Growth hormone inhibits hepatic de novo lipogenesis in adult mice

**Running title:** GH inhibits hepatic DNL

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**Word count:** 3920

**Number of Tables:** 2; **Number of figures:** 5
Abstract

Patients with nonalcoholic fatty liver disease (NAFLD) are reported to have low growth hormone (GH) production and/or hepatic GH resistance. GH replacement can resolve fatty liver in diet-induced obese rodents and in GH-deficient patients. However, it remains to be determined if this inhibitory action of GH is due to direct regulation of hepatic lipid metabolism. Therefore, an adult-onset, hepatocyte-specific, GH receptor knock-down (aLivGHRkd) mouse was developed to model hepatic GH resistance in humans that may occur after sexual maturation. Just seven days after aLivGHRkd, hepatic de novo lipogenesis (DNL) was increased in male and female chow-fed mice, compared to GHR-intact littermate controls. However, hepatosteatosis only developed in male and ovariectomized female aLivGHRkd mice. The increase in DNL observed in aLivGHRkd mice, was not associated with hyperactivation of the pathway by which insulin is classically considered to regulate DNL. However, glucokinase mRNA and protein levels as well as fructose-2,6-bisphosphate levels were increased in aLivGHRkd mice suggesting enhanced glycolysis drives DNL in the GH resistant liver. These results demonstrate hepatic GH actions normally serve to inhibit DNL, where loss of this inhibitory signal may explain, in part, the inappropriate increase in hepatic DNL observed in NAFLD patients.
Introduction

Patients with NAFLD show a higher prevalence of insulin resistance, characterized by increased fasting glucose and insulin, as well as an increase in non-esterified fatty acids (NEFA) (1). Nonalcoholic fatty liver disease (NAFLD) affects up to 30% of adults and represents a high risk factor in the progression to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocarcinoma (2; 3). Hepatic re-esterification of NEFA is considered a major contributor to NAFLD (4; 5). However, it is now evident that increased \textit{de novo} lipogenesis (DNL, production of new fatty acids from glucose (6)) also plays a significant role in NAFLD progression (5; 7-9), which may in part be driven by hyperinsulinemia (1).

Liver is a major target of growth hormone (GH). GH is required to maintain hepatic production of insulin-like growth factor I (IGF-I), where the liver is the primary source of circulating IGF-I (10). GH and IGF-I work together to promote longitudinal growth during the adolescent period and to support metabolic function in adults (11). Circulating GH levels are reduced by weight gain and decline progressively with age, independent of weight (12-14). A reduction in circulating GH levels or defects in hepatic GH signaling have been associated with NAFLD (15). Specifically, subjects with primary GH deficiency have a higher incidence of NAFLD, that can be reversed with GH replacement (16-18). Also, subjects with inactivating mutations in the GH receptor (GHR; Laron Syndrome) have a higher incidence of NAFLD, which cannot be reversed by IGF-I treatment (19), further suggesting GH plays a key role in regulating hepatic lipid processing. The negative association between GH and NAFLD is not limited to these rare conditions. In a large cross-sectional study, individuals with lower GH levels exhibited a higher prevalence of NAFLD (20). Since, GH is released in a pulsatile and diurnal fashion; a single GH measurement may not accurately represent cumulative GH released.
Therefore, many studies have used IGF-I as a surrogate marker of GH secretion. These studies demonstrate that circulating IGF-I levels are negatively associated with NAFLD (21-25). The reduction in GH levels that occur with weight gain and obesity could certainly contribute to the low IGF-I levels associated with NAFLD. However, the fact that IGF-I levels remain significantly lower in NAFLD after corrections for weight, waist circumference and diabetes (25), suggests their livers are resistant to the actions of GH. In fact, mice made obese by high-fat feeding fail to respond to an acute GH injection by increasing hepatic pStat5b (26), where the GHR/Jak2/Stat5b signal transduction pathway is required for GH-mediated regulation of IGF-I gene expression (27). In addition, rats fed a high-fat, low-carbohydrate diet exhibited a decrease in hepatic expression of the GHR (mRNA and protein), pStat5b protein and IGF-I mRNA levels (28). Also hepatic insulin resistance, characterized by impaired IRS/Akt inactivation of Foxo1, could lead to hepatic GH resistance since Foxo1 has been shown to decrease hepatic GHR expression (29).

The reduction in GH production/signaling and subsequent fall in IGF-I levels may not simply be the consequence of NAFLD, but could actually contribute to the progression of NAFLD, based on studies showing an increase in hepatic triglyceride (TG) content in humans treated with the GHR antagonist, pegvisomant (30), and in mice expressing a GHR antagonist (31). In fact, raising GH decreases hepatic TG content in animal models with hepatosteatosis (32; 33), as well as GHD patients (16; 17). However, questions regarding how GH mediates this inhibitory effect still remain. Therefore, our laboratory has conducted a series of studies to address whether GH directly regulates hepatic fat production/accumulation in adults. Specifically, a mouse model was generated with adult-onset liver-specific GHR knock-down (aLivGHRkd). The rate of hepatic DNL more than doubled, just 7 days after induction of hepatic
GH resistance. The increase in DNL and hepatoesteatosis in aLivGHRkd mice was not associated with an increase in insulin’s actions on the liver or an increase in white adipose tissue (WAT) lipolysis, but was associated with endpoints suggesting that glycolysis is enhanced.

