Production of Biological Active Single Chain Bovine LH and FSH

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ABSTRACT : Luteinizing hormone as other glycoprotein hormones is characterized by a heterodimeric structure composed a common α-subunit noncovalently linked to a specific β-subunit. The correct conformation of the heterodimer is important for efficient secretion, hormonal-specific post-translational modifications, receptor binding and signal transduction. To determine whether α- and β-subunits can be synthesized as a single polypeptide chain (tethered-bLH and -bFSH) and also display biological activities, the tethered-bLH and -bFSH molecules were constructed and transfected into chinese hamster ovary (CHO-K1) cells. LH and FSH activities were assayed by using the human embryonic kidney (HEK) 293 cells expressing rat LH and FSH receptor genes. The tethered-bLH and -bFSH proteins were efficiently secreted and showed a similar activity to the dimeric bovine LH and FSH α/β wild type and native purified from bovine pituitary. The tethered-molecules can be permit development of potent new analogues that stimulate ovarian development. Taken together, a single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds. These data indicate the potentiality of the single chain approach to further investigate structure-function relationships of LH and FSH. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 4 : 498-503)***

Key Words : Tethered-bLH and -FSH, Biological Activity

INTRODUCTION

Luteinizing hormone (LH) belongs to the glycoprotein hormone family, which includes follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) of the pituitary and chorionic gonadotropin (CG) of the placenta. This hormone family is characterized by a heterodimeric structure composed a common α-subunit noncovalently linked to a hormone specific β-subunit (Pierce and Parsons, 1981). LH is synthesized in and secreted from the anterior pituitary, and plays important roles in the regulation of gonadal functions. In women, LH promotes ovulation and luteinization of the ovarian follicle and enhances steroid production in the ovaries (Suganuma et al., 1996). Purified FSH administrated alone or in combination with semipurified human menopausal gonadotropins containing a mixture of FSH and LH has been used to stimulate the development of ovarian follicles for *in vitro* fertilization (Keene et al., 1989).

There have two N-glycosylation sites (Asn 56 and Asn 82) on the α-subunits of all these glycoproteins, whereas the numbers of such sites on their β-subunits differ depending on the hormone: LH β- and FSH β-subunits bear one and two sites, respectively, there are two on hCG, whereas eCG has only one (Crawford et al., 1986; Min, 2000a,b). Analysis of a purified preparation of eCG revealed that its β-subunit consists of 149 amino acids (Sugino et al., 1987) which was confirmed by the molecular cloning of its cDNA described previously (Min et al., 1994, 1996). There seem to be at least four to six, or even as many as 11, O-glycosylation sites on the extended C-terminal region of the eCG β-subunit (Min et al., 1996, 1997; Bousfield et al., 1992). In that point, the role of oligosaccharides in the function of glycoprotein hormone is a pivotal roles. These oligosaccharides of hFSH, eCG and eFSH are required for efficient signal transduction (Sairam et al., 1985; Calvo et al., 1986; Min 2001; Saneyoshi et al., 2001). We also have studied the signal transduction and function/roles of glycoprotein hormone receptor to analyze the gonadotropin biological activity (Min et al., 1998; Min, 1999, 2000c).

To determine bovine α-and β-subunits can be synthesized as a single polypeptide chain (tethered-bLH and FSH) and also display biological activity, the tethered-molecules by fusing the amino terminus of the α-subunit to the carboxyl terminus of the each β-subunit were constructed and transfected into chinese hamster ovary (CHO-K1) cells. These results indicate that the tethered-molecules were secreted efficiently, and that is similar to wild type glycoprotein hormones in the LH-and FSH-like activities.

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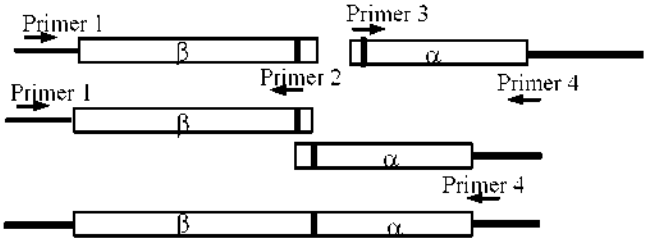

Figure 1. Construction of tethered-bLH and -bFSH by overlapping PCR mutagenesis.
PCR was carried out the tethered-genes as described in *Materials and Methods*.

