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Assessment of Growth Parameters and Life Span of GHR/BP Gene-Disrupted Mice*

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ABSTRACT

GH has many biological roles, including promotion of growth. Most, if not all, of its roles are achieved through interaction with its receptor. We chose to study the effects of loss of GH signaling on growth and aging in a mouse model for Laron Syndrome (LS) in which the GHR/BP gene has been disrupted. We observed that mice homozygous for the disruption (−/−) were significantly smaller than normal wild-type (+/+) mice as well as mice heterozygous for the disruption, even at 1.5 yr of age. IGF-I levels were also significantly lower in the −/− mice and remained low as the mice aged. IGFBP-3 levels were severely reduced in the −/− mice, whereas IGFBP-1, -2, and -4 levels remained unchanged. Finally, the −/− mice lived significantly longer than +/+ and +/− mice. The latter result contradicts the anti-aging GH data and suggests the need for further analysis of GH and aging. (*Endocrinology* **141:** 2608–2613, 2000)

GH IS A protein produced and secreted by a set of specialized cells in the anterior pituitary. GH has direct and indirect effects on many tissues, such as stimulating bone and soft tissue growth and influencing carbohydrate, protein, and lipid metabolism. Direct biological activities of GH include receptor binding, internalization of the hormone/receptor complex, and activation of proteins involved in signal transduction (for recent reviews, see Refs. 1, 2, 3).

Protein and RNA transcripts for receptors of GH (GHR) have been detected in many of the tissues influenced by the hormone (4–7). It was determined that a single molecule of GH binds sequentially to two receptor molecules, forming an active complex (8). This complex, in turn, signals stimulation of other genes, including insulin-like growth factor I (IGF-I). IGF-I, produced and secreted by the liver and other target tissues, mediates some of the indirect effects of GH on growth and development (9, 10). Other intracellular events occurring after the GH/GHR interaction include activation of tyrosine kinases such as Janus kinase 2 (Jak-2), which leads to phosphorylation and activation of other proteins including signal transducer and activator of transcription 5A (STAT 5A) and mitogen activated protein (MAP) kinase that, in turn, activate other proteins and genes (2, 11).

The cDNA encoding the GHR has been cloned from many species (5, 6). The receptor consists of an extracellular hormone-binding region (exons 2–7), a single membrane spanning region (exon 8), and an intracellular region (exons 9–10) (12). GHR has no intrinsic kinase domain, but the intracellular region plays a major role in the signal transduction process (13). A truncated form of the receptor, known as GH binding protein (GHBP), lacks the transmembrane and intracellular regions of GHR and is secreted into the serum (14). The truncated protein is produced by one of two different processes, depending on the animal species. In mice and rats, alternative splicing of GHR precursor messenger RNA replaces the transmembrane and intracellular regions with a very short hydrophilic tail (encoded by exon 8A; 15, 16). In humans, cows, and pigs (among others), no alternative RNA splicing is apparent but instead the GHBP is produced by proteolysis of the GHR (17). The function of the binding protein is not clear, but it appears to modulate the level of circulating GH (18).

In an attempt to understand the actions of GH, an animal that is resistant to GH action would be of value. Previously, we generated GH resistant animals by expression of a GH antagonist gene in transgenic mice (19–22). These mice are smaller than control mice, with reduced levels of IGF-I. They also are resistant to diabetes-induced end organ damage (23). In an alternative approach, we disrupted the mouse GHR/BP gene, mimicking the primary defect causing Laron syndrome in humans (24). Mice homozygous for the gene disruption are smaller in size with reduced levels of IGF-I but increased levels of GH. We have recently demonstrated that these mice are also resistant to diabetes-induced end organ damage (25).

One question that has remained controversial is the role of GH in aging. It has been reported that dwarf mice deficient in GH have longer life expectancies (26). However, the mice used in those studies also had other hormonal deficiencies. We have extended our initial investigations to assess the

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combined effects of the GHR/BP gene disruption and advancing age on weight gain, IGF-I and IGF binding protein (IGFBP) levels, and longevity.