Research Design and Methods

Generation of aLivGHRkd and littermate controls

All mouse studies were approved by the IACUC of the Jesse Brown VA Medical Center. C57Bl/6J GHR-floxed (34) mice were housed in a temperature (22-24°C) and humidity controlled specific-pathogen free barrier facility with 12h/12h light/dark cycle (lights on at 0600h) and fed standard laboratory rodent chow, unless otherwise indicated. Ten to twelve week-old mice were injected in the lateral-tail vein with 100µl saline containing 1.5x10^{11} genome copies of an adeno-associated virus (AAV) bearing a liver-specific thyroxine-binding globulin (TBG)-promoter driving a Cre recombinase transgene (AAV-TBGp-Cre, Cat#AV-8-PV1091, AAV8.TBG.PI.Cre.rBG, Penn Vector Core, University of Pennsylvania) or a null allele (AAV-TBGp-Null, Cat#AV-8-PV0148, AAV8.TBG.PI.Null.bGH, Penn Vector Core). TBGp is a hepatocyte-specific promoter that allows expression of Cre recombinase exclusively in hepatocytes (see Supplemental Figure 1B-E), leading to recombination of the Ghr allele, and knockdown of the hepatic Ghr mRNA and protein (Figure 1A,B and Supplemental Figure 1C and 2). GHR-floxed littermate mice injected with AAV-TBGp-Null served as controls. Injection of AAV vectors did not increase markers of inflammation (Supplemental Figure 3). Mice were killed seven days after AAV injection.

Hormone and metabolites

Plasma GH, insulin (Millipore, Billerica, MA), IGF-I (Immunodiagnostic Systems, Gaithersburg, MD), ALT (Biovision, Milpitas, CA), TG and NEFA (Wako Diagnostics,
Richmond VA) were measured following the manufacturer’s instructions. Hepatic TG levels were measured after extraction of neutral lipids (35) using Wako Diagnostics reagents. Hepatic glycogen was measured by the Yale Metabolic Mouse Phenotyping Center (MMPC; Yale School of Medicine, New Haven CT). Hepatic F2,6BP was measured using the method described by Van Schaftingen et al. (36). Hepatic acyl-CoA species (Acetyl-CoA, Malonyl-CoA, β-hydroxybutyryl-CoA (BHB-CoA)) were determined by LC/MS by CASE MMPC (Case Western Reserve University, Cleveland OH).

**Hepatic de novo lipogenesis (DNL).**

To measure hepatic DNL, food was removed at 0600h and mice injected at 1200h with 30µl/g BW 0.9% NaCl deuterated water (²H₂O, to enrich body water up to 4%, Sigma-Aldrich, Madison, WI). 4h-later (1600h), mice were killed by cervical dislocation under isofluorane anesthesia and blood was collected from the inferior cava into heparinized syringes (~50 µl). This condition represents the natural post-absorptive state of mice, where mice consume less than 20% of their total food intake during the day. Livers were collected and snap-frozen in liquid nitrogen. Blood and livers were sent to Case Western MMPC for the determination of the amount of newly synthetized fatty acids bound to TG. Total TG-bound fatty acids were isolated from tissues by chemical hydrolysis and extraction procedures. The percent ²H-labeled fatty acids were analyzed by gas chromatography mass spectrometry (GC-MS). The ²H-labeled fatty acid (palmitate, stearate or oleate) covalently attached to glycerol indicates the amount of new fatty acids. The contribution of DNL to the pool of triglyceride and palmitate was calculated using the following equation: % ²H-labeled palmitate = [total ²H-labeled palmitate × (²H-labeled body water × n) -1] × 100, where n is the number of exchangeable hydrogens, which is assumed to be 22 (37; 38), and ²H-labeled total body water enrichment was measured from the blood.
collected from each mouse and determined using the acetone exchange method and measured by GC-MS (39).

**Ovariectomy**

Eight to nine week-old GHR-floxed female mice underwent sham-operations or bilateral ovariectomy under isoflurane anesthesia. A subset of mice were implanted (sc) with a slow-release pellet containing 0.1mg 17β-estradiol (#SE-121, Innovative Research of America, Saratoga, FL), at the time of the ovariectomy. Two weeks later, mice were injected with AAV-TBGp-Null or AAV-TBGp-Cre in the lateral-tail vein, and females were killed 7d later.

