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Function of Constitutively Activating Lutropin/Choriogonadotropin Receptor

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지속적으로 발현되는 융모성 성선자극호르몬 수용체의 기능

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ABSTRACT

Gonadotropin receptors are members of the seven-transmembrane (TM) receptor family. Point mutations in the lutropin/choriogonadotropin receptor (LH/CGR) have been shown to cause constitutive activation which results in precocious puberty in affected males. We introduced one of the mutation, D556Y, into the LH/CG receptor and the same high affinity binding mutant (D556Y) receptor clone cell for wild type LH/CGR (LH/CGR-wt) was chosen for further analysis. In contrast to cells expressing LH/CGR-wt, it was demonstrated that the mutant receptor exhibited markedly increased basal cAMP production in the absence of agonist, suggesting that autonomous Leydig cell activity in familial male-precocious puberty (FMPP) is caused by a constitutively activating LH/CGR.

(Key words : LH/CG receptor, Constitutively Activating LH/CG receptor)

I. INTRODUCTION

The lutropin/choriogonadotropin receptor (LH/CGR), a membrane glycoprotein that is present on testicular Leydig cells and ovarian theca, granulosa, luteal, and interstitial cells, plays a pivotal role in the regulation of gonadal development and function in males as well as in nonpregnant and pregnant females (Wang et al., 1993). The LH/CGR is a member of the family of G protein-coupled receptors (GPCRs) and its structure is predicted to consist of a large extracellular domain connected to a bundle of seven membrane-spanning a-helices (Segaloff and Ascoli, 1993).

We previously studied the regulation of rat and equine placental function at different stages of pregnancy and identified pregnancy-stage specific placental functions which include secretion of growth modulators called placental lactogens (Hirosawa et al., 1994; Shiota et al., 1997), equine choriogonadotropin (eCG), equine relaxin (Min et al., 1994, 1996a, 1996b), rat CG (rCG) (Shinozaki et al., 1997), and leukemia inhibitory factor receptor (Aikawa et al., 1997).

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Recently, constitutively activating mutations of the receptor have been identified that are associated with familial male-precocious puberty (FMPP) (Shenker et al., 1993; Yano et al., 1996). A FMPP is a form of isosexual precocious puberty in boys in which testosterone levels are elevated independent of changes in luteinizing hormone-releasing hormone and serum luteinizing hormone levels (Kraaij et al., 1995). The affected males manifest pubertal development between the ages of 1 and 4 years (Kremer et al., 1993). One of the constitutively activating mutations involves a single base transition from A to G in the Asp-564-Tyr mutation of LH /CG receptor gene (Shenker et al., 1993). Although many activating GPCR mutations have now been described, the molecular basis of the activating effects has only been explored in a few cases.

In the present study, to address these further functions, we used site-directed mutagenesis to substitute Tyr for Asp at 556 amino acid residue. The mutant receptor gene was expressed in human embryonic kidney 293 cells, and hCG binding, cAMP response were measured in wild type and activating mutant receptors transfected cells.

Ⅱ. MATERIALS AND METHODS

1. Materials

The expression vector pcDNA3 was purchased from Invitrogen (San Diego, CA, USA). Endonucrease, Primers for point mutation, and Agarose were from Takara (Japan). Lipofectamine, DMEM, Wa /BSA, fetal bovine serum (FBS) and hepes were from Gibco BRL (MD, USA). Purified hCG (CR-127) was obtained from the National Hormone and Pituitary Agency of the NIDDK. [^{125}I]hCG was prepared as described (Min et al., 1996b). The QIAprep-spin plasmid kit was from QIAGEN Inc. (Hilden, Germany) and all the other reagents were from Wako Pure Chemicals (Osaka, Japan).

2. Cell Culture and Expression

A LH /CGR-wt cDNA (Min et al., 1999) was subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). A cDNA encoding for the LH /CGR was constructed using the polymerase chain reaction method with two mutant primers to substitute the Tyr for amino acid residue 556 with Asp codon. The identity of this construct was verified by sequencing.

