BRIEF REPORT

HYPOGONADISM CAUSED BY A SINGLE AMINO ACID SUBSTITUTION IN THE β SUBUNIT OF LUTEINIZING HORMONE

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INFERTILITY occurs in approximately 10 to 20 percent of couples and can be attributed to a reproductive disorder in the man or woman with equal frequency.[1,2] Among men, specific causes of infertility can be identified in only a minority.[1] In a previously identified kindred in which several men were infertile, the proband had not undergone spontaneous puberty and had low testosterone and elevated luteinizing hormone concentrations in serum, a pattern of hormone values indicative of primary hypogonadism.[3] However, testosterone secretion in response to the administration of exogenous luteinizing hormone and human chorionic gonadotropin was normal. Thus, the proband appeared to secrete luteinizing hormone that was not active, a suggestion supported by the demonstration that the hormone was biologically inactive when tested in in vitro bioassays.[4] The occurrence of infertility in three maternal uncles and a family history of consanguinity suggested that members of the family may have had an inherited defect in the structure of luteinizing hormone.

Luteinizing hormone is a member of the glycoprotein hormone family that also includes follicle-stimulating hormone, thyroid-stimulating hormone, and chorionic gonadotropin.[5,6] Each of these hormones is a heterodimer composed of a common α subunit and a specific β subunit that confers specificity for the receptors in the target organ for each hormone. The intact heterodimer is required for biologic activity, and both subunits are glycosylated. Because the abnormality in this family appeared to be restricted to luteinizing hormone, a defect in the common α subunit (LHα) was unlikely and a structural alteration was hypothesized to reside either in the coding sequence of the β subunit (LHβ) or in its post-translational processing. Delineation of the specific defect in this family therefore provided an opportunity to identify a region of the luteinizing hormone molecule that is critical for its biologic activity. In this article, we report a mutation in the coding sequence of the LHβ gene that eliminates the ability of luteinizing hormone to bind to its receptor.

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Performed as part of the National Cooperative Program on Infertility Research and supported by grants (HD 29164 and HD 23519) from the National Institute of Child Health and Human Development, by a Medical Research Council Travelling Fellowship, and by a grant from Ares Serono.

Methods

Subjects

The clinical history, physical findings, and endocrinologic features of the proband and his family have been described.[3] In brief, the proband presented at the age of 17 years because of pubertal delay. He was treated for two years with testosterone, and there was no evidence of spontaneous puberty after its withdrawal. His serum immunoreactive luteinizing hormone concentration was twice as high as normal, and his serum follicle-stimulating hormone concentration was normal, but the serum testosterone concentration was low. His karyotype was 46,XY. A testicular biopsy revealed an arrest of spermatogenesis; no Leydig cells could be identified. Long-term treatment with chorionic gonadotropin resulted in testicular enlargement, normal virilization, and a sperm count of 11 million per milliliter with 50 percent motility. He subsequently was unable to father children despite courses of chorionic gonadotropin and testosterone therapy. Recent serum hormone values for the proband, his mother, three maternal uncles, and his sister are shown in Table 1. A portion of the pedigree is shown in Figure 1. The proband's parents, as well as his maternal grandparents, were first cousins. There was a history of infertility in three maternal uncles (III-3, III-4, and III-5), each of whom underwent normal puberty, reported normal libido and sexual performance, and had a normal physical examination. Their serum testosterone concentrations were low or in the lower range of normal on multiple occasions[3] (Table 1). The serum luteinizing hormone concentrations, measured by radioimmunoassay, were normal in Subjects III-3 and III-5 and slightly elevated (22.9 U per liter) in Subject III-4. The serum follicle-stimulating hormone concentrations were normal in Subject III-3, but elevated in Subjects III-4 and III-5. Semen analyses were not performed in these three men. The proband's mother (III-2) and his sister (IV-2) had normal pubertal development and regular menstrual cycles, and both were fertile. Their serum gonadotropin concentrations were normal for their ages. All family members studied were euthyroid and had normal serum thyroid-stimulating hormone concentrations.