**Assessment of other metabolic endpoints**

*Triglyceride clearance* was assessed in overnight fasted mice (1700h-0900h) after an oral gavage of 200μl of food-grade olive oil (Oleoestepa, Estepa, Spain). Rate of *hepatic VLDL-TG secretion* was assessed after tyloxapol injection (Sigma-Aldrich, 500mg/kg, ip). *Glucose tolerance tests* were performed in overnight fasted mice injected with 2mg/g-BW glucose ip. *Insulin tolerance tests* were performed in mice 4h after food removal starting at 0700h by injecting 1.5 mU/g-BW insulin ip (Novolin, Novo Nordisk, Bagsvaerd, Denmark). Blood samples were taken from lateral tail vein and blood glucose measured (Alphatrack 2, Abbott laboratories, Abbott Park, IL). To assess *ex vivo WAT lipolysis*, 50-60mg of tissue from urogenital fat pads was washed in cold-PBS and minced in small pieces, then incubated in 500μl Krebs-Ringer Heps Buffer without or with 1μM isoproterenol for 2h at 37°C 5%CO₂. Media was collected to measure glycerol production (Free glycerol reagent, Sigma-Aldrich). *Hepatic insulin sensitivity* was assessed in overnight fasted mice injected with saline or 2mU/g-BW insulin ip. Twenty min later, liver was collected for Western blot analysis.

*Gene expression analysis.*
Tissues were processed as previously described (35). qPCR primers used are indicated in Supplemental Table 3.

**Western-blot.**

Livers were homogenized using buffers containing protease inhibitors. Equal amounts of denatured proteins were separated by SDS-PAGE gels (Bio-Rad Laboratories), transferred to nitrocellulose membranes and incubated with primary and secondary antibodies shown in Supplemental Table 4.

**Statistics**

Two-tailed t-student's tests were performed to analyze the effect of aLivGHRkd, isoproterenol-stimulated lipolysis and insulin-mediated AKT phosphorylation. Two-way ANOVA followed by Bonferroni post hoc test were performed to compare glucose changes in response to glucose and insulin tolerance tests, and TG changes after tyloxapol injection or oral lipid load. p-values less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**Results**

**Knock-down of the hepatic GHR, in adult mice, rapidly leads to fatty liver in males, while estrogen protects females from lipid accumulation.**

Treatment of adult GHR-floxed mice with a single injection of AAV-TBGp-Cre, resulted in liver-specific recombination of the Ghr allele (Supplemental Figure 1C), leading to aLivGHRkd, as confirmed by a dramatic reduction in Ghr mRNA and GHR protein levels, compared to AAV-TBGp-Null treated, GHR-floxed littermate controls (Figure 1A, B and Supplemental Figure 2). Consistent with the requirement for GH to maintain hepatic IGF-I production, aLivGHRkd mice exhibited a reduction in hepatic Igf1 mRNA and circulating IGF-I
levels, compared to controls (Figure 1C, D). However, the suppression of IGF-I in female aLivGHRkd mice was not as dramatic as that observed in males, which may be due to the GH-independent effects of estrogen on IGF-I expression (40). Since IGF-I inhibits pituitary GH production, we examined the expression of pituitary GH, GH releasing hormone receptor (GHRH-R), and ghrelin receptor (GHS-R) and all were increased in aLivGHRkd male, but not female mice (Supplemental Figure 4). Nonetheless, these pituitary changes did not lead to a significant increase in circulating GH levels (Supplemental Figure 4B).

Seven days after induction of aLivGHRkd, liver weight was increased in both male and female aLivGHRkd mice that were maintained on a standard chow diet (Figure 1E). Hepatic glycogen and TG content increased in male, but not female, aLivGHRkd mice (Figure 1F, G, I-K). Since it has been reported that premenopausal women are protected from NAFLD (41; 42), and ovary-intact, but not ovariectomized (OVX) mice are protected from diet-induced fatty liver (43), we examined the impact of aLivGHRkd in OVX mice, with and without estrogen (E2) replacement (Fig. 1H, L-N). Three weeks after OVX in GHR-intact mice, hepatic TG content did not differ from sham-operated controls. However, induction of aLivGHRkd two weeks after OVX, doubled hepatic TG content one week later, and this effect was blocked by estrogen replacement (Fig. 1H, L-N). Therefore, estrogen protects female mice from excess hepatic TG accumulation induced by hepatic GH resistance.

