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Deletion, But Not Antagonism, of the Mouse Growth Hormone Receptor Results in Severely Decreased Body Weights, Insulin, and Insulin-Like Growth Factor I Levels and Increased Life Span

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GH participates in growth, metabolism, and cellular differentiation. To study these roles, we previously generated two different dwarf mouse lines, one expressing a GH antagonist (GHA) and the other having a disrupted GH receptor and binding protein gene (GHR −/−). In this study we compared the two dwarf lines in the same genetic background (C57BL/6J). One of the most striking differences between the mouse lines was their weight gain profile after weaning. The weights of the GHA dwarfs gradually approached controls over time, but the weights of the GHR −/− dwarfs remained low throughout the analysis period. Additionally, fasting insulin and glucose levels were reduced in the GHR −/− mice but normal in the GHA mice. IGF-I and IGF binding protein 3 (IGFBP-3) levels were significantly reduced, but by different degrees, in both mouse lines, but IGFBP-1 and -4 levels were reduced and IGFBP-2 levels increased in GHR −/− mice but unaltered in GHA mice. Finally, life span was significantly extended for the GHR −/− mice but remained unchanged for GHA dwarfs. These results suggest that the degree of blockade of GH signaling can lead to dramatically different phenotypes. (*Endocrinology* 144: 3799–3810, 2003)

GH IS A PROTEIN produced and secreted by the somatotrophic cells of the anterior pituitary. Although it was originally identified for its effects on growth, GH has been found to influence many other processes including protein, lipid, and carbohydrate metabolism (1–3). GH signaling begins with a single GH molecule binding to two GH receptor molecules (GHR), which are found in many tissues (4, 5). A cascade of intracellular signaling ultimately results in a myriad of physiological effects (6, 7).

We have generated two different transgenic mouse lines with altered GH signaling. One line expresses a GH antagonist (GHA) that competes with endogenous GH resulting in a reduction of GH-induced intracellular signaling (8–12). The other line carries a disruption of the GHR/GH binding protein (GHBP) gene that, in the homozygous state (herein designated GHR −/−), lacks expression of the GHR and GHBP (13). Both of these GH-related signaling alterations have a similar effect on growth resulting in dwarf mice (8–10, 13). Over the years we and others have examined additional parameters of these lines including whole body, organ and bone growth, insulin and glucose levels, and kidney resistance to diabetic damage, finding both similarities and differences between the lines (14–22). It was unclear, however, whether the differences were due to the different modes of altered GH signaling or merely to the difference in genetic background between the two strains.

In this study, we sought to eliminate the genetic background difference between the two dwarf lines and then compare several parameters to determine whether the previously observed differences were still apparent. After multiple backcrosses of each line to C57BL/6J mice, we assessed genotype ratios, weight gain profiles, tissue weights, food consumption, IGF-I and IGF binding protein (IGFBP) levels, fasting plasma insulin and glucose levels, and longevity. Despite the similar genetic backgrounds, striking differences were seen for several parameters.

Materials and Methods

Animals

The GHA mouse line used in this study has been described previously (9). It was generated by pronuclear microinjection of a bovine GH minigene with a mutation in the third α helix resulting in glycine 119 being replaced by lysine. Expression of this GHA minigene is driven by the mouse metallothionein-I transcriptional regulatory element. The original genetic background for these mice was a heterogeneous B6-SJL background. They were subsequently backcrossed for greater than 20 generations to C57BL/6J mice, resulting in mice that were greater than 99.99% congenic. The GHA mice and nontransgenic (NT) control littermates for this study were generated by crossing GHA males in the C57BL/6J background with C57BL/6J females purchased from The Jackson Laboratory (Bar Harbor, ME) or female NT littermates.

The genotypes of the mice were determined by PCR analysis of genomic DNA obtained from tail clips using a modified version of the procedure described by Chandrashekar *et al.* (23). In brief, after extraction and purification of genomic DNA, PCR was performed on the resuspended, purified genomic DNA using three primers: IN A-2 (−)

Abbreviations: BAT, Brown adipose tissue; FIRKO, fat-specific insulin receptor knockout; GHA, GH antagonist; GHBP, GH binding protein; GHR, GH receptor molecule; IGFBP-3, IGF binding protein 3; NT, nontransgenic; WAT, white adipose tissue.

(5′-AGCCCAAAGCTCTGAACACATA-3′), MT3T (+) (5′-CTGAGTACCTTCTCCTCACTTAC-3′) and MMTI (−) (5′-GTAAGTTTAGTAATGCCTGGGACT-3′). The sequence for IN A-2 (−) occurs in the noncoding strand of the bovine GH intron A found in the transgene. The sequence for MT3T (+) occurs in the coding strand of the mouse metallothionein transcriptional regulatory element found in the transgene and found endogenously in the mouse genome. The sequence for MMTI (−) occurs in the noncoding strand of the endogenous mouse metallothionein gene. Each sample [10 mmol/liter Tris-HCl (pH 9.0), 50 mmol/liter KCl, 0.1% Triton X-100, 2 mmol/liter $MgCl_2$, 0.2 mmol/liter deoxy(d)-ATP, 0.2 mmol/liter dCTP, 0.2 mmol/liter GTP, 0.2 mmol/liter TTP, 0.46 μmol/liter In A-2 primer, 0.88 μmol/liter Mt3t primer, 0.84 μmol/liter MMTI primer, 0.05 U *Taq* polymerase, and 0.013 μl genomic DNA/μl reaction] was amplified in a GeneAmp (Applied Biosystems, Foster City, CA) (1 cycle of 94 C for 2 min followed by 30 cycles of 94 C for 15 sec, 67 C for 20 sec, and 72 C for 30 sec) and then separated by electrophoresis through a 1× TAE (40 mmol/liter Tris, 20 mmol/liter acetic acid, and 1 mmol/liter EDTA), 1% agarose, 1% Metaphor agarose (FMC, Rockland, ME), 0.2 μg/ml ethidium bromide gel. NT animals produced a single fragment of approximately 400 bp because of amplification of the endogenous metallothionein gene by MT3T (+) and MMT1 (−). GHA animals produced two fragments: an approximately 400-bp fragment amplified from the endogenous metallothionein gene by MT3T (+) and MMT1 (−) and an approximately 200-bp fragment amplified from the transgenic GHA gene by MT3T (+) and IN A-2 (−).

The GHR −/− mouse line used in this study also has been described previously (13). It was derived from a founder animal created by homologous recombination resulting in deletion and gene substitution of most of the fourth exon and part of the fourth intron of the GHR/GHBP gene. The original heterogeneous genetic background for these mice resulted from 129 Ola-derived embryonic stem cells and BALB/cJ blastocysts. Subsequently heterozygous (+/−) males from this line were backcrossed for eight generations to C57BL/6J females, resulting in mice that were 99.61% congenic. After the eighth backcross, +/− males were crossed to +/− females in subsequent generations to maintain the line. The GHR −/− and +/+ control littermates used for this study were generated by three different crosses: +/− males × +/− females, −/− males × +/− females and +/+ males × +/+ females. The genotypes of the mice were determined by PCR using genomic DNA isolated from tail clips as described previously (23).