The expression vectors of the wild type or mutant LH /CG receptor cDNAs were transfected into 293 cells by the liposome formulation (Lipofectamine) transfection method as previously described (Min et al., 1996b). The cells were maintained by incubation in growth medium (DMEM containing 10 mM HEPES, 50 μg /ml gentamicin, and 10% fetal bovine serum, pH 7.4) in a humidified atmosphere containing 5% CO_2. Selection was then started by supplementing the growth medium with 700 μg /ml geneticine (G418). The surviving colonies were then picked up individually with the aid of a pipetman. A series of clonal cell lines expressing wild type or mutant receptors were screened using a radioligand binding assay as described previously (Min et al., 1999).

3. Saturation Receptor Binding Assays

The methodology used to measure the saturation receptor binding was adapted from the previously used methods (Min et al., 1999). Briefly, [^{125}I]hCG was prepared as previously reported (Min et al., 1996b). The cells (2×10^5) were plated in 6-well plates coated with gelatin. The wells were placed on ice and the cells were washed twice with 2 ml aliquots of Wa /BSA and placed in 1 ml of the same medium contain-

ing a trace amount of [^{125}I]hCG (0.1~1,000 ng/ml). After an overnight incubation at 4℃, the cells were scraped into a small volume of cold Wa/BSA and centrifuged at 1,500×g for 10 min at 4℃. The supernatants were aspirated and the cells were resuspended in 2 ml of cold Wa/BSA and collected by centrifugation again before counting in a gammer-counter. All determinations were performed in duplicate, the binding affinity and maximal binding capacity were calculated by analyzing the data using the computer program LIGAND and Deltagraph (Munson and Rodbard, 1980).

4. cAMP Assays

Cells were preincubated with 0.5 mM isobutyl-methylxanthine (MIX) for 15 min and then incubated with increasing concentrations of hCG (30 min) and cholera toxin 100 ng/ml (2 hours) at 37℃. The wells were then placed on ice, and after adding 1 ml of the cold stop solution (1 N perchloric acid with 360 μg/ml theophylline), the cells were scraped and transferred to glass tubes. The wells were washed with 0.5 ml of 0.5 N perchloric acid with 180 μg/ml theophylline and this combined with the previous extract. One ml of the supernatants was neutralized with 0.5 ml of 0.72 M KOH/0.6 M $KHCO_3$ and centrifuged again to remove the salt precipitates. The samples were then used for cAMP measurement by RIA as previously described (Min et al., 1999).

Ⅲ. RESULTS

1. [^{125}I]hCG Binding Properties in Cell lines Expressing LH/CGR-wt and Mutant LH/CGR

Previous studies have resulted in the isolation of clonal 293 cell lines expressing LH/CGR and FSHR (Min et al., 1998). We have also shown that cells expressing these receptors bind hCG and FSH with high affinity and respond to these hormone with cAMP accumulation.

Embryonic kidney 293 cells were stably transfected with the cDNAs encoding the LH/CGR-wt and activating mutant (D556Y) LH/CGR, and the schematic representation of the membrane topology of the LH/CGR was shown (Fig. 1). Several clonal cell lines expressing different numbers of cell surface receptors were obtained. Only one cell lines for LH/CGR-wt and activating mutant were chosen, respectively and a corresponding cell line expressing the LH/CGR-wt was selected based on the number of cell surface receptors for the particular mutant LH/CGR. A clonal line of the cells ex-