**Hepatic GH resistance increases DNL in both male and female mice.**

A series of studies were conducted to identify the source of excess TG accumulation in livers of male aLivGHRkd mice, and to begin to explore why ovary-intact aLivGHRkd females are protected. The rate of hepatic VLDL-TG released, after inhibition of systemic lipoprotein lipase activity by tyloxapol, did not differ between aLivGHRkd mice and controls (Figure 2A).
Also, the rate of TG clearance following an oral gavage of olive oil was not altered by aLivGHRkd (Figure 2B). Although enhanced GH-mediated WAT lipolytic activity is thought to contribute to fatty liver in mouse models with congenital defects in hepatic GH signaling (44; 45), we found no evidence to support enhanced WAT lipolysis in aLivGHRkd mice. Specifically, there was no increase in plasma NEFA levels, under different conditions (Table 1, and Supplemental Table 1). Also there was no reduction in WAT sub-depot weights or alterations in WAT lipolytic/lipogenic gene expression (Table 1). Consistent with these observations, ex vivo explants of WAT from aLivGHRkd mice did not release more glycerol under basal or isoproterenol-stimulated conditions (Figure 2C, and Supplemental Table 1). Taken together, these results demonstrate that the increase in hepatic TG observed in male aLivGHRkd mice is unlikely due to a decrease in hepatic lipoproteins release, increases in hepatic TG uptake or re-esterification of NEFA derived from peripheral sources.

Common to both males and females, hepatic GH resistance more than doubled the rate of DNL (Figure 2D). These findings clearly demonstrate that intact GHR signaling is required to suppress hepatic DNL, independent of sex. The reason why females are protected from hepatic TG accumulation, despite enhanced DNL, remains to be determined. However, recent studies suggest estrogen promotes the flux of lipids from the liver into adipose tissue stores (46)(47). This would be consistent with the observation that urogenital fat pad weight increased in female, but not male aLivGHRkd mice (Table 1). In addition, circulating TG levels increase in male, but not female, aLivGHRkd mice (Supplemental Table 1). Although intriguing, further studies are required to better define these sexually dimorphic responses. Nonetheless, given the profound phenotype observed in male mice after aLivGHRkd, subsequent studies used male mice to begin to explore how GH regulates hepatic DNL.
The canonical pathway by which insulin drives lipogenesis is not enhanced by hepatic GH resistance.

Since insulin is a key driver of hepatic DNL through AKT-mediated maturation of the lipogenic transcription factor, sterol regulatory element binding protein 1c (SREBP1c) (6), we examined the impact of short-term hepatic GH resistance on circulating insulin levels, as well as systemic and hepatic insulin sensitivity. Insulin levels did not differ from controls under conditions used to study DNL (10h after food removal starting at 0600h, Table 2) or after an overnight fast (16h after food removal starting at 1700h, Supplemental Table 2). However, insulin was elevated under basal conditions (4h food removal starting at 0800h, Supplemental Table 1), but was not associated with systemic insulin resistance, as measured by insulin or glucose tolerance tests (Figure 3A,B). In addition, hepatic levels of mature SREBP1c (mSREBP1c) were not elevated, and in fact were reduced in the same conditions used to assess DNL (Figure 3C). This disconnect between insulin and mSREBP1c, could be explained in part by the observation that insulin-mediated phosphorylation of AKT was impaired in the livers of aLivGHRkd mice (Figure 3D). These results suggest that increased hepatic DNL rate in aLivGHRkd mice is not due to hyperactivation of the canonical insulin signaling associated with DNL.

Hepatic gene profile, cytosolic GCK and F2,6BP levels suggest glycolysis is driving enhanced DNL in the GH resistant liver.

The expression of key genes and metabolites involved in hepatic lipid and glucose metabolism, as illustrated in Figure 4A (6), were measured in the same livers in which DNL was evaluated, in order to ascertain the potential point of control of enhanced DNL by hepatic GH resistance. Consistent with the observation that mSREBP1c was not increased, the expression of
SREBP1c lipogenic target genes (Acc-1, Fasn and Elovl6) were also not elevated above control levels (Figure 4B). Interestingly, Scd-1 expression was dramatically increased in aLivGHRkd mice, where an increase in SCD-1 activity has been shown to promote DNL, by decreasing the level of saturated fatty acids that are known inhibitors of ACC-1 activity (48). In addition, reduced Cpt1a mRNA (Figure 4B) and BHB-CoA levels and a tendency (p=0.06) for lower plasma ketone levels (Figure 4C,D) indicate fatty acid oxidation is suppressed in the aLivGHRkd mice, which could contribute to the hepatic TG accumulation. However, acetyl-CoA and malonyl-CoA levels were normal or tended to be increased, respectively (Figure 4E,F), where malonyl-CoA may serve to suppress CPT1a activity (6). In addition, expression of the gluconeogenic gene, Pck-1, was suppressed (Figure 4B) and is consistent with previous reports showing that GH/JAK2/STAT5b directly stimulates hepatic Pck-1 expression (49).

Normal acetyl-CoA levels in aLivGHRkd livers, in presence of enhanced DNL and suppressed fatty acid oxidation/gluconeogenesis, suggest that glycolysis likely provides carbons to maintain acetyl-CoA pools. Increased expression of glucose-uptake related genes (Glut2 and Gck, Figure 4B) and total GCK protein levels (Figure 4G) suggest that glucose is taken up more efficiently by the aLivGHRkd livers to feed the glycolytic route and provide carbons for DNL.