Because of the difficulty of getting a large number of GHA or GHR −/− dwarf mice at any one time (see *Results*), smaller groups of dwarf and control mice were studied as they became available. The results were pooled to obtain the number of animals used in each experiment. Mice were weaned onto a standard rodent chow (Prolab RMH 3000, PMI Nutrition International, Inc., Brentwood, NJ; 14% of calories from fat, 16% from protein, and 60% from carbohydrates) at 28 d of age and housed, two per cage, in a temperature-controlled room at 22 C with a 14-h light/10-h dark cycle. Food and water were supplied *ad libitum*. Protocols were approved by the Ohio University Institutional Animal Care and Use Committee and followed federal, state, and local guidelines.

Assessment of genotype ratios

The genotype and gender of progeny born between January 2001 and November 2002 of GHA male × NT female crosses and GHR +/− male × GHR +/− female crosses in the homogeneous C57BL/6J genetic background were tallied upon weaning at 4 wk of age. For comparison, progeny from GHR +/− male × GHR +/− female crosses in the heterogeneous Ola-BALB/cJ genetic background also were counted.

Weight gain profiles

Animals were weighed every 2 wk throughout the course of the study. Means for each genotype at each age were determined.

Food consumption measurements

Food consumption was monitored at two different time points during the study; at 2 months of age and at 8 or 9 months of age. Food was measured twice a week for at least 1 wk at the earlier time point and for at least 3 wk at the later time point. The average amount of food consumed per mouse was calculated by dividing the amount of food consumed each week by the number of mice in the cage and then averaging the weekly measurements. Food consumption was normalized to body weight by dividing the average weekly food intake per mouse by the average weight of the mice in the cage at the time point indicated.

Blood glucose, serum insulin, IGF-I, and IGFBP measurements

Food was removed in the morning, and mice were fasted for 8 h before blood collection at the indicated ages. Mice were briefly warmed under a heat lamp for less than 1 min to vasodilate the tail vein. Blood glucose concentrations were determined from tail blood samples using a Lifescan One Touch glucometer (Johnson & Johnson, New Brunswick, NJ). Blood was then collected from the tip of the tail using heparinized capillary tubes. Whole blood was centrifuged at 7000 × *g* for 10 min at 4 C and serum collected. Serum insulin concentrations were determined using the Mercodia Ultrasensitive rat insulin ELISA kit (ALPCO, Windham, NH). Values were corrected for mouse insulin by multiplication by a factor of 1.23 as recommended by the manufacturer. Serum IGF-I levels were measured after extraction using acid-ethanol as previously described (24). The intra- and interassay coefficients of variation were less than 5% and less than 10%, respectively. Serum IGFBP-1, -2, -3, and -4 levels were assessed by SDS-PAGE and Western ligand blot analysis according to the method of Hossenlopp *et al.* (25) as described previously (18, 26). Quantification of Western ligand blots was done by densitometry using a Shimadzu CS-9001 PC dual-wavelength flying spot scanner (Shimadzu Europe GmbH, Duisburg, Germany). The relative densities of the bands are expressed as pixel intensity.

Tissue weights

All animals were killed at about 11 months of age and kidney, liver, gastrocnemius muscle, epididymal fat, heart, brain, and stomach were collected and weighed. The percent of body weight was calculated for each tissue by dividing the absolute tissue weight by the body weight for each individual mouse and multiplying by 100. Means were determined for each genotype.

Life span analysis

An analysis of life span was carried out for each dwarf line by recording the age of spontaneous death of GHA and NT littermates born between September 1997 and May 1999, GHR −/− and +/+ littermates in the C57BL/6J background born between March 1999 and April 2000, and GHR −/− and +/+ littermates in the Ola-BALB/cJ background born between May and December of 1997. Means, medians, and the percent living beyond 1000 d were calculated for each genotype and gender. At the time of analysis, two GHR −/− females in the C57BL/6J background were still alive, and, therefore, a date of death of May 12, 2003, was assigned to each. Both mice were more than 1000 d of age.

Statistical analysis

All parameters were statistically evaluated using ANOVA (analyzing genders separately) except for the genotype assessments, which were analyzed using the χ^2 test for goodness of fit. Results were considered statistically significant if $P < 0.05$. Unless otherwise indicated, data are presented as mean ± SEM.

Results

Genotype assessments

Over a 2-yr time span, from January 2001 through November 2002, the gender and genotype of pups resulting from GHA or GHR +/− matings were tallied and compared after weaning at 4 wk of age (Table 1). A total of 421 pups were born in 74 litters resulting from crosses of GHA males and NT females in the homogeneous C57BL/6J genetic background in the 2-yr time span. Considering both genders, a

TABLE 1. Gender and genotype assessment of weaned pups from the GHA and GHR −/− mouse lines

Background	Gender	Genotype	Expected (%)	Observed[a] (%)	Degrees of freedom	*P* value
C57BL/6J	Male	NT	25	30 (128)	3	<0.0005
		GHA	25	18 (74)		
	Female	NT	25	30 (127)		
		GHA	25	22 (92)		
	Male	GHR +/+	12.5	16 (47)	5	<0.0005
		GHR +/−	25	30 (87)		
		GHR −/−	12.5	5 (14)		
	Female	GHR +/+	12.5	16 (47)		
		GHR +/−	25	27 (79)		
		GHR −/−	12.5	7 (20)		
Ola-BALB/cJ	Male	GHR +/+	12.5	10 (82)	5	<0.05
		GHR +/−	25	26 (207)		
		GHR −/−	12.5	10 (80)		
	Female	GHR +/+	12.5	13 (104)		
		GHR +/−	25	28 (224)		
		GHR −/−	12.5	12 (92)		

[a] The actual number of mice for each gender and genotype is indicated *in parentheses.*

statistically significant deviation from Mendelian ratios was observed such that the dwarfs were underrepresented and the NT mice were overrepresented ($P < 0.0005$). A similar deviation was also observed within the 294 pups born in 52 litters resulting from crosses of GHR +/− males and GHR +/− females in the C57BL/6J background. Once again, the dwarf (−/−) mice were underrepresented and the normal-sized (+/+ and +/−) mice were overrepresented ($P < 0.0005$). This deviation was much less pronounced within the 789 pups born in 123 litters resulting from crosses of GHR +/− males and GHR +/− females in the heterogeneous Ola-BALB/cJ background ($P < 0.05$). Similar statistical significance was observed for all lines when the genders were pooled together for analysis (data not shown).

