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ORIGINAL PAPER

Role of the GH/IGF-I axis in the growth retardation of weaver mice

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Abstract IGF-I is a well-established anabolic growth factor essential for growth and development. Although the role of the GH/IGF-I axis is established for normal postnatal growth, its functional state in neurodegenerative diseases is not fully characterized. The weaver mutant mouse is a commonly used model for studying hereditary cerebellar ataxia and provides an opportunity to investigate the function of IGF-I in postnatal growth following neurodegeneration. Previously, we reported that weaver mice are growth retarded and their body weights correlate with a decrease in circulating IGF-I levels. Because weaver mice have the same food intake/body weight ratios as their wild type littermates, our observation suggests that an impairment of the GH/IGF-I axis, rather than poor nutrition, likely contributes to their growth retardation. This study further investigated the etiology of reduced circulating IGF-I levels. We found that GH levels in weaver mice were reduced following acute insulin injection, but the hepatic GH receptor transduction pathway signaled normally as evidenced by increased STAT5b phosphorylation and IGF-I mRNA levels in response to acute GH administration. In addition, 2-week GH treatment induced a significant increase in body weight and circulating IGF-I levels in homozygous weaver mice but not in wild type littermates. In summary, a deficiency in the GH/IGF-I axis may be partially responsible for postnatal growth retardation in weaver mutant mice. This deficiency may occur at the level of the pituitary and/or hypothalamus and can be improved with GH administration.

Keywords Weaver mice · IGF-I · GH administration · Stat5b

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Introduction

The growth hormone (GH) and IGF-I axis plays a critical role in postnatal growth and development. The majority of circulating IGF-I is produced in the liver [1, 2] under the control of pituitary GH. The synthesis and release of GH is regulated by several hypothalamic hormones and by a negative feedback mechanism [3]. Data from genetically modified mice generally corroborates clinical observations concerning the importance of the GH/IGF-I axis in postnatal growth. Mice with GH deficiency (GHD) or GH receptor deficiency (GHRD) have dramatic decreases in their circulating IGF-I levels and body weights [4–7]. Conversely, expression of a GH transgene in mice induced increases not only in GH levels, but also in circulating IGF-I levels and body weights [8, 9]. Although GH also has IGF-I-independent effects on the growth of certain organs, most of GH's growth promoting effects are mediated by IGF-I [10]. GH administration did not increase postnatal growth in IGF-I deficient mice or human patients [10, 11]. On the other hand, administration of GH to GHD, but not GHRD, mice or humans led to an increase in circulating IGF-I levels and growth [12, 13], suggesting that GH stimulates IGF-I production via activation of the GHR.

Although the role of the GH/IGF-I axis is established for normal postnatal growth, its functional state in neurodegenerative diseases has not been fully characterized. The

weaver mouse is a commonly used ataxic model, which is a result of the depletion of most cerebellar granule neurons due to premature apoptosis [14–16]. We previously reported that this abnormal apoptosis may involve a decrease in IGF-I biological activity due to the overexpression of IGFBP5 by weaver granule neurons [17, 18]. Furthermore, we found expression of an IGF-I transgene in weaver mice resulted in improved muscle strength and a reduction in ataxia [19]. During the course of our study, we also found that weaver mice were growth retarded and their body weights correlated with a decrease in circulating IGF-I levels [20]. Although normal pituitary GH levels were previous reported in weaver mice [21], the circulating GH levels as well as the functional status of the GH/IGF-I axis has not been examined.

To investigate the cause of low circulating IGF-I levels and the functional states of the GH/IGF-I axis in weaver mice, we examined their circulating GH levels and the activity of the hepatic GH receptor-signaling pathway in response to exogenous GH stimulation. The body weights and circulating IGF-I levels were also measured following 2-week GH administration. Our results demonstrated that a deficiency of the GH/IGF-I axis is responsible for postnatal growth retardation in weaver mice and, therefore, maintaining normal function of GH/IGF-I axis may be important for patients suffering from neurodegenerative diseases.

