The cells (1×10^4) were seeded in the 384 wells and 5 μL compound medium buffer was added to each well as described by the supplier. The plate was read using a compatible homogeneous-resolved fluorescence microplate reader. The results represented the 665 nM/620 nM ratio and were expressed as Delta F% (cAMP inhibition), which was calculated as:

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

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

Data analysis

Dose-response curves were fit with nonlinear regression and a variable slope equation using GraFit 5.0 (Erithacus Software Limited, Surrey, UK) and GraphPad Prism 6.0 (GraphPad Software, Inc). Curves fit in a single experiment were normalized to the background signaling measured for mock-transfected cells (0%). Each sum curve was calculated from three independent experiments performed in triplicate.

RESULTS

eFSHR-wt has a long C-terminal region containing approximately 10 phosphorylation sites. As shown in Fig. 1, the potential phosphorylation sites were deleted by PCR

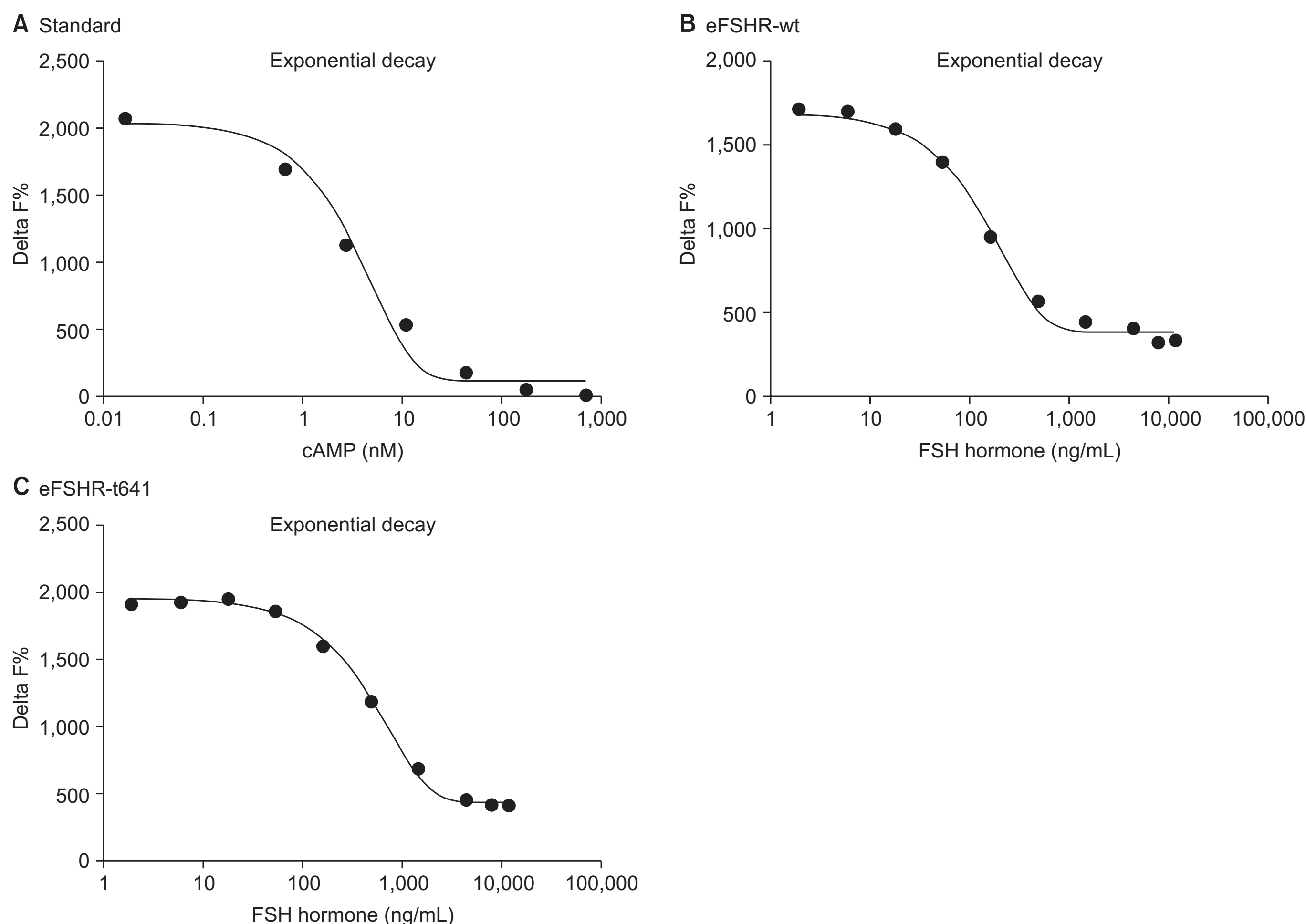

Fig. 2. Dose-dependent inhibition of cAMP accumulation by agonist-induced treatment (hFSH) in PathHunter CHO Parental cells expressing β-arrestin. Cells were transfected with eFSHR-wt and eFSHR-t641 cDNAs. The cells were adjusted for cAMP analysis 48-72 h after transfection (see Materials and Methods for details). (A) Standard curve. (B) Delta F% value of eFSHR-wt determined by inhibition. (C) Delta F% of eFSHR-t641. The standard samples were prepared to cover an average range of 0.17-712 nM. Data are expressed as the mean ratio relative to the vehicle control ± SEM.

to assess the function of phosphorylation in the signal transduction through second messenger cAMP responsiveness.

