

Growth Hormone-Releasing Hormone Resistance in Pseudohypoparathyroidism Type Ia: New Evidence for Imprinting of the $Gs\alpha$ Gene

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Heterozygous inactivating mutations in the $Gs\alpha$ gene cause Albright's hereditary osteodystrophy. Consistent with the observation that only maternally inherited mutations lead to resistance to hormone action [pseudohypoparathyroidism type Ia (PHP Ia)], recent studies provided evidence for a predominant maternal origin of $Gs\alpha$ transcripts in endocrine organs, such as thyroid, gonad, and pituitary. The aim of this study was to investigate the presence of pituitary resistance to hypothalamic hormones acting via $Gs\alpha$ -coupled receptors in patients with PHP Ia. Six of nine patients showed an impaired GH responsiveness to GHRH plus arginine, consistent with a complete GH deficiency (GH peak from 2.6–8.6 $\mu\text{g/liter}$,

normal > 16.5), and partial (GH peak 13.9 and 13.6 $\mu\text{g/liter}$) and normal responses were found in two and one patient, respectively. Accordingly, IGF-I levels were below and in the low-normal range in seven and two patients. All patients had a normal cortisol response to 1 μg ACTH test, suggesting a normal corticotroph function that was confirmed by a normal ACTH and cortisol response to CRH test in three patients. In conclusion, we report that in addition to PTH and TSH resistance, patients with PHP Ia display variable degrees of GHRH resistance, consistent with $Gs\alpha$ imprinting in human pituitary. (*J Clin Endocrinol Metab* 88: 4070–4074, 2003)

PSEUDOHYPOPARATHYROIDISM (PHP) REFERS to a heterogeneous group of rare metabolic disorders characterized by hypocalcemia and hyperphosphatemia caused by PTH resistance (1, 2). Heterozygous loss of function mutations in stimulatory guanine nucleotide binding protein α -subunit gene inherited from the mother leads to PHP type Ia (PHP Ia). PHP Ia is a disease in which Albright's hereditary osteodystrophy (AHO), a disorder characterized by a constellation of physical features (short stature, obesity, round face, brachydactyly, and sc calcifications), is associated with end organ resistance to the action of different hormones that activate Gs-coupled receptors, primarily PTH, TSH, and gonadotropins (reviewed in Refs. 3–5 and the Web site: <http://mammary.nih.gov/aho/>). Interestingly, when the same mutations are inherited from the father, patients show the physical abnormalities of AHO, without evidence of hormone resistance (pseudo-pseudohypoparathyroidism) (6, 7). This pattern of inheritance is consistent with a tissue-specific $Gs\alpha$ paternal allele imprinting, an epigenetic phenomenon by which the paternal allele undergoes partial or total loss of expression (8, 9). The human $Gs\alpha$ gene *GNAS* maps on chromosome 20q13, and there is increasing evidence that this locus is under complex imprinting control with multiple maternally, paternally, and biallelically alternatively spliced

transcripts encoding multiple products in humans and animals (10–14). Considering the PHP Ia phenotype, $Gs\alpha$ gene imprinting is predicted to be limited mainly to tissues, such as the renal proximal tubule, thyroid, and gonad, in which there is a parent-of-origin-specific difference in hormone responsiveness (15–17). Indeed, although previous expression studies failed to demonstrate monoallelic $Gs\alpha$ expression in various human fetal tissues (18, 19), recent reports demonstrated that in the thyroid, gonad, and pituitary $Gs\alpha$ transcription mainly derives from the maternal allele (20–22).

Because the pituitary is not classically included among the target organs resistant to hormone action in PHP Ia, the aim of the study was to evaluate the presence of pituitary defects involving pituitary hormones, primarily GH and ACTH that are regulated by neuropeptides acting through $Gs\alpha$ -coupled receptors, in patients with PHP Ia.