Genetic Analyses

DNA was isolated from peripheral-blood leukocytes.[7] The LHβ and β subunit of chorionic gonadotropin (CGβ) genes from the proband and unrelated normal subjects were amplified by the polymerase chain reaction (PCR).[8] After the identification of a mutation, a 300-base-pair (bp) region of exon 3 that included the mutation site was amplified in additional family members. A map of the LHβ/CGβ gene cluster and the strategy for PCR amplification are shown in Figure 2. The primers used contained artificial restriction-enzyme sites (*Bam*HI: primers A, B, and D; *Eco*RI: primer C) to facilitate subcloning into the pGEM-7z (Promega Biotec, Madison, Wis.) or pM^{2}α (kindly provided by I. Boime, Washington University, St. Louis) plasmids. The LHβ and CGβ clones were distinguished by filter hybridization with oligonucleotide probes that were specific for nonhomologous regions of the genes.[11] DNA sequencing was performed with the chain-termination method.[12] Genotypes were confirmed by sequencing a minimum of six clones.

Characterization of the Functional Properties of Mutant Luteinizing Hormone

The mutant and normal LHβ genes were subcloned into the pM^{2}α expression vector as described above. The LHβ gene sequence extends from 32 bp upstream from the translational

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Table 1. Serum Hormone Values in Members of a Family with a Mutation in LHβ.*

VARIABLE	AGE	LH	FSH	TESTOSTERONE
	yr	*U/liter*		*nmol/liter*
Family member				
Proband (IV-1)	44	64.2	113.6	1.7
Sister (IV-2)	47	9.7	8.8	ND
Mother (III-2)	80	67.0	145.2	ND
Uncle (III-3)	77	15.6	8.5	9.2
Uncle (III-4)	70	22.9	31.3	7.4
Uncle (III-5)	61	11.6	35.7	5.6
Normal range				
Men	—	3–18	3–18	10.4–38.1
Premenopausal women	—	2.4–34.5	4.6–22.4	ND
Postmenopausal women	—	30–150	30–150	ND

*The pedigree is shown in Figure 1. LH denotes luteinizing hormone, FSH follicle-stimulating hormone, and ND not done.

initiation site to 101 bp downstream from the stop codon and includes the polyadenylation signal. The subclones were sequenced to verify that *Taq* polymerase errors had not occurred during PCR amplification. The pM^{2}α/LHβ expression vector contained the LHα and LHβ genes, each under the control of a separate Harvey murine-sarcoma-virus promoter. The plasmid also contained a selectable marker gene for neomycin resistance.[13] Chinese hamster–ovary cells (American Type Culture Collection, Rockville, Md.) were maintained in modified Eagle's medium that contained 5 percent fetal-calf serum (GIBCO Laboratories, Grand Island, N.Y.) and was supplemented with penicillin (100 U per milliliter) and streptomycin (100 μg per milliliter). The cells were transfected with the calcium phosphate technique.[14] Stable clones were selected with Geneticin (0.30 mg per milliliter) (GIBCO Laboratories), an analogue of neomycin. Luteinizing hormone activity in the medium from the cultured cells was measured by radioimmunoassay and by an immunoradiometric assay that uses antiserum specific for LHα and LHβ.[15] The reactivity of uncombined LHα and LHβ was less than 5 percent in this assay. Radioreceptor assays were performed to measure the ability of unlabeled hormone to inhibit the binding of radiolabeled hormone to the luteinizing hormone receptor.[16]

