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Distinct Mechanisms of cAMP Induction by Constitutively Activating LH Receptor and Wild-Type LH Receptor Activated by hCG

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Asp^{578}Gly is the major mutation of luteinizing hormone (LH) receptors in humans. It is a dominant mutant, constitutively activates Gαs, and induces cAMP production in the absence of the cognate hormone, causing the familial male precocious puberty. The mechanism of the elevated basal cAMP level is unclear. Our data show strikingly different mechanisms between the elevated basal cAMP induced by the activating mutant and the cAMP induced by the wild-type receptor activated by human chorionic gonadotropin (hCG) binding. The study suggests an approach to attenuating the elevated basal cAMP of the activating mutant LH receptor, which could be useful for controlling the familial male precocious puberty. For the study, we used the C-terminal peptides of Gαs and Gαi2, which couple to the receptor.

Key Words: LH; hCG; FSH; LH receptor; activating mutant; G protein.

Introduction

The luteinizing hormone (LH) receptor, a G protein–coupled receptor, is expressed primarily in the gonads of both sexes *(1,2)* as well as in other tissues including the brain *(3)*. The receptor regulates the development of the gonads of both sexes and the production of gametes. In the ovary, it induces ovulation from a mature follicle and stimulates progesterone production in the corpus luteum, which is responsible for preventing the onset of the ovulation cycle and preparing implantation and pregnancy. Therefore, defective LH receptors lead to disorders in ovulation and pregnancy. In fact, numerous mutations cause infertility by rendering the receptor recessively inactive *(4,5)* or dominantly active *(4,6)*.

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The first human mutant LH receptor was discovered in 1993, which is Asp^{578}Gly, in the sixth transmembrane helix and causes familial male precocious puberty *(7)*. It is the most frequently encountered LH receptor mutant *(8)* and induces cAMP production, as a dominant mutant, in the absence of the cognate ligands, LH and human chorionic gonadotropin (hCG). As it is not clear how the mutant receptor constitutively stimulates cAMP production, a specific question is raised whether the activating mutant induces the elevated basal cAMP level in the same mechanism as the wild-type receptor induces cAMP upon hCG binding. It is known that when hCG binds to the LH receptor, the receptor activates Gαs that in turn activates adenylyl cyclase to convert ATP to cAMP *(1,2)*. A simple explanation for the constitutive activity is that the Asp^{578}Gly mutant interacts with Gαs and activates it, as the liganded wild-type receptor would do.

To test the hypothesis, we decided to utilize the C-terminal region of Gα subunits. It is known that G protein–coupled receptors interact with the C-terminal region of Gα subunits and the corresponding C-terminal minigenes *(9)*. Using the C-terminal minigenes, we have compared the wild-type and Asp^{578}Gly receptors for their capacity to induce cAMP. Surprisingly, we discovered remarkably different mechanisms for the two types of LH receptors.

Results

The focus of this study was to determine whether the wild-type LH receptor (LHR^{WT}) activated by hCG and the activating Asp^{578}Gly mutant (LHR^{D578G}) interact with Gαs in the same mode or different modes. Because there is no standard assay to distinguish the interactions of the receptors with Gα subunits, we had to devise a new method. After trials and errors, we found that the Gα C-terminal minigenes encoding the C-terminal peptides of Gαs (GαsCP) and Gαi2 (GαiCP) distinctly interacted with the wild-type receptor and the mutant and distinguished them.

