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An Activating Mutation of the Follicle-Stimulating Hormone Receptor Autonomously Sustains Spermatogenesis in a Hypophysectomized Man*

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ABSTRACT

As both gonadotropins, LH and FSH, are required for normal spermatogenesis, patients with pituitary insufficiency need hCG plus human menopausal gonadotropin therapy to induce spermatogenesis and establish fertility. In a patient hypophysectomized because of a pituitary tumor, who, despite undetectable serum gonadotropin levels, had normal testis volume and semen parameters and fathered three children under testosterone substitution alone, we hypothesized an activating mutation of the FSH receptor. Exon 10 of the FSH receptor gene was amplified from genomic DNA by PCR, screened by single stranded conformation polymorphism gel electrophoresis, and sequenced. We identified a heterozygous A→G base change at nucleotide position 1700, leading to an Asp→Gly transition in codon 567 in the third intracytoplasmatic loop. COS-7 cells transiently transfected with the mutated receptor displayed a 1.5-fold increase in basal cAMP production compared to wild-type receptor, indicating that this mutation leads to ligand-independent constitutive activation of the FSH receptor. We conclude that this activating mutation of the FSH receptor, the first ever described, autonomously sustains spermatogenesis in the absence of gonadotropins. (*J Clin Endocrinol Metab* **81:** 1367–1370, 1996)

THE ROLE OF FSH in spermatogenesis is still a contentious issue (1). Although the production of mature fertile sperm can be induced in gonadotropin-deficient rats with testosterone alone (2) and in hamsters with FSH alone (3, 4), primate spermatogenesis apparently requires both hormones (for a review, see Ref. 5). Clinical experience shows that fertility cannot be achieved in hypogonadotropic patients solely with testosterone.

The FSH receptor, a member of the G protein-coupled receptor family, is highly homologous to the receptors of the other glycoprotein hormones, and its activation triggers the cAMP pathway. It has been recently reported that inactivating mutations of the FSH receptor result in inherited primary hypergonadotropic amenorrhea, but the corresponding reproductive phenotype in males has not been identified (6). Mutations of the FSH receptor that abolish (inactivating) or augment (activating) FSH function would be helpful for understanding the role of this gonadotropin in human spermatogenesis.

In this paper we describe the case of a hypophysectomized hypogonadotropic patient treated solely with testosterone who was unexpectedly fertile despite his gonadotropin deficiency. The recent description of activating mutations of the LH and TSH receptors (7–12) prompted us to investigate whether a constitutive activation of the FSH receptor could be the cause of his unexplained fertility.

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Subjects and Methods

Case report

A 28-yr-old male patient attended our clinic 8 yr ago requiring substitution therapy after hypophysectomy and repeated radiotherapy because of a pituitary chromophobe adenoma. He had no residual pituitary function and was adequately substituted with conventional doses of glucocorticoids, T_4, and testosterone enanthate (250 mg, im, every 3 weeks). His clinical data are summarized in Table 1. Serum gonadotropins, measured either by RIA using the WHO reagents or by immunofluorometric assay (13) were undetectable before and after a standard GnRH stimulation test. Serum testosterone concentrations (14, 15) were in the normal range while the patient was receiving substitution therapy, but became frankly hypogonadal when testosterone treatment was withdrawn. No FSH-like bioactivity was detected in serum using an *in vitro* FSH bioassay (16). Testicular volume was in the upper normal range, with normal sperm concentrations and slightly subnormal morphology and motility (17, 17a). The sperm parameters also remained normal after prolonged withdrawal of testosterone therapy. In that condition, the patient had very low serum testosterone concentrations and overt signs of androgen deficiency, including lack of libido and potency and undetectable serum gonadotropins, and did not respond to GnRH. When long term testosterone substitution was resumed, the patient had regular intercourse, and his wife became pregnant four times. Although the first pregnancy ended in spontaneous abortion, three sons were born 6, 5, and 2 yr ago. To analyze his FSH receptor, the patient was asked to provide a blood sample for DNA analysis and gave informed consent for this investigation.