MATERIALS AND METHODS

Materials

The expression vector pcDNA3 was purchased from Invitrogen (Groningen, Netherlands). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The bLH (H055/H) and bFSH (H058/H) for radioiodination, anti-bLH (A555/R1H) and -bFSH (A558/P1H) polyclonals, anti-rabbit precipitating reagent (A223/SH) for bLH and anti-guinea-pig IgG for bFSH were purchased from Biogenesis Ltd (England, UK). Radioisotope ^{125}I was obtained from ICN Pharmaceutics, Inc. (California, USA). Endonucleases were purchased frm Takara Shuzo (Kyoto, Japan). Polymerase reagents were from Stratagene (La Jolla, CA). Trizol reagent, the Superscript preamplification system, Ham's F-12 and lipofectamine were from Gibco BRL (Gaithersburg, MD). All the other reagents used were from Wako Pure Chemicals (Osaka, Japan) unless otherwise noted.

Isolation of RNA and cDNA synthesis

Bovine pituitary tissue was obtained by operation from Korean Native cows (3 years old). The tissue was kept at -80°C until RNA extraction. Total RNA was extracted according to the method previously described (Min et al., 1996). First-strand cDNA was synthesized by using the Superscript preamplification system according to the manufacture's instructions.

Construction of the tethered-bLH and -bFSH

Expression vectors for the wild type bLH and bFSH were constructed according to the method previously described (Min et al., 1996; Min, 2001). The following primers were used in the construction of the tethered-bLH:

Primer-1, (M13 RV), 5'-CAGGAAACAGCTATGAC-3':
Primer-2, 5'-TCCATCAGGAAAGAGGAAGAGGATGTC-3':
Primer-3, 5'-ATCCTCTTCCTCTTTCCTGATGGAGAG-3':
Primer-4, (M13 M4), 5'-GTTTTCCCAGTCACGAC-3'.

tethered-bFSH:

Primer-1 and primer 2 is same as bLH:
Primer-2, 5'-TCCATCAGGAAATTCTTTGATTTCCCT-3':
Primer-3, 5'-GAAATCAAAGAATTTCCTGATGGAGAG-3':

Primer 2 contains the first four codons of the mature α-subunit and the five codons of the LH and FSH β-subunits carboxyl-terminal codons. Primer 3 also contains the sequence corresponding to the last four carboxyl-terminal codons of the LH and FSH β-subunits and the first five codons of the α-subunit form which signal sequence was removed.

The first PCR for tethered-bLH and -bFSH was carried out by using primer sets (1 and 2), (3 and 4), respectively. The fragments were annealed and subjected to the second PCR using primers 1 and 4 to generate the tethered-bLH (731 bp) and -bFSH (695 bp) as shown in figure 1. This fragment was digested by Kpn I/Xba I and ligated into the same sites of pUC119 (pUC-bLH and -bFSH) and was sequenced to ensure no errors were introduced during the PCR. Following Kpn I/Xba I digestion, the fragment encoding tethered-bLH and -bFSH was ligated into the same sites of the pcDNA3 expression vector. The direction of expression transfer vectors (pcDNA3-bLHβ/α and pcDNA3-bFSHβ/α) was confirmed by restriction mapping.

Cell culture and transfection

The expression vectors (pcDNA3-bLHβ/α and pcDNA3-bFSHβ/α) were transfected into CHO-K1 cells by the liposome formulation (Lipofectamine) transfection method according to the supplier's instruction. Stable cell transfectants were selected by incubation in growth medium [Ham's F12 media containing penicillin (50 U/ml), streptomycin (50 mg/ml), glutamine (2 mM) and 10% FCS] supplemented with G418 (800 ug/ml) for 2 weeks post-transfection according to the method reported previously (Min, 2001). After incubation of selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37°C for 48 h, the culture media were collected and centrifuged at 100,000×g, 4°C for 60 min to remove the cell debris. The amount of recombinant wild type and tethered-bLH and -bFSH was quantified by RIA (Biogenesis Ltd).