Human embryonic kidney 293 cells (HEK293) were transfected with pcDNA3 vector carrying LHR^{WT} or LHR^{D578G}, and stable cell lines were established as described in Materials and Methods. The cell lines expressing similar concentrations of receptors per cell are desirable for comparative

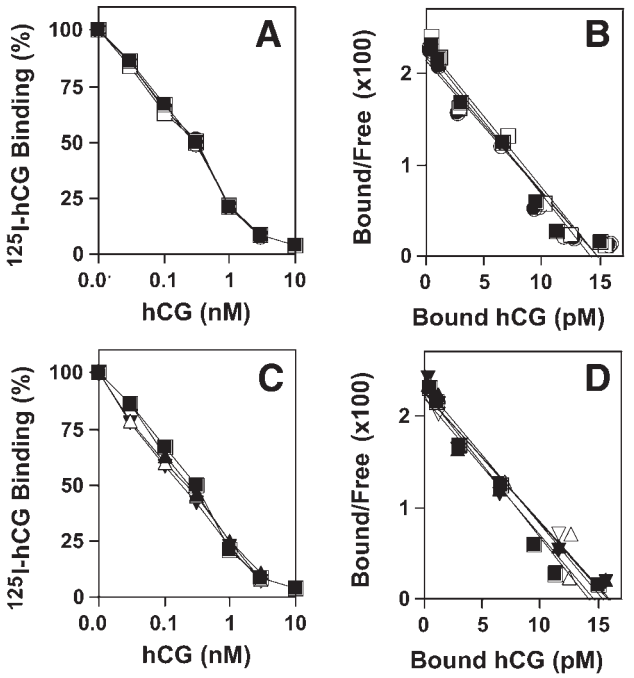
studies, because the surface concentration of a receptor could impact cAMP induction. Furthermore, overexpression of receptors can disrupt the cellular activities and distort cAMP induction. Therefore, it is necessary to limit the expression levels near the in vivo receptor concentration, and we selected the cell lines expressing approx 15,000 receptors per cell and used them in further studies (Fig. 1).

Intact cells stably expressing LHR^{WT} or LHR^{D578G} were assayed for ^{125}I-hCG binding in the presence of increasing concentrations of nonlabeled hCG (Fig. 1A,C) and the results were converted into Scatchard plots (Fig. 1B,D). The plots show that LHR^{WT} and LHR^{D578G} were, respectively, expressed 14,300 and 16,100 receptors per cell (tabular section of Fig. 1). In addition to the similar concentrations, their binding affinities were similar in the Kd range of 633–714 pM. These two cell lines were transiently transfected with blank pcDNA3, the pcDNA3 carrying the C-terminal 93 amino acids of Gαs (GαsCP) or the pcDNA3 carrying the C-terminal 76 amino acids of Gαi (GαiCP). The resulting cells with double transfections were assayed for ^{125}I-hCG binding. The ^{125}I-hCG displacement and Scatchard plots in Fig. 1 show that there were no significant changes in the receptor concentrations and binding affinities after the second transfection. To estimate the relative expression levels of GαsCP and GαiCP, the cells were probed for the HA epitope that is linked to GαsCP and GαiCP. ^{125}I-antiHA antibody binding showed the similar expression levels of GαsCP and GαiCP within the range of 94–126% (tabular section of Fig. 1).

Next, the cells were assayed for cAMP. As shown in Fig. 2, the cells stably expressing LHR^{WT} transfected with blank pcDNA3 produced cAMP in the hCG dose dependent manner (Fig. 2A). The maximum cAMP level was similar to the wild-type level (data not included) as previously described *(10)*. The maximum cAMP level was, however, significantly suppressed when the cells were transiently transfected with GαsCP or GαiCP.

The cells stably expressing LHR^{D578G} produced a significant amount of cAMP in the absence of hCG (Fig. 2B), consistent with the previous report of a high level of the basal cAMP *(7)*. The cells produced additional cAMP in response to hCG, although the maximum cAMP level was less than the wild-type level. The transient transfection of the cells with GαsCP dampened the basal and maximum cAMP levels. Surprisingly, the transient transfection of LHR^{D578G} with GαiCP raised the basal cAMP level. In addition, the cells responded to hCG and induced additional cAMP, as LHR^{D578G} did.

These results are summarized in Fig. 3 and show significant differences in the cAMP levels produced by LHR^{WT} and LHR^{D578G}. The most striking difference occurs when GαiCP was coexpressed, which increased the basal cAMP level of LHR^{D578G} to 53% of the maximum cAMP level, as compared with a marginal increase in the case of LHR^{WT}.