Materials and Methods

Animals

The mice used in this study were derived from a founder created by deletion and gene substitution of most of the fourth exon and part of the fourth intron of the GHR/BP gene (24). Their genetic background was a mix of 129Ola and BalbC. Nontransgenic (+/+), heterozygous (+/−) and homozygous (−/−) progeny were generated by +/− × +/− matings and genotyped after weaning by PCR (27). The mice were ear notched for identification purposes, housed in groups of mixed genotype of up to four mice per cage, provided with standard rodent chow (26%, 14%, and 60% of the calories were provided by protein, fat and carbohydrates, respectively; 5P00 Prolab RMH 3000, PMI Nutrition International, Inc., Brentwood, MO) and water *ad libitum*, and maintained on a 14-h light, 10-h dark cycle. All procedures were approved by Ohio University's Institutional Animal Care and Use Committee and complied with federal, state, and local laws.

Weight gain profiles

Progeny of multiple +/− × +/− matings were weighed at weekly intervals starting at weaning (four weeks of age). Mean weights for each mouse were calculated at 4-week intervals (termed weighing intervals) for statistical analysis. Thus, mice averaged 5.5 weeks of age for weighing interval 1, 9.5 weeks of age for weighing interval 2, etc. Means of the weighing interval means were determined and plotted for each gender (male and female) and genotype (+/+, +/−, −/−). One −/− male was excluded from the final analysis since his weight differed from the mean by greater than 2 standard deviations (SD) from the age of 5 weeks onward.

Plasma IGF-I measurements

Blood was collected into heparinized tubes from the tails of five mice of each gender (male and female), genotype (+/+, +/−, and −/−), and age (averaging 1, 10 and 23 months). After centrifugation, the plasma was transferred to a new tube and stored at −20 C. IGFBPs were removed from duplicate samples using an acid-ethanol extraction kit essentially as described by the manufacturer (Nichols Institute Diagnostics, San Juan Capistrano, CA) except that extractions were scaled down 20-fold and the total dilution for samples from −/− mice was only 25-fold, whereas the total dilution for samples from +/+ and +/− mice was the standard 225-fold. These changes were necessary due to the reduced body size and, thus, reduced blood volume of the −/− mice as well as due to the extremely low levels of IGF-I in the −/− mice. Tests were performed to ensure that the results were not altered by the changes. IGF-I levels were measured using a human IGF-I RIA kit with human IGF-I standards (Nichols Institute Diagnostics, San Juan Capistrano, CA). Values between assays were normalized by use of two control plasma samples included in each assay. Means were determined for each gender, genotype and age.

Plasma IGFBP analysis

Blood was collected into heparinized tubes from the tails of 60-day-old male and female +/+, +/− and −/− mice. After centrifugation, the plasma was transferred to a new tube and stored at −20 C. IGFBP levels were assessed by ligand blotting (28) and quantified by scanning densitometry (29).

Longevity analysis

An average lifespan was calculated for +/+, +/− and −/− male and female mice using mice born between July and December of 1996 that had spontaneously died.

Statistical analyses

Weights at specific time points or as differences between two time points, as well as lifespans, initially were analyzed by two-way (genotype × gender) ANOVA using Quick Statistica for Macintosh (StatSoft; Tulsa, OK). IGF-I levels initially were analyzed by three-way (genotype × gender × age) ANOVA. As no statistically significant interactions ($P < 0.05$) were observed, significant main effects of independent variables were analyzed by one-way ANOVA followed by posthoc comparisons using Tukey's HSD test, collapsing across the other independent variable(s) when it was not differing significantly or separately when it was differing significantly. Student's *t* test for nonpaired samples was used to assess age of attainment of final weight and also IGFBP-3 levels.

Results

GHR/BP −/− mice grow more slowly and remain significantly smaller than their +/+ and +/− littermates

Growth, as assessed by weight gain, is dramatically different in GHR/BP gene-disrupted −/− mice as compared with +/+ and +/− mice (Figs. 1 and 2). At 4 weeks of age, which was when the pups were weaned and this study initiated, weights did not differ significantly between genders or between +/+ and +/− mice, but −/− mice weighed significantly less than +/+ mice (~45% less; $P < 0.0002$).