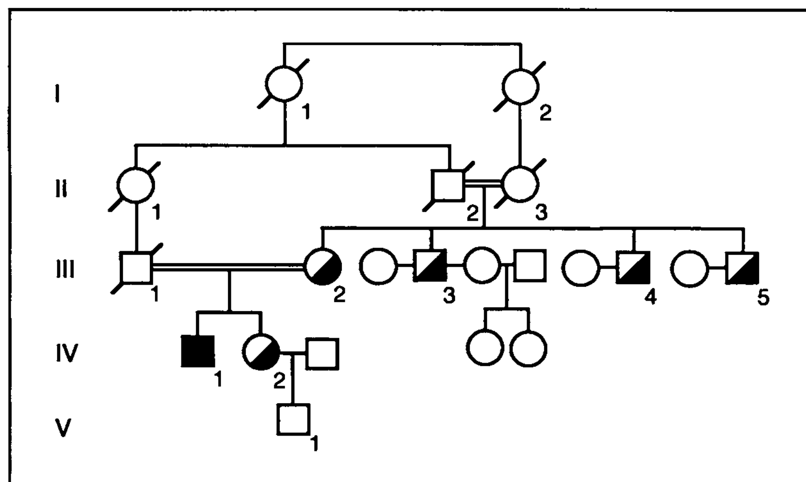

Figure 1. Kindred with the LHβ Mutation.

Squares denote male family members, circles female family members, slashes deceased members, double horizontal bars consanguineous relationships, the solid symbol a member homozygous for the LHβ mutation (the proband), and half-solid symbols heterozygous members. The genotype of the proband's father (III-1) could not be determined, but he was presumed to have been an obligate heterozygote because of the transmission of the mutation to his son. The genotype of the proband's nephew (V-1) was not examined.

RESULTS

Characterization of a Mutation in LHβ

The LHβ protein is encoded by a single gene that is located adjacent to several highly homologous CGβ genes and pseudogenes on chromosome 19.[9] The structure and arrangement of the LHβ and CGβ genes in several affected family members and in unrelated normal subjects were assessed by Southern blot analyses. The LHβ gene and each of the six CGβ genes were present, and there was no evidence of a gene rearrangement in any of the affected family members (data not shown).

The PCR was then used to clone the individual LHβ and CGβ genes (Fig. 2). The LHβ genes were distinguished from the structurally similar CGβ genes with sequence-specific oligonucleotides.[11] In the proband (IV-1), a single-base substitution was identified in the LHβ gene. This substitution converts codon 54 from glutamine to arginine (Fig. 3). The mutation was verified in three independent PCR reactions. The genotypes of the proband and additional family members were determined by analyzing the ratio of normal and mutant LHβ clones (Table 2). The proband (IV-1) was homozygous for the mutation, whereas his mother (III-2), sister (IV-2), and three of his maternal uncles (III-3, III-4, and III-5) were heterozygous for the mutation. Although DNA was not available from the proband's father, he was presumably an obligate heterozygous carrier of the mutation.

In addition to a spontaneous mutation in the LHβ gene, the presence of a cluster of LHβ and CGβ genes also raised the possibility that a gene conversion could have occurred in which the LHβ mutation was derived from the transfer of DNA sequences from one of the CGβ genes. To address this possibility, CGβ clones were also screened for the mutation in codon 54 (Table 2). Among the 248 CGβ genes analyzed, none contained the mutation that was found in the LHβ gene. Thus, the alteration in the LHβ gene sequence probably arose as a spontaneous mutation.

Functional Properties of the Mutation in Luteinizing Hormone

Because the LHβ mutation caused hypogonadism in the homozygous state and resulted in a hormone that was immunologically active in vivo but biologically inactive,[4] we performed additional studies to define the functional consequences of the substitution of arginine for glutamine at amino acid

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54. Mutant or normal LHβ genes were inserted into an expression vector that also contained a copy of the human LHα gene.[17] The expression vectors were transfected into a cell line (Chinese hamster ovary) that does not normally produce this hormone, but secretes biologically active luteinizing hormone after the introduction of the glycoprotein hormone LHα and LHβ genes.[17] The expression of mutant luteinizing hormone was demonstrated with a dimer-specific immunoradiometric assay (data not shown), indicating that the mutant LHβ formed a heterodimer with the α subunit. The mutant hormone was undetectable in the radioreceptor assay, whereas normal luteinizing hormone expressed in Chinese hamster–ovary cells was readily measured (Fig. 4). These findings demonstrate that the absence of biologic activity in the mutant luteinizing hormone is due to its inability to bind to its receptors.