Patients and Methods

Patients

The study included nine patients affected with PHP Ia, six of whom have already been described in a previous report by our group (23). Four of these patients are related to each other: in particular, patients I and II are mother and daughter, respectively, and patients III and IV are siblings. In all patients (seven females and two males, aged 7–51 yr), the diagnosis was based upon the occurrence of AHO manifestations together with PTH resistance (*i.e.* hypocalcemia, hyperphosphatemia, and raised serum PTH levels) and hypothyroidism because of resistance to TSH (documented by raised serum TSH levels with an exaggerated response to TRH, in the absence of antithyroid antibodies and in the

Abbreviations: AHO, Albright's hereditary osteodystrophy; BMI, body mass index; IGFBP, IGF-binding protein; PHP, pseudohypoparathyroidism; PHP Ia, PHP type Ia.

presence of a normal thyroid scan). Oligoamenorrhea was present in three of the four females in the postpubertal age. The required therapy (calcium, vitamin D, L-thyroxine, and hormone replacement therapy) was not discontinued during the study. Dysarmonic short stature (height below the fifth percentile for chronological age in all patients except for patient VI, whose height was at the 20th percentile; see Table 1 for details) and overweight [body mass index (BMI), ranging from 25.0–29.5 kg/m², median 27.05 ± 1.8 kg/m², with the exception of patient VII, whose BMI was 19.4 kg/m²] were present in all patients as part of AHO features. No patient had signs or symptoms of adrenal insufficiency. Clinical and biochemical details are shown in Table 1. Informed consent was obtained from all subjects involved in the study. The study was approved by the local Ethical Committee.

Methods

Sequencing analysis

Genomic DNA was extracted with the phenol-chloroform method from peripheral blood leukocytes (Nucleon-Amersham Life Science, Aylesbury, UK). The Gsa gene (exons 1–13, GenBank accession no. AH002748) was then amplified by PCR using the specific primers previously described (23). Amplification of exons 2–12 included each bordering intron region, and for exon 1, because of the abundance of guanine and cytosine in the bordering regions, a DNA fragment from 20 bp downstream of the initiation codon to the donor site of intron 1 was amplified. Direct sequencing of the amplified fragments was then performed using the AmpliTaq BigDye Terminator kit and 310 genetic analyzer (Perkin-Elmer Corp., Applied Biosystems, Foster City, CA).

Testing

All subjects were tested with GHRH (GHRH 1–29; Geref, Serono, Italy; 1 µg/kg body weight iv at time 0) plus arginine (0.5 g/kg body weight, L-ARG monohydrochloride iv, from time 0 over 30 min). Blood samples were drawn from an indwelling catheter inserted in an antecubital vein at –15 min and times 0, 15, 30, 45, 60, and 90 min. All studies started between 0800 and 0900 h after overnight fasting according to the accepted criteria (24–26): normal GH peak after stimulation (>16.5 µg/liter); partial GH deficiency (9–16.5 µg/liter); complete GH deficiency (<9 µg/liter). On another day, patients were subjected to a low-dose ACTH test (Synacthen, Novartis, Origgio, Italy; 1 µg iv at time 0) with cortisol evaluation at times –30, 0, 30, 40, and 60 min. Normal values of cortisol peak after ACTH injection were more than 500 nmol/liter. In three patients CRH (ovine-CRH, Calbiochem, 1 µg/kg body weight iv at time 0) was injected, and ACTH and cortisol levels were determined at times –30, 0, 20, 30, 60, 90, and 120 min.

Assays

Serum GH levels were measured by an immunofluorimetric assay method supplied by AutoDelfia (Wallac OY, Turku, Finland). The sensitivity is 0.01 µg/liter, the intra- and interassay coefficients of variation are 2% and 1.7%, respectively. Serum IGF-I was measured by a RIA (commercial kit supplied by Medagnost, Tübingen, Germany) able to measure total IGF-I by separating IGF-I from IGF-binding protein (IGFBP) by acidification in IGF-II excess. IGF-II cross-reactivity was less than 0.05%; the intra- and interassay coefficients of variation was 3.2% and 7.4%, respectively. Plasma ACTH and serum cortisol levels were measured by immunoluminometric assay and luminescence immunoassay methods, respectively (Nichols Institute, San Juan Capistrano, CA). The sensitivity was 0.22 pmol/liter and 22 nmol/liter, respectively, and the inter- and intraassay coefficients of variation were less than 10% and less than 4.8%, respectively, for both methods. Serum IGFBP3 levels were measured by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX). The sensitivity was 0.004 nmol/liter, and the intra- and interassay coefficients of variation were 3.25 and 5.6%, respectively.

