Effects of long-term treatment with growth hormone-releasing peptide-2 in the GHRH knockout mouse

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Submitted 5 May 2005; accepted in final form 20 May 2005

Alba, Maria, Danilo Fintini, Cyril Y. Bowers, A. F. Parlow, and Roberto Salvatori. Effects of long-term treatment with growth hormone-releasing peptide-2 in the GHRH knockout mouse. Am J Physiol Endocrinol Metab 289: E762–E767, 2005. First published June 28, 2005; doi:10.1152/ajpendo.00203.2005.—Growth hormone (GH) secretagogues (GHS) stimulate GH secretion in vivo in humans and in animals. They act on the ghrelin receptor, expressed in both the hypothalamus and the pituitary. It is unknown whether GHSs act predominantly by increasing the release of hypothalamic GH-releasing hormone (GHRH) or by acting directly on the somatotroph cells. We studied whether a potent GHS could stimulate growth in the absence of endogenous GHRH. To this end, we used GHRH knockout (GHRH-KO) mice. These animals have proportionate dwarfism due to severe GH deficiency (GHD) and pituitary hypoplasia due to reduced somatotroph cell mass. We treated male GHRH-KO mice for 6 wk (from week 1 to week 7 of age) with GH-releasing peptide-2 (GHRP-2, 10 μg sc twice a day). Chronic treatment with GHRP-2 failed to stimulate somatotroph cell proliferation and GH secretion and to promote longitudinal growth. GHRP-2-treated mice showed an increase in total body weight compared with placebo-treated animals, due to worsening of the body composition alterations typical of GHD animals. These data demonstrate that GHRP-2 failed to reverse the severe GHD caused by lack of GHRH.

The issue of whether GHSs need GHRH to exert their stimulatory effect on GH secretion is of clinical importance, since these compounds have been considered a potential alternative to GH therapy in patients with GH deficiency (GHD). Indeed the vast majority of cases of idiopathic isolated GHD (IGHD) are believed to be due to immaturity or deregulation of GHRH production rather than to intrinsic abnormalities in the somatotroph cells (28). Unfortunately, most of the studies that have investigated the mechanisms and effects of GHSs on GH secretion have been done on isolated somatotroph cells or in animals with intact GHRH function. An ideal model that would allow determination of the intrinsic ability of GHSs to stimulate GH would be an animal with a normal somatotroph cell population despite the lack of endogenous GHRH. Unfortunately, such a model is not yet available. A recent report of viral ablation of the GHRH neurons in mice showed that lack of GHRH causes GHD and selective inability to respond with GH secretion to acute stimulation by GHS (17). There is, however, no report of the effects of long-term GHRP administration in this model.

We (2) recently have created a mouse model of isolated GH deficiency (IGHD) caused by disruption of the GHRH gene (GHRH knockout, GHRH-KO). These mice have the ideal features listed above with the exception of dramatically reduced somatotroph cell mass. They can secrete GH and partially reverse anterior pituitary hypoplasia in response to long-term treatment with a GHRH analog (3). All of the phenotypic abnormalities (except pituitary hypoplasia) of the GHRH-KO mouse are corrected by the administration of exogenous GH, suggesting that the lack of GHRH has no additional effect on the mouse phenotype that is not mediated by the lack of GH (1).

To determine whether long-term treatment with a GHS would be able to increase GH secretion, we treated GHRH-KO animals with GHRP-2 for 6 wk. We here demonstrate that chronic GHRP-2 does not stimulate GH production or have any effect on somatotroph cell mass. Treatment with GHRP-2 causes worsening of the already altered body composition of the GHRH-KO mice.

MATERIALS AND METHODS

Animals. Male offspring derived from KO breeding pairs on a hybrid C57BL/6-SV129 background were used for both GHRH-KO treated and placebo groups, whereas male wild-type animals (WT;

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also on C57BL76-SV129 background) served as a normal control group. Mice were weaned during the treatment onto a standard mouse/rat chow (Prolab RMH2500; PMI Nutrition International, Brentwood, MO) at 4 wk of age and were housed on the basis of genotype and treatment. All animals experienced controlled environment with 14:10-h light-dark cycles at 21°C and 23% humidity. Food and tap water were supplied ad libitum. All procedures were approved by the Johns Hopkins Institutional Animal Care Committee.

