CLINICAL STUDY

Serum GH response to pharmacological stimuli and physical exercise in two siblings with two new inactivating mutations in the GH-releasing hormone receptor gene

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Abstract

Objective: Inactivating mutations of the GH-releasing hormone receptor (*GHRHR*) gene (*GHRHR*) cause familial isolated GH deficiency (IGHD) type IB. The GH response to physical exercise (PE) in patients lacking *GHRHR* has never been studied. We hypothesized that subjects lacking functional *GHRHR* may be a model to study GH response to PE.

Design: We have analyzed peripheral genomic DNA of a family with two sibs affected by IGHD IB for mutations in the *GHRHR*, studied the patients' GH response to different GH secretagogues and to PE, and examined the morphology of their pituitary gland by magnetic resonance imaging (MRI).

Methods: The *GHRHR* was analyzed by direct sequencing of the 13 exons, intron–exon boundaries, and of the proximal 327 bp of the promoter region in the index case. The patients' GH response to GHRH and standardized PE was studied twice, using a GH ultrasensitive assay in the second round of testing.

Results: Both subjects were compound heterozygotes for two previously undescribed mutations in the *GHRHR* that are predicted to cause complete lack of functional *GHRHR* protein: a nonsense mutation in codon 43 (Q43X), and a splice mutation at the beginning of intron 3 (IVS3 + 1G → A). MRI showed hypoplasia of their anterior pituitaries. Both subjects had a small but detectable increase in serum GH after maximal PE.

Conclusions: *GHRHR* mutations need to be considered in IGHD IB patients even in the absence of parental consanguinity, and patients lacking *GHRHR* may provide a model to study the mechanism by which PE influences GH secretion.

European Journal of Endocrinology 147 591–596

Introduction

Pituitary secretion of growth hormone (GH) and somatotroph cell proliferation are under hypothalamic control. They are stimulated by GH-releasing hormone (GHRH) and inhibited by somatostatin (1). These two hormones act on the somatotroph cells by binding to specific cell surface receptors, which have opposite effects on intracellular cAMP production. The physiological role of ghrelin (recently identified from the rat stomach), the natural ligand for the GH-releasing peptide (GHRP) receptor, is not yet well defined (2).

Isolated GH deficiency (IGHD) type IB, the most prevalent form of familial IGHD, is transmitted as an autosomal recessive trait (3). Unique mutations in the gene encoding the GHRH receptor (*GHRHR*) (*GHRHR*, Online Mendelian Inheritance in Man gene no.

139191) have been recently described by others and us in kindreds with IGHD IB (3–11). Although most commonly *GHRHR* mutations have been reported in families in which the homozygous affected subjects had consanguineous parents, we have previously described three kindreds in which affected subjects are compound heterozygotes for different mutations in this gene (7, 8, 11).

Patients with two faulty *GHRHR* alleles have severe IGHD, and their serum GH response to conventional GH stimulation tests is frankly subnormal (3–11). However, their GH response to physical exercise (PE) has never been studied.

We report here that the two affected subjects from a newly identified IGHD IB family are compound heterozygotes for two new null mutations in the *GHRHR*, both predicted to cause completely non-functional or absent

receptor proteins. We describe clinical and molecular findings in these subjects, their pituitary imaging and their GH response to chemical stimuli and to maximal PE.

Patients and methods

Patients

Two brothers presented to medical attention in 1992 for evaluation of short stature. They were the products of normal uncomplicated pregnancies that ended in spontaneous deliveries from non-blood related, normal-stature parents (father 175 cm, mother 171 cm, by report). There were no reports of neonatal hypoglycemia. The pedigree of the family is shown in Fig. 1.

At presentation, subject II-1 was 11 years old, measured 108.2 cm (-5.5 SDS), and had a bone age (calculated via Greulich and Pyle atlas) of 8 years. Subject II-2 was 8 years and 9 months old, measured 101.1 cm (-5.5 SDS) and had a bone age of 6 years. Both patients were pre-pubertal (Tanner 1 stage). Physical examination was normal, except of short stature. They both had normal hemoglobin levels, blood chemistries and thyroid function tests. They had normal prolactin and normal thyrotrophin (TSH) response to thyrotrophin-releasing hormone (TRH; data not shown). Serum insulin-like growth factor-I (IGF-I) was low in both subjects: $20 \mu\text{g/l}$ (age-appropriate normal value (NV) $110\text{--}565$) in subject II-1 and $16 \mu\text{g/l}$ (NV $77\text{--}437$) in subject II-2.