Iodination and chromatography of ^{125}I-bLH and -bFSH proteins

Chloramine-T method (Hunter and Greenwood, 1962) was adopted to prepare ^{125}I bLH and bFSH trace. The separation of iodinated bLH and bFSH from free iodine was carried out on Sephadex G25 colum (Pharmacia). The reaction volume was applied on the colum and fractions (0.5 ml) were collected in tubes containing 1% BSA-PBS. All the iodinated fractions (5 ul) were counted in Gamma Counter. Elution pattern on Sephadex G25 has been shown in Figure 2. The first peak was the iodinated bLH and bFSH. The second peak represented free ^{125}I (Singh and Madan, 1998). The first peak fraction was divided in aliquots and

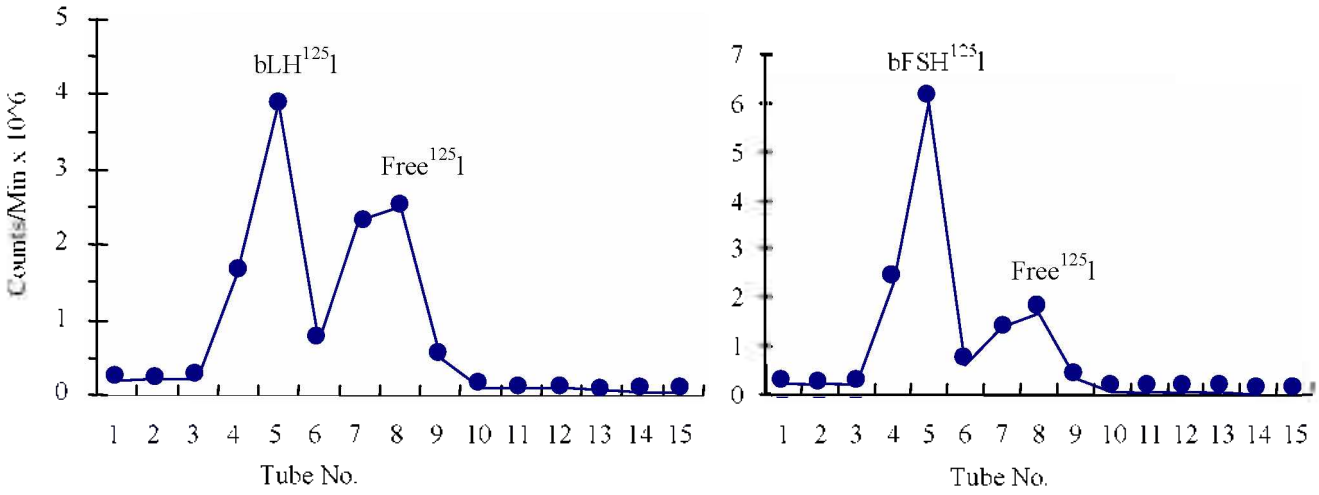

Figure 2. Elution diagram of reaction mixture for bovine LH and FSH iodination (^{125}I).

stored at -20°C. RIA determined the concentrations, expressed as native bLH and bFSH equivalents, of tethered recombinant bLH and bFSH, in triplicate. The quantity of recombinant protein was estimated as equivalents of the native standard as previously described (Min et al., 1996; Min, 2001).

cAMP assay

Dose-response curves for the recombinant tethered-bLH and -bFSH induced increases in cAMP accumulation were obtained by measuring total cAMP levels in cells that had been incubated with at least five different concentrations of recombinant hormones for 30 min at 37°C in the presence of a phosphodiesterase inhibitor described previously (Min et al., 1998). The wells were then placed on ice, and the total cAMP in the cells and medium was extracted by adding 1 ml of 2 N perchloric acid containing 360 ug/ml theophylline. The samples were subjected to rapid cycle of freezing and thawing, and the debris was collected by centrifugation. The supernatants were neutralized and then used for cAMP measurement by RIA (Min et al., 1998).

RESULTS

Cloning of bovine α, LHβ and FSHβ genes

The bovine α, LH β and FSH β primers which previously reported were synthesized by DNA synthesizer (Corebio System, Seoul, Korea). The PCR products were analyzed by electrophoresis (Figure 3). The amplified products of α (380 bp), LH β (443 bp) and FSH β (407 bp) were ligated into the Kpn I and Xba I sites of pUC119. The tethered-bLH (731 bp) and -bFSH (695 bp) genes were constructed by using the method previously described (Min, 2001). This fragment was digested by Kpn I/Xba I and ligated into the same sites of pcDNA3. The plasmid DNAs were prepared from QIAprep-spin plasmid kit. The tethered-bLH and -bFSH genes were sequenced to ensure no errors were introduced during the PCR. DNA sequencing was performed by the dideoxy chain-termination method using AutoRead sequencing kit and A.L.F DNA sequencer (Pharmacia LKB, Uppsala, Sweden). Sequence data were analyzed with MacMollyTetra computer software (Soft Gene, Berlin, Germany).