**GHRP-2 treatment.** GHRP-2 [Pramorelin, KP-102: d-alanyl-3-(2-naphthyl)-d-alanyl-L-lysophenylalanyl-L-lysinoamide dihydrochloride] was injected subcutaneously (10 μg sc) twice a day (8 AM and 6 PM) into GHRH-KO mice (n = 11) from day 7 of life for 6 wk. Sex- and age-matched GHRH-KO (n = 13) and WT mice (n = 8) were used as controls and injected with placebo (normal saline).

Total body weight (TBW) and body length (nose-anus distance, N-A) were measured at day 7 and once a week thereafter, using a daily calibrated electronic balance (Scout Pro Balance; Ohaus, Pine Brook, NJ) and an Electronic Digital Caliper (Control, Friendswood, TX).

At the end of the treatment, an acute-stimulation test was performed in all mice (including placebo and WT) by injecting subcutaneously 10 μg of GHRP-2. Animals were anesthetized using Avertin (trihaloethanol; Sigma-Aldrich, St. Louis, MO), and blood was collected with a heparinized capillary tube from a retroorbital vein at 0 and 30 min. Blood samples were centrifuged, and plasma was stored at −20°C until assay.

**Auxological data and body composition.** At the end of the treatment period, final TBW and N-A were measured. Femur and tibia lengths were measured after dissection of the surrounding muscle tissues and disarticulation without disturbing the articular cartilages. Femur length was measured, using the Electronic Digital Caliper, as the maximal distance between the head of the great trochanter and the distal condyles, and tibia length was considered as the maximal distance between proximal condyles and malleolus.

Organs from the thoracic and abdominal cavities and brain were dissected, and their wet weight was measured using a precision electronic balance (AG104; Mettler-Toledo, Columbus, OH).

To determine the effects of GHRP-2 on body composition, weights of subcutaneous fat pad (SF), visceral fat pad (VF), and lean mass (LM) were assessed separately. Animals were deskinned, and the perirenal and epidydimal fat pads were pooled (VF), whereas the sum of the fat pads from the interscapular and axillary region, thighs, and inguinal region were considered SF. LM was determined by weighing the animals deprived of tail, skin, adipose tissue, and organs. LM, VF, and SF weights of each animal were normalized to TBW by calculating the percentage as follows: [weight (g)/TBW (g)] × 100.

**Pituitary immunohistochemistry.** Heads were deskinned, parietal and temporal bones removed, and tissues fixed in 10% buffered formalin for 24 h and decalcified (Cal-Ex; Fisher Scientific, Fair Lawn, NJ) for 1 wk. By cutting through the dorsal sagittal sinus, hemispheres were embedded in paraffin and cut into 5-μm sections. The sections were then immunostained for GH with rabbit anti-mouse GH antibody (National Hormone and Peptide Program, Harbor UCLA Medical Center, Torrance, CA) (1:3,000 dilution) using the avidin-biotin-peroxidase complex technique with diaminobenzidine substrate (brown, DAKO, Carpinteria, CA). Cell nuclei were counterstained using hematoxylin (blue).

**Liver IGF-I mRNA and serum IGF-I.** Total liver RNA was extracted using TRIzol. Measurement of mRNA for IGF-I was performed by Northern blotting (20 μg of RNA/lane) using a 510-bp 32P-labeled mouse IGF-I cDNA probe generated by PCR amplification of mouse genomic DNA (sense primer 5′-ATGACCGCACCT-GCAATAAAAG-3′, antisense primer 5′-CTAAGCCAGTCTTTT-TCTCTG-3′). We consistently detected one predominant mRNA species (of ~0.9 kb). The levels of mRNA were quantified by PhosphorImager analysis, and the results were normalized to GAPDH mRNA after stripping of the membrane.

Serum IGF-I was measured using rat-mouse IGF-I RIA (DSL-2900; DSL, Webster, TX), after acid-ethanol extraction following the manufacturer’s recommendations. All samples were analyzed in duplicate. The IGF-I assay included quality controls provided by the manufacturer. The standard curve of the assay performed in accordance to the manufacturer’s provided samples.

**Statistical analysis.** Results are expressed as means ± SE. Parameters were statistically analyzed by ANOVA using the SPSS statistical package, with post hoc analysis by Bonferroni’s method. Results were considered statistically significant at P values <0.05.

**RESULTS**

**Effects of GHRP-2 on linear growth and organ weight.** Initial N-A length (day 7) was not statistically different among any of the groups (Fig. 1). However, by the end of the 1st wk of treatment, both placebo-treated and GHRP-2-treated GHRH-KO animals were significantly smaller compared with WT mice. All the GHRH-KO mice (placebo and GHRP-2 treated) displayed similar growth patterns throughout the
study, so that at the end of the 6-wk treatment the N-A distance in the GHRP-2-treated group showed no significant difference from the placebo-treated group.