DNA isolation, PCR, and DNA sequencing

DNA was obtained from the patient and from two volunteers with normal hormone levels and normal sperm parameters according to WHO criteria (17, 17a). Genomic DNA from peripheral blood was isolated and purified by using anion exchanger columns (Quiagen, Düsseldorf, Germany). Exon 10 of the human FSHR gene (18) was subdivided into seven overlapping subfragments (Fig. 1) individually amplified by PCR and screened by single stranded conformation polymorphism (SSCP)-gel electrophoresis. Each PCR reaction (50 μL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% gelatin, 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxy-NTPs, 2.5 U *Taq* polymerase (Promega, Heidelberg, Germany), and 100 nmol/L primer. Denaturation at

TABLE 1. Summary of the clinical data

Date	Feb 1987	Feb 1987	⇓	Apr 1987	May 1987	Jun 1987	Jul 1987	1988	⇓⇓⇓	1995
Testosterone therapy	Yes	Yes		No	No	No	No	Yes		Yes
Serum hormones										
Testosterone (nmol/L)	33	18.8		6.3	4.9	7.7	5.4	17.2		12.8
LH (IU/L)	<1.5	<1.5		<1.5	<1.5	<1.5	<1.5	<1.5		<0.5
FSH (IU/L)	<1.0	<1.0		<1.0	<1.0	<1.0	<1.0	<1.0		<0.5
Sperm parameters										
Conc. (millions/mL)	16.8	27.4		36.9	40.6	52.0	79.4	83		14.2
Count (millions/ejaculate)	50.4	54.8		129.2	121.8	156.0	238	265.6		79.5
% Motility a + b	47	58		40	40	73	74	32		35
Normal morphology (%)	43	40		52	38	35	58	62		14

Semen parameters were analyzed according to the WHO recommendations (19). It should be noted that due to stricter criteria, the lower limit of normal morphology was changed from 50% to 30% from 1987 to 1992. *Arrows* indicate induction of pregnancy.

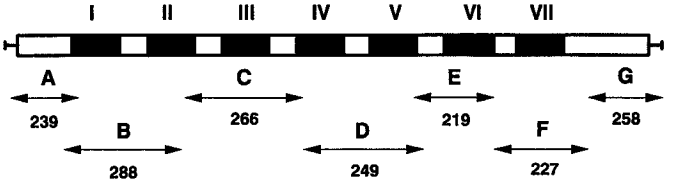

FIG. 1. Schematic representation of exon 10 of the FSH receptor with the seven transmembrane domains (■). For PCR amplification, exon 10 was subdivided into seven fragments given below in alphabetical order with their corresponding nucleotide sizes.

94 C for 2 min was followed by 35 cycles at 94 C for 50 s, 58 C for 30 s, and 72 C for 1 min and 30 s and a final elongation step at 72 C for 5 min. The different subfragments were screened for mutations by SSCP. Two microliters of the amplified DNA were mixed with 8 μL formamide mix (94% formamide, 5% glycerol, 10 mmol/L ethylenediamine tetraacetate, and 0.01% bromophenol blue), denatured at 95 C for 2 min, and applied to a 6% mutation detection enhancement gel (AT-Biochem, Heidelberg, Germany). The gels were run overnight at 10 C at 70–120 volts. DNA was visualized by silver staining. Fragment E was amplified using the following primers: forward primer, 5′-CCTTGTGCTCAATGTC-CTGG-3′ (corresponding to nucleotide position 1602–1621 of the FSH receptor sequence) (18); and reverse primer, 5′-GCTTTGGACACAGT-GATGAC-3′ (corresponding to nucleotide position 1801–1820). The amplified fragment was directly sequenced using a PCR sequencing kit (U.S. Biochemical Corp., Braunschweig, Germany) with the same primers.

Mutagenesis

The human FSH receptor complementary DNA (19) was cloned into the *Eco*RI restriction site of pSG5 (Stratagene, Heidelberg, Germany). The Asp^{567} was converted into Gly by oligonucleotide-mediated site-directed mutagenesis using the Transformer Site-Directed Mutagenesis kit (Clontech, ITC Biotechnology, Heidelberg, Germany). The selection primer (5′-GAGTGCACCATGGGCGGTGTGAAAT-3′) converted the unique *Nde*I restriction site of pSG5 into *Nco*I. The mutagenic primer was 5′-CCTCCTCTAGTGGCACCAGGATCGCC-3′. The mutation was confirmed by direct PCR sequencing. Plasmids were isolated and purified by anion exchange columns (Quiagen, Düsseldorf, Germany).