Weight gain comparisons

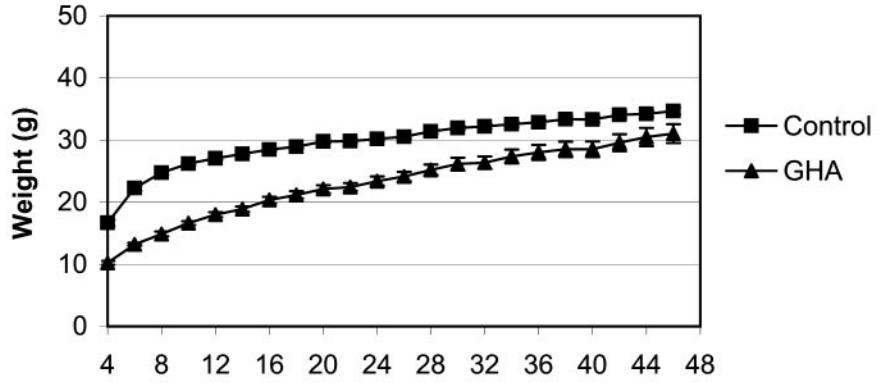
GHA, GHR −/−, and control (NT or +/+) mice differ substantially in size, both in length (Fig. 1) and weight (Fig. 2). The differences in total body weights of the GHA and GHR −/− mice and their respective control littermates were assessed biweekly from weaning at 4 wk of age until the end of the study 42–44 wk later (Fig. 2). Despite having the same C57BL/6J genetic background, a significant difference between body weights for the control littermates of the two different transgenic lines was sometimes seen ($P < 0.05$ at 6 wk and then from 14 wk onward). Therefore, for these and all other experiments described herein, the GHA and the GHR −/− mice were compared with their respective control littermates.

Upon weaning at 4 wk of age, the control mice of the two transgenic lines were similar in weight (Fig. 2). However, the weights of the GHA and GHR −/− mice were 61% and 41% the weights of controls, respectively ($P < 0.0001$). Furthermore, the weights of the GHA and GHR −/− mice differed significantly from each other ($P < 0.0001$). The difference in weights between the GHA and GHR −/− mice increased dramatically with age as the weights of the GHA mice steadily approached those of their control littermates, but the weights of the GHR −/− mice remained low (Fig. 2).

FIG. 1. Size comparison of the two dwarf mice. Three representative 11-month-old adult female mice, all in the same C57BL/6J genetic background, were photographed to show their differences in length. *Left*, Normal genotype. *Middle*, GHA mouse. *Right*, GHR −/− mouse.

Food consumption comparisons

Food consumption was monitored at 2 months of age and again at 8 or 9 months of age (Fig. 3). GHA mice consumed the same mass quantity of food as their control littermates. In contrast, GHR −/− mice consumed 52% the amount of food as their control littermates ($P < 0.0005$). When normalized to body weight, GHA and GHR −/− mice consumed significantly more than the control mice at 2 months of age (43% more, $P < 0.05$ for GHA; 37% more, $P < 0.001$ for GHR −/−). Food consumption normalized to body weight for GHA and GHR −/− mice was similar to the controls at the later age.



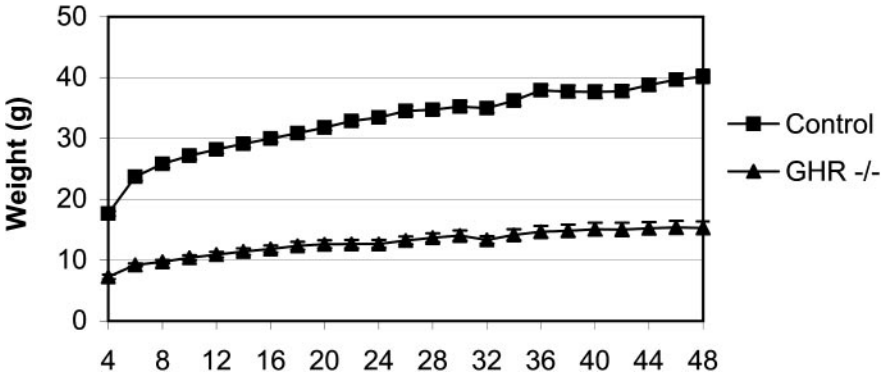


FIG. 2. Body weight gain profiles for the two dwarf lines. Means obtained from biweekly weighings of the GHA and GHR −/− lines in the C57BL/6J background were plotted for each genotype. *Top*, GHA males (▲, n = 17) and their control male littermates (■, n = 20). *Bottom*, GHR −/− males (▲, n = 10) and their control male littermates (■, n = 11). *Vertical bars*, SEM.

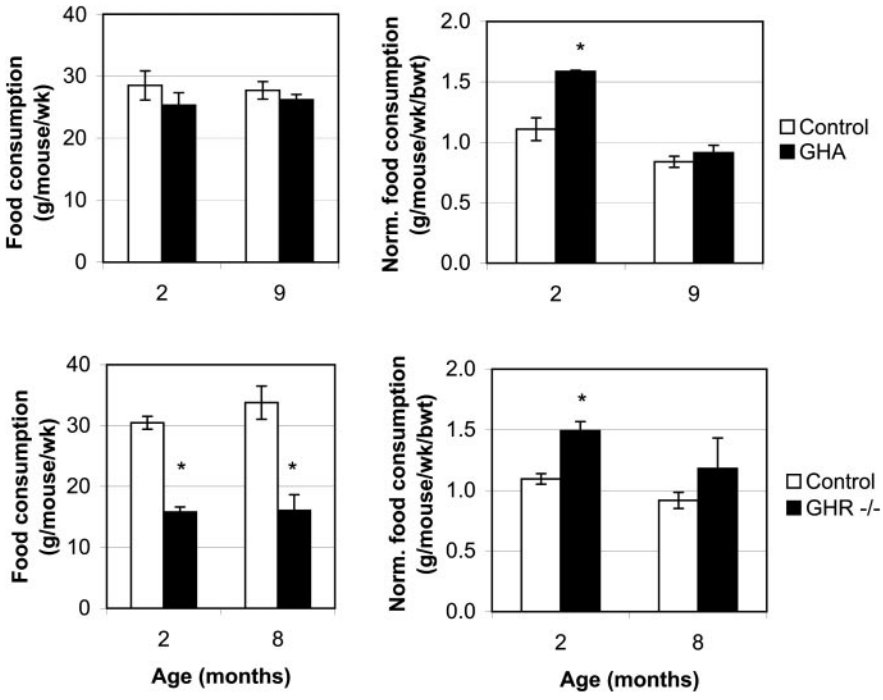


FIG. 3. Food consumption comparisons for the two dwarf lines. Means obtained from measuring the amount of food consumed at 2 months and 8 or 9 months of age of the GHA and GHR −/− lines in the C57BL/6J background were plotted for each genotype. *Left panels*, Absolute amount of food consumed per mouse per week. *Right panels*, Food consumption normalized to body weight. *Top panels*, GHA males (■, n = 2 at 2 months and n = 4 at 9 months) and their control littermates (□, n = 3 at 2 months and n = 5 at 9 months). *Bottom panels*, GHR −/− males (■, n = 5 at 2 months and n = 6 at 8 months) and their control littermates (□, n = 6 at 2 months and n = 6 at 8 months). n refers to independent measurements, not individual mice. *Vertical bars*, SEM. *, Significantly different from control ($P < 0.05$).