Final tibia lengths of both GHRH-KO groups were significantly shorter compared with WT (placebo 14.1 ± 0.5, GHRP-2 14.3 ± 0.3, WT 17.2 ± 0.6 mm). Interestingly, although no difference was found in final tibia length between the two GHRH-KO groups, the GHRP-2-treated animals achieved a mildly but significantly higher final femur length compared with the GHRH-KO placebo-treated mice (placebo 10.6 ± 0.4, GHRP-2 11.2 ± 0.3 mm, P < 0.05). However, they were both significantly shorter than WT animals (13.8 ± 0.5 mm, P < 0.0001).

Analysis of the wet organ weights (liver, spleen, kidneys, testes) showed significantly higher values in WT animals compared with both GHRH-KO mouse groups.

Effects of GHRP-2 on body weight and body composition. As shown in Fig. 2, similar to N-A measurements, TBW of both GHRH-KO groups diverged significantly from the WT group by the end of the 1st wk of treatment and remained significantly lower throughout the rest of the treatment period compared with WT animals. On the contrary, although the GHRP-2-treated group started with a slightly lower weight compared with the placebo group, their TBW became significantly higher than that of the placebo-treated animals already by the end of the 5th wk of treatment.

To determine how treatment with GHRP-2 affects body composition, we measured the weights of the individual body compartments (Fig. 3A). GHRH-KO mice have altered body composition with reduced lean mass and increased adipose tissue due to the lack of the anabolic and lipolytic effects of GH.

LM was significantly reduced in both GHRH-KO groups compared with WT animals, whereas no difference was seen between placebo- and GHRP-2-treated animals. Relative LM (expressed as %TBW) was reduced in GHRP-2-treated mice compared with the placebo-treated GHRH-KO group and to WT group (Fig. 3B).

Absolute VF was significantly higher in WT compared with both GHRH-KO groups, and higher in GHRP-2-treated animals than in placebo animals (Fig. 3A). When adjusted for TBW (Fig. 3B), no difference was seen between WT and the two other groups. Relative VF remained higher in the GHRP-2-treated group compared with placebo.

The increase in adipose tissue was especially apparent for SF. Although the absolute weights of SF in WT animals and GHRH-KO placebo-treated mice were identical, GHRP-2-
treated mice had twice as much SF (Fig. 3A). Adjustment for TBW revealed that SF had a twofold increase in GHRH-KO placebo-treated mice and threefold in GHRP-2-treated GHRH-KO mice compared with WT (Fig. 3B).

These data demonstrate that the increase in body weight observed in the GHRP-2-treated group was due mainly to apposition of adipose tissue, especially in the subcutaneous compartment, causing a worsening of the already altered body composition observed in the GHRH-KO placebo group.

**Pituitary total RNA and GH mRNA.** Average total RNA content per pituitary in GHRP-2-treated mice showed no difference compared with placebo-treated mice (1.98 vs. 1.74 μg/pituitary) and both groups had markedly lower total RNA yield compared with WT (9 μg/pituitary), consistent with pituitary hypoplasia (Fig. 4A).

Determination of GH mRNA pituitary content by Northern blot demonstrated that GHRP-2 had failed to stimulate GH transcription (Fig. 4B).

**Serum GH.** The lack of a GHRP-2 effect on GH was confirmed by the acute GHRP-2 stimulation test. As shown in Fig. 4C, baseline serum GH concentrations were similar among all groups. Thirty minutes after GHRP-2 injection, WT animals showed a mean serum peak of GH of 15 ng/ml, whereas none of the GHRH-KO groups responded to the stimulus.

**Pituitary immunohistochemistry.** Chronic treatment with GHRP-2 was not able to reverse somatotroph cell hypoplasia. Both GHRH-KO groups display an overall smaller pituitary size compared with WT. The amount of GH-positive cells is reduced, although not completely absent (Fig. 5).

**Liver IGF-I and serum IGF-I.** Liver IGF-I mRNA revealed no significant increase in GHRP-2-treated mice compared with placebo-treated GHRH-KO mice. As expected, both GHRH-KO groups had significantly lower levels compared with WT mice (Fig. 6A).