Cell lines of stably expressing tethered-bLH and bFSH

The expression vectors were transfected into chinese hamster ovary (CHO-K1) cells by the liposome formulation (Lipofectamine) transfection method according to the supplier's instruction. Stably cell transfectants were selected for G418 (800 ug/ml) resistance. The clonal cell lines isolated with G418 were subjected to reverse transcription

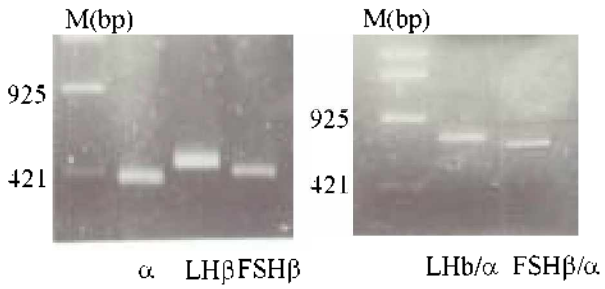

Figure 3. PCR amplification and construction of transfer vector. The PCR products were analyzed by agarose electrophoresis. The major fragments obtained, which comprised α(380 bp), LHβ(443 bp) and FSHβ(407 bp), were amplified (left). The transfer vectors of tethered-bLH (731 bp) and -bFSH (695 bp) genes were constructed into the *Kpn* I and *Xba* I sites of pcDNA3 and the fragments were digested by the same enzymes (right). M: λ-EcoT14I digest marker.

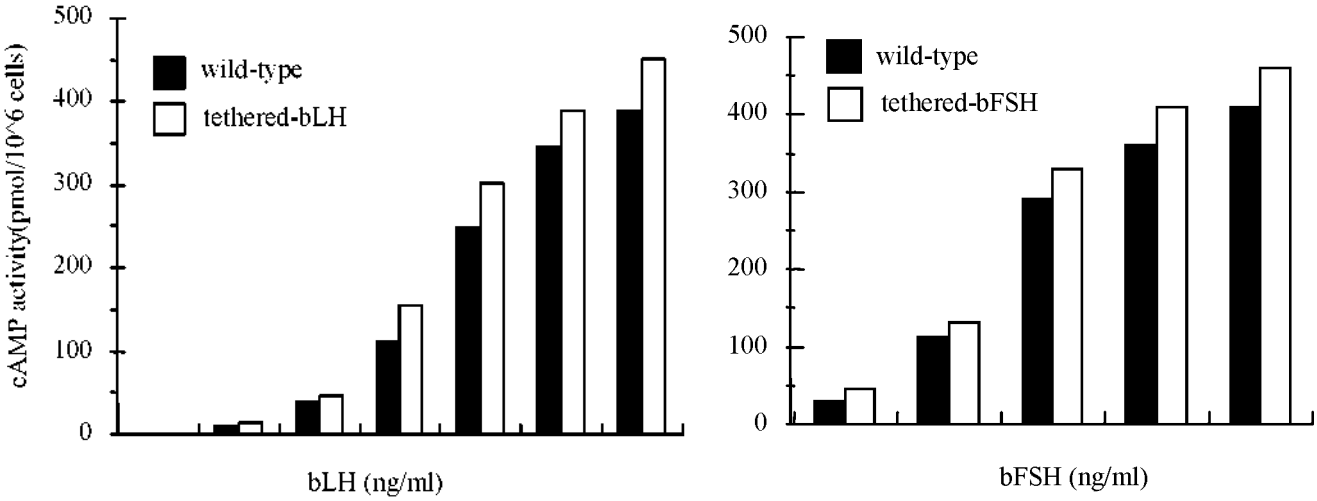

Figure 4. Tethered bLH and bFSH stimulated cAMP accumulation in stably transfected 293 cells expressing the wild type of rat LH and rFSH receptor genes.
Total levels of cAMP were determined in cells indicated in the presence of a phosphodiesterase inhibitor and increasing concentrations of the recombinant hormones for 30 min at 37°C as described in *Materials and Methods*.

polymerase chain reaction to select and establish those which stably expressed α- and β-subunit mRNAs as data previously described (data not shown). Recombinant bLH and bFSH protein secreted into the medium were collected and quantified by RIA. These competitive curves were directly proportional to each other, showing that there were no quantitative differences in the recognition of native, wild type and tethered-bLH and -bFSH by the antibody as the method previously described (Min, 2001).