Because the rate of glycolysis-driven DNL is highest following a meal, the same gene panel measured in Fig. 4B was measured in a different set of mice that were fasted overnight and then fed with a standard chow-diet for 6h (6h-refed; Figure 5A). The expression of Glut-2, Gck, Pklr, G6pc, Fasn and Scd-1 was greater in refed aLivGHRkd mice, as compared to refed controls. Of note, cytosolic GCK protein levels remained elevated in aLivGHRkd in both fasted and refed conditions (Figure 5B). Since GCK is active in the cytosol, it can be concluded that GCK activity is sustained in the GH resistant liver. Importantly, fructose 2,6-bisphosphate
(F2,6BP), the most potent activator of glycolysis and inhibitor of gluconeogenesis (50), was increased in aLivGHRkd livers (Figure 4E). Taken together, these results suggest that hepatic GH resistance enhances hepatic glucose uptake (GCK) and glycolysis (F2,6BP), thereby supplying substrate for glycogenesis and DNL.

Discussion.

Existing literature cannot readily explain why GH protects the adult liver from excess fat accumulation. It has been previously reported that mouse models with congenital, liver-specific knockout of the GHR or its downstream effectors (JAK2 and Stat5) develop hepatosteatosis as adults (44; 45; 51-53). In these mice, hepatosteatosis is thought to be mediated by the indirect actions of GH since circulating IGF-I was reduced, leading to a rise in GH which is thought to drive systemic insulin resistance and WAT lipolysis, thereby shifting the flux of fatty acids to the liver, independent of changes in DNL (45). However, mice with congenital liver-specific knockout of IGF-I [LID mice; (54)] do not exhibit hepatosteatosis relative to intact controls, despite elevated GH levels and systemic insulin resistance. In fact, the livers of LID mice are protected from fat accumulation that develops with age (55), suggesting GH may act directly on the liver to block excessive fat accumulation. Unfortunately, the direct effect of GH on hepatic metabolism is difficult to assess using congenital models, in that low IGF-I / high GH levels during development alters the development of other tissues, including muscle, fat and bone (44; 54), which could indirectly contribute to the adult metabolic phenotype.

To test the direct effect of GH on adult liver metabolism, independent of the confounding factors that arise in congenital knockout models, we generated a mouse model with adult-onset, liver-specific knockdown of the GHR (aLivGHRkd). In sharp contrast to previous models, aLivGHRkd mice exhibit an increase in hepatic DNL sufficient to increase hepatic TG content,
independent of changes in systemic insulin sensitivity or WAT lipolysis. These observations reveal, for the first time, that hepatic GHR is critical to keep DNL under control in order to prevent fatty liver development. Since NAFLD patients have low GH production/signaling (21-25), as well as elevated DNL (5; 8), our current data suggest that the reduction in hepatic GH action may directly contribute to inappropriate DNL in this patient population.

Both male and female aLivGHRkd mice exhibited an increase in hepatic DNL, but only males rapidly accumulate lipids. The first reports examining the liver phenotype of mice with congenital hepatic GH signaling defects focused on males and did not indicate any sex differences (44; 45; 51). More recently, List et al, (56) reported that female LivGHRKO mice accumulate less TG in the liver, when examined at six months of age, consistent with our observations in aLivGHRkd mice. In the current study, we found that intact-ovarian function and estrogen protects female aLivGHRkd mice from excess hepatic fat accumulation. These results are consistent with reports indicating premenopausal women show a lower prevalence of NAFLD, as compared to postmenopausal women and men (41; 42) and ovaries (estrogen) protect female mice from diet-induced hepatosteatosis (43; 46; 57). Estrogens may protect the liver from hepatic lipid accumulation at multiple levels (47). Estrogen improves whole body insulin sensitivity in humans and rodents, favoring fat deposition in adipose tissue (47). However, recent studies suggest that estrogen acting through hepatic ERα, blocks insulin-mediated suppression of hepatic TG secretion, thereby reducing hepatic TG accumulation (46). Therefore, estrogen may protect the female aLivGHRkd liver from excessive fat accumulation by shifting the flux of newly formed TG from the liver to the adipose tissue, as supported by our observation that urogenital fat pad weights were increased in female aLivGHRkd mice. However, future studies will be required to specifically test this hypothesis.
Despite the sexually dimorphic impact of aLivGHRkd on hepatic TG accumulation, loss of hepatic GH signaling clearly increased DNL in both male and female mice. GH is commonly thought to antagonize the actions of insulin (58). Therefore, it might be assumed that a reduction in GH signaling would simply enhance the ability of insulin to promote lipid production. However, in livers of aLivGHRkd mice, proximal insulin signaling, SREBP1c maturation, or expression of SREBP1c target genes (Acc-1, Fasn, Elovl6) were not increased in association with enhanced DNL that was measured in the post-absorptive state. Since SREBP1c is considered the canonical target of insulin-mediated increases in hepatic TG production, the normal/low levels of mSREBP1c in aLivGHRkd mice suggests GH acts independently of classic insulin-mediated lipogenic pathways to modulate hepatic DNL.