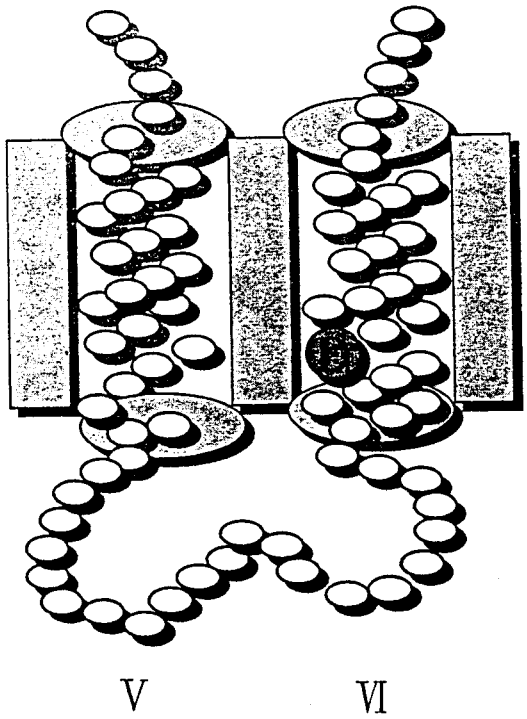

Fig. 1. Schematic representation of the membrane topology of the LH/CGR. Position of known constitutively activating mutation causing FMPP; Tyr(D)-556 in the sixth transmembrane helix.

pressing LH /CGR (D556Y) bound hCG with an affinity comparable to that of the LH /CGR-wt. This clonal cell line, designated LH /CGR-D556 Y, expressd approximately 155,000 cell surface receptors (Fig. 2).

2. hCG-stimulated cAMP Accumulation in Cell lines Expressing -WT and Activating Mutant LH/CGRs

The ability of the mutant LH /CGR-D556Y to transduce the hCG signal was measured by quantitating cAMP accumulation in cells incubated with increasing concentrations of hCG (Fig. 2). Since the chosen receptor mutation does not affect the binding affinity for hCG, and we reported corresponding cell lines expressing equivalent numbers of wild type receptors (Min et al., 1999). Since the maximal response elicited by an agonist is dependent on the binding affinity and the number of receptors, it is important to make these cell line expressing similar numbers of receptor. The basal cAMP levels were 0.

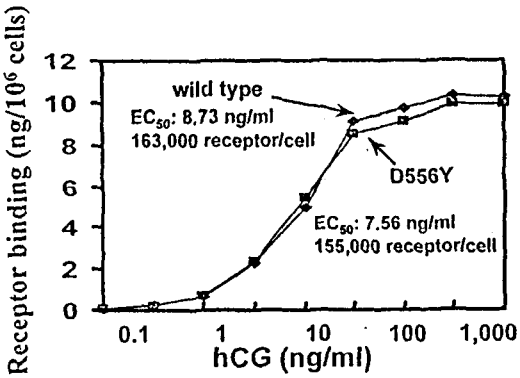

Fig. 2. Binding of ^{125}I-hCG to cell surface LH/CGR-wt and activating mutant LH/CGR-D556Y. 293 cells were transfected with LH/CGR-wt and activating mutant LH/CGR-D556Y cDNAs and subjected to the ^{125}I-hCG binding assay described under "Materials and Methods".

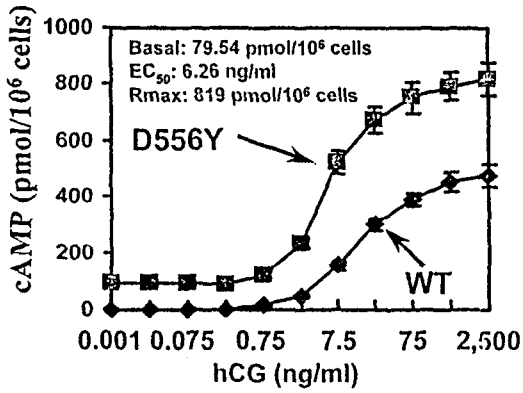

Fig. 3. Effects of hCG on cAMP accumulation in stable transfectants of 293 cells expressing LH/CGR-wt and mutant LH/CGR-D556Y. Cells were incubated with the indicated concentrations of hCG in medium containing 0.5 mM 3-isobutyl 1-methyl xanthine for 30 min at 37℃ before total cAMP was assayed (see Materials and Methods for details). Each point represents the average±SEM of three independent experiments. Duplicate dishes were used for each experiment.