First, eFSHR-wt and eFSHR-t641 plasmids were transfected into PathHunter-EA CHO Parental cells that expressed β-arrestin. The cells were subjected to cAMP analysis following agonist-mediated induction. The standard curve was appropriately inhibited by concentration increase (0.17-712 nM).

The Delta F% in cells expressing the eFSHR-wt and eFSHR-t641 was also similar to the standard curve (Fig. 2A). The eFSHR-t641 plot was shift slightly to right compared to the eFSHR-wt plot. The Delta F% IC_{50} values of eFSHR-wt and eFSHR-t641 were 144 ± 11.8 ng/mL and 501 ± 38.6 ng/mL, respectively (Fig. 2B and 2C). The IC_{50} value of eFSHR-t641 was approximately 28.7% relative to eFSHR-wt. Thus C-terminal region deletion was drastically decreased in the cAMP response. Next, the Delta F% data was amended to correct for the cAMP level by dose-dependent concentration.

Based on the Delta F%, we calculated the data as cAMP concentration (nM/10^4 cells) as shown in Fig. 3. There was no difference in the basal cAMP accumulation between eFSHR-wt (0.8 ± 0.2 nM) and eFSHR-t641 (0.5 ± 0.2 nM). The EC_{50} value calculated by cAMP stimulation in cells transfected with eFSHR-wt and eFSHR-t641 was 1,079 ng/mL and 1,834 ng/mL, respectively (Table 1). The truncation of the C-terminal region was approximately 0.58-fold compared with the wild type receptor. The Rmax value of wild type receptor was 24.7 ± 4.0 nM, but eFSHR-t641 was decreased to 16.9 ± 0.3 nM by the agonist treatment (8,000 ng/mL). Thus, the maximal response in cells expressing eFSHR-t641 was remarkably lower than that in cells expressing eFSHR-wt. The data suggested that the phosphorylation sites in the eFSHR C-terminal region play a pivotal role in signal transduction, internalization, and recycling.

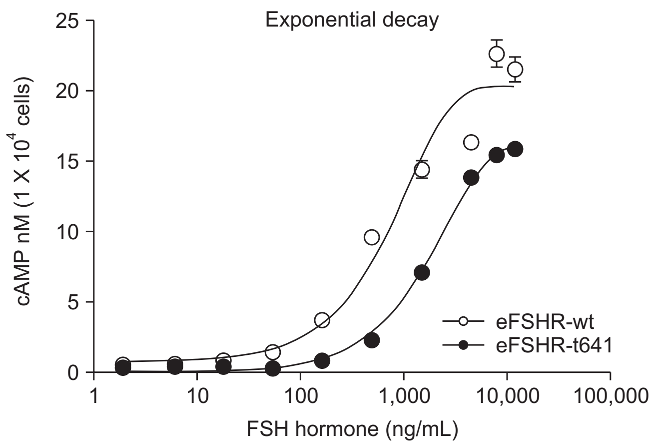

Fig. 3. Dose-dependent increase in cAMP accumulation of eFSHR-wt and eFSHR-t641 induced by agonist treatment. The cells (10,000 cells per well) were dispensed in 384 well in a plate. The plate was incubated for 30 min at room temperature the addition of follicle stimulating hormone (0 to 12,000 ng/mL). cAMP d2 and anti-cAMP-cryptate were added and incubated at room temperature for 1 h. Inhibition of cAMP accumulation was expressed as Delta F%. The cAMP concentration for Delta F% value was calculated using GraphPad Prism software. The cAMP value in nM from 1 × 10^4 cells value is shown.

DISCUSSION

GPCR signal by coupling to G proteins and arrestins, which alternatively activate beta-adrenergic-receptor kinase (βARK) and G-protein-coupled kinase (GRK). Second messenger kinases phosphorylate the serine and/or threonine residues in the intracellular region of GPCR in the agonist-induced treatment. The present study analyzed the roles of the phosphorylation sites and their responsibility for the uncoupling of eFSHR from cAMP accumulation in cells expressing eFSHR-wt and eFSHR-t641. The EC_{50} value in the truncated potential phosphorylation sites of the C-terminal cytoplasmic tail was approximately 58% of that in the eFSHR-wt. The maximal response was also considerably decreased similar to the EC_{50} value by the C-terminal deletion in eFSHR.

In glycoprotein hormone receptors, rFSHRs mutated in

Table 1. Bioactivity of eFSH receptors in cells expressing eFSHR-wt and eFSHR-t641

eFSH receptors	cAMP responses		
	Basal[a] (nM/10^4 cells)	EC_{50}[b] (ng/mL)	Rmax[c] (nM/10^4 cells)
eFSHR-wt	0.8 ± 0.2	1079 (1.0-fold) (301 to 3863)[d]	24.7 ± 4.0 (1-fold) (14.8 to 34.6)[d]
eFSHR-t641	0.5 ± 0.2	1834 (0.58-fold) (1680 to 2001)[d]	16.9 ± 0.3 (0.68-fold) (16.3 to 17.6)[d]

Values are the mean ± SEM of triplicate experiments. EC_{50} values were determined from the concentration-response curves from *in vitro* bioassays.