Results

Mutational analysis

Direct sequencing of the amplified genomic DNA fragments revealed heterozygous frameshift mutations in seven

TABLE 1. Clinical, biochemical, and molecular data of PHP Ia patients included in the study

Patient	Sex	Age (yr)	BMI (kg/m ²)	Height (cm)	Target height (cm)	PTH (pg/ml) (nv, 10–65)	TSH (mU/liter) (nv, 0.3–4.2)	GH peak (µg/liter) (nv > 16.5)	IGF-I (nmol/liter) (nv)	Cortisol peak (nmol/liter) (nv > 500)	Gsa gene mutations
I	F	51	29.5	148 (–2.3 SDS)	160 (–2.1 SDS)	219	4.7	3.1	12.1 (13–38)	582	Exon 11, 2-bp deletion
II	F	22	29	146 (–2.6 SDS)	151.5 (–0.9 SDS) ^a	87	8.2	3.0	8.9 (15–44)	556	Exon 11, 2-bp deletion
III	F	9 _{8/12}	25.0	125 (–1.5 SDS)	149.9 (–0.6 SDS) ^a	346	12.6	8.2	8.9 (13–48.7)	593	Exon 1, 1-bp deletion
IV	F	29	26.1	139 (–3.7 SDS)	164 (–4.1 SDS) ^a	198	9.6	13.9	22 (15–44)	549	Exon 5, 1-bp deletion
V	M	22	28.9	158.5 (–2.5 SDS)	177 (–2.7 SDS) ^a	250	11.5	25	15.0 (15–44)	772	Exon 5, 1-bp deletion
VI	F	8 _{9/12}	25.1	124 (–0.7 SDS)	175 (–2.7 SDS)	263	5.4	13.6	11.1 (13–48.7)	624	Exon 7, 4-bp deletion
VII	M	8 _{4/12}	19.4	119 (–1.6 SDS)	176.5 (–1.8 SDS)	303	7.7	8.2	12.2 (13–48.7)	657	Intron 2, 4-bp deletion splicing donor site
VIII	F	20	26.5	150 (–2.0 SDS)	164.5 (–2.4 SDS)	188	75.7	8.6	14.3 (15–44)	589	None
IX	F	7 _{7/12}	26.3	112 (–2.2 SDS)	143 (1.0 SDS) ^a	923	83	2.6	9.7 (13–48.7)	600	None

F, Female; M, male; nv, normal values; SDS, SD score.

The age and height refer to the actual ones (when pituitary secretion was evaluated). PTH and TSH levels refer to the time of diagnosis. GH and cortisol peaks refer to the highest values after respective stimulation tests (GHRH plus arginine and 1 µg ACTH).

^a Patient whose mother is affected with PPHP and thus with short stature.

of nine patients. In particular, mutations were found in six affected members of the four families previously described (23): a 4-bp deletion in exon 7, a 1-bp deletion in exon 5, a 1-bp deletion in exon 1, and a 2-bp deletion in exon 11, all causing a premature stop codon in the mutant allele, thus giving rise to a truncated nonfunctional protein. Moreover, a novel 4-bp deletion in intron 3 involving and eliminating the donor splicing site was detected in patient VII. No mutations in the protein-coding region of the gene were found in patients VIII and IX.