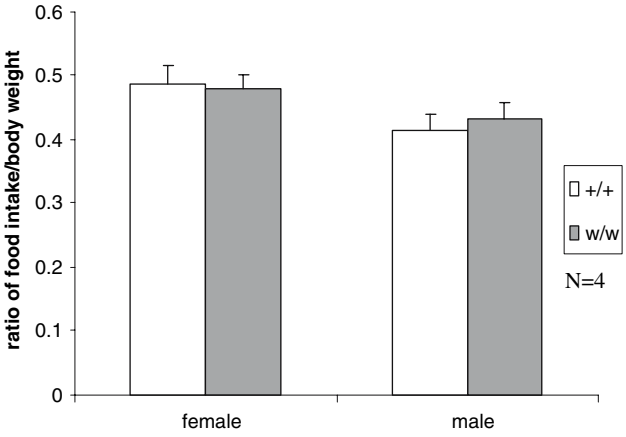

Fig. 1 Ratios of food intake/body weight in weaver mice. The ratio of food intake/body weight is measure in male and female weaver mice (w/w) and their wild type littermates (+/+) from D21 to D60. Each value represents means ± SEM (n = 4). No significant difference is found between weaver and wild type control mice, as assessed by Student's t-test

Results

Ratio of food intake/body weight of weaver mice

To evaluate whether poor nutrition due to movement disorders affects the postnatal growth of weaver mice, we compared the ratio of food intake/body weight from D21 to D60 between homozygous weaver mice and their wild type littermates. Our results showed no significant difference in the ratio of food intake/body weight between weaver and wild type mice (Fig. 1), suggesting nutrient intake is not a factor affecting their postnatal growth.

GH stimulated STAT5b phosphorylation in weaver mice

To examine the functional states of the hepatic GH signaling pathways in weaver mice, Western blot was used to measure levels of phosphorylated Stat5a and Stat5b levels in homozygous weaver mice (D21) and their wild type littermates at 1 h following vehicle or GH administration (Genotropin, 3 mg/kg, i.p.). As shown in Fig. 2, basal levels (saline treated) of phosphorylated Stat5b and Stat5a (data not shown) were higher in wild type control littermates than they were in weaver mice, indicating that GH signaling in weaver hepatocytes is subnormal. Moreover, levels of phosphorylated Stat5b, but not total Stat5b, were increased in both weaver homozygous mice (~59%) and their wild type littermates (~15%) following GH treatment.

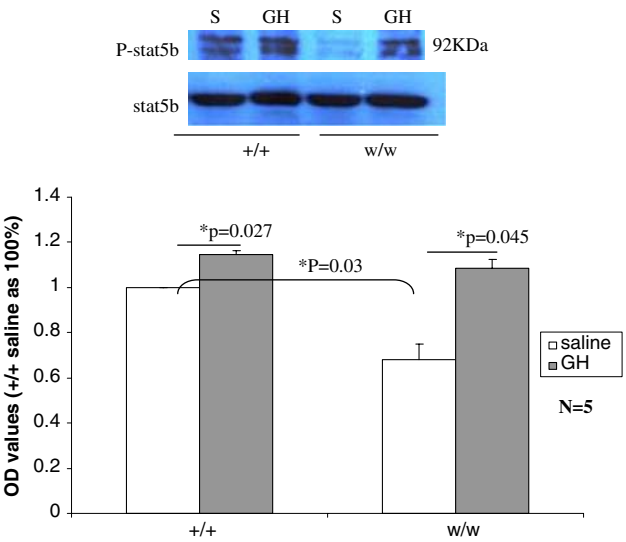

Fig. 2 Phosphorylated STAT5b levels in weaver mice after GH stimulation. Hepatic phosphorylated and total Stat5b proteins of +/+ and w/w D21 mice are measured by Western blot 1 h after saline/GH stimulation. S: saline (vehicle) treated; GH: growth hormone treated. Each value represents means ± SEM (n = 5). P-value is assessed by Student's t-test

GH promoted hepatic IGF-I gene expression in weaver mice

To see if the same signaling events occur following Stat5b phosphorylation in both weaver mice and their wild type littermates, Northern blot was used to measure hepatic IGF-I mRNA levels in liver samples collected at 1 h following GH administration. As shown in Fig. 3, basal hepatic IGF-I mRNA levels were higher in wild type littermates than they were in weaver mice, which was consistent with our previous report that weaver mice have lower hepatic IGF-I mRNA levels [20]. Following GH administration hepatic IGF-I mRNA showed a significant increase in weaver mice ($P < 0.019$) and a trend of increase in wild type mice ($P < 0.059$).