Tissue weight comparisons

At the end of the study, the mice were killed and their organs weighed. The absolute weights of all tissues examined for the GHR −/− mice were significantly less than their control littermates ($P < 0.0001$; data not shown). The weights averaged about 20–40% those of controls, except for the brain, which was about 80% the weight of the controls. The absolute weights of all but one tissue, the epididymal fat pad, for the GHA mice were significantly less than their control littermates, averaging about 60–90% the weights of the controls ($P < 0.05$; data not shown).

When normalized to body weight (Fig. 4), the weights of the kidney and liver were significantly decreased in both transgenic lines relative to their control littermates ($P <$

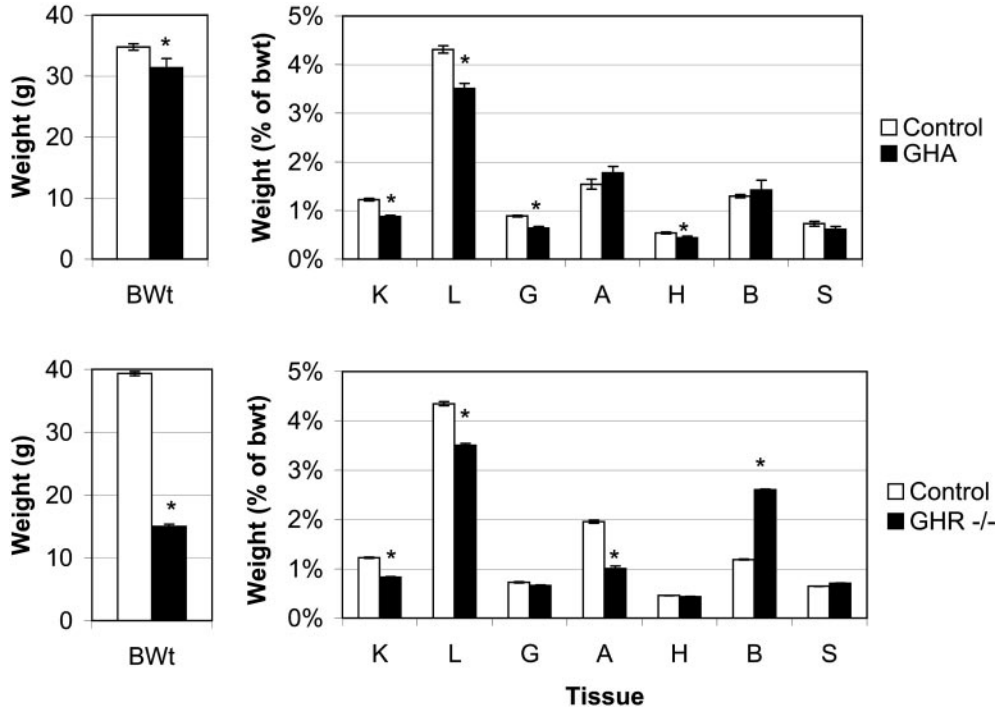


FIG. 4. A comparison of organ weights for the two dwarf lines. Means obtained from weighing the various organs of the GHA and GHR −/− lines in the C57BL/6J background on killing at about 11 months of age were plotted for each genotype. *Left panels*, Body weight (BWt). *Right panels*, Organ weights expressed as a percent of body weight. K, kidney; L, liver; G, gastrocnemius muscle; A, epididymal adipose pad; H, heart; B, brain; S, stomach. *Top panels*, GHA males (■; n = 17 for BWt, K, L, G, and A; n = 15 for H; n = 4 for B and S) and their control littermates (□; n = 19 for BWt, K, L, G, and A; n = 17 for H; n = 5 for B and S). *Bottom panels*, GHR −/− males (■; n = 10 for all measurements) and their control littermates (□; n = 11 for all measurements). *Vertical bars*, SEM. *, Significantly different from control ($P < 0.05$).

0.0001). The weights of the gastrocnemius muscle and the heart were decreased only in the GHA mice ($P < 0.01$), and the epididymal fat pad was decreased only in the GHR −/− mice ($P < 0.0001$). Brain weight was increased in the GHR −/− mice ($P < 0.0001$) but not in the GHA mice.

Serum IGF-I and IGFBP comparisons

As described in *Weight gain comparisons*, unexplainable differences were again seen between the control groups for the two dwarf lines with regard to the IGF-I levels and several IGFBP levels (Fig. 5). For this reason, only comparisons between the dwarf mice and their respective controls were made, not between lines.

Serum IGF-I levels were significantly reduced in both GHA and GHR −/− mice, compared with controls, at all ages examined (Fig. 5). In the GHA mice, levels were reduced to about 75–80% the level of controls ($P < 0.05$). In the GHR −/− mice, levels were reduced to about 20% the level of controls ($P < 0.0001$).

IGFBP-3 levels were also significantly reduced in the GHA and GHR −/− mice but only in the older mice (Fig. 5). In the GHA mice, levels were reduced to about 30% the levels of controls at 11 months of age ($P < 0.05$). At the same age in the GHR −/− mice, levels were reduced to less than 10% of the controls ($P < 0.0005$).

IGFBP-1, -2, and -4 levels were not significantly altered in the GHA mice (Fig. 5). In contrast, IGFBP-1 and -4 levels were significantly reduced, but levels of IGFBP-2 were significantly elevated in the GHR −/− mice relative to control animals ($P < 0.05$).

Serum insulin and blood glucose comparisons

Fasting serum insulin levels in the GHA mice for the most part did not differ significantly from the controls (Fig. 6, *top left panel*). At early ages, there was a tendency for the insulin levels in the GHA mice to be lower than the controls, but at later ages the trend switched with the GHA mice having higher levels than controls. The only time point that showed a statistically significant difference was at 1.5 months of age ($P < 0.005$). There was a tendency for fasting blood glucose levels in the GHA mice to be lower than the controls, but the difference was statistically significant only at 1, 1.5, 5, and 7 months of age ($P < 0.05$; Fig. 6, *top right panel*). In contrast, the fasting insulin levels in the GHR −/− mice were severely reduced compared with the controls at all ages (26–10% the level of controls, $P < 0.0001$; assays were not performed at 1 month of age because of their small body size; Fig. 6, *bottom left panel*). Fasting glucose levels in the GHR −/− mice were also significantly reduced, compared with controls, at all but one time point, 11 months of age (65–86% the level of controls, $P < 0.05$; Fig. 6, *bottom right panel*).