Transfection and cAMP assay

COS-7 cells were grown in six-well plates in DMEM and 10% heat-inactivated FCS until they reached 50–70% confluency. Transfection of wild-type or mutated FSH receptor was performed using the Lipofectamine reagent (Life Technologies, Eggenstein, Germany). Forty-eight hours after transfection, cells were washed and incubated for 2 h at 37 C in Dulbecco's phosphate-buffered saline, 0.2% glucose, 0.1% BSA, and 0.1 mmol/L isobutylmethylxanthine in the absence or presence of increasing doses of recombinant human FSH (Serono Laboratories, Aubonne, Switzerland). Media were collected for cAMP RIA (20) using the cAMP antiserum obtained from NIDDK (Bethesda, MD). Cells were scraped and lysed for protein determination.

Results

SSCP analysis of exon 10 showed a pattern similar to that found in normal men in every amplified fragment except fragment E, which displayed an additional band indicating the presence of a heterozygous mutation (Fig. 2). Upon sequencing, a heterozygous point mutation of nucleotide 1700 (from A to A/G) was detected, resulting in an amino acid transition from Asp (GAC) to Gly (GGC) at position 567 (Fig. 3). The mutation is located at the C-terminal part of the third intracytoplasmic loop in the codon corresponding to codon 619 of the TSH receptor and codon 564 of the LH receptor that have been reported to be affected by the same Asp→Gly transition in cases of thyroid hyperfunctioning adenoma (10) and pseudoprecocious puberty (21). The region at the boundary between the third intracytoplasmic loop and the sixth transmembrane domain is highly conserved among the glycoprotein hormone receptors, and mutations therein lead to constitutive activation (Fig. 4).

COS 7 cells transiently transfected with the mutant receptor produced cAMP concentrations consistently higher than those produced by wild-type receptor in the absence of FSH stimulation (Fig. 5, *left*). When increasing amounts of the two receptor constructs were used, the maximal production of cAMP (1.5-fold increase compared to wild-type) was obtained with 100 ng plasmid DNA. cAMP production in the absence of the ligand indicates that the mutated receptor is constitutively active. When 2 μg plasmid DNA were trans-

FIG. 2. SSCP-gel electrophoresis of fragment E from two fertile men (N1 and N2) and the affected patient (P). In addition to the normal migration pattern, an abnormal band in the patient's DNA indicates the presence of a heterozygous mutation.

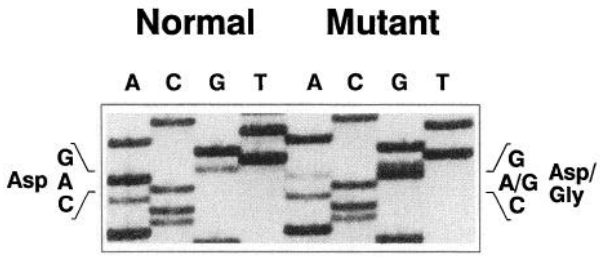

FIG. 3. Partial sequence of fragment E from exon 10 of the FSH receptor. The mutated FSH receptor of the patient (*right*) displays an additional G at the second position of codon 567, resulting in the heterozygous Asp/Gly formation. The sequence of the wild-type FSH receptor obtained from one fertile man can be seen on the *left*.

fected, both wild-type and mutated FSH receptor reacted to increasing concentrations of FSH with a similar dose-dependent increase in cAMP production (Fig. 5, *right*). Moreover, in these experiments, cAMP production by the mutated receptor was higher in the absence of stimulation than that by the wild-type cells.

Discussion

Hypophysectomized men are normally substituted with testosterone to maintain androgenization, but if fertility is required, testosterone alone cannot restore spermatogenesis, and gonadotropin therapy has to be implemented. Studies in nonhuman primates have shown that, unlike in rodents, only extremely high doses of exogenous testosterone, which cannot be administered to humans, are capable of initiating, maintaining, and reinitiating spermatogenesis (22–24). The doses of testosterone given to this patient are not sufficient to stimulate spermatogenesis. On the other hand, FSH immunoneutralization in monkeys leads to a reduction of spermatogenesis and loss of fertility (25, 26), whereas exogenous FSH alone can maintain spermatogenesis in monkeys pharmacologically hypophysectomized by GnRH antagonists (27). Based on this evidence, we propose that the activating mutation of the FSH receptor can sustain fully developed spermatogenesis in the absence of gonadotropins in the patient investigated. It is noteworthy that the patient also had normal sperm production when testosterone substitution therapy was withdrawn. Although his serum testosterone levels never dropped below 4.9 nmol/L, gonadotropins