[a]Basal cAMP level is the average in the absence of agonist.

[b]EC_{50} of best-fit values (95% confidence intervals).

[c]Rmax average cAMP level/10^4 cells.

[d]95% Confidence interval.

the additional phosphorylation sites on the second and third intracellular loops were reportedly not essential for internalization, while the interaction with a nonvisual arrestin was essential for internalization (Nakamura et al., 1998). Another study described that FSHR was phosphorylated by the addition of hFSH and PMA, while a calcium ionophore (A23187), 8-bromoadenosine 3′, 5′-cyclic adenosine monophosphate, and prostaglandin E_2 resulted in little or no increase in the phosphorylation of the FSHR in ^{32}P-labeled cells (Quintana et al., 1994). Our results in cells expressing the β-arrestin 2 kinase gene are consistent with prior results indicating that the phosphorylation sites are essential in the signal transduction through G proteins.

We observed that the rFSHR-t635 mutant, in which the phosphorylation sites in the C-terminal cytoplasmic tail had been deleted, was highly phosphorylated by hFSH and PMA. Cells in expressing rFSHR-t635 also effectively uncouple the truncated receptor from the cAMP response (Hipkin et al., 1995). Thus, the role of the phosphorylation sites in response to PMA and FSH are not identical to FSH stimulation (Quintana et al., 1994; Hipkin et al., 1995). However, not all the potential phosphorylation sites were deleted in FSHR-t653. One potential site remained in the intracellular C-terminal region. The hFSHR-t678 mutant, which was truncated by 678 residues of the C-terminal tail, displayed a greater degree of down-regulation of cell surface receptors compared with hFSHR-wt expressing human kidney 293 cells (HEK-293) (Krishnamurthy et al., 2003).

Increasing hFSH concentration produced approximately 40% loss of cell surface receptors in hFSHR-wt transfected KK1 mouse granulosa cells, whereas hFSHR-t678 transfected KK-1 cells experienced approximately a 60% loss (Bhaskaran and Ascoli, 2005). The researchers did not measure the estradiol levels in KK-1 cells transfected with hFSHR-wt. These results are not consistent with our results in PathHunter-EA CHO Parental cells expressing β-arrestin 2 kinase. Thus, we suggest that eFSHR in phosphorylation sites of the C-terminal cytoplasmic tail are necessary for signal transduction in cells expressing β-arrestin.

In an rLHR mutant, the change of serine and threonine to alanine in the C-terminal region considerably reduced the agonist-induced cAMP responsiveness (Wang et al., 1997). The cell lines expressing rLHR-t631 or rLHR-t628, in which all the potential phosphorylation sites in the intracellular domain of the C-terminal cytoplasmic tail were deleted, showed a delay in the early phase of agonist-induced desensitization, complete loss of PMA-induced desensitization, and increased rate of agonist-induced receptor down-regulation (Wang et al., 1996). The deletion of 11 serine/threonine phosphorylation sites in cells expressing rLHR-t631 impaired the receptor phosphorylation by hCG- and PMA-increased treatment. However, the cAMP response of cells expressing the truncated receptors to Chorela toxin was also slightly elevated and the Rmax level was 1.5-fold higher than that of cells expressing the wild type receptor (Hipkin et al., 1995). The findings suggest that LHR-t631 is not phosphorylated in response to hCG and PMA and does not arise from insufficient hormone binding or from diminished second messenger generation upon the binding of hCG to this truncated receptor.

Recently, we also reported that eelFSHR-t635, in which all potential phosphorylation sites in the intracellular cytoplasmic tail are deleted, displayed and EC_{50} approximately 60.4% that of eelFSHR-wt (Kim et al., 2018). The truncation of the cytoplasmic tail of the β2-adrenergic receptor was delayed at the onset of agonist-induced uncoupling (Bouvier et al., 1988). Although the rFSHR-truncated mutant does not affect phosphorylation or uncoupling, our results obtained with eFSHR-t641 were considerably affected concerning cAMP responsiveness using cells expressing eFSHR-t641 with β-arrestin.

In conclusion, eFSHR-wt in PathHuner Parental cells expressing β-arrestin responded to increased cAMP activity in a dose-dependent manner. The cAMP-mediated receptor was greatly reduced in the FSHR-t641mutant that lacked the phosphorylation sites in the cytoplasmic tail. Thus, we suggest that the C-terminal phosphorylation sites in eFSHR are necessary in cells expressing β-arrestin. Further studies are required to elucidate the functional mechanisms that regulate the internalization, loss of cell surface receptor, and recycling in these cells.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

HKS and MB conducted and designed all experiments; SHC rearranged the data; KSM analyzed, interpreted the data and wrote the paper. All authors contributed to its critical review and agreed on the final version.

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