Both sets of control mice also displayed an age-associated rise in fasting insulin levels (3- to 5-fold increase between 1 and 11 months of age, $P < 0.0005$; Fig. 6, *left panels*). A similar age-associated rise in insulin levels was seen in the GHA mice (nearly 8-fold increase between 1 and 11 months of age,

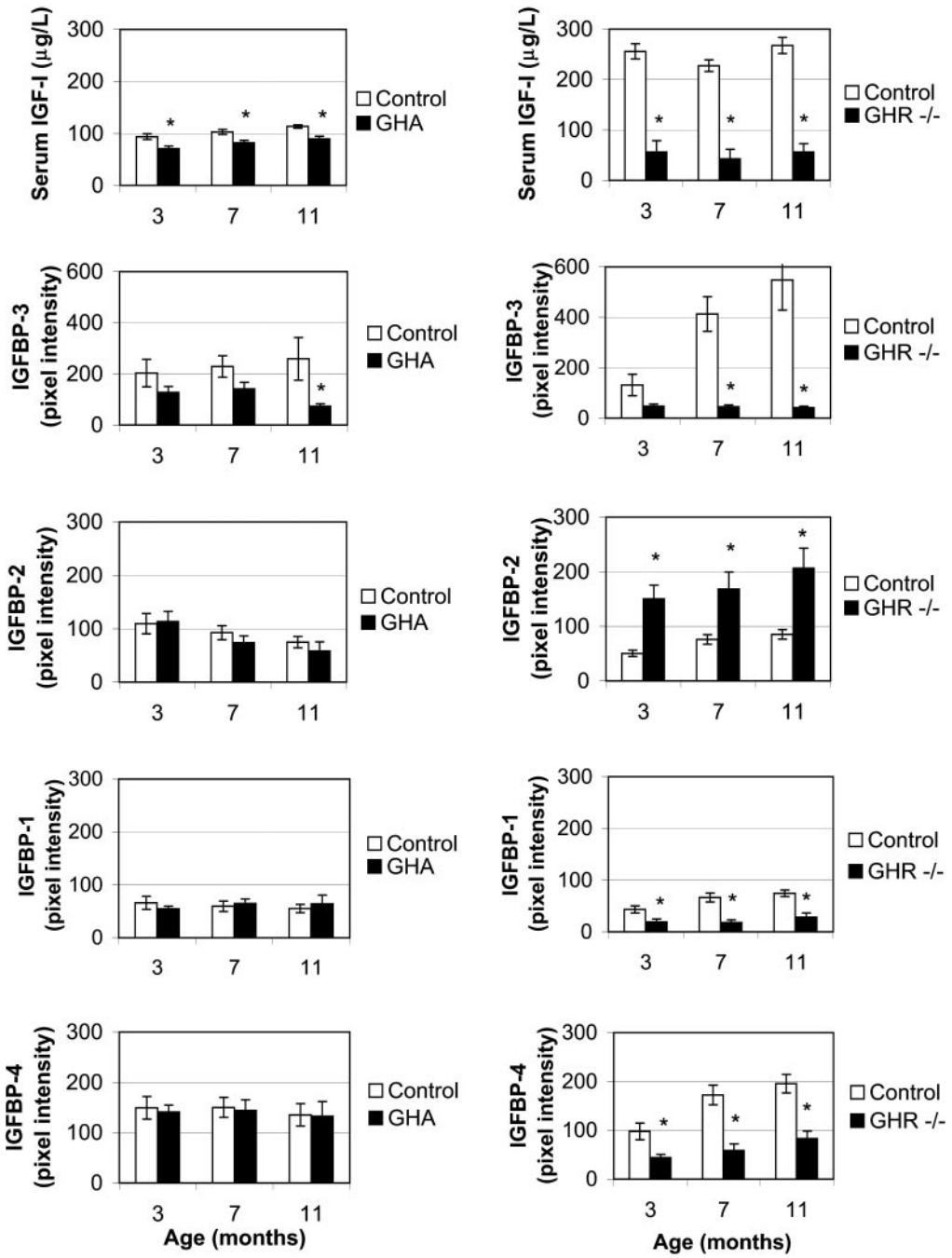


FIG. 5. Serum IGF-I and IGFBP profiles for the two dwarf lines. Means obtained for serum levels of IGF-I, IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 of the GHA and GHR −/− lines in the C57BL/6J background at three different ages (3, 7, and 11 months of age) were plotted for each genotype. *Left panels*, GHA males (■, n = 9) and their control littermates (□, n = 10). *Right panels*, GHR −/− males (■; n = 10) and their control littermates (□; n = 11). *Vertical bars*, SEM. *, Significantly different from control ($P < 0.05$).

$P < 0.0005$; Fig. 6, *top left panel*), but not in the GHR −/− mice (Fig. 6, *bottom left panel*).

Life span comparison

Mean and median life spans as well as the percent surviving beyond 1000 d were calculated for the GHA and GHR −/− lines in the C57BL/6J background and for additional mice from the GHR −/− line in the Ola-BALB/cJ background to assess the effect of different levels of GH signaling on longevity (Table 2 and Fig. 7). Although there was a tendency for the GHA mice to live longer than their controls, especially for the females, this difference did not reach statistical significance for either gender. In contrast, the average lifespan of GHR −/− males in either genetic background was significantly longer than their controls (26% increase in the C57Bl/6J background and 40% increase in the Ola-BALB/cJ background; $P < 0.05$; see *Materials and Methods*). GHR −/− females in the Ola-BALB/cJ background also

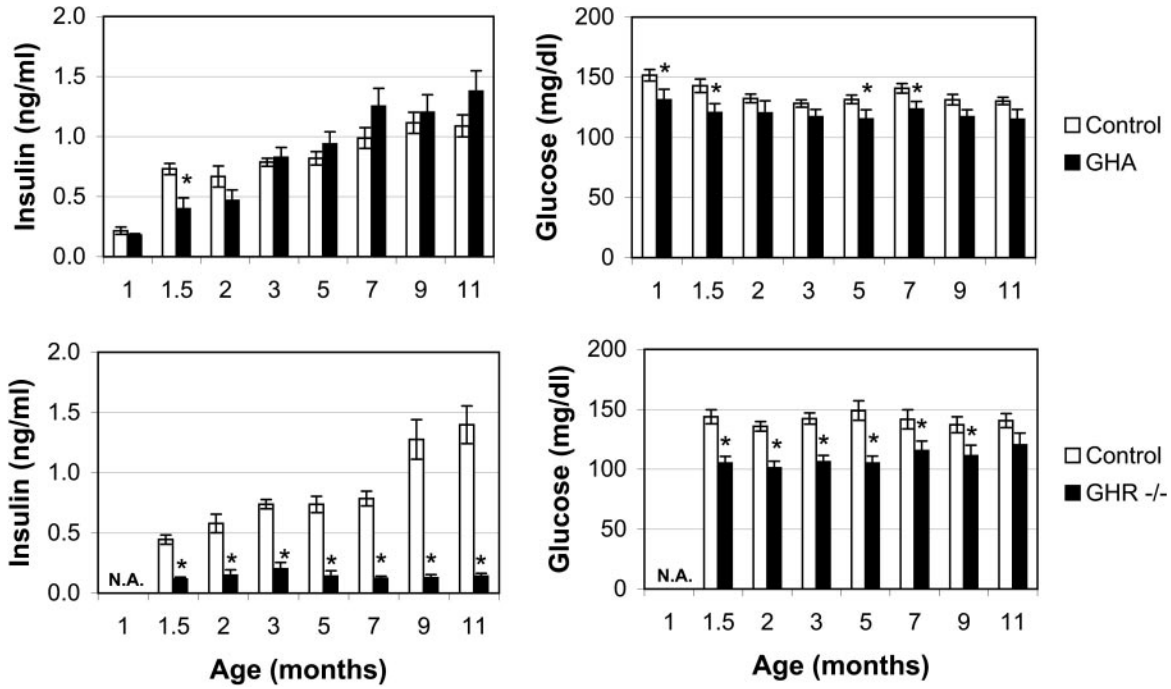


FIG. 6. Fasting serum insulin and blood glucose levels at different ages for the two dwarf lines. Means obtained for serum insulin and blood glucose levels after an 8-h fast of the GHA and GHR −/− lines in the C57BL/6J background at different ages were plotted for each genotype. *Left panels*, insulin levels. *Right panels*, glucose levels. *Top panels*, GHA males (■; n = 8 for the first three ages; n = 17 for the rest) and their control littermates (□, n = 10 for the first three ages; n = 20 for the rest). *Bottom panels*, GHR −/− males (■; n = 10 for all ages) and their control littermates (□; n = 11 for all ages). N.A., Not assayed. *Vertical bars*, SEM. *, Significantly different from control ($P < 0.05$).