To assess rates of growth immediately after weaning, the weight differences between weighing intervals 1 and 2 (a 4-week increase in age) were analyzed (Fig. 2). Significant differences were seen between male and female +/+ mice ($P < 0.004$) and male and female +/− mice ($P < 0.02$), but not between male and female −/− mice, indicating a loss of gender difference in the −/− mice. For both genders, the rates of growth for +/+ and +/− mice did not differ, but the −/− mice grew significantly slower ($P < 0.0002$).

Although they have a slower rate of growth, it might be expected that the −/− mice eventually attain a final weight similar to +/+ mice but that it just takes longer. However,

FIG. 1. Size comparison of GHR/BP gene disruption littermates. Three female mice from the same litter were photographed at 5 months of age to show their differences in size. *Left*, Normal genotype (+/+). *Middle*, Homozygous for the GHR/BP gene disruption (−/−). *Right*, Heterozygous for the GHR/BP gene disruption (+/−).

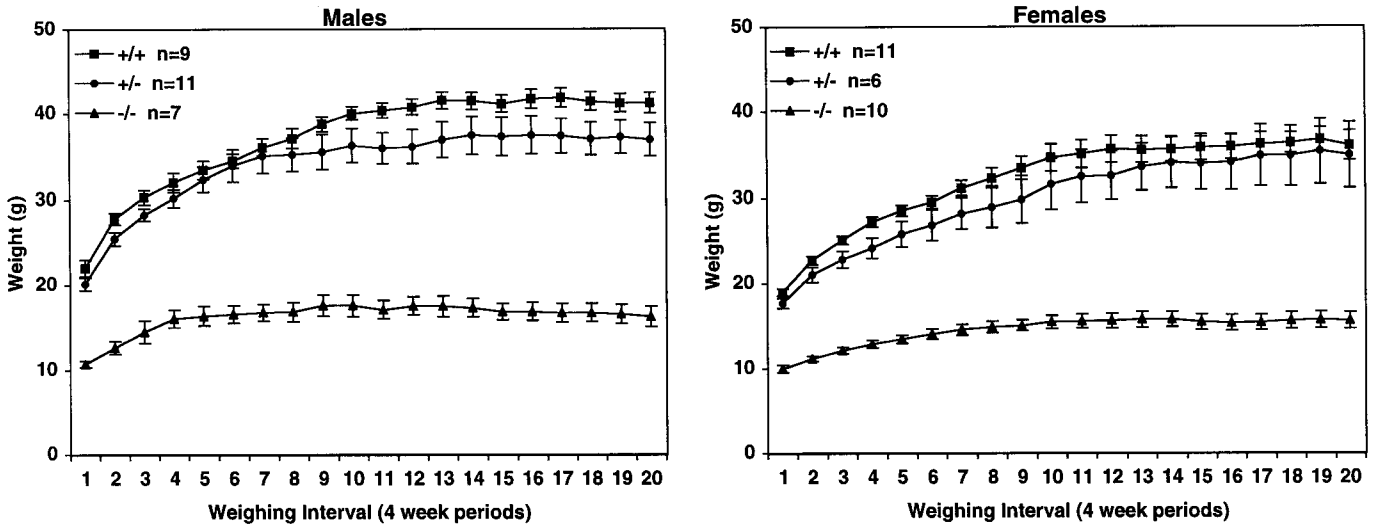

FIG. 2. Weight gain profiles for the various GHR/BP gene-disrupted mice. Means derived from weekly weights averaged at 4-week intervals were plotted for each genotype and gender.

this was not observed. In fact, −/− mice reached their maximum weight at an earlier age than did the +/+ and +/− mice (Fig. 2). Male −/− mice reached their maximum weight at weighing interval 3 (an average age of 14 weeks), whereas male +/+ mice reached their maximum weight 28 weeks later (weighing interval 10). Female −/− mice reached their maximum weight at weighing interval 8 (an average age of 34 weeks), whereas female +/+ mice reached their maximum weight 12 weeks later (weighing interval 11). Heterozygous mice reached plateaus at weighing interval 8 for males and weighing interval 11 for females. The maximally attained weights, analyzed at weighing interval 20, did not differ significantly between genders or between +/+ and +/− mice, but −/− mice were significantly smaller ($P < 0.0002$), attaining a final weight that was approximately 40% that of +/+ mice.