These findings were confirmed by the measurements of the circulating serum IGF-I. As shown in Fig. 6B, serum IGF-I levels in GHRP-2-treated mice were not significantly different from those found in GHRH-KO mice, and both groups had significantly lower levels compared with WT animals.
GHRP-2 THERAPY IN GHRH-KO MICE

**DISCUSSION**

GHRP-2 is among the most potent ghrelin receptor agonists (21). Studies on rats have shown that its gh-secretory potency is even higher than that of injected GHRH (10). Numerous studies have aimed to attain a better understanding of the interdependency between GHRH and GHSs in stimulating GH secretion. It has been demonstrated that in vitro GHRH is able to induce GH secretion only through its own receptor (GHRH-R), and others have confirmed that GHRP-2 acts primarily through GHS-R but the presence of GHRH-R potentiates its stimulus (12, 21, 25).

To determine whether long-term GHRP-2 treatment could cause GH secretion in GHRH-KO mice, we treated mice for 6 wk with a dose of GHRP-2 that has been proven to be effective to increase GH secretion in normal animals (10). GHRP-2 failed to determine acceleration in longitudinal whole body growth and tibial length. Although mice treated with GHRP-2 achieved a slightly higher femur length (11.2 ± 0.3 vs. 10.6 ± 0.4 mm), this difference was small (0.6 mm), and the femur length was still very far from normal (13.8 ± 0.5). The lack of growth stimulation was reflected by a complete absence of GH response to both chronic and acute administration of GHRP-2. In addition, immunohistochemistry of the pituitary showed no change in pituitary size. Both GHRH-KO groups displayed no GHPR-2 induced change in total pituitary RNA. In addition, both GH mRNA expression and serum GH did not increase in GHRP-2 treated mice, whereas WT animals responded with a sevenfold rise in serum GH after acute injection of the GHS.

The present study shows that, in vivo, the absence of GHRH completely abolishes the ability of GHS (even in long-term treatment) to stimulate GH secretion. In a similar human model of GHD with pituitary hypoplasia (due to a null mutation of the GHRH receptor), acute treatment with GHRP-2 has shown a small but significant peak of GH after acute challenge (12). It is possible that a slight increase in serum GH levels may have occurred in GHRP-2-treated mice, but it has remained undetected because of the sensitivity of the assay we used or because it occurred at different time points than the one we chose. However, GH mRNA expression was also not increased by GHRP-2, making the possibility of an increase in GH transcription unlikely. This model, however, does not allow us to rule out that this result may be due, in part or entirely, to reduced somatotroph cell mass. To circumvent this problem, one would need an animal without GHRH and with intact somatotroph cell mass. As we (3) have previously reported that long-term treatment with a GHRH analog is able to partially revert pituitary hypoplasia, further experiments are planned to treat GHRH-KO mice with GHRP-2 after (or simultaneously with) long-term treatment with a GHRH analog.

Similarly to other models of GHD, the GHRH-KO mouse has an aberrant body composition with reduced lean mass and increased adipose tissue, particularly at the subcutaneous level as early as 5 wk of age (1). GHRP-2-treated mice gained more weight compared with their placebo-treated littermates. When we measured the three body compartments (LM, VF, SF) we saw that the absolute weights of the adipose pads were significantly higher in GHRP-2-treated mice compared with the placebo animals, and this was also confirmed after adjustments for TBW. Although the relative LM was reduced in GHRP-2-treated mice, the absolute LM was not influenced by GHRP-2. Taken together, these results show that a 6-wk treatment with GHRP-2 caused further worsening of the body composition of GHRH-KO animals by increasing visceral and subcutaneous adipose tissue. Due to their small size in relation to the drinking water supply, GHRH-KO animals cannot be weaned before the 4th wk of age. Therefore, we could not assess food intake in these animals throughout the study. Nevertheless, it is noteworthy that the TBW of the GHRP-2-treated group started to increase at the 3rd wk of age, when the animals begin to eat some solid food, having therefore unlimited access to food intake. Our findings may be due to the recently appreciated orexigenic and lipogenic effects of GHS (5, 11, 19, 24, 29, 30, 31). This may be a direct hypothalamic effect rather than the result of an alteration in the GH homeostasis (11, 24) and therefore not influenced by the absence of GHRH. Indeed, lack of a GHRH effect does not alter endogenous ghrelin levels (6).

In conclusion, we demonstrate that long-term therapy with the potent secretagogue GHRP-2 does not stimulate longitudinal growth in GHRH-KO mice.

**GRANTS**

This work was supported in part by National Institute of Child Health and Human Development Grants 1R03 HD-042465-01 and 1R03 HD-046641. M. Alba was supported in part by a fellowship from Pfizer.
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