TABLE 2. Analysis of lifespan for the GHA and GHR −/− mouse lines

Background	Gender	Genotype	n	Lifespan (days)	Median	% surviving beyond 1000 d
C57BL/6J	Male	NT	24	758 ± 40	797	0
		GHA	22	790 ± 41	823	14
	Female	NT	28	771 ± 26	770	4
		GHA	33	839 ± 25	872	9
	Male	GHR +/+	22	756 ± 68	866	5
		GHR −/−	14	951 ± 50[a]	941	43
	Female	GHR +/+	17	821 ± 49	850	18
		GHR −/−	19	956 ± 80[b]	1023	63
Ola-BALB/cJ	Male	GHR +/+	15	656 ± 67	698	0
		GHR −/−	11	917 ± 55[c]	888	27
	Female	GHR +/+	24	759 ± 41	765	13
		GHR −/−	30	921 ± 41[c]	981	43

[a] $P < 0.05$ compared with +/+.
[b] Two mice are still living.
[c] $P < 0.01$ compared with +/+.

demonstrated a significantly longer average life span, compared with the corresponding control females (21% increase, $P < 0.01$). However, the same was not true for the GHR −/− females in the C57BL/6J background. The lack of significant difference in average life span for the females in the C57BL/6J background was most likely due to the fact that two GHR −/− females were still alive at the time of analysis and thus had not yet reached their maximum life span, affecting the statistics (see *Materials and Methods*). The increased median and percent surviving beyond 1000 d suggest that the GHR −/− females in the C57BL/6J background also live longer than the controls. Furthermore, if the statistical analysis is performed on females that lived more than 1 yr (excluding one GHR −/− female that died at a very young age), a significant difference in life span is seen (19% increase, $P < 0.01$). A statistically significant result was not obtained if a similar analysis was performed on the GHA mice.

Discussion

GH, acting through its receptor, initiates a cascade of intracellular signaling events that ultimately affect a multitude of physiological parameters such as growth, fat depletion, and glucose metabolism. Based on their genetic alterations as well as their differences in body size, we assumed that the mice used in this study exhibited different degrees of GH signaling, from reduced GH signaling in the GHA mice to a

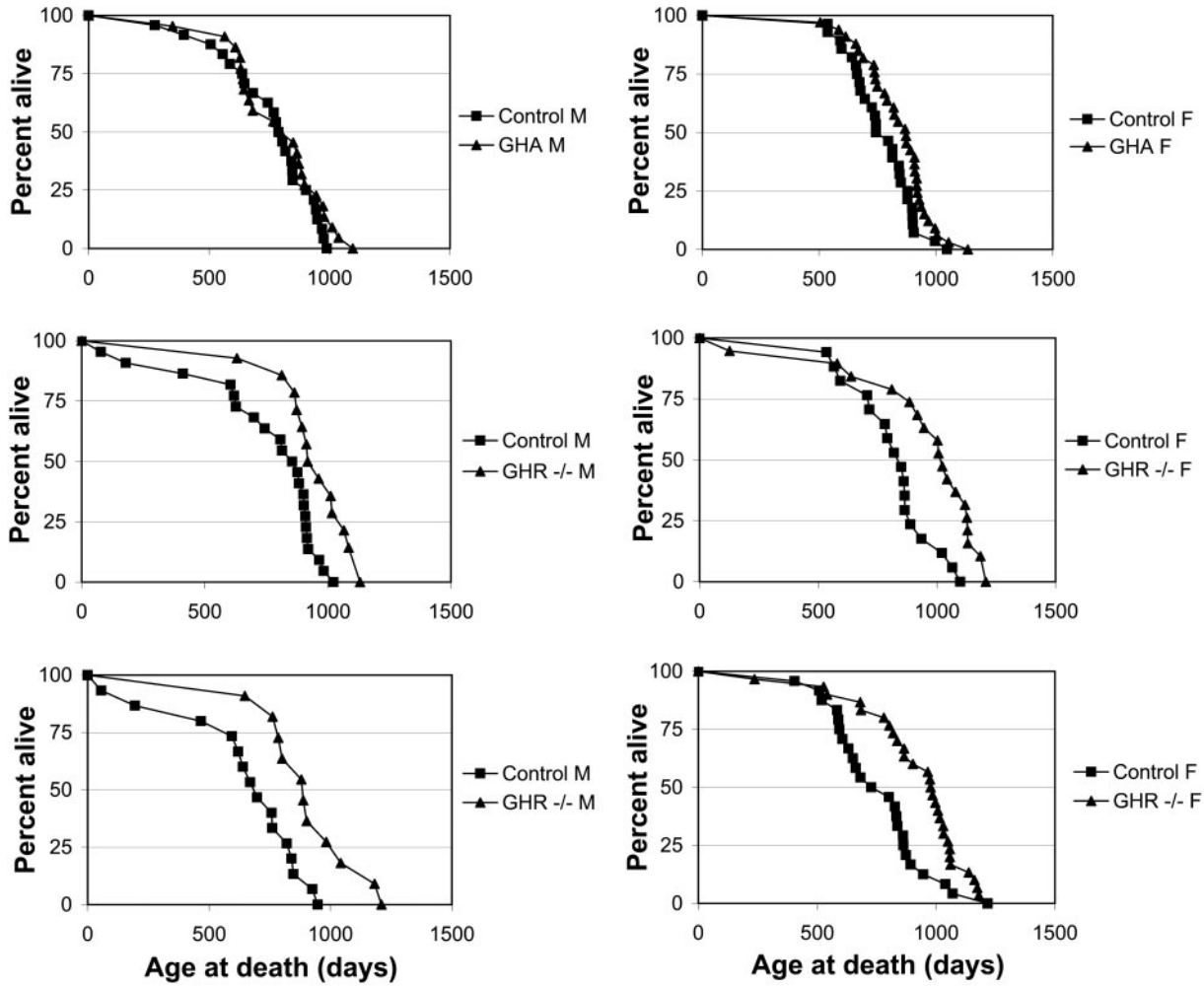


FIG. 7. Survival curves for the dwarf lines. The age at death of each individual mouse was plotted against the percent of mice still alive for each gender and genotype of the GHA and GHR −/− lines in the C57BL/6J background (*top* and *middle panels*, respectively) and the GHR −/− line in the Ola-BALB/cJ background (*bottom panels*). *Left panels*, Males; *right panels,* females; ▲, dwarfs; ■, control littermates.

complete absence of GH signaling in the GHR −/− mice. This modulation of GH signaling revealed both similar and differing phenotypic effects.