Biological activities of recombinant bLH and bFSH

The effects of the recombinant protein were determined to evaluate the cAMP secretion in LH and FSH receptor cells. The bLH and bFSH wild type and the single chains were examined for their ability to stimulate cAMP from rat LH and FSH receptor cells. The cAMP concentrations resulted in similar dose-dependent increase in cAMP formation (Figure 4). The recombinant bLH and bFSH produced by the CHO-K1 cells showed LH- and FSH-like activities *in vitro* bioassays system. Taken together, these data show that both LH and FSH single chains exhibit signal transducing response to the corresponding heterodimer.

DISCUSSION

We have been investigated the roles of oligosaccharides on glycoprotein hormones in the culture system of rat Leydig cells and granulose cells by using recombinant eCG (Min et al., 1996, 1997), eFSH (Saneyoshi et al., 2001) and tethered-eCG (Min, 2001). Converting multisubunit complexes into single chains was previously attempted increase stability/activity of the parent compounds or to fuse complementary functional domains into a single molecule. To determine whether α and β subunits can be synthesized as a single polypeptide chain (tethered-LH and FSH) and also display biological activity, the tethered-LH and FSH by fusing the carboxyl terminus of the β-subunit to the amino terminus of the α-subunit was constructed. The dose-response curves of the recombinant tethered-LH and FSH were shown in figure 4. The results indicate that heterodimer can be expressed as a single chain encoding both subunits: α and β. Thus, the noncovalent heterodimeric structure is not critical for the function and secretion of the glycoprotein hormone family.

The tethered molecule has been proven by several experiments on the hCG and hFSH. Sugahara et al. (1995, 1996) suggested that the tethered hCG and FSH not only was efficiently but also displayed an increased biological activity *in vitro* and *in vivo* and retain a biologically active conformation similar to that seen in the heterodimer. Narayan et al. (1995) and Furuhashi et al. (1995) reported a similar result that the receptor binding and signal transduction of the two single chain hCGs (single chain hCG or single chain hCG devoid of C-terminal peptide) were as active as wild type heterodimeric hCG that was expressed in baculovirus-infected insect cells. Garcia-Campayo et al. (1997) suggested that receptor binding of the single chains is not impaired by changes in the heterodimeric configuration resulting from tethering the subunits. In addition, single chains exhibited a remarkably greater *in vitro* stability than the heterodimer, implying that these analogs will be useful as diagnostic reagents and that their purification will be facilitated. Fares et al. (1998)

suggested that the secretion rate of the single chain (TSH) also was 3-fold higher than that of hTSH wild-type. Moreover, the secretion of the single chain in the presence of the C-terminal peptide linker was dramatically increased. On the other hand, receptor binding and *in vitro* bioactivity of the single chains were similar to that of hTSH wild-type. Puett et al. (1998) reported that single chain hCG-receptor complex has been proven very useful gonadotropin and receptor structure-function studies, as well as studies aimed at elucidating the mechanism of transmembrane signaling. We determined the roles of oligosaccharide for mutant eCGs. eCG α56/β mutant, subunit Asn^{56} for Gln, did not show any LH-like activity, indicating that the carbohydrate residue at position 56 on the α-subnit of eCG is indispensable for such activity (Min et al., 1996, 1997). However, eFSHα56/β did not show any FSH activity, indicating the oligosaccharide at Asn^{56} was necessary for eFSH (Saneyoshi et al., 2001). Thus, FSH-like activities of two gonadotropins, eCG and eFSH, are evoked through the distinct molecular mechanisms regarding the biological role of oligosaccharide at Asn^{56} of α-subunit. We also reported that single chain eCG had a biological dual-activity of LH and FSH *in vitro* system (Min, 2001).

The target disruption of the α-subunit gene was proved hypogonadal and exhibited profound hypothyroidism, resulting in dwarfism (Kendall et al., 1995), and FSH β deficient female mice were infertile due to a block in folliculogenesis prior to antral follicle formation. However, FSH β-deficient male mice are fertile despite having small testes (Kumar et al., 1997). The deletion mutant of FSH β-subunit gene was proved to be a significant cause of reproductive dysfunction (Matthews et al., 1993). Thus, it was confirm that FSH mediated signaling is essential for normal ovarian follicular maturation, but dispensable for male fertility in mammals. Finally, tethered-molecules can be permit development of potent new analogues that stimulate ovarian development. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds. These data indicate the potentiality of the single chain approach to further investigate structure-function relationships of LH and FSH.

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