Although the exact control points remain to be established, three pieces of evidence suggest that glycolysis is increased in the aLivGHRkd liver. First, the increase in Gck mRNA levels was reflected by an increase in total GCK protein levels, as well as an increase in cytosolic GCK protein levels (active form). An increase in active GCK would trap glucose as glucose-6-phosphate to favor glycolysis (6). Consistent with the increase in Gck expression in the aLivGHRkd liver, it was observed that two days of GH-treatment (ip twice daily) suppressed Gck mRNA in diet-induced obese male mice (personal communication of Dr. Edward O. List, Ohio University), where GH treatment ultimately led to a reduction in hepatic TG content (33). Second, the level of fructose 2,6 bisphosphate (F2,6BP) was increased in fasted and refed aLivGHRkd mice. F2,6BP is a potent inhibitor of fructose-1,6-bisphosphatase and activator of phosphofructokinase-1, so an increase of F2,6BP levels would serve to inhibit gluconeogenesis and promote glycolysis (6). Third, the expression of Pklr and G6pc is increased in aLivGHRkd livers after refeeding, above that observed in refed controls. These genes are unique targets of ChREBP, where ChREBP requires
glucose metabolites produced during glycolysis for its activation (6). These results, coupled with experimental and clinical reports showing overexpression or pharmacologic activation of GCK can increase DNL and hepatic TG accumulation (59; 60), and patients with NAFLD exhibit an increase in Gck expression (61) and DNL (8), raise the possibility hepatic GH resistant might contribute to NAFLD by increasing glycolysis-mediated DNL.

This study demonstrates for the first time that GH directly regulates hepatic TG content by keeping DNL under control. This information is highly translational and provides a new molecular mechanism controlled by GH in the liver which may help to understand how GH therapy reverses NAFLD in individuals with GH deficiency and may provide insight into an ongoing clinical trial examining if low-dose GH therapy can reverse NAFLD in the general population (NCT 02217345).

Acknowledgements

We thank Dr. Rafael de Cabo (Experimental Gerontology Section, Translational Gerontology Branch, National Institute on Aging, National Institutes of Health, Baltimore, MD) for his assistance in the protein analysis of the aLivGHRkd livers. We thank Dr. Gary Cline (Yale MMPC, Yale Medical School, New Haven, CT), for the analytical measurements to determine glycogen concentrations.

Author contributions: JCC and RDK designed, performed the experiments, analyzed the data and wrote the manuscript; NM, performed experiments; ADR and MP performed experiments and provide key technical assistance; RB and AM measured F2,6BP; SJF provided the GHR antibody, EOL and JJK provided GHR floxed mice. All authors reviewed and approved the final version of the manuscript.
Duality of interest: JCC (Scholar Award Recipient, Genentech, Inc/Endocrine Society). Other authors do not have any conflict of interest.

Funding sources: This work was supported by Endocrine Scholar Award in Growth Hormone Research (Endocrine Society) (to JCC), Intramural Research Program of the NIH, National Institute of Aging (to ADR), CASE MMPC U24 DK76174 (to MP), Department of Veterans Affairs, Office of Research and Development Merit Award BX001114, National Institutes of Health R01DK088133 (to RDK), Instituto de Salud Carlos III, Spain, PI13-00096 (to RB), and State of Ohio’s Eminent Scholar Program that includes a gift by Milton and Lawrence Goll and National Institutes of Health P01AG031736 (to JJK).

Guarantor statement: RDK serves as guarantor of this manuscript.

Part of this work have been presented at the 16th International Congress of Endocrinology & the Endocrine Society’s 96th Annual Meeting & Expo held in Chicago, IL (June 2014) and at the Endocrine Society’s 97th Annual Meeting & Expo held in San Diego, CA (March 2015).


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Table 1. aLivGHRkd does not increase WAT lypolysis

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<td>PlasmNEFA (mEq/L)</td>
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Fat depot weight/BW

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Lipolytic genes mRNA

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<td>Hsl</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td>100.00 ± 2.56</td>
<td>111.31 ± 4.30</td>
</tr>
<tr>
<td>Atgl</td>
<td>100.00 ± 16.76</td>
<td>100.67 ± 8.63</td>
</tr>
<tr>
<td>Adrb3</td>
<td>100.00 ± 26.14</td>
<td>101.64 ± 8.83</td>
</tr>
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</table>

