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The Serum Growth Hormone (GH) Response to Provocative Tests Is Dependent on Type of Assay in Autosomal Dominant Isolated GH Deficiency because of an ARG183HIS (R183H) GH-I Gene Mutation, Roxana Marino, Eduardo Chaler, Monica Warman, Marta Ciaccio, Esperanza Berenzweig, Marco A. Ricardo, and Alicia Belgorovsky (Research Laboratory and Endocrinology Service, Garrahan Pediatric Hospital, Buenos Aires, Argentina 1245; * address correspondence to this author at: Coordinación de Investigación, Hospital de Pediatria Garrahan, C. de los Pozos 1881-I2, Buenos Aires, Argentina 1245; fax 5411-4308-5325, e-mail abelgo@elsitio.net)

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We sequenced GH-1 (16). The gene was specifically PCR-amplified with use of sense and antisense primers corresponding to nucleotides 5101–5136 (gh1) and the complement of nucleotides 7255–7226 (gh2). The resulting GH-1 PCR product (2155 bp) was used as template for a nested PCR. For a description of the primer composition, see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue6/.

We studied two prepubertal girls (P1 and P2) who presented severe short stature (below −3 SDS) and poor growth rate. Their main anthropometric characteristics are listed in the Data Supplement. They were monitored for 1 and 6 years, respectively, without any improvement in height. Characteristic clinical features in the absence of organic disease, skeletal dysplasia, or eating disorders and in the presence of normal intestinal, renal, and hepatic function, suggested GHD. Tests of corticotropin and thyrotropin function were normal. Serum IGF-I and IGF-binding protein-3 (IGFBP-3) values were low: serum IGF-I was 2.49 and 4.08 μg/L and serum IGFBP-3 was 0.65 and 0.6 mg/L in P1 and in P2, respectively. Several family members had severe short stature, including one parent of each index case. The pedigrees of the two nonconsanguineous families are shown in the Data Supplement. The study was approved by the Internal Review Board of the Garrahan Pediatric Hospital.

In the two patients, GH secretion was evaluated after two pharmacologic tests of provocative secretion by determining serum GH in the same samples with use of the assays for total and 22-kDa GH. The maximum response of serum total GH in P1 and P2 was 10.9 and 10.4 μg/L, respectively, above the cutoff for normal response. These results did not support the diagnosis of GHD. However, the test was reassessed by determining the serum concentration of 22-kDa GH in the same samples. Maximum responses were 2.5 and 1.5 μg/L, respectively, below the cutoff for a normal response. In contrast to the first assay, results from the second assay supported the diagnosis of GHD.

P1 and P2 were treated with 0.17 mg of recombinant human GH (rhGH) per kg of body weight per week for 1.7 and 3.7 years, respectively. For both patients, the height SDS improved markedly after treatment.

The time course of serum GH response in P1 and P2 to clonidine as determined by two immunoassays and the bioassay is shown in Fig. 1. The relative pattern of the response was similar for the three assays, but the response was above the cutoff limit when serum total GH was assayed, whereas it was below the cutoff when 22-kDa GH or GH bioactivity was determined.

DNA sequence analysis of PCR amplification products of the GH-1 gene is shown in the Data Supplement. A heterozygous point mutation in exon 5, which causes an Arg-to-His mutation at position 183 of the GH protein, was present in both P1 and P2. The father of P1 and the mother of P2 carried the same GH-1 mutation. Furthermore, we found two additional base changes in intron 1, at positions +52 (A-G) and +56 (A-T), in all affected members.

<table>
<thead>
<tr>
<th>Day</th>
<th>P1</th>
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<tr>
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*Normal minimum ΔIGF-1 is 15 μg/L. Median (range) in nine patients with idiopathic GHD was 39.3 (21.1–85.0) μg/L.
We report here on two prepubertal girls, belonging to two different families, with severe short stature and autosomal dominant IGHD. The initial work up was misleading because they had normal responses to two provocative tests of GH secretion, as measured by the SER 66/217 GH assay. Although we used stimulation tests that are weak for adults (17), it is accepted that they are informative tests in children (18). We have used these tests in children for more than 20 years with good results (11). We have found that our patients responded normally to stimulation tests when total GH was determined but below normal when 22-kDa GH or bioactive GH was determined. Our data indicate that secretion of the active 22-kDa GH isoform is diminished and that, consequently, a high proportion of non-22-kDa GH molecular forms circulates in the blood. Apparently these molecular forms have poor biological activity, as indicated by the Nb2 cell proliferation bioassay.

In our study, the two patients failed to respond to an acute IGF-I generation test. The reliability of the IGF-I generation test has been questioned because of lack of reproducibility (19) and overlapping responses in GH-insensitive and GHD patients (7). However, short-term increments of IGF-I predict growth response to GH therapy (20). Moreover, because positive responses are found in most GHD patients (7), some degree of insensitivity to GH was present in our two patients. As in a patient reported by others (21), our patients responded to treatment with increased growth velocity and serum IGF-I after prolonged rhGH treatment. This suggests that there was a reversible inhibition of GH biological action, i.e., prolonged exogenous rhGH might have overcome GH insensitivity by decreasing secretion of all endogenous GH isoforms. The molecular mechanism of the dominant-negative effect is unknown. As suggested by Wada et al. (22), the altered 22-kDa GH/non-22-kDa GH isoform ratio in blood could impair receptor dimerization. The 20-kDa GH isoform has low affinity for GHR site 1, forming no detectable 1:1 complex but forming a 1:2 complex efficiently. Different GH analogs and fragments may interact as weak antagonists or agonists of the GHR, depending on the relative affinities of sites 1 and 2 to the receptor (23).