18 pmol /10^6 cells for the wild type LH /CGR and maximal cAMP responses were 485 pmol / 10^6 cells (Fig. 3). The maximal cAMP response seems directly proportional to the number of receptors. The basal cAMP levels for the activating mutant were 79.54 pmol /10^6 cells and maximal cAMP responses were 819 pmol /10^6 cells (Fig. 3).

In the absence of hCG, LH /CGR-D556Y cDNA exhibited a higher basal cAMP level than cells transfected with wild type receptor cDNA, indicating that it was constitutively active. Elevated basal cAMP production should result in elevated androgen production by Leydig cells from FMPP patients in the absence of LH.

Ⅳ. DISCUSSION

The present studies were designed to investigate the function on the signal transduction of the clonal cell lines of activating mutant LH /CGR. To pursue this goal we prepared mutant LH /CGR-D556Y containing single point mutation in trasnmenbrane Ⅵ among the seven transmenbrane. The mutant cell line we selected had no effect on hCG binding (Fig. 2), but had profound effects on the ability of LH /CGR to transduce the hCG signal (Fig. 3).

We conclude that the constitutively higher cAMP levels caused by the D556Y mutation led to Leydig cell activation and male-limited precocious puberty. Kraaij et al. (1995) described the activating mutant (M398T) in a European boy and his father with FMPP. They also showed *in vitro* that this mutation elevates cAMP production in the absence of hormone. Two constitutively activating mutations were reported in the second transmembrane domain of the mouse melanocyte-stimulating hormone (MSH) receptor gene, which resulted in sombre coat color (Robbins et al., 1993). Constitutive activation of the Glu to Lys (E92K) mutation in the second transmembrane domain of MSH receptor was explained by the change of negative to positive charge at position 92.

In the subfamily of glycoprotein hormone receptors, hormone binding and receptor activation are probably sequential events. It is not likely that activating site will be found in the large N-terminal extracellular domain, because this domain is involved mainly in hormone binding and probably in proper positioning of the hormone relatively to the transmembrane domain. Such positioning may enhance the interaction of the hormone with the transmembrane domain.

In fact, it has been reported that hCG can activate a truncated LH /CGR lacking the extracellular domain (Ji and Ji, 1991), suggesting an interaction site for the hormone with the transmembrane domain. However, high concentrations of hCG had to be used and similar experiments with the TSH receptor did not result in receptor activation (Paschke et al., 1994).

Inactivating mutations of GPCRs can serve as a mechanism of human disease (Rosenthal et al., 1992), and the identification of amino-acid substitutions that cause constitutive activation of adrenergic receptors *in vitro* led to the prediction that such mutations might also be pathogenic (Kjeisberg et al., 1992). FMPP provides the first example of an inherited human disease that is due to a constitutively activating mutation in a GPCR.

In summary, we present evidence that the constitutively activating mutant LH /CGR was produced highly basal cAMP response in the absence of hCG. We are on way to identify why this activting mutation (D556Y) appeared an elevated cAMP levels in the absence of ligand. Future studies using gonadotropin receptors could provide useful informations regarding the structure-function relationship of seven-TM receptors in signal transduction.

Ⅴ. 요 약

성선자극호르몬 수용체(LH /CGR)는 7번 막을 통과하는 수용체의 일종이다. LH /CGR의 유전자 돌연변이 질환은 남성에 있어서 이들 수용체가 조기에 발현되어 조기 성숙의 원인이 된다. 이러한 수용체의 기능을 분석하기 위하여 556번째의 아미노산(D)을 Y로 치환한 돌연변이 수용체(D556Y)를 만들었다. 이러한 돌연변이 수용체를 동물세포에 발현시켜 cAMP의 분석결과 Ligand(호르몬)가 없어도 지속적으로 정보전달을 세포내부로 보내 cAMP 발현을 현저히 증가시켰다. 따라서 남성의 조기성숙과 관련된 질환은 지속적으로 발현하는 LH /CGR에 의한 원인 때문일 것이다.

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