Their GH secretion was evaluated at the time of presentation by sleep test (blood drawn every 30 min for 10 h during night-time sleep), arginine infusion (0.5 g/kg intravenously over 30 min), and L-dopa ($0.5 \text{ mg}/1.73 \text{ m}^2$ orally) plus propranolol (0.75 mg/kg orally). Serum GH levels were at all points below the sensitivity of the assay used at that time ($0.1 \mu\text{g/l}$), consistent with severe GH deficiency (GHD). Both

subjects were treated with GH replacement therapy (0.18 mg/kg per week), with good growth response. They underwent spontaneous puberty, and attained adult stature of 170.5 cm (subject II-1) and 166.8 cm (subject II-2). At adult age, 1 year after discontinuing exogenous GH therapy, subject II-1 had unmeasurable serum IGF-I ($<15 \mu\text{g/l}$, NV $182\text{--}780$) and low IGF-binding protein-3 (1.0 mg/l , NV $2.5\text{--}4.8$), confirming persistence of severe GHD. These tests were not done in subject II-2.

The subjects gave informed written consent for the studies described below. The protocol was approved by the local ethics committee.

Testing of GH secretion

GH secretion in both subjects (II-1 and II-2) was re-studied after they reached adult ages (20 and 18 years old respectively). They were both at Tanner stage 5 of sexual development and had normal body mass index: 25.0 and 26.6 respectively. Neither of them had taken exogenous GH for at least 1 month. They underwent arginine, GHRH and PE stimulation tests. Each GH test was performed on separate days after an overnight fast. An indwelling catheter was placed in a forearm vein. Clonidine was administered orally (0.15 mg/m^2) and blood samples were obtained at 0, 30, 60, 90 and 120 min. Arginine was administered as an intravenous infusion at the dose of 30 g over 30 min and blood samples were obtained at 0, 15, 30, 45, 60 and 90 min. 1–29 GHRH (Geref, Serono Norwell, MA, USA) $1 \mu\text{g/kg}$ was administered as an i.v. bolus and blood samples were obtained at 0, 15, 30, 45, 60, 90 and 120 min.

A standardized bicycle protocol was used for exercise testing (12). An indwelling catheter was placed in the forearm and blood was drawn for baseline GH. After subjects warmed up for 3 min, resistance was increased to keep heart rate at or above 70% of predicted maximum work rate. Both subjects cycled for more than 15 min to exhaustion. Blood samples were obtained immediately at the end of exercise, and 5, 10 and 20 min later.

Serum was immediately separated, and kept frozen until assayed. GH was assayed via commercially available immunoradiometric assay (Immulite Growth Hormone, DPC, Los Angeles, CA, USA) with a sensitivity limit of $0.05 \mu\text{g/l}$.

Several months later, the subjects underwent repeated GHRH and PE testing (on different days), following the same protocol described above. For this round of tests, serum GH was measured with an ultrasensitive assay, with a detection limit of $0.002 \mu\text{g/l}$, intra-assay coefficients of variation of 8.5%, and inter-assay coefficients of variation of 4.6% (13–15).

Figure 1 Pedigree of the family with mutations in the *GHRHR*. Squares denote male family members, ovals female family members. The white half symbols represent the wild-type allele; the gray half symbols represent the IVS3 + 1G $\rightarrow$ A mutation, and the horizontal line half symbols represent the Q43X mutation.

Amplification of the GHRHR and mutation detection

Genomic DNA was extracted from peripheral blood leukocytes by standard techniques. The 13 exons and the corresponding intron–exon boundaries and the proximal 327 bases of the promoter of the *GHRHR* from the index case (II-1) were individually amplified via PCR as in previous studies (6, 7).

PCR products were separated by electrophoresis through 8% acrylamide gels, and bands were isolated and sequenced directly. Sequencing was performed using the Amersham Thermo-Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After the identification of a mutation in the index patient, all other members of the family were genotyped via direct sequencing of the appropriate gene regions.

We then tested whether the newly identified mutations (see below) alter the migration pattern of the respective amplicon (amplified with one of the primers containing a 5' GC-rich 40-base tail) when analyzed by denaturing gradient gel electrophoresis (DGGE) (16, 17). As they both did, we used DGGE to test the prevalence of the newly discovered changes in a commercial panel of genomic DNAs from 44 normal subjects (88 chromosomes) obtained from the DNA Polymorphism Discovery Resource, which includes DNA from anonymous unrelated individuals with diverse ethnicity (18).

Results

Mutations identification

We found two heterozygous nucleotide changes in the *GHRHR* of the two affected boys when compared with the normal sequence (19). One is a C to T transition that results in substitution of glutamine (CAA) in position 43 with a stop codon (TAA) (Q43X) (Fig. 2),

which was also found in heterozygous fashion in the subjects' mother and in one of the normal stature siblings (II-4).