Weaver mice had lower serum GH levels following insulin injection

To see whether decreased circulating IGF-I levels in weaver mice were due to GHD, circulating GH levels were measured after insulin stimulation in male and female D21 homozygous weaver mice and their wild type littermates. As shown in Fig. 4, serum GH levels in weaver mice after insulin stimulation were 47% (female) and 52% (male) less than in their wild type littermates. This observation supports the hypothesis that GHD caused the decrease in circulating and hepatic IGF-I levels.

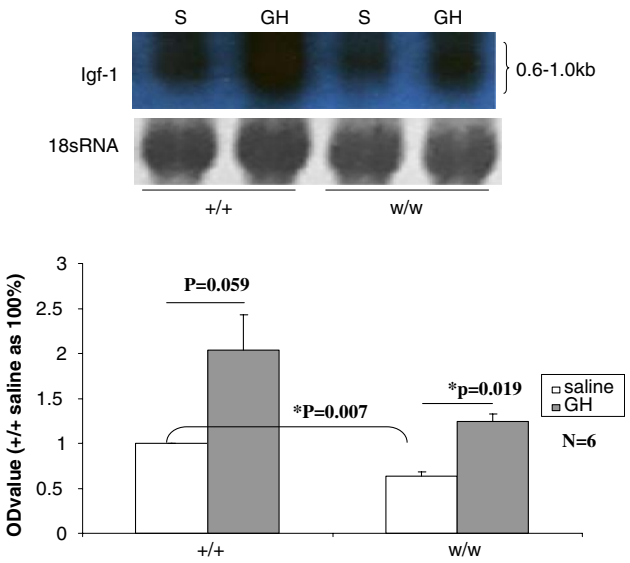

Fig. 3 Hepatic IGF-I mRNA levels in weaver mice after GH stimulation. Hepatic IGF-I mRNA levels of +/+ and w/w D21 mice are measured by Northern blot 1 h after saline/GH stimulation. The lower panel shows the quality and evenness of sample loading by ethidium bromide staining of the 18S on the agarose gel used for the same Northern blot. Changes in the hepatic IGF-I mRNA (0.6–1.0 kb) levels are quantified by densitometry analysis of autoradiography, within a linear range. Each value represents means ± SEM ($n = 6$). *P*-value is assessed by Student's *t*-test

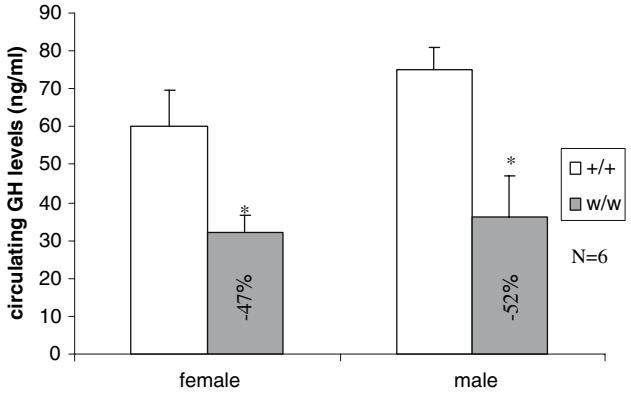

Fig. 4 Circulating GH levels in weaver mice. Serum GH levels of D21 male and female weaver mice (w/w) and their wild type littermates (+/+) 45 min after insulin injection are measured with an EIA kit. Value represents means ± SEM ($n = 6$). *$P < 0.05$ compared to their corresponding gender-matched +/+ mice, as assessed by Student's *t*-test