	Stable/Transient	Kd (pM)	Receptors/Cell	Gα (%)
■	Wild Type	633 ± 52	14,300 ± 700	–
□	Wild Type/pcDNA3	645 ± 57	14,500 ± 600	0
●	Wild TypeGαsCP	694 ± 74	14,900 ± 800	100 ± 14
○	Wild Type/GαiCP	680 ± 68	14,800 ± 900	126 ± 18
▲	$Asp^{578}Gly$	714 ± 63	16,100 ± 1,200	–
△	$Asp^{578}Gly$/pcDNA3	667 ± 71	15,500 ± 900	7 ± 5
▼	$Asp^{578}Gly$/GαsCP	709 ± 82	15,900 ± 800	110 ± 15
▽	$Asp^{578}Gly$/GαiCP	676 ± 59	14,800 ± 1,100	94 ± 24

Fig. 1. Hormone binding of LH receptors. HEK293 cells were transfected with pcDNA3 carrying the wild-type LH receptor (LHR^{WT}) or $Asp^{578}Gly$ mutant LH receptor (LHR^{D578G}). The cells were selected for those stably expressing the receptors in the concentration of 15,000–16,000 copies per cell. These cells were transfected again with blank pcDNA3, pcDNA3 carrying the hemagglutinin epitope (HA) and C-terminal peptide of Gαs (GαsCP), or pcDNA3 carrying the HA and C-terminal peptide of Gαi2 (GαiCP). The cells were assayed for ^{125}I-hCG binding in the presence of increasing concentrations of unlabeled hCG (**A,C**). The results were converted to Scatchard plots (**B,D**). To determine the relative expression levels of GαsCP and GαiCP, the cells that were transiently transfected with blank pcDNA, GαsCP or GαiCP were solubilized in nonionic detergent, the solutions were spotted on membranes and probed with ^{125}I-antiHA antibody. The binding to the cells transfected with the wild-type LHR and blank pcDNA3 was used as nonspecific binding. The binding to the cells expressing the wild-type LHR and GαsCP was subtracted with the nonspecific binding and was considered 100%. The experiments were performed in duplicate and repeated three times. The means and standard deviations are presented in the tabular section.

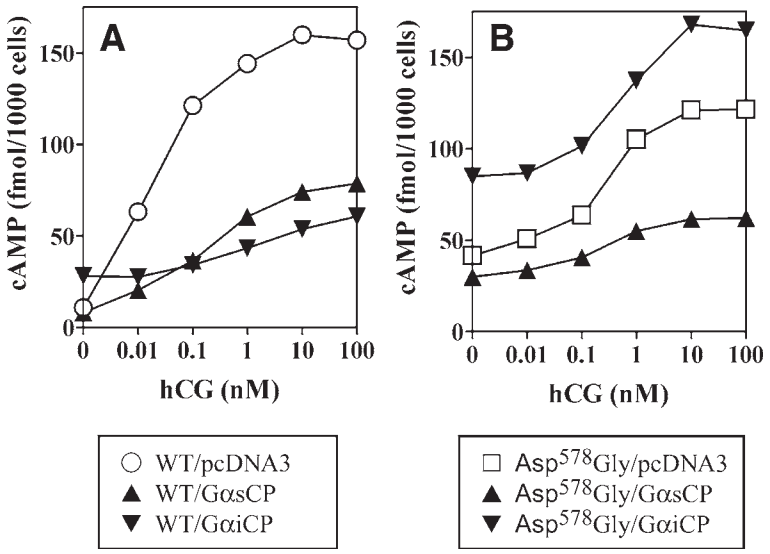


Fig. 2. Effects of GαsCP and GαiCP on cAMP induction. The HEK293 cells described in the legend to Fig. 1 were tested for cAMP induction as described in Materials and Methods.