Our study showed that affected members of the two families carry the R183H point mutation in exon 5. We also detected two additional point mutations at positions +52 and +56 of intron 1. This novel finding suggests that genotype and phenotype variations exist among different families. These intronic mutations could represent normal polymorphism variants, or they might determine alternative splicing, generating GH isoforms that could contribute to changes in the ratio between 22-kDa GH and non-22-kDa GH.

We conclude that the evaluation of serum GH response to provocative stimuli depends on the assay selected to measure serum GH and suggests that altered circulating GH isoforms may be important in defining the GHD phenotype in these patients. GH isoforms might have a reversible dominant-negative effect at the GHR level.

Impaired GH secretion may present also, as suggested by the absence of GH hypersecretion. Interestingly, the dominant-negative effects were overcome by administration of rhGH at a dose similar to that used to treat nonfamilial GHD.

We thank the National Pituitary Agency for the generous supply of IGF-I RIA reagents. This work was supported by grants from CONICET, FONCYT, and Ministerio de Salud (Beca Carrillo-Oñativia) of Argentina and from Pharmacia Endocrine Care International Fund.

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**Fasting and Post-Methionine Loading Concentrations of Homocysteine, Vitamin B2, and Vitamin B6 in Patients on Antiepileptic Drugs, Terje Apeland,1* Mohammad Azam Mansoor,2 Kristina Pentieva,4 Helene McNulty,4 and Roald E. Strandjord1**

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Patients on antiepileptic drugs (AEDs) frequently have low serum folate and high plasma total homocysteine (tHcy) (1–4). This metabolic disturbance has been implicated in an increased rate of cardiovascular disease, fetal malformations, dementia, and neuropsychiatric symptoms among patients on AEDs (5–7). Homocysteine metabolism is dependent on four B vitamins as cofactors: The methylation of homocysteine to methionine requires folate and vitamin B12, and the irreversible transsulfuration to cysteine requires vitamin B6 (8). The recycling of folate cofactors is dependent on vitamin B6 and B12, and vitamin B6 is necessary for activating vitamin B12 to pyridoxal 5'-phosphate (PLP) (9). Vitamin B12 and B6 status thus may have an impact on tHcy concentrations in patients taking AEDs, but few studies have reported on this matter (3, 4, 10, 11). The methionine loading test detects individuals with impaired homocysteine metabolism not detected by fasting Hcy concentrations alone and is often considered primarily a test of the transsulfuration pathway (1, 8, 12). We therefore determined the fasting and 6 h post-methionine loading (postload) plasma concentrations of thiol and B vitamins in patients taking AEDs.

We recruited 101 patients with symptomatic, cryptogenic, or primary generalized epilepsy from our outpatient clinic. None of them had epilepsy secondary to ischemic stroke or other conditions considered to be associated with high tHcy, and none were on prescribed vitamin supplements. We recruited 101 controls among blood donors and hospital employees. All participants gave written informed consent before entering the study, and The Regional Ethics Committee (University of Bergen, Norway) approved the study. The number of male patients and controls was identical (n = 53), as was the mean (SD) age [37.9 (15.0) and 37.3 (10.3) years for patients and controls, respectively; difference not significant]. There were 34 and 30 smokers among patients and controls, respectively (difference not significant). The patients had been on AEDs for 13 (11) years, and their medication had been unchanged the last 6 months before inclusion. Of the 101 patients, 43 were on carbamazepine monotherapy (group 1) and 24 were on valproate monotherapy (group 2); 21 received phenytoin, phenobarbital, and/or primidone (group 3); and 13 were on various combinations of the five AEDs (group 4). Twenty-four patients reported supplementing their diet with over-the-counter multivitamins: 10 in group 1, 3 in group 2, 7 in groups 3 and 4, and 4 in group 4. The controls were not stratified for intake of over-the-counter multivitamins. Seven patients and eight controls were homozygous (TT genotype) for the C677T mutation of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, whereas 34 patients and 42 controls were heterozygous (CT genotype; difference not significant) (1, 2, 13).

Fasting blood samples were collected in the morning after an overnight fast. Thereafter, the participants were given L-methionine (100 mg/kg of body weight) and a standardized breakfast (13). A postload blood sample was collected 6 h later. The vials were immediately cooled on ice, protected from daylight, centrifuged at 2000g for 10 min at 4 °C, and stored at −70 °C until analysis. The plasma concentrations of tHcy, cysteine, and cysteinylglycine were determined by HPLC (14). For the determination of vitamin B2 status, plasma concentrations of riboflavin and flavin nucleotides (FNs; i.e., the combined concentration of the coenzymes FAD and flavin mononucleotide) were determined by a modified HPLC method; CVs were <6% and <4%, respectively (15). Plasma PLP and pyridoxic acid were determined by HPLC (16). The serum concentrations of folate and cobalamin were determined by immunoassay (Access; Beckman Instruments). Plasma concentrations of thios and vitamins did not follow a gaussian distribution as raw data; they therefore were log-transformed before calculations and are presented as geometric means with 95% confidence intervals. Before logistic regression analysis, continuous variables were divided into five groups corresponding to the quintiles. Statistical significance was set at P < 0.05.

In the total sample, both fasting and postload, the mean plasma concentration of riboflavin (i.e., the precursor of FNs) was similar in patients and controls (Table 1). However, analysis of patient (sub)groups and controls showed that fasting riboflavin concentrations were borderline lower in the patients in groups 1 and 3 (Table 1). When the patients on inducer AEDs (groups 1 and 3; n = 64) were analyzed as a single group and compared with controls, they had a low mean fasting riboflavin concentration (4.8 nmol/L; 95% confidence interval, 3.9–5.8 nmol/L; P = 0.02). Logistic regression analysis of patients and controls revealed that fasting riboflavin concentrations below the 20th percentile were more likely to