GH treatment increased circulating IGF-I levels and body weights of weaver mice

To investigate whether decreased circulating GH is responsible for weaver mouse growth retardation, we treated D21 weaver and wild type mice with GH (Genotropin, 3 mg/kg, s.c. twice/day) for 2 weeks. Circulating IGF-I levels and body weights were measured at the end of the GH treatment (D35). As shown in Fig. 5, GH administration significantly increased circulating IGF-I levels as well as the body weights of both male and female weaver mice, compared to their corresponding saline controls. The circulating IGF-I levels of homozygous weaver mice were approximately 42% (female) and 67% (male) higher, and their body weights were approximately 69% (female) and 42% (male) higher than their corresponding saline controls. In comparison, circulating IGF-I levels and body weights did not increase in wild type mice following GH administration using the same dosing regimen.

Discussion

This is part of a comprehensive study focusing on the functional role of the GH/IGF-I axis in neurodegenerative disease using the weaver cerebellar mutant mouse as the animal model. The growth retardation of weaver mice occurs soon after birth, simultaneous to the premature apoptosis of their cerebellar granule neurons [18, 20]. The weight gains of homozygous weaver mice consistently lag

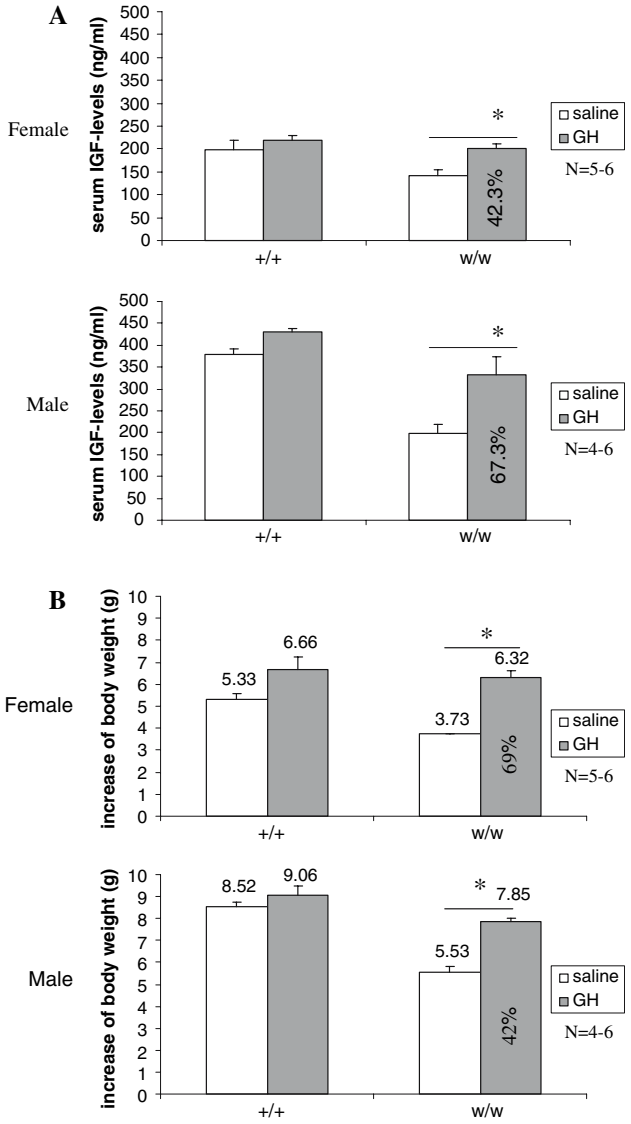

Fig. 5 Circulating IGF-I Levels and body weights in Weaver Mice after 2-week GH Treatment. Serum IGF-I levels (A) and the increase in body weights (B) of male and female weaver mice (w/w) and their wild type control littermates (+/+) are measured after 2 weeks saline or GH treatment. Each value represents means ± SEM (n = 4–6). *$P < 0.05$ compared to their corresponding control mice, as assessed by Student's t-test

behind those of their heterozygous and wild type littermates as they enter adulthood [20]. We previously showed that this postnatal growth retardation is associated with decreased hepatic and circulating IGF-I levels [20]. We now provide evidence suggesting that the decreased hepatic IGF-I synthesis is due to a decrease in circulating GH levels, while hepatic GH receptors function normally when stimulated.