Endocrine testing

The mean GH response after GHRH plus arginine was significantly lower in the patient population ($9.5 \pm 7.1 \mu\text{g/liter}$; range 2.6–25), compared with the response in the normal population ($>16.5 \mu\text{g/liter}$) (Fig. 1). In particular, six patients showed an absent/blunted GH response (peak $< 9 \mu\text{g/liter}$, normal values $> 16.5 \mu\text{g/liter}$) that is considered indicative of complete GH deficiency, and two patients (IV and VI) showed a peak of 13.9 and 13.6 $\mu\text{g/liter}$, respectively, indicative of partial deficiency (24–26). Patient V had a normal GH response (GH peak 25 $\mu\text{g/liter}$) (Table 1). The presence of a complete or partial GH deficiency in these patients was confirmed by the determination of IGF-I levels that were below the normal limits according to age and sex in seven patients and in the low-normal range (22.2 nmol/liter; normal values 15–44) in patients IV and V (Table 1). Determination of IGFBP3 showed low-normal levels in all of them, ruling out any elevation that might account for the reduced IGF-I levels. It is worth noting that patients IV and V were siblings, probably indicating the presence in this family of a genetic background somehow protective with respect to GH secretion.

Basal serum ACTH and cortisol levels were within the normal range. Cortisol response elicited by 1 μg ACTH injection was normal in all patients (Table 1) (normal response $> 500 \text{ nmol/liter}$). Similarly, in the three patients tested (I, II, IV), CRH injection caused a normal increase in both plasma ACTH ($\Delta\% = 85, 330, 870$, respectively, normal

value > 50) and cortisol ($\Delta\% 25, 40, 212$, respectively, normal value > 20).

Discussion

The present study provides evidence for the presence of GH deficiency, probably because of resistance to the action of GHRH, in patients with PHP Ia. The study was performed on a series of patients with PHP Ia who displayed the typical AHO features together with PTH and TSH resistance. By analyzing the entire *Gs α* gene-coding sequence, heterozygous mutations were detected in seven of nine patients (77%), consistently with previous mutational analysis (27). To evaluate the presence of resistance to GHRH, these patients were submitted to a combined provocative test, that explores the maximal secretory response to GHRH occurring in association with the inhibition of somatostatin release by arginine infusion (24, 25). This test, which is one of the most reliable tests to distinguish normal subjects from GH-deficient patients during the whole lifespan (24–26), showed impaired responsiveness to GHRH in almost all patients. Although it is well known that obesity (BMI $\geq 30 \text{ kg/m}^2$) is associated with low spontaneous and stimulated GH secretion (28, 29), it is very unlikely that the severely reduced GH secretion observed in PHP Ia patients may be due to their increased body mass because they were only overweight (BMI between 25 and 29.9 kg/m^2), as typically occurs in AHO syndrome. Moreover, the presence of GH deficiency was confirmed by the low IGF-I levels found in these patients.

Consistent with a normal pituitary-adrenal function, all patients had normal cortisol response to 1 μg ACTH test, a low dose that appears to detect mild forms of secondary hypoadrenalism (30, 31). Moreover, the marked ACTH and cortisol response to CRH test seems to rule the occurrence of resistance to CRH in PHP Ia patients, although criteria for normal ACTH responses to the peptide are lacking.

Our data on GH deficiency in patients with PHP Ia are in agreement with some anecdotal case reports (32–34) and in contrast with those by Faull *et al.* (35), who described a large family with five affected members who all displayed normal GH responses to a variety of stimuli. It is possible that a specific mutation able to prevent *Gs α* binding to some receptors but not to others, as it has already been demonstrated in one family (36), could explain why in this particular family *Gs α* binding to the GHRH receptor appears to be normal, despite a defective coupling to PTH and TSH receptors. Alternatively, in this family a protective genetic background may be hypothesized, in analogy with the two siblings here reported.