IGF-I levels remain significantly lower in GHR/BP −/− mice than in +/+ and +/− mice

As indicated by the decrease in body size, GHR/BP gene-disrupted −/− mice of both genders have severely reduced levels of plasma IGF-I, measured at less than 10% the levels found in +/+ mice (Fig. 3). A small but significant difference between +/+ and +/− mice was also observed ($P < 0.005$), mainly driven by the difference in levels at 10 months of age. There were no significant differences seen between genders or with age, although there was a slight tendency for +/+ levels to decrease and −/− levels to increase at the 23-month time point.

IGFBP-3 levels are severely reduced in GHR/BP −/− mice

Because IGF-I levels were dramatically affected in the GHR/BP gene-disrupted −/− mice, IGFBP profiles were also assessed (Fig. 4 and Table 1). As no significant changes in IGF-I levels were seen at different ages for the gene-disrupted mice, the IGFBP profile was examined at a single age of 60 days. Ligand blotting using radiolabeled IGF-I revealed no changes in plasma levels of IGFBP-1, IGFBP-2,

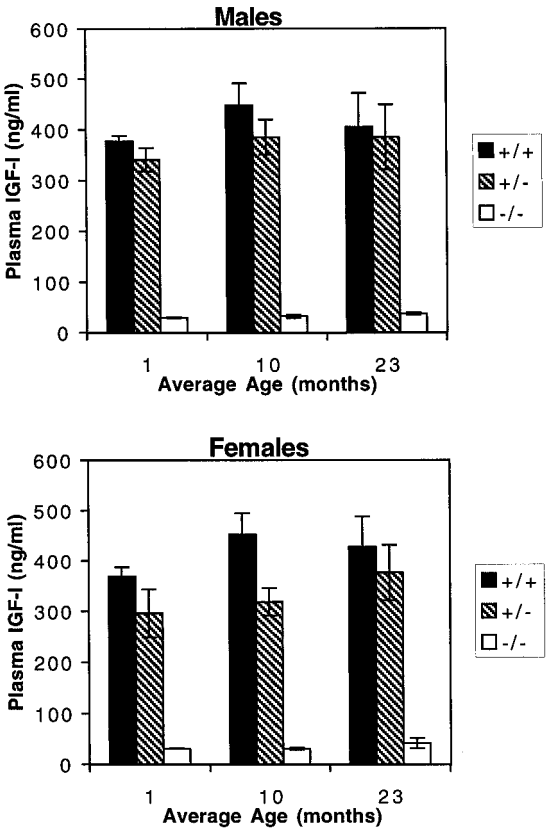

FIG. 3. Plasma IGF-I concentrations determined at three different ages. IGF-I concentrations were measured by RIA after acid-ethanol extraction of plasma from five mice of each indicated gender, genotype and age. *Top panel*, Males. *Bottom panel*, Females.

and IGFBP-4 in 60-day-old mice, but a severe reduction in IGFBP-3 levels was detected in male and female −/− mice (Fig. 4). Scanning densitometry of the IGFBP profiles indicated an approximately 20-fold decrease in IGFBP-3 levels in −/− mice in comparison to +/+ mice ($P < 0.001$; Table 1).