Despite a wide variety of etiologies, low serum IGF-I levels were found in most mouse models of ataxia and human ataxic patients [22]. The low IGF-l levels are thought to contribute to the neurodegeneration of cerebellar neurons. For example, we found reduced levels of hepatic IGF-I mRNA and circulating IGF-I in weaver mutant mice [20] as well as Purkinje cell degeneration mice [23, 24]. Both mouse models have cerebellar ataxia but the etiology is caused by the death of different cerebellar neuronal populations. To date, no study has been performed to identify the cause of the low IGF-I levels associated with cerebellar ataxia or to assess the functional status of the GH/IGF-I axis in ataxic mice or human patients. We recently found that decreased circulating IGF-I levels are likely responsible for the poor growth of weaver mice, since homozygous weaver mice expressing an IGF-I transgene reach the same adult body weights as their wild type littermates (manuscript submitted). The current investigation is the first intending to identify the cause of decreased circulating IGF-I.

Most circulating IGF-I (~75%) was synthesized in and released from the liver [1, 2] under the control of GH. When GH binds to its receptor (GHR) on hepatocytes, multiple intracellular signaling pathways are activated, especially the classic JAK2-STAT5 signal transduction pathway [25]. Activated JAK2, a receptor-associated intracellular tyrosine-protein kinase, phosphorylates and activates the transcription factor STAT5 which then translocates into the nucleus, where it binds a specific DNA sequence in the IGF-I promoter region and activates IGF-I gene transcription [26–28]. Although both STAT5a and STAT5b are activated after GH binding as in this study, STAT5b, the predominant Stat5 mRNA (over 90%) in the liver [29], is believed to be responsible for hepatic IGF-I gene transcription [30]. Most recently, two distinct conserved GH response elements were identified in the rat IGF-I locus that contains paired Stat5b sites [26, 28]. Each pair consists of one high affinity and one lower affinity Stat5b site and is able to mediate GH-stimulated IGF-I transcription [26]. Furthermore, serum IGF-I and hepatic IGF-I mRNA levels were decreased in both STAT5b knockout (Stat5b−/−) and STAT5a/b knockout (Stat5a/b−/−) mice, but not in STAT5a knockout (Stat5a−/−) mice [31]. GH administration did not induce the expression of liver IGF-I mRNA in STAT5b−/− mice [32] and patients with mutations in the STAT5b gene have decreased serum IGF-I levels [33, 34]. The above observations suggest STAT5b plays a key role in coupling GH action and IGF-I synthesis.

Following insulin stimulation, levels of hepatic phosphorylated Stat5b and IGF-I mRNA levels were lower in homozygous weaver mice than in their wild type littermates. On the other hand, both were increased substantially upon acute GH administration. These results indicate that the GHR signaling pathway functions at subnormal levels in weaver mice, but can operate at the same capacity as their wild type littermates if exogenous GH is available. In

addition, we also found that IGF-I levels after GH administration were still lower in the weaver mouse than in wild type littermates. This might be due to the fact that the samples were obtained after only 1 h of GH administration. It has previously been shown that IGF-I levels are just beginning to increase 1 h after GH administration in vitro [30]. This probably relates to the need for Stat5b levels to increase before hepatic IGF-I synthesis increases. Regardless, this result demonstrated that the GHR signaling pathway functioned normally in weaver mice. On the other hand, the subnormal GHR signaling activity in weaver hepatocytes may be due to lower GH circulating levels in weaver mice than those in their wild type littermates.