The second mutation, a G to A transition located in the splice donor site at the beginning of intron 3 (IVS3 + 1G → A) (Fig. 3), was also found in heterozygous fashion in the subjects' father and in the other normal sibling (II-3).

The G nucleotide of the splice donor site (GT) is conserved in 100% of mammalian genes and mutations are consistently associated with a non-functional allele (20). The IVS3 + 1G → A mutation is predicted to cause the retention of part or all of intron 3 and a severe alteration of protein structure. No potential cryptic splice site has been identified downstream of the normal donor splice site, and an in-frame TAG stop codon is located 123 nucleotides from the end of the exon 3 coding region.

Neither of these two new mutations was found in 88 normal chromosomes from the Polymorphism Discovery Resource (18), screened by DGGE.

GH testing

In both subjects, GH remained below the assay sensitivity at all points during arginine, clonidine and GHRH testing when tested with an immunoradiometric assay with a detection limit of 0.05 µg/l. By contrast, PE caused a detectable increase in GH secretion in both subjects at all points after exercise completion (Table 1, upper rows). This increase is significantly lower than the increase reported for normal adult subjects (peak GH after PE = 7.8 ± 1.3 µg/l) (21).

We then repeated GHRH and PE testing, and measured the GH response using an ultrasensitive GH assay. Both subjects' serum GH was confirmed to be above 0.05 µg/l at all points after PE. However, although in both subjects the absolute GH peak after exercise was higher than after GHRH stimulation, the

Figure 2 Sequence analysis of a portion of exon 2 of the *GHRHR* from genomic DNA of the index patient (PATIENT). The normal sequence (CONTROL) is from an unrelated normal subject. The heterozygous C to T transition is indicated by the arrow.

Figure 3 Sequence analysis of a portion of exon 3–intron 3 junction of the *GHRHR* from genomic DNA of the index patient (PATIENT). The normal sequence (CONTROL) is from an unrelated normal subject. The heterozygous G to A transition is indicated by the arrow. The exon 3 sequence is shown in capital letters, and the intron 3 sequence in lower case letters.

difference between the two stimuli was much less evident, with individual peak GH levels that for patient II-2 were quite similar during the two tests (Table 1, lower rows).

Radiological evaluation

Magnetic resonance imaging (MRI) of the sellar region in both patients revealed hypoplastic anterior pituitary glands (2.5 and 3 mm in height respectively), similar to what has been described in patients with other homozygous null mutations of the *GHRHR* (7, 11, 22, 23).

Discussion

Mutations in the *GHRHR* are being recognized with increasing frequency in patients with familial IGHD. So far, one nonsense, five missense, two splice mutations, two small deletions (one of them with possible dominant negative effect), and a promoter mutation have been reported (3–11). In most families, parents of affected subjects are either consanguineous or are connected by common ancestry even when they live in different geographical areas (24). Our two

patients have no family history of consanguinity. Accordingly, they are compound heterozygotes for two different, previously undescribed, mutations in the *GHRHR*. The first one is a nonsense mutation in exon 2 (Q43X), which, if mRNA is translated, would cause the generation of a severely truncated protein (only containing a small fragment of the extracellular domain). The second one is a splice site mutation (IVS3 + 1G → A) at the beginning of intron 3. The IVS3 + 1G → A mutation alters the first base of the splice donor site (GT), which is conserved in 100% of mammalian genes (20). As a consequence, it is predicted that part or the entire intron 3 is retained in the mature mRNA. An in-frame stop codon localized in intron 3 (123 bases from the mutation) would cause protein truncation. Although a *GHRHR* splice variant lacking exon 1–3 coding sequences has been described in prostate cancer cells and in a pituitary adenoma (25), it is unlikely that this variant would be expressed in the patient's pituitary, as mRNAs containing premature stop codons are likely to be degraded by nonsense-mediated mRNA decay (26). Therefore, both of these two mutations are likely to cause total absence of the *GHRHR* protein. The absence of either mutation from 88 normal chromosomes demonstrates that they are not highly prevalent in the general population.

Table 1 Serum GH response (in $\mu\text{g/l}$) to PE and GHRH in two sibs (II-1 and II-2) with mutated *GHRHR* analyzed by an IRMA with a sensitivity limit of 0.05 $\mu\text{g/l}$ (upper rows), and by a chemiluminescence ultrasensitive assay (CLU) with a sensitivity limit of 0.002 $\mu\text{g/l}$ (lower rows). Pre = pre-exercise; End = end of maximal exercise. Each assay relates to tests done on separate days.