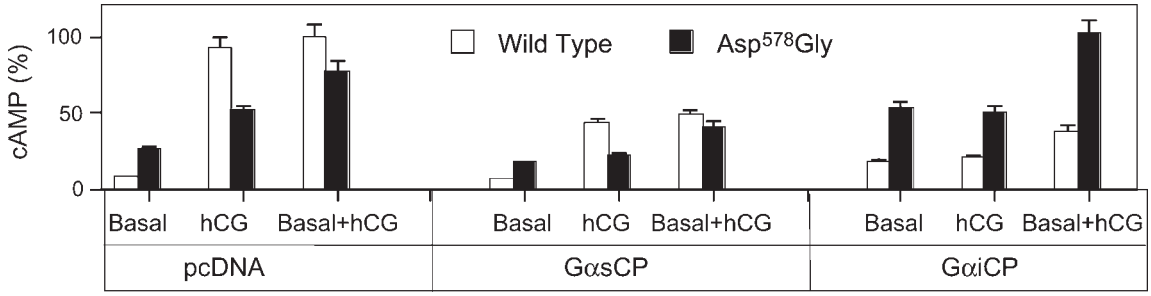


Fig. 3. Comparison of cAMP induction by the wild type and mutant receptors. The basal cAMP (basal), hCG-induced cAMP (hCG), and the sum of the basal cAMP and hCG-induced cAMP (basal + hCG) by the wild-type receptor and the $Asp^{578}Gly$ receptor were compared in tandem.

In addition, hCG induced a considerable amount of cAMP production by LHR^{D578G}, but not for LHR^{WT}. GαsCP caused a less dramatic difference in the hCG-induced cAMP levels of LHR^{WT} and LHR^{D578G}, as LHR^{D578G} produced less cAMP than LHR^{WT} did. This effect of GαsCP is opposite to the effect of GαiCP on the hCG-induced cAMP induction. These results taken together show remarkable differences in the association of GαsCP and GαiCP with LHR^{WT} and LHR^{D578G}.

Discussion

LHR^{D578G} *(7)* is the major human LHR mutant *(8)*. It is a dominant mutant, activates Gαs, and induces cAMP production in the absence of the cognate hormone, causing the familial male precocious puberty *(7)*. The C-terminal peptides of the Gα subunits are responsible for the interaction with G protein–coupled receptors *(9)*, and, indeed, Gαs and Gαi2 couple to the LH receptor *(11,12)*. Therefore, we used the C-terminal peptides of Gαs (GαsCP) and Gαi2 (GαiCP) and investigated their effects on activation of the Gαs subunit for cAMP production by LHR^{WT} and LHR^{D578G}.

The wild-type LH receptor and LHR^{D578G} responded differently to GαiCP. The different responses are not due to the secondary transfection per se, because it was not seen in the secondary transfection of LHR^{WT} and LHR^{D578G} with GαsCP or the blank vector. The effect is specific, because it was seen upon the secondary transfection with GαiCP, not with GαsCP. The difference is not caused by unlikely putative differences at the surface of the receptors. Their concentration and affinity for hCG were similar (Fig. 1).

In addition to the different basal cAMP level, there is a notable difference in the cAMP induced by hCG. Whereas the coexpression of GαiCP attenuated the hCG-induced cAMP of LHR^{WT} by 4.6-fold, it did not impact the hCG-induced cAMP of LHR^{D578G}. For example, LHR^{D578G} responded to hCG and induced cAMP production oblivious of GαiCP (Figs. 2 and 3). Compared with the effects of GαiCP, GαsCP had less dramatic yet sufficiently contrasting effects

on LHR^{WT} and LHR^{D578G}. GαsCP nearly abolished the hCG-induced cAMP of LHR^{D578G}, whereas it allowed a notable amount of the hCG-induced cAMP by LHR^{WT}.