Circulating GH levels after insulin stimulation were about 50% lower in male and female homozygous weaver mice on D21, compared with their corresponding wild type controls. Furthermore, administration of GH for 2 weeks induced significantly more weight gain and a parallel increase in circulating IGF-1 levels in both female and male homozygous weaver mice than that in saline treated weaver mice. These results are reminiscent of the parallel increases in circulating IGF-I levels and body weights in GH-treated hypophysectomized rats [35, 36] or GHD patients [37–40]. Unlike weaver mice, GH treatment for 2 weeks did not induce a significant increase in circulating IGF-I levels or body weight gain in wild type mice. This is likely due to the normal inhibitory feedback mechanism of the GH/IGF-I axis in wild type mice that prevents excess weight gain. In other words, exogenous GH stimulated hepatic IGF-I synthesis and release in the early period as demonstrated by increased hepatic Stat5b and IGF-I mRNA at 1 h following GH treatment. Soon after, increased IGF-I circulating levels inhibit endogenous pituitary GH synthesis. By the end of the 2-week treatment, the GH/IGF-I axis adapted to daily exogenous GH to reach a new balance. As a result, there was very little change in body weight gains and circulating IGF-I levels in the wild type littermates. On the other hand, the GH-induced coordinated increase in IGF-I circulating levels and body weights in weaver mice implied a lack of the normal inhibitory feedback mechanism or an impairment of the GH/IGF-I axis.

Why do weaver mice have lower circulating GH levels? The GH in circulation is synthesized and released from the anterior pituitary. Since homozygous weaver mice are reported to have normal pituitary GH mRNA levels [21], the cause of low GH circulating levels is likely related to the regulation of its release, which is under the control of a complex neuroendocrine network comprising hypothalamic GH releasing hormone, somatostatin, and ghrelin [3]. While GHRH has not been measured in weaver mice, our results do not support the involvement of ghrelin, which stimulates GHRH secretion and amplifies GHRH's effects on the somatotroph [41]. This is deduced from the fact that weaver mice have normal food intake, which is also known to be regulated by ghrelin [41]. On the other hand, somatostatin mRNA levels are 20–87% higher in the weaver mouse hypothalamus as demonstrated by in situ hybridization [42]. The excess hypothalamic somatostatin levels are likely one mechanism by which GH release from the anterior pituitary is inhibited, leading to a decrease in circulating GH levels. Alternatively, a decrease in GH binding protein levels may reduce circulating GH levels, even though pituitary GH expression is normal. GH binding protein consists of the GHR extracellular ligand-binding domain [43, 44] and functions as a physiological buffer to stabilize GH in plasma [45]. Further studies are needed to investigate these interesting hypotheses.

In conclusion, our study demonstrates that a deficiency of the GH/IGF-I axis is responsible for postnatal growth retardation in weaver mice. This deficiency is secondary to the weaver gene mutation. These data are consistent with a defect at the level of the pituitary and/or the hypothalamus. Because IGF-I circulating levels are decreased in most cerebellar ataxic mice and human patients, our study provides a pathological mechanism for IGF-I deficiency as well as a rationale for GH and/or IGF-I therapy for the treatment of ataxic patients.

Materials and methods

Animal and tissue preparation

The care and maintenance of the mice were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were obtained from a colony of mice maintained at the Indiana University Medical Center. This colony was established from mice heterozygous for the weaver gene purchased from the Jackson Laboratory (Bar Harbor, ME). Mutant and wild type (+/+) mice were maintained on a hybrid B6CBA-AW-J/A stock. Homozygous weaver mutants (w/w) were obtained by mating heterozygous or homozygous females with heterozygous males. Animals were provided with food and water ad libitum and placed on a 12:12 h light/dark cycle. Mice were housed two per cage with the same genotype. Wet-feed was always provided to weaver mice to maximize nutrition.

The ratio of food intake to body weight

Homozygous weaver mice ($n = 4$) and their wild type ($n = 4$) littermates were provided with wet-feed and housed in pairs of the same genotype. To monitor their

food intake, fresh pre-weighed wet-feed was provided daily and the leftover wet-feed was collected and weighed. The daily food intake was calculated by subtracting the weight of the leftover from the weight of fresh. This procedure was repeated from D21 to D60 and daily body weights were recorded for each mouse. The ratio of food intake to body weight was calculated by dividing the daily food intake by the daily body weight.