The demonstration of GH deficiency in patients with PHP Ia provides new support to the view that tissue-specific imprinting of *Gs α* may be the potential mechanism responsible for end-organ resistance to the action of different hormones that characterizes this disease. Indeed, recent reports demonstrated that in the thyroid and gonad, *Gs α* transcription mainly derived from the maternal allele (21, 22), thus explaining why only patients with maternally inherited mutations are resistant to TSH and gonadotropins. The observation of an almost exclusive maternal origin of *Gs α* transcription in the human normal pituitary (20, 21) triggered

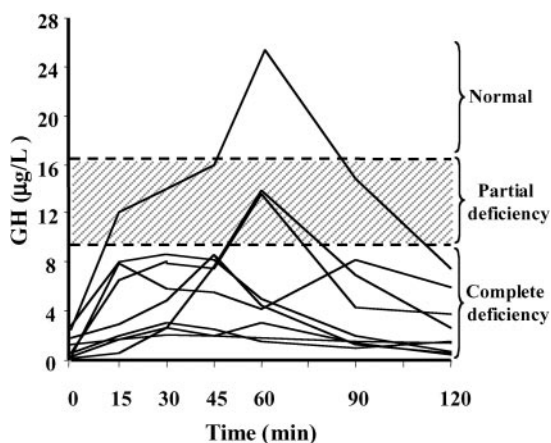


FIG. 1. GH response to GHRH plus arginine injection in the nine PHP Ia patients. The shaded area indicates partial GH deficiency (GH peak between 9 and 16.5 $\mu\text{g/liter}$). As described in *Results*, except for one patient, who showed a normal GH response to stimuli, all the other patients displayed an impaired GH response.

the question why this gland was not classically included among the target organs resistant to hormone action in PHP Ia patients. Indeed, because it is well established that both GHRH and CRH mediate their action on hormone secretion and cell differentiation by activating specific receptors that are coupled with G_{α} , the maternal origin of G_{α} transcription in the pituitary would be expected to result in impaired function of somatotroph and/or corticotroph in PHP Ia patients. Our data demonstrating a defective GH secretion and normal hypothalamic-pituitary-adrenal axis are only apparently in contrast with this finding of G_{α} imprinting in the pituitary taken as a whole. In fact, the predominance of the maternally derived transcript observed in pituitary tissues occurred at a variable extent (21), probably because of the cellular heterogeneity of this gland. Taking into account that somatotroph cells account for about 50% of the adenohypophysial population and corticotrophs represent no more than 10–15% (37), it is tempting to speculate that G_{α} imprinting was indeed present in the somatotroph population, but not in the corticotroph cells, in which the maternal and paternal allele would be equally represented but contributed to a lesser extent to the overall expression of the gene in the total gland. This interpretation is also consistent with the lack of reports of overt or subclinical hypoadrenalism in PHP Ia patients.

To date, the relevancy of GH deficiency on final height in patients with PHP Ia is unknown. However, taking into consideration that patients with pseudo-pseudohypoparathyroidism, although not displaying any endocrine abnormality, are characterized by short stature as their relatives with PHP, a crucial role of GH deficiency in the determination of short stature in these patients seems unlikely. However, although we may hypothesize only a minor effect on growth, PHP Ia patients might benefit from GH replacement therapy for amelioration of other parameters, such as body composition, lipid profile, bone mineral density, physical performance, and quality of life (26).

A last consideration can be made on the age of onset of pituitary resistance. All the patients considered in this study have been investigated for GH and ACTH deficiency at least 5 yr after the diagnosis of PHP Ia, and it is possible that subclinical GH deficiency might have developed over the years in these patients. Consistent with this view, it has been demonstrated that imprinting can be a process beginning and evolving in postnatal life (38–40), and the observation that PHP Ia patients do not show any sign of hormone resistance at birth but generally develop hypocalcemia, hypothyroidism, and hypogonadism over childhood or adolescence supports this hypothesis.

In conclusion, we report nine patients with PHP Ia displaying, in addition to PTH and TSH resistance, also resistance to the action of GHRH. These data are consistent with the recent demonstration of G_{α} gene imprinting in the human pituitary gland and suggest that patients with known mutations in this gene should be carefully monitored for GH deficiency. Further studies will be necessary to determine the possible beneficial effects of GH replacement in these patients.

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