Taken together our data show that distinct mechanisms exist for the cAMP induction of the wild-type LHR and the activating mutant, and suggest that the difference involves the interaction of the receptors with Gα subunits. In particular, the elevated basal cAMP of the activating mutant is differently induced from the cAMP induced by hCG binding to the wild-type receptor. A simple explanation for the increase in the basal cAMP level of LHR^{D578G} in the presence of GαiCP as compared with that of LHR^{WT} is that GαiCP facilitates the activation of Gαs by LHR^{D578G}. It would be interesting to see whether the activation involves a better interaction of the receptor with Gαs or a better modulation of Gαs by the receptor. In fact, these observations underscore the fact of how little we know about the interaction and modulation of G proteins. Our data suggest that the contact site(s) for Gαs and Gαi in the activated LHR complexed with hCG assumes a conformation distinctly different from the conformation of the constitutively active $Asp^{578}Gly$ mutant. Furthermore, the results imply that the interactions of Gαs and Gαi with the LH receptor allosterically affect each other. The allosteric effect appears to be more pronounced with the $Asp^{578}Gly$ mutant. Beyond these observations, our data offer an approach to attenuating the elevated basal cAMP of the activating mutant LH receptor. This could be useful for controlling the familial male precocious puberty.

Materials and Methods

Mutagenesis and Functional Expression of Receptors

Wild-type and mutant LH receptors were engineered to carry the prolactin signal sequence and the flag epitope. Mutant cDNAs were prepared in a pSELECT vector using the nonpolymerase chain reaction–based Altered Sites Mutagenesis System (Promega), sequenced, and subcloned into pcDNA3 (Invitrogen) as described previously *(13–15)*. After subcloning pcDNA3, the mutant cDNAs were sequenced again. The C-terminal peptide of 93 amino acids of Gαs (GαsCP) and the 76 residues of Gαi (GαiCP) *(16)* were individually cloned into the pcDNA3 vector carrying the hemaggulutinin (HA) epitope (YPYDVPDYS) at the N-terminus. Varying concentrations (2–12 μg) of plasmids were transfected into human embryonic kidney (HEK) 293 cells by the calcium phosphate method *(17)*. Transiently transfected cells were assayed 60–72 h after transfection. Stable cell lines were established in minimum essential medium containing 8% horse serum and 500 μg/mL G-418.

Assays for Hormone Binding, cAMP, and Expression of GαsCP and GαiCP

hCG was purchased from the National Hormone and Pituitary Program and radioiodinated as described previously *(18)*. Cells were assayed for ^{125}I-hCG (150,000 cpm) binding in the presence of increasing concentrations of nonradioactive hormone. Kd values were determined by Scatchard plots. For intracellular cAMP assay, cells were washed twice with minimum essential medium and incubated in the medium containing isobutylmethylxanthine (0.1 mg/mL) for 15 min. Increasing concentrations of hCG were then added, and the incubation was continued for 45 min at 37°C. After removing medium, the cells were rinsed once with fresh medium without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000*g* for 10 min at 4°C, the supernatant was collected, dried under vacuum, and resuspended in 10 μL of the cAMP assay buffer that was provided by the manufacturer. cAMP concentrations were determined with an ^{125}I-cAMP assay kit (Amersham Pharmacia Biotech) following the manufacturer's instruction and validated for use in our laboratory. Cells transfected with GαsCP or GαiCP were solubilized on ice for 30 min in buffer A containing 1% NP-40, 20% glycerol, 150 m*M* NaCl, and 20 m*M* HEPES, pH 7.4, containing protease inhibitors (1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* *N*-ethylmaleimide, and 10 m*M* EDTA). The detergent solutions were centrifuged at 30,000*g* for 30 min, and the supernatants were spotted onto polyvinylidene difluoride membrane. The membranes were incubated overnight at 4°C with ^{125}I-HA antibody, 0.5% nonfat dry milk, and 0.1% NP-40 in 150 m*M* NaCl and 20 m*M* HEPES, pH 7.4, and washed three times, and counted for radioactivity. All assays were carried out in duplicate and repeated three to four times, and means and standard deviations were calculated and presented.

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