Genotyping

Genomic DNA was isolated from tails and the genotypes of weaver mice were determined by polymerase chain reaction (PCR) with primers as previously reported [46]. A point mutation of the K^+ ion channel (Girk2) [47] is associated with the weaver mutation [48, 49]. The DNA was amplified in a 25 µl reaction volume using primers AgeI-1 (5′-GCT TTT TTA TTC TCC ATA GAG ACA GAA ACC ACC ACC-3′) and AgeI-2 (5′-AAC ACG GAC TGG ATT AAG AAG-3′). In the weaver mouse, the AgeI primer has a C at the penultimate base of the 3′ end instead of a T, which is present in wild type DNA. In the final amplification product, this results in the loss of an AgeI site if the weaver mutation is present. The cycling parameters are: denaturation at 95°C for 2 min, followed by 35 amplification cycles (30 s at 95°C, 30 s at 66°C, 1 min at 72°C) and a final extension at 72°C for 5 min. After amplification, 10 µl of the PCR products were digested with AgeI in a 20 µl volume and evaluated by electrophoresis in an 8% polyacrylamide gel. The resulting restriction fragments were determined for mutant and normal alleles.

Acute GH administration

Twenty-one-day-old (D21) male and female homozygous mice and their wild type littermates were fasted overnight before the study. Animals were injected with recombinant human GH (Genotropin, 3 mg/kg body weight, i.p.) [50, 51]. One hour after injection, mice were sacrificed and their livers were harvested. Liver protein and total RNA were extracted and used for Western blot and Northern blot, respectively.

Western immunoblot

Total proteins were extracted from fresh livers by homogenization in a lysis buffer (10 mM Tris (pH 7.4), 1 mM $NaVO_4$, 1% SDS). The cell lysates were centrifuged for 10 min at 15,000*g* at 4°C. After discarding the cell debris in the bottom of the tubes, the supernatants (total proteins) were used for Western blot. To extract the nuclear protein, fresh livers were homogenized in Buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT (dithiothreitol), and 0.5 mM PMSF (phenylmethylsulphonyl fluoride)) and centrifuged for 10 min at 15,000*g* at 4°C. The cell pellet containing the nuclear proteins was suspended in Buffer C (20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% glycerol) and incubated for 15 min. The suspension was then centrifuged for 15 min at 15,000*g* at 4°C and the supernatant containing the nuclear proteins was analyzed. Total and nuclear proteins were separated on a 6% PAGE SDS gel simultaneously in different wells and then transferred to PVDF (polyvinylidene fluoride) membrane according to standard protocols. The membranes were blocked with 5% nonfat milk in TBST (Tris-Buffered Saline Tween-20) for 1 h at room temperature. The blots were then incubated with a mouse monoclonal antibody against STAT5b and phosphorylated-STAT5b (1:1000; Santa Cruiz) overnight at 4°C. After washing in TBST three times, the blots were incubated with goat anti-mouse peroxidase-conjugated secondary antibodies at 1:2000 dilution at room temperature, and the results were visualized using ECL plus (Amersham Biosciences). The data were analyzed by comparing each phosphorylated STAT5b to its corresponding total STAT5b on the same membrane.

Northern hybridization

Total hepatic RNA was prepared by the RNAzolTM B method according to the manufacturer's instructions, modified from the acid guanidinium thiocyanate–phenol–chloroform extraction method [52]. Briefly, 100 mg of frozen liver was homogenized in 2 ml of RNAzolTM B solution (Tel-Test Inc., Cat. No. CS-105), vigorously mixed with 0.2-ml chloroform, cooled 5 min on ice, and then centrifuged at 12,000*g* at 4°C. The aqueous layer was transferred to a new tube, mixed with an equal volume of isopropanol, stored at 4°C for 15 min, and then centrifuged at 12,000*g* for 15 min at 4°C. After centrifuging, the RNA pellet was washed in ice-cold 75% ethanol and then dissolved in diethylpyrocarbonate-treated water. After measuring the concentration, total RNA (10 µg) was electrophoresed on a 1.2% agarose formaldehyde gel at 20–25 V overnight. Photographs of the ethidium bromide-stained gel were taken under UV light. The quality and quantity of the 18S and 28S were used to assess the RNA integrity and 18S rRNA was used to normalize the loading. Separated RNA was transferred overnight onto nitrocellulose membranes by capillary transfer in 20× SSC (sodium