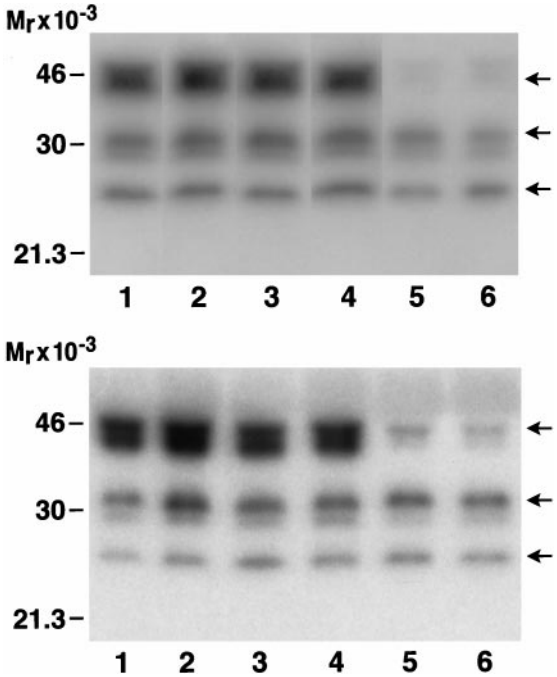

FIG. 4. Plasma IGFBP profiles for the GHR/BP gene-disrupted mice. Plasma from 60-day-old GHR/BP gene-disrupted mice and their controls were analyzed for IGFBPs by ligand blot. *Top panel*, Males. *Bottom panel*, Females. Lanes 1 and 2, Normal genotype (+/+). Lanes 3 and 4, Heterozygous genotype (+/−). Lanes 5 and 6, Homozygous genotype (−/−). Molecular weight standards are shown on the left, $M_r \times 10^{-3}$. The *arrows* on the *right* indicate, from top to bottom respectively, the positions of IGFBP-3 (double bands), IGFBP-1 and -2 (double bands), and IGFBP-4 (single band).

TABLE 1. Scanning densitometry values for IGFBP-3

Gender	Genotype	N	Scanning units[a]
Males	+/+	7	5512 +/− 164
	−/−	7	210 +/− 72[b]
Females	+/+	7	6125 +/− 492
	−/−	6	310 +/− 44[b]

[a] Mean ± SE.
[b] $P < 0.001$ compared with +/+.

Levels of IGFBPs, including IGFBP-3, in +/− mice were similar to +/+ mice and thus were not extensively analyzed.

GHR/BP −/− mice live longer than their +/+ and +/− littermates

Because a decreased body size has been suggested to result in a longer lifespan (26), average lifespans were calculated for each genotype and gender to assess longevity in the GHR/BP gene-disrupted mice (Table 2). Statistical analysis indicated that males and females had similar lifespans. There were also no differences in lifespans of +/+ and +/− mice, but a significant increase in lifespan of nearly a year was observed for −/− mice ($P < 0.0002$). Thus, loss of one GHR/BP allele had no effect on lifespan but loss of both GHR/BP alleles resulted in a significant extension of lifespan, regardless of gender.

TABLE 2. Analysis of lifespan in GHR/BP gene-disrupted mice

Gender	Genotype	N	Lifespan (days)[a]
Males	+/+	7	629 +/− 72
	+/−	8	668 +/− 51
	−/−	7	975 +/− 106[b]
Females	+/+	13	749 +/− 41
	+/−	19	701 +/− 36
	−/−	11	1031 +/− 41[c]

[a] Mean ± SE.
[b] $P < 0.01$ compared with +/+.
[c] $P < 0.0002$ compared with +/+.

Discussion

Until creation of the GHR/BP gene-disrupted mouse, studies investigating the effects of lack of GH signaling employed mainly hypophysectomized animals or genetic dwarfs such as the Ames, Snell, or little (lit/lit) mice. Of these models, only the lit/lit mouse exhibited isolated GH deficiency with no alteration in the levels of the other pituitary hormones. One study involving the lit/lit mouse examined several parameters over the course of a year (30). As was seen in the GHR/BP −/− mice in the present study, serum IGF-I levels were extremely low in the lit/lit mice throughout the time period examined. Likewise, expression of IGFBP-3, which is regulated by GH, was severely reduced while expression of the other IGFBPs remained unaffected. Curiously, there was a difference in weight gain between the lit/lit mice and the GHR/BP −/− mice. While the mean weights of the male and female GHR/BP −/− mice leveled out at approximately 15 g, the mean weights of the male lit/lit mice continued to increase such that the lit/lit males caught up with +/lit female controls (24 g). This additional weight gain in the lit/lit mice appeared to be due to an increased accumulation of fat. Although a few obese GHR/BP −/− mice have been seen in our colony, this does not appear to be the norm.