Lipogenic genes mRNA
<table>
<thead>
<tr>
<th>Gene</th>
<th>Baseline</th>
<th>SD</th>
<th>Control</th>
<th>SD</th>
<th>aLivGHRkd</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
<td>Srebp1c</td>
<td>100.00</td>
<td>± 12.19</td>
<td>97.50 ± 7.36</td>
<td>100.00 ± 23.13</td>
<td>101.58 ± 19.13</td>
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<tr>
<td>Pparg</td>
<td>100.00</td>
<td>± 22.70</td>
<td>132.69 ± 4.66</td>
<td>100.00 ± 15.06</td>
<td>95.82 ± 10.38</td>
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</tr>
<tr>
<td>Fasn</td>
<td>100.00</td>
<td>± 18.55</td>
<td>118.11 ± 26.93</td>
<td>100.00 ± 21.69</td>
<td>92.76 ± 27.34</td>
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<tr>
<td>Scd1</td>
<td>100.00</td>
<td>± 18.06</td>
<td>103.36 ± 8.20</td>
<td>100.00 ± 18.22</td>
<td>92.62 ± 22.21</td>
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<tr>
<td>Gpat</td>
<td>100.00</td>
<td>± 10.72</td>
<td>88.28 ± 8.18</td>
<td>100.00 ± 15.02</td>
<td>119.08 ± 14.34</td>
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<tr>
<td>Dgat2</td>
<td>100.00</td>
<td>± 38.35</td>
<td>131.35 ± 13.96</td>
<td>100.00 ± 21.14</td>
<td>91.15 ± 20.29</td>
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Plasma NEFA and WAT (unilateral subdepot mg/g BW x 10^3) weight (UG, urogenital; RP, retroperitoneal; SC, subcutaneous) and UG-WAT lipolytic/lipogenic gene expression: Hsl, hormone sensitive lipase; Atgl, adipose triglyceride lipase; Aдрb3, adrenergic receptor, β3; Srebp1c, sterol regulatory element binding transcription factor 1c; PPARγ, peroxisome proliferator activated receptor gamma; Fasn, fatty acid synthase; Scd1, steroyl-CoA desaturase 1; Gpat, glycerol-3-phosphate acyltransferase; Dgat2, diacylglycerol O-acyltransferase 2. Asterisk indicates differences between control and aLivGHRkd. Mice were killed 4h after food removal starting at 0800h. Males and females were analyzed at separated times and they were not littermates, therefore values between males and females should not be directly compared. *, p<0.05
Table 2. Plasma NEFA and insulin levels in male and female control and aLivGHRkd mice.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>aLivGHRkd</td>
<td>Control</td>
<td>aLivGHRkd</td>
</tr>
<tr>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
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<tr>
<td></td>
<td>SEM</td>
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<td>SEM</td>
<td>SEM</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>1.05±0.07</td>
<td>0.91±0.07</td>
<td>0.62±0.06</td>
<td>0.66±0.05</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.04±0.21</td>
<td>1.37±0.14</td>
<td>0.65±0.07</td>
<td>0.78±0.08</td>
</tr>
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</table>

Mice were killed 10h after food removal at 0600h. Blood was taken from inferior cava vein. Males and females were analyzed at separated times and they were not littermates, therefore values between males and females should not be directly compared n/group= 7-8.
Figure legends:

**Figure 1. Phenotype of adult-onset liver-specific GHR knock-down (aLivGHRkd, Kd) mice.** Hepatic A) Ghr mRNA, B) GHR protein, C) Igf1 mRNA, D) plasma IGF-I level, E) liver weight, F) glycogen and G,H) TG content in male and female mice. I) 20X Oil Red O, J) 40X hematoxylin-eosin and K) 40X PAS-stained liver sections of male control and aLivGHRkd mice. L-N) Oil Red O stained liver sections of female control and aLivGHRkd mice. Females (H,L-M) were operated at 8 wks of age (sham and ovariectomy [OVX]), a subset of OVX females received a sc pellet with 17β-estradiol (E2), 2 wks later females were injected with AAV-TBGp-Null (C-controls) or AAV-TBGp-Cre (aLivGHRkd -kd) and killed 7d afterward. Asterisks indicate difference between control and aLivGHRkd within group. “a” indicates difference between OVX-kd and OVX-E2-kd females (H). Insets (100X) in J and K. Arrow indicates fat filling and # indicates glycogen deposition.* p<0.05; **,p<0.01;***,p<0,001. nd: below the detection limit of the assay. n/group=4-7 (males), 4-8 (females).

**Figure 2. Potential sources of hepatic fat accumulation in aLivGHRkd mice.** A) Rate of hepatic VLDL-TG secretion after tyloxapol injection (time 0, 500mg/kg ip) in mice after 4h food removal starting at 0800h, B) Plasma TG clearance after an oral gavage of olive oil (200µl) in overnight fasted mice, C) ex vivo basal and 1µM isoproterenol-stimulated urogenital-fat lipolysis assessed by the amount of glycerol in the media and D) the amount of newly formed palmitate associated with TG as an indicator of de novo lipogenesis. These measurements were done in male (left) and intact-female (right) control (dotted lines and open circles/columns) and aLivGHRkd (solid lines and circles/columns) mice. Asterisks indicate differences between control and aLivGHRkd within experiment. †,‡ indicate differences between basal and
isoproterenol-stimulated glycerol production within group (C) *, p<0.05; **,p<0.01, †,p=0.0063;‡,p=0.0006. n/group=8-10 (A,B); 7-8 males and 3 females (C); 6-9 (D).