chloride/sodium citrate) overnight. They were then cross-linked to the membranes by exposure to UV light. Membranes were prehybridized (50% formamide, 0.4 M Na_2PO_4, 1 mM EDTA, 5% SDS, and 1% BSA, pH 7.2) for 1–2 h at 42°C and then probed with a 32P-cDNA probe labeled with a Random primer DNA labeling kit (Invitrogen, Cat. No. 18187-013). The cDNA template was a 362 bp PCR product amplified from the RT product of the total mouse hepatic RNA:

Forward: 5′-ttc aca tct ctt cta cct ggc-3′;
Reverse: 5′-tct tgt ttc ctg cac ttc ct-3′.

The membranes were incubated at 42°C overnight, then washed sequentially in 2× SSC, 1% SDS, 0.1× SSC, 1 mM EDTA, and 0.5% SDS all at room temperature. Buffer used in the final two washes consisted of 0.1× SSC, 1 mM EDTA, and 0.5% SDS for 30 min at 70°C. The membranes were then air dried, exposed to X-ray film at − 80°C for 2 weeks, and analyzed using scanning densitometry. Northern hybridization was performed twice with different RNA extractions of the same tissue sample, and the results were reproducible.

Enzymeimmunoassay (EIA)

Male and female D21 homozygous weaver mice and their wild type littermates were fasted overnight before the injection of porcine insulin (1.5 IU/kg body weight, i.p.). Forty-five minutes after injection, blood was collected from each mouse for GH measurement using the reagent and protocol provided in an enzymeimmunoassay (EIA) kit (ALPCO Diagnostics Inc., Windham, NH). Briefly, 25 μl sera from each mouse were diluted 1:5 in 100 μl dilution buffer. Next, 50 μl diluted samples and 50 μl rat GH antiserum were aliquoted into the 96-well plate. After 20 h incubation at room temperature, 50 μl AChE-labeled rat GH was dispensed to each well. Following 20 h incubation at room temperature, 200 μl Ellman's reagent was added and incubated in the dark at room temperature. When the maximum binding wells reached an absorbance of 0.6 unit, the plates were read at 410 nm and the resulting data were analyzed by a weighted four-parameter logistic algorithm method.

Long-term GH administration and radioimmunoassay

Male (n = 4–6) and female (n = 5–6) D21 homozygous weaver mice and their wild type littermates were injected with Genotropin (3 mg/kg body weight, s.c.) and vehicle (saline) twice a day for 2 weeks. On D35, their body weights were measured and blood was collected for measuring circulating IGF-I using a commercial radioimmunoassay (RIA) kit. All reagents and RIA procedures for determining mouse IGF-I were described in manufacturer instructions (ALPCO Diagnostics, Inc. Windham, NH). Before RIA, serum IGF-I was dissociated from the IGFBPs by diluting the serum (10 μl) in an acidic buffer (1 ml; 1:101 dilution) and adding excess recombinant human IGF-II (rhIGF-II) in the reaction mixture. The reaction mix consisted of 100 μl diluted sample, 100 μl anti-hIGF-I primary antibody, and 100 μl ^{125}I-IGF-I. The mixture was incubated for 2 days at 4°C. Then, 500 μl cold precipitation buffer containing anti-rabbit secondary antibody was added, mixed, and then incubated for 1 h at 4°C. After adding 1 ml ice-cold distilled water, the reaction mixtures were centrifuged at 3,000g for 30 min at 4°C. The supernatants were then carefully aspirated and the radioactivity in precipitates was measured in a gamma counter.

Statistical analysis

All values are presented as mean ± SEM. The difference between two groups was assessed by Student's t-test (two tails). Two mean values are considered statistically different if a P-value is less than 0.05.

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