	PE					GHRH						
	Pre	End	5 min	10 min	20 min	0 min	15 min	30 min	45 min	60 min	90 min	120 min
IRMA												
II-1	<0.05	0.14	0.13	0.11	0.08	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
II-2	<0.05	0.26	0.36	0.26	0.22	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
CLU												
II-1	0.036	0.112	0.106	0.088	0.060	0.022	0.022	0.023	0.024	0.033	0.077	0.038
II-2	0.007	0.109	0.132	0.130	0.101	0.061	0.027	0.014	0.008	0.006	0.088	0.104

The phenotype in our patients closely resembles previously described patients with different null *GHRHR* mutations, including severe GHD and MRI evidence of anterior pituitary hypoplasia, likely due to reduced somatotroph cell mass similar to findings in the *little* mouse, a murine model of *GHRHR* gene mutation (27).

We have previously reported that two sibs who are compound heterozygotes for two missense mutations in the *GHRHR* presented a notable GH increases after PE (peak GH 14.3 and 13.9 µg/l), despite frankly subnormal GH responses to insulin-induced hypoglycemia (peak GH 3.8 and 2.3 µg/l despite frank hypoglycemia) and absent night-time GH peaks (7). However, these patients were studied in the 1970s, using an old polyclonal GH radioimmunological assay with limited sensitivity and specificity (28), and the exercise test was not standardized. Furthermore, missense mutations may allow partial residual receptor function. Conversely, the patients described in this report are predicted to completely lack functional *GHRHR* protein. Therefore, the analysis of their GH response to standardized PE by a sensitive modern assay may help to shed some light on the mechanism by which PE causes GH secretion, as it has been speculated that PE may act by removing somatostatin inhibition on somatotroph cells rather than by increasing hypothalamic GHRH secretion (29–31). The limitation of this model is the severely decreased somatotroph mass that greatly reduces the potential of their pituitaries of producing GH. However, patients lacking functional *GHRHR* have detectable GH pulses and a detectable rise in serum GH to acute stimulation with the GH secretagogue GHRP-2, proving that their somatotrophs (although reduced in number) are properly differentiated and able to respond to stimuli that may, at least in part, not depend on an intact GHRH pathway (10, 32, 33).

We found that, using an IRMA assay with a sensitivity of 0.05 µg/l, serum GH levels were below detection at all points in both patients after GHRH or arginine or clonidine. These three tests sum to a total of 15 post-stimulus points per patient, although it must be kept in mind that clonidine is a notoriously poor GH stimulant in adults (34). By contrast, GH was detectable (albeit low) at all four points after maximal PE. When we sought to confirm these findings by repeating the GHRH and PE testing measuring GH by ultrasensitive chemiluminescence assay, we found that the GH was confirmed to be above 0.05 µg/l at all points after PE, and the absolute GH peaks were higher after PE than after GHRH (0.112 vs 0.077 and 0.132 vs 0.104 respectively). However, the difference between response to PE and GHRH was not fully confirmed. In the repeated GHRH testing, both subjects had one or more time points (90 min for II-1, baseline, 90 min and 120 min for II-2) that were above 0.05 µg/l, partially invalidating the results of the less sensitive assay. In addition, as the peak after GHRH in subject

II-2 is reached at the last time point (120 min), we cannot rule out the possibility that, had we continued the test for a longer time, his serum GH level would have further increased. It is likely that that these 'post-GHRH' peaks are actually unrelated to GHRH injection, and are just an expression of spontaneous GH pulsatility (10, 33). This is less likely for the peaks reached after exercise, as the peaks' profiles are very similar for the two patients, and are sustained for several minutes after completion of PE.

Arginine is believed to act by removing somatostatin's influence on somatotroph cells rather than stimulating GHRH secretion (35). No GH response to arginine (measured by IRMA) was detected in our patients, arguing against the hypothesis that PE works via the same pathway. Regrettably, no sera are available to repeat the measurements using the ultrasensitive assay.

Although these results do not allow us to draw any definitive conclusion, this initial observation creates the rationale for studying GH response to PE in larger kindreds with mutated *GHRHR*, such as the ones from Brazil and Pakistan (5, 6), where the high number of subjects may allow us to determine if any difference in GH secretion after PE and chemical secretagogues is real.

In conclusion, we have described two brothers with IGHD caused by compound heterozygosity for two new null mutations in the *GHRHR*. These findings indicate that faulty *GHRHR* alleles may be quite prevalent, that *GHRHR* mutations need to be considered in IGHD IB patients even in the absence of parental consanguinity, and suggest that patients lacking functional *GHRHR* may provide a natural model to study the mechanism by which PE influences GH secretion.

Acknowledgements

We wish to thank the patients who volunteered their time for the studies. This work was supported by NIH-NCRR GCRC-CAP award 3 M01 RR000052-38S1 (R S), and by a grant from the Genentech Center for Clinical Research and Education (R S).

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Received 3 April 2002

Accepted 29 May 2002