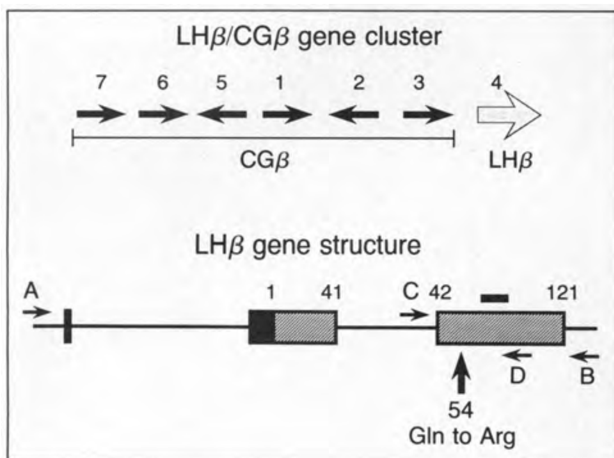

Figure 2. Arrangement of the LHβ/CGβ Gene Cluster on Chromosome 19 and the Structure of the LHβ Gene and Mutation in Codon 54.

There are six CGβ genes and pseudogenes (dark arrows) and a single LHβ gene (shaded arrow) in the gene cluster, which spans approximately 52 kb. The CGβ gene nomenclature has been changed: the gene previously[9] identified as gene 8 is now[10] referred to as gene 6. The diagram of the LHβ gene structure also shows the strategy for PCR amplification. The three exons are indicated by boxes; the signal peptide sequence is black, and the mature LHβ peptide sequence is hatched. The positions of the boundaries of the LHβ coding sequence are shown above the exons. The sites of action of the PCR primers are indicated by arrows (A, B, C, and D). The location of the mutation at amino acid 54 (substitution of arginine for glutamine) is indicated. The location of an oligonucleotide probe that distinguishes LHβ and CGβ sequences is denoted by a bar above exon 3.

The immunologic and receptor-binding properties of serum luteinizing hormone from the homozygous proband and his heterozygous relatives are summarized in Table 3. As was consistent with the data in the cell-expression system, the proband's serum luteinizing hormone was undetectable in the radioreceptor assay, despite the fact that concentrations of luteinizing hormone were elevated on radioimmunoassay. The proband's heterozygous mother also had elevated immunoreactive serum concentrations of luteinizing hormone, consistent with her age. The activity of her serum luteinizing hormone in the radioreceptor assay was reduced, producing a decreased ratio of radioreceptor-assay to radioimmunoassay results (0.65). This ratio was 50 percent of the value (1.3) in normal postmenopausal women, suggesting that her circulating luteinizing hormone contained equal amounts of mutant and normal forms of the hormone. In family members III-3 and IV-2, the activity of luteinizing hormone in serum was below the detection limits of the radioreceptor assay.

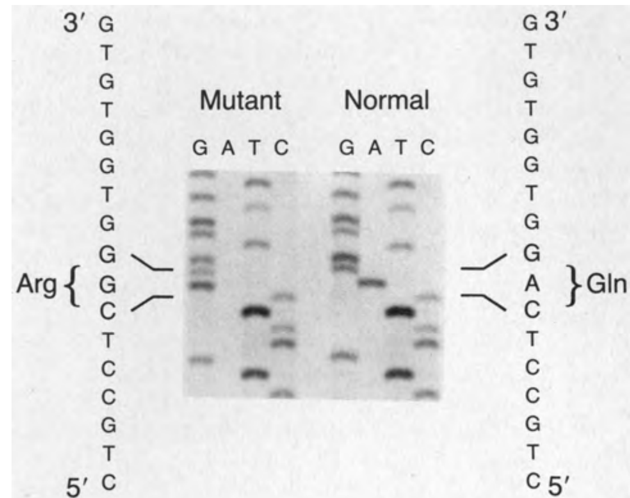

Figure 3. DNA Sequence of the LHβ Gene Mutation.

The mutation that changes codon 54 from glutamine to arginine is indicated.