Examination of genotype ratios revealed a deviation from Mendelian ratios for the GHA line and the GHR −/− line in the C57BL/6J genetic background. In both cases, the number of dwarf mice was reduced in relation to the number of normal sized NT, +/+ or +/− mice. Interestingly, although this deviation was also observed in the GHR −/− line in the mixed Ola-BALB/cJ background, it was not as pronounced as in the C57BL/6J background. In general, GH is thought to play a very minor role in fetal growth and development. In support of this, we and others have not observed any phenotypic differences in GHA or GHR −/− mice, compared with controls, until approximately 2 wk after birth, even though we expect expression of GHA RNA driven by the metallothionein-I transcriptional regulatory element to be high in the fetus (27) and the GHR gene disruption is present at conception, thus potentially altering GH action early in development. However, expression of endogenous GHR has been detected as early as d 12 in rat embryos (28) and has been shown to respond to retinoic acid (an important regulator of embryonic development) in embryonic stem cells such that GHR mRNA levels increased more than 100-fold (29). Endogenous GH has also been detected in embryos and is proposed to act as an autocrine/paracrine factor during early chick embryogenesis (30). As further evidence for an early role, Hikida *et al.* (31) reported alterations in muscle fiber number in GHA mice, compared with both wild-type and GH transgenic mice, and suggested that the effects of GH on muscle fiber number must occur in the fetus because the number of muscle fibers found in adults is determined early in fetal development and changes little postnatally (32). Thus, the presence of both GH and GHR during embryo development and the early effect of GH on muscle fiber number suggest that GH could have a role in development in mice much earlier than 2 wk after birth. If this is the case, fetal expression of GHA or the lack of GHR in the fetus could be affecting the viability of the underrepresented GHA and GHR −/− mice. To answer this, fetal and neonatal studies in these dwarf models are needed.

The body weight profiles and fat accumulation measurements exhibited surprisingly different responses to the modulation of GH signaling. It is well known that GH acts to

suppress fat accumulation and increase muscle mass. Thus, the increased weight gain and epididymal fat mass seen in the GHA mice was not surprising. Similar results have also been seen in GHA mice in a mixed background (75% C57BL/6J and 25% SJL) that showed a significant increase in percent body fat, compared with nontransgenic littermates (14, 33), and in GH-deficient lit/lit mice that showed pronounced weight gain with increasing age (34). Interestingly, the opposite trend was seen in the GHR −/− mice. This was surprising because patients with GHR deficiency, or Laron syndrome, who lack GH signaling and thus are the human equivalent of the GHR −/− mice, exhibit obesity (35–38). This is often not reflected in their weight gain profiles because of a significant decrease in bone density and muscle mass that offsets the increase in adipose weight (35). Thus, it is possible that a similar phenomenon is occurring in the GHR −/− mice. In support of this are two reports of decreased bone density in the GHR −/− mice (20, 39).

It has also been suggested that GH has different effects on different fat depots (40–43). It is therefore possible that the epididymal fat pad weight did not accurately reflect the accumulation of other fat depots. A report by Li *et al.* (22) shows differences in accumulation of interscapular and epididymal white adipose tissue (WAT) in GHA and GHR −/− mice, compared with controls, supporting this possibility. Alternatively, it is possible that increased adiposity as seen in the GHA mice requires a low level of GH action during development to promote/induce differentiation of preadipocytes into adipocytes. Oberbauer *et al.* (44, 45) have demonstrated, using an inducible GH transgene, that an early transient exposure to elevated GH in mice results in an approximately 300% increase in WAT. No increase was seen for wild-type or continuously activated transgenic mice. Thus, the reduced level of GH action found in the GHA mice may still be enough to trigger adipocyte differentiation, but the complete lack of GH action found in the GHR −/− mice could limit adipocyte formation. Consistent with the induction of preadipocyte differentiation by GH (46–49), we have shown that the GHA inhibits mouse 3T3-F442A preadipocyte differentiation (11, 12). Therefore, GH may play a paradoxical role in fat metabolism with GH being required for adipocyte formation initially and then playing an important role in decreasing fat deposition in mature adipocytes. Future detailed analysis using the GHA and GHR −/− mice may help to resolve this paradox.

Differences in food consumption did not seem to account for the difference in weight or fat pad mass for the two dwarf mouse models. Although this study did show that the GHR−/− mice but not the GHA mice ate less than their littermate controls, when the food consumption was normalized to body weight, young GHA and GHR −/− mice ate significantly more than the controls. A possible explanation for the increased food intake/body weight in the GHA and GHR −/− mice may be due to an increase in brown adipose tissue (BAT). BAT is involved in nonshivering thermogenesis and exerts a larger energy expenditure than WAT. Our laboratory has recently demonstrated that BAT depots are enlarged in GHA and GHR −/− mice, compared with littermate controls (22). In addition, levels of uncoupling protein-1 were found to be higher in the GHA and GHR −/− mice (22). Thus, the GHA and GHR −/− mice may be expending more energy than the control mice, perhaps to compensate for a greater surface:volume ratio and therefore need more food. Previous reports of GH effects on feed efficiencies, determined by comparing the amount of weight gained per amount of food consumed (as opposed to food intake/body weight as reported in our study), indicate that GH increases feed efficiency (14, 50). Whether the increased BAT and uncoupling protein-1 observed in the GHA and GHR −/− mice alone accounts for the increased food intake/body weight remains unclear.

Examination of the other tissue weights for proportional changes also revealed several unexpected results. All tissue weights, with the exception of the epididymal fat pad as already discussed, were decreased for the GHA and GHR −/− mice, compared with their respective controls. However, when normalized to body weight, disproportional changes were observed for several of the tissues. As reported previously for the GHR −/− mice, the liver was disproportionately smaller and the brain disproportionately larger than control mice (20). The liver was also smaller for the GHA mice, but the brain was proportional. It is possible that the brain increase was negated by the increased body mass of the GHA mice. Kidney weights were also disproportionately decreased for the two dwarf lines. A similar tendency was seen by Sjögren *et al.* (20) for GHR −/− mice in a different genetic background, but the difference did not reach statistical significance. The gastrocnemius muscle and heart weights were disproportionately decreased for the GHA mice, a difference perhaps exaggerated by the increased body weight of the GHA mice. These differences in tissue weights suggest that the degree of GH signaling has tissue-specific effects.