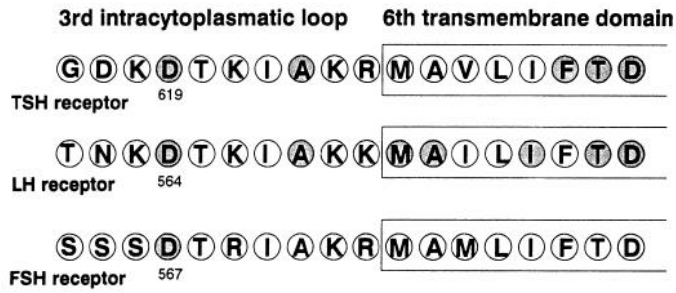

FIG. 4. Alignment of the boundary region of the third intracellular loop and the six transmembrane domain of the three glycoprotein hormone receptors. Amino acids found to be mutated, thereby leading to a constitutive activation, in cases of thyroid hyperfunctioning adenoma (16, 28, 29) and male pseudoprecocious puberty (13, 15, 27, 30, 31) are indicated by *filled circles*.

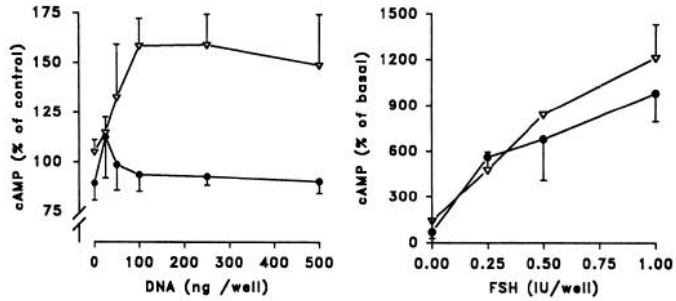

FIG. 5. Basal and FSH-stimulated cAMP production by COS-7 cells transiently transfected with wild-type (●) or mutated (▽) FSH receptor constructs. *left*, Mean ± SEM of four individual experiments in duplicate, considering the cAMP production in mock-transfected cells as the control. *right*, cAMP production in response to increasing concentrations of FSH. Results are expressed as the mean ± SEM of two individual experiments performed in duplicate. Basal cAMP production was evaluated by transfecting the cells with pSG5 vector in which the FSH receptor was cloned in reverse orientation.

remained undetectable, and ethical considerations prevented us from extending testosterone deprivation for a longer period.

The discovery of this patient sheds a new light on our understanding of dual hormonal regulation of spermatogenesis. Intriguingly, this case would suggest that FSH can sustain sperm production in man permanently, even in the absence of adequate concentrations of intratesticular testosterone. To reconcile this finding with the clinical experience that LH/hCG is also required for fertility in hypogonadotropic patients, we must assume that a crucial role of testosterone in spermatogenesis is to prepare the ground for FSH, facilitating its action through a mechanism involving the FSH receptor. Such a permissive role would not be necessary in the case of an autonomous activation of the receptor.

The activating mutation of the FSH receptor in this patient was discovered because of the concomitance with hypophysectomy, and the possible phenotype in the absence of other abnormalities of the reproductive system can only be a matter of speculation. Considering the importance of FSH for Sertoli cells and spermatogonia development and proliferation, we hypothesized that a chronic FSH-like hyperstimulation would eventually result in large testicles, but results from screening of exon 10 of the FSH receptor in 13 male patients with megalotestis were negative (our unpublished observation). It is possible that activating mutations of the FSH receptor do not lead to any specific pathology in the presence of normal pituitary function. Alternatively, the effects of such mutations could be sex limited, in analogy with the phenotype associated with activating mutations of the LH receptor that becomes manifest only in one gender. Possibly, activating mutations of the FSH receptor could lead to some familial mild disturbance of female sexual maturation and/or reproductive function still unrecognized as a separate nosological entity. The patient described in this paper, however, was not aware of any peculiar reproductive disorder in his mother and his only sister, who has two children, and other members of his family were not available for genetic analysis.

If activating mutations of the FSH receptor are not detri-

mental to reproductive function, *i.e.* are not self-limiting in their transmission, they might be not so rare. In this case, they could remain unrecognized unless a concomitant reproductive dysfunction is present. We suggest that activating mutations of the FSH receptor could underlie some cases of inappropriately normal testis size, sperm output, and fertility, *e.g.* in hypogonadotropic patients reported to develop normal spermatogenesis with LH/hCG alone (28–31).

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