Discussion

We describe a mutation that causes a substitution of arginine for glutamine in amino acid 54 of the LHβ subunit. This mutation eliminates the ability of luteinizing hormone to bind to its receptors, resulting in the failure of puberty to develop spontaneously, with infertility in the homozygous state. In male heterozygotes, the effect of the mutation is more subtle, causing impaired steroidogenesis and a high incidence of infertility (three of four men), despite the develop-

Table 2. Summary of Mutations in the PCR-Amplified Clones of the LHβ and CGβ Genes from Members of a Family with a Mutation in LHβ.

Family Member	LHβ Clones		CGβ Clones		Genotype
	Normal	Mutant	Normal	Mutant	
	no. of genes				
III-2	12	20	35	0	Heterozygous
III-3	19	28	39	0	Heterozygous
III-4	9	23	48	0	Heterozygous
III-5	19	17	31	0	Heterozygous
IV-1	0	50	58	0	Homozygous
IV-2	4	2	37	0	Heterozygous

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ment of normal secondary sex characteristics. Female heterozygotes have normal sexual development and are fertile.

A number of endocrine disorders are characterized by elevated serum hormone concentrations with no evidence that these elevations have any biologic effects. In most of these disorders, end-organ failure results in a compensatory increase in the level of stimulatory hormone. There are also examples of inherited hormone resistance syndromes that are caused by mutations of hormone receptors.[18] In contrast to mutations in receptors, few mutations in hormones resulting in defective hormone action have been identified. One such example consists of mutations in the insulin gene that either impair the processing of proinsulin to insulin or reduce its affinity to bind to the insulin receptor.[19] The mutation in the LHβ gene described here represents a second example of a hormone mutation that causes a deficiency syndrome, despite the finding of normal or elevated serum hormone concentrations on radioimmunoassay. As a result of these properties, this mutation in the LHβ gene causes a rare syndrome that should be distinguished from hypergonadotropic hypogonadism due to primary gonadal failure.

The clinical features of the members of the kindred with the LHβ mutation allow one to infer the physiologic role of luteinizing hormone during development and sexual maturation. Luteinizing hormone first appears in the pituitary during the 10th week of gestation, and serum levels reach a maximum (approximately 20 to 40 U per liter) around week 16.[20] One of the potential functions of fetal luteinizing hormone is

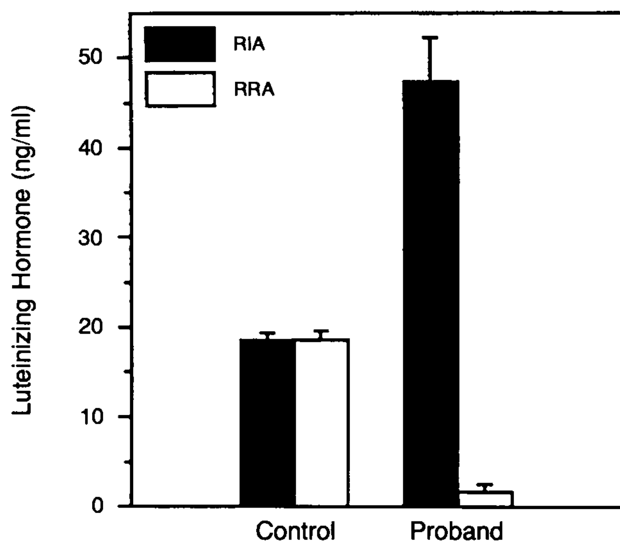

Figure 4. Receptor-Binding Activity of the Mutant Luteinizing Hormone Expressed in Chinese Hamster–Ovary Cells.

Chinese hamster–ovary cells were transfected with luteinizing hormone expression vectors containing the LHα and LHβ genes. Luteinizing hormone activity in medium from stable cell lines expressing either the normal LHβ (control) or the mutant LHβ (proband) gene was measured by radioimmunoassay (RIA) and radioreceptor assay (RRA). Values are the means ±SD of duplicate samples of medium.