Even though in previous experiments we had shown little difference between GHR/BP +/+ and +/− mice (24), we included the +/− mice in our present study to characterize them more extensively. Once again, in all the parameters tested, there was little difference between +/+ and +/− mice. This further supports the observation that only one functional GHR/BP allele is necessary for full GHR/BP activity.

An area of study that has received limited attention in isolated GH-deficient animal models is an assessment of longevity. Alterations in longevity, or life expectancy, cannot easily be assessed in humans, but this is not the case in mice. We show here that the lifespan of −/− mice is significantly increased in comparison to +/+ and +/− mice. This analysis is the first documented study of life expectancy in animals or humans with mutations that cause isolated GH deficiency or altered/disrupted GH signaling. These results are in agreement with the increased longevity seen in hypophysectomized rats that were provided replacement therapy with glucocorticoids or thyroxine and seen in Ames and Snell dwarf mice as well as humans that are deficient in GH, PRL, and TSH (reviewed in Refs. 26 and 31). The results implicate GH deficiency as the major factor in increased longevity and suggest use of a cautionary approach

to the therapeutic administration of GH, especially as an anti-aging agent, until more studies can be completed.

Although the GHR/BP −/− mice appear to have a longer life expectancy, it is not clear whether they have a prolonged senescence or whether their entire development proceeds more slowly. We had previously observed that the first conception was somewhat delayed in matings between GHR/BP −/− males and females (24). Danilovich *et al.* (32) further examined the GHR/BP −/− females and found that their sexual maturity was delayed by approximately one week. These results suggest that the GHR/BP −/− mice may age more slowly than their +/+ counterparts, but this hypothesis needs to be further tested.

While the mechanism of aging remains elusive, one aging theory purports that exposure to growth factors and the rate of decline in reserve capacity influence lifespan (33). Support of this comes from caloric restriction studies in mammals, which result in decreased exposure to growth stimulus (*e.g.* GH, IGF-I, and insulin) and an increase in lifespan (33). At the molecular level, in addition to the GHR/BP gene disruption results presented here, two other genes have been identified that suggest involvement of the GH signaling pathway in determination of lifespan. *daf-2*, an insulin receptor-like gene from *Caenorhabditis elegans*, controls growth in a manner that may be homologous to the mammalian IGF-I receptor that acts downstream of GHR in the GH signaling pathway (34). Mutation of *daf-2* results in a marked increase in longevity (34). To distinguish direct effects of GH from effects of IGF-I, it would be interesting to add back IGF-I to the GHR/BP −/− mice, either genetically or by IGF-I administration, and assess the effect on aging. In another report, gene disruption of the $p66^{shc}$ gene in mice results in an increased lifespan (35). It also enhances resistance to environmental stresses such as UV light and reactive oxygen species (35). The authors cite an expanding list of references that suggest a correlation between enhanced resistance to environmental stresses and an extended lifespan. It is of interest to note that GH regulates the phosphorylation status of two other SHC proteins, p52shc and p46shc (36). If disruption of either of these genes also resulted in enhanced resistance to environmental stresses, the same could hold true for disruption of the GHR/BP gene and GH signaling. Testing the resistance of the GHR/BP gene-disrupted mice to environmental stress would support or refute this idea.

In summary, disruption of the gene for GHR/BP results in −/− mice that are significantly smaller than their +/+ and +/− littermates. This difference, as assessed by weight gain, as well as their IGF-I levels, remains constant well into old age. IGFBP-3 levels are also significantly reduced in the GHR/BP −/− mice. Despite, or perhaps as a result of, their decreased growth, GHR/BP −/− mice have a longer life expectancy. Further experiments are in progress to elucidate the role of GH in aging.

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