The effects of GH on growth are in large part mediated by IGF-I, whose expression is regulated by GH signaling. Thus, as shown previously, IGF-I levels were reduced in GHA and GHR −/− mice (13, 15, 16, 18). The reduction was greater in GHR −/− mice. IGF-I action is regulated by a family of high-affinity IGFBPs. In states of GH deficiency in humans, IGFBP-1 and -2 levels are elevated, but IGFBP-3 and -4 levels are reduced (51, 52). In the GHA mice, IGFBP-3 levels were reduced, but the other IGFBPs remained unchanged. This was also seen in the GH-deficient lit/lit mice (34). The decrease in IGFBP-3 was expected because a correlation between IGF-I and IGFBP-3 levels has been observed that was caused, in part, by an increase of IGFBP-3 mRNA stability by IGF-I (53, 54), and IGF-I levels are significantly decreased in the GHA mice. In contrast, levels of all four binding proteins were altered in the GHR −/− mice. This differs from what was seen previously (18) but may be related in part to age when assayed, the overall decrease in body size, or the altered genotype distribution seen in the C57BL/6J background. As observed in states of GH deficiency in humans, IGFBP-2 levels were increased, but IGFBP-3 and -4 levels were decreased in the GHR −/− mice. Surprisingly, IGFBP-1 levels were reduced rather than increased as seen in humans. This may be related to the increased insulin sensitivity of the GHR −/− mice. Fasting plasma insulin levels were severely reduced in the GHR −/− mice and did not increase with age. However, GHR −/− mice tended to be slightly hypogly-

cemic, suggesting that they were more sensitive to insulin. This differs from the human Laron dwarfs who have increased levels of insulin relative to their glucose levels and, thus, are insulin resistant (55). It also differs from what was seen for the GHA mice. They maintained nearly normal levels of insulin and glucose and exhibited insulin resistance as they aged, similar to the control mice.

Because insulin is a strong inhibitor of IGFBP-1 expression (56–60) and the GHR −/− mice have extremely low insulin levels, the observed decrease in IGFBP-1 levels was opposite of expected. A possible explanation may be that, just as the GHR −/− mice exhibit an increased response to insulin, they may also exhibit an increased response to the inhibitory regulators of IGFBP-1. Alternatively, because many known positive regulators of IGFBP-1 exist, such as cAMP (56, 57), glucocorticoids (57, 58), progesterone (61), IL-1 (62), and relaxin (63), it is possible that the level of one or more of these compounds is decreased in the GHR −/− mice.

The last difference seen between the GHA and GHR −/− mice was the effect of altered GH signaling on lifespan. Caloric restriction, a lack of GH or GH signaling, reduced insulin levels, decreased body size, and reduced adiposity have all been correlated with an extension of life span (64–68). GHR −/− mice exhibit an increase in life span, even with different genetic backgrounds (18). Surprisingly, this extension of lifespan is not seen for the GHA mice. GHA mice have a dwarf phenotype, but it is not as pronounced as for GHR −/− mice. GHA mice have essentially normal levels of insulin and an increased caloric intake. Because moderate caloric restriction has been shown to increase life span in all species studied to date (69), one of our speculations before this study was that the GHR −/− mice eat less than their littermate controls, and therefore it was the effect of caloric restriction on longevity. But as discussed earlier, when the food consumption was normalized to body weight, both dwarf mouse models actually eat more. Therefore, when considering food consumption, caloric restriction does not appear to be the mode by which longevity is increased in the dwarf GHR −/− mice. A similar finding was recently reported for the fat-specific insulin receptor knockout (FIRKO) mice (68). Like the GHR −/− mice, the FIRKO mice have an extended life span as well as increased food consumption relative to body weight. Carter *et al.* (70) have recently argued that GH also plays an important role in gastrointestinal development and thus the increased life spans of the GH-related dwarf models may still be due to caloric restriction if nutrient absorption was adversely affected in the absence of GH action. GH levels were not reported for the FIRKO mice. Based on their studies of the FIRKO mice, Bluher *et al.* (68) suggested that leanness, not food restriction, is a key contributor to increased longevity. Our studies with the GHR −/− and GHA mice support this view in that the GHR −/− mice appeared to be leaner (*i.e.* less fat) than the GHA mice, and only the GHR −/− mice exhibited an increase in longevity.

Another key player in life span extension appears to be insulin exposure. Curiously, caloric-restricted animals and GHR −/− mouse models exhibit severely decreased insulin levels and live longer, but the GHA mice have normal levels of insulin and do not live longer. It is therefore possible that the lower insulin levels are responsible for the increased longevity. The theory of decreased insulin exposure and increased longevity is not new (71, 72). According to this theory, a decreased level of insulin is also associated with an overall decrease in growth factors such as GH (73, 74) and IGF-I (75). Interestingly, a reduction in insulin levels also occurs in Snell and Ames dwarf mice (66, 76). Both are long-lived mouse lines that are deficient in GH, prolactin, and thyroid hormone (21, 65, 66, 76–79). It is therefore tempting to speculate that the decreased insulin common to all four animal models for extended longevity (GHR −/− dwarf, Ames dwarf, Smell dwarf, and caloric-restricted mice) is indeed an important factor in life expectancy. Although insulin and IGF-I are decreased in these models, supporting the insulin exposure theory, the role of GH is more controversial. In contrast to early reports that caloric restriction decreases GH levels in rodents (73, 74), a more recent study indicates that moderate caloric restriction actually increases GH levels (69).

According to Sonntag *et al.* (69), moderate caloric restriction resulted in increased GH with decreased IGF-I and decreased insulin levels. Furthermore, Sonntag *et al.* suggested that this compensatory endocrine state (increased GH/decreased IGF-I axis) could possibly mediate the increased longevity that occurs during caloric restriction. Moreover, because GHR −/−, Ames, and Snell dwarf mice and even caloric-restricted mice live significantly longer and all three have decreased GH signaling with a subsequent decrease in IGF-I production, this suggests that GH administration in humans (such as GH replacement therapies in elderly humans) may actually decrease life span. However, there is at least one reported case that contradicts this, a study of mice treated with GH to normalize IGF-I levels in 29-month-old mice to that of 3-month-old mice (80). When this replacement therapy was given to 50 female mice, they had similar median and maximal life spans to nontreated female mice. Therefore, although the lack of GH signaling leads to a longer life span in the GHR/BP −/− mice and not in GHA mice, the precise role of GH in longevity remains unclear. Although low insulin in the GHR −/− mice is a likely candidate to explain the difference in longevity, a more severe decrease in IGF-I in the GHR −/− mice, compared with GHA mice, might also be involved. Further studies such as IGF-I replacement or restoring insulin levels to normal levels in the GHR/BP −/− mice may help elucidate such a mechanism.

In summary, although the GHA and GHR −/− mice shared a dwarf phenotype, differences were apparent between the two lines. Some of their differences, such as in life span extension, may be explained by their different degrees of GH signaling. This life span extension has also been seen in the Ames and Snell dwarf mice in which the GH signaling pathway has been disrupted (64, 65). Other differences, such as in fat accumulation, are harder to explain. Insulin levels are also different between the two dwarf lines. They are suppressed in the GHR −/− mice but normal in the GHA mice. Apparently, a complete lack of GH signaling is required for a significant decrease in insulin levels. These two models of reduced and absent GH signaling, in comparison

with the normal controls, provide an interesting assessment of the roles of GH signaling.

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