Table 3. Immunologic and Receptor-Binding Properties of Serum Luteinizing Hormone in Members of a Family with a Mutation in LHβ.*

Family Member	Luteinizing Hormone		RRA:RIA Ratio	
	RRA†	RIA‡	Family Member	Normal Subjects§
	U/liter			
III-2	31.5	48.7	0.65	1.3±0.3
III-3	<7.2	8.2	<1	1.1±0.1
IV-1	<7.2	47.3	<0.15	1.1±0.1
IV-2	<7.2	9.7	<1	1.2±0.1

*Plus–minus values are means ±SD. RRA denotes radioreceptor assay, and RIA radioimmunoassay.

†The second international reference preparation of luteinizing hormone was used for both the RRA and the RIA. Serum concentrations of luteinizing hormone measured in the immunoradiometric assay (data not shown) were similar to those measured by the RIA.

‡The normal ranges for serum luteinizing hormone are given in Table 1.

§Ratios are for age-matched and sex-matched normal subjects described previously.[16]

to stimulate androgen production by the testes in conjunction with the production of chorionic gonadotropin. Androgens are required for the development of a normal male phenotype, as revealed by persons with testicular feminization, a disorder caused by mutations in the androgen receptor. The proband (IV-1) in this family lacked functional luteinizing hormone and consequently had a testosterone deficiency. Nevertheless, he had a normal prepubertal male phenotype, including descended testes. These findings indicate that luteinizing hormone is not required for male sexual differentiation. Androgens derived either from the adrenal gland or from chorionic gonadotropin–induced stimulation of the testes in utero were apparently sufficient to mediate these developmental events.

In addition to its role as a secretagogue, luteinizing hormone is a tropic hormone and may have an important role in Leydig-cell division and steroidogenesis during fetal development.[20] Consistent with this view was the finding that Leydig cells were not seen in a testicular-biopsy specimen from the proband (IV-1).[3] Fetal exposure to luteinizing hormone, as well as to chorionic gonadotropin, may be important for the normal development of the Leydig-cell population. Although the proband received chorionic gonadotropin and testosterone therapy on several occasions, he had decreased spermatogenesis and was infertile. It is possible that the absence of fetal exposure to luteinizing hormone altered his subsequent capacity for spermatogenesis, a process that is critically dependent on high intratesticular concentrations of testosterone.[1]

One of the important issues raised by this study is whether this mutation in the LHβ gene caused clinical manifestations in the heterozygotes. None of the three heterozygous men (III-3, III-4, and III-5) who were studied had any clinical evidence of hypogonadism. Although each of these men was apparently infertile, the cause of their infertility is not known. Their serum

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testosterone concentrations were either low or in the low-normal range, suggesting a less severe defect in steroidogenesis than in the proband. The reduced intratesticular and circulating testosterone concentrations in these men may have contributed to their infertility. Because the proband's father was presumably an obligate heterozygote, the heterozygous condition is not invariably associated with infertility in men, and fertility was not affected in the two heterozygous women (III-2 and IV-2).

In addition to their potential clinical importance, naturally occurring mutations provide valuable insights into the relations of structure and function in the glycoprotein hormones. A mutation in amino acid 29 in the β subunit of the human thyroid-stimulating hormone gene has been described that prevents dimerization with the α subunit and causes hypothyroidism in homozygotes.[21] The mutation in the LHβ gene that we described occurs at an amino acid (glutamine 54) that is conserved in all β subunits of the glycoprotein hormones,[5] suggesting that this residue may have an important role in hormone structure or function. The substitution of arginine for glutamine at this site in the luteinizing hormone did not affect dimerization or immunoreactivity, but it eliminated receptor binding. It is not known whether this mutation identifies an actual contact site with the receptor or whether it causes a structural alteration that secondarily interferes with receptor binding. It will be of interest to determine the frequency of mutations in the gonadotropin genes in patients with abnormal receptor binding, especially in view of the fact that heterozygosity may not be detected during routine infertility evaluations.

We are indebted to Dr. I. Boime for the expression vector pM$^2\alpha$.

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