**Figure 3. Peripheral glucose homeostasis and hepatic insulin sensitivity in male control and aLivGHRkd mice** A) Insulin tolerance tests (1.5 mU insulin/g BW, ip in mice after 4h food removal) and B) glucose tolerance test (2g glucose/g BW, ip in mice after overnight food removal), of control (dotted lines) and aLivGHRkd (solid lines) mice. C) Hepatic mature SREBP1c (mSREBP1c) protein levels, in mice after 10h or 4h food removal, of control (C) and aLivGHRkd (Kd) livers measured by western-blot analysis using housekeeping proteins: β-tubulin for SREBP1c antibody C-20 and Sypro staining for SREBP1c antibody H-160. D) Insulin-mediated hepatic AKT phosphorylation (S473 and T308) in overnight fasted mice 20 minutes after an ip bolus of 2mU insulin/g BW measured by western-blot analysis. Asterisks indicate differences between vehicle- and insulin-mediated AKT phosphorylation. †,‡ indicate difference between control and aLivGHRkd within experiment or withing insulin treatment. NS: non-specific band. *, p<0.05; **,p<0.01; ***,p<0.001; †, p<0.05. ‡, p<0.001. n/group= 7-10 (A,B); 5-6 (C); 3 (D).

**Figure 4. Hepatic gene expression profile, acyl-CoA, GCK and plasma ketone levels in male control and aLivGHRkd mice, measured in the same mice used to assess DNL.** A) Diagram of key hepatic metabolic pathways. B) Hepatic gene expression in aLivGHRkd (kd, closed columns) mice relative to control mice (set at 100%, dotted line). C) Hepatic BHB-CoA, D) plasma ketones (3-HB or β-hydroxybutyrate), E) hepatic acetyl-CoA, F) malonyl-CoA and G) GCK protein levels from control (c) and aLivGHRkd (kd) male mice. Mice were killed 10h after
food removal, starting at 0600h. Asterisks indicate differences between control and aLivGHRkd within experiment. -*, p<0.05; **,p<0.01; ***,p<0.0001. n=7-8 mice/group. Glut-2: glucose transporter 2; Gck: glucokinase; Pklr: pyruvate kinase liver and red blood cell; G6pc: glucose-6-phosphatase, catalytic; Pck1: phosphoenolpyruvate carboxykinase 1; Acc1: acetyl-CoA carboxylase 1; Fasn: fatty acid synthase; Elovl6: fatty acid elongase 6; Scd1: stearoyl-CoA desaturase 1.

**Figure 5. Hepatic gene expression profile, cytosolic GCK and F2,6BP levels in fasted and refed male control and aLivGHRkd mice.** A) Hepatic gene expression in aLivGHRkd (kd, closed columns) mice relative to control mice 6h after refeeding a standard chow diet, following an overnight fast (1700h-0800h) (refed controls set at 100% as a dotted line). B) Hepatic cytosolic GCK protein and C) F2,6BP levels in mice after an overnight fast or in 6h-refed mice following an overnight fast. Asterisks indicate differences between control and aLivGHRkd within experiment. “a” indicates differences between fast and refed mice within group. *, p<0.05; **,p<0.01; ***,p<0.0001; a, p<0.05; a, p<0.05. n=4-6 mice/group.
Figure 1
Figure 2

A) Males and Females

B) Plasma TG (mg/dL) over Time (h)

C) Glycerol (μg/g fat/h)

D) μmol new TG-bound palmitate/g liver
Figure 3

A) Glucose level (mg/dL) vs. Time (min)

- Control
- aLivGHRkd

B) Glucose level (mg/dL) over time

- Control
- aLivGHRkd

C) SREBP1 expression

- C-20
- H-160

D) Insulin-induced P-Akt/Akt

- Control
- aLivGHRkd

- P-Akt (Ser473)
- P-Akt (Thr308)

SREBP1 (C-20) and (H-160)

- NS
- 68KDa

mSREBP1/HKP

- C-20
- H-160

Total

- Veh-treated mice

Insulin-induced P-Akt/Akt (% of Veh-treated mice)

- C
- V

- ***
- **
- *
- †
- ‡
Figure 4
Figure 5

A

mRNA copy #/NF (C set at 100%)

Glut-2 Gck Pklr G6pc Pck1 Acc-1 Fasn Elovl6 Scd1 Cpt1a

0
100
200
300
400
*
*
*
*
*
*

B

Cytoplasmic GCK/β-actin

Fast Refed

Control aLivGHRkd

0.0
0.2
0.4
0.6
a
**

C

F2,6BP pmol/mg protein

Fast Refed

Control aLivGHRkd

0.0
0.2
0.4
0.6
a
**