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Growth Hormone Inhibits Hepatic De Novo Lipogenesis in Adult Mice

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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 the fatty liver condition in diet-induced obese rodents and in GH-deficient patients. However, it remains to be determined whether this inhibitory action of GH is due to direct regulation of hepatic lipid metabolism. Therefore, an adult-onset, hepatocyte-specific, GH receptor (GHR) knockdown (aLivGHRkd) mouse was developed to model hepatic GH resistance in humans that may occur after sexual maturation. Just 7 days after aLivGHRkd, hepatic de novo lipogenesis (DNL) was increased in male and female chow-fed mice, compared with GHR-intact littermate controls. However, hepatosteatosis developed only 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 that enhanced glycolysis drives DNL in the GH-resistant liver. These results demonstrate that 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.

Patients with nonalcoholic fatty liver disease (NAFLD) show a higher prevalence of insulin resistance, which is characterized by increased fasting glucose and insulin levels, as well as an increase in nonesterified fatty acids (NEFAs) (1). NAFLD affects up to 30% of adults and represents a high-risk factor in the progression to nonalcoholic steatohepatitis, 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 de novo lipogenesis (DNL [i.e., the production of new fatty acids from glucose]) (6) also plays a significant role in progression to NAFLD (5,7–9), which may in part be driven by hyperinsulinemia (1).

The liver is a major target of growth hormone (GH). GH is required to maintain hepatic production of 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 has been associated with NAFLD (15). Specifically, subjects with primary GH deficiency have a higher incidence of NAFLD, which can be reversed with GH replacement therapy (16–18). Also, subjects with inactivating mutations in the GH receptor

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(GHR; Laron Syndrome) have a higher incidence of NAFLD, which cannot be reversed by IGF-I treatment (19), further suggesting that 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 (20), individuals with lower GH levels exhibited a higher prevalence of NAFLD. Since GH is released in a pulsatile and diurnal fashion, a single GH measurement may not accurately represent the cumulative amount of GH that has been released. Therefore, many studies have used IGF-I as a surrogate marker of GH secretion. These studies (21–25) demonstrate that circulating levels of IGF-I are negatively associated with NAFLD. The reduction in GH levels that occurs 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 individuals with NAFLD after corrections for weight, waist circumference, and diabetes (25) suggests that their livers are resistant to the actions of GH. In fact, mice made obese by high-fat feeding fail to respond to an acute injection of GH by increasing the level of 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). Hepatic insulin resistance, which is characterized by impaired IRS/Akt inactivation of FOXO1, also could lead to hepatic GH resistance, since FOXO1 has been shown to decrease hepatic GHR expression (29).

The reduction in GH production/signaling and the 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 levels decreases hepatic TG content in animal models of hepatosteatosis (32,33) as well as in GH-deficient 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 knockdown (aLivGHRkd). The rate of hepatic DNL more than doubled just 7 days after the induction of hepatic GH resistance. The increase in DNL and hepatosteatosis in aLivGHRkd mice was not associated with an increase in the actions of insulin on the liver or with an increase in white adipose tissue (WAT) lipolysis but was associated with end points suggesting that glycolysis is enhanced.

RESEARCH DESIGN AND METHODS

Generation of aLivGHRkd and Littermate Controls

All mouse studies were approved by the Institutional Animal Care and Use Committee of the Jesse Brown VA Medical Center. C57BL/6J GHR-floxed (34) mice were housed in a temperature-controlled (22–24°C) and humidity-controlled, specific pathogen–free barrier facility with a 12-h light-dark cycle (lights on at 0600 h) and were fed standard laboratory rodent chow, unless otherwise indicated. The 10- to 12-week-old mice were injected in the lateral tail vein with 100 μL saline containing 1.5×10^{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, catalog #AV-8-PV1091, AAV8.TBG.PI.Cre.rBG; Penn Vector Core, University of Pennsylvania) or a null allele (AAV-TBGp-Null, catalog #AV-8-PV0148, AAV8.TBG.PI.Null.bGH; Penn Vector Core). TBGp is a hepatocyte-specific promoter that allows the expression of Cre recombinase exclusively in hepatocytes (Supplementary Fig. 1B–*E*), leading to recombination of the Ghr allele and knockdown of the hepatic Ghr mRNA and protein (Fig. 1*A* and *B* and Supplementary Figs. 1*C* and 2). The GHR-floxed littermate mice injected with AAV-TBGp-Null served as controls. The injection of AAV vectors did not increase levels of the inflammation markers (Supplementary Fig. 3). Mice were killed 7 days after AAV injection.

Hormone and Metabolites

Plasma GH and insulin (Millipore, Billerica, MA), IGF-I (Immunodiagnostic Systems, Gaithersburg, MD), alanine aminotransferase (BioVision, Milpitas, CA), and TG and NEFA (Wako Diagnostics, Richmond, VA) were measured following the manufacturers' instructions. Hepatic TG levels were measured after the extraction of neutral lipids (35) using reagents obtained from Wako Diagnostics. Hepatic glycogen was measured by the Yale Metabolic Mouse Phenotyping Center (MMPC; Yale School of Medicine, New Haven, CT). Hepatic fructose 2,6-bisphosphate (F2,6BP) was measured using the method described by Van Schaftingen et al. (36). Hepatic acyl-CoA species (acetyl-CoA, malonyl-CoA, β-hydroxybutyryl [BHB]-CoA) were determined by liquid chromatography-mass spectrometry (MS) at the MMPC of Case Western Reserve University (Cleveland, OH).

Hepatic DNL

To measure hepatic DNL, food was removed at 0600 h, and mice were injected at 1200 h with 30 μL/g body wt (BW) 0.9% NaCl deuterated water (^2H_2O; Sigma-Aldrich, Madison, WI) to enrich body water up to 4%. Four hours later (at 1600 h), mice were killed by cervical dislocation under isoflurane anesthesia, and blood was collected from the inferior cava into heparinized syringes (~50 μL). This condition represents the natural postabsorptive state of mice, where mice consume <20% of their total food intake during the day. Livers were collected and snap frozen in liquid nitrogen. Blood and livers were sent to the Case Western Reserve University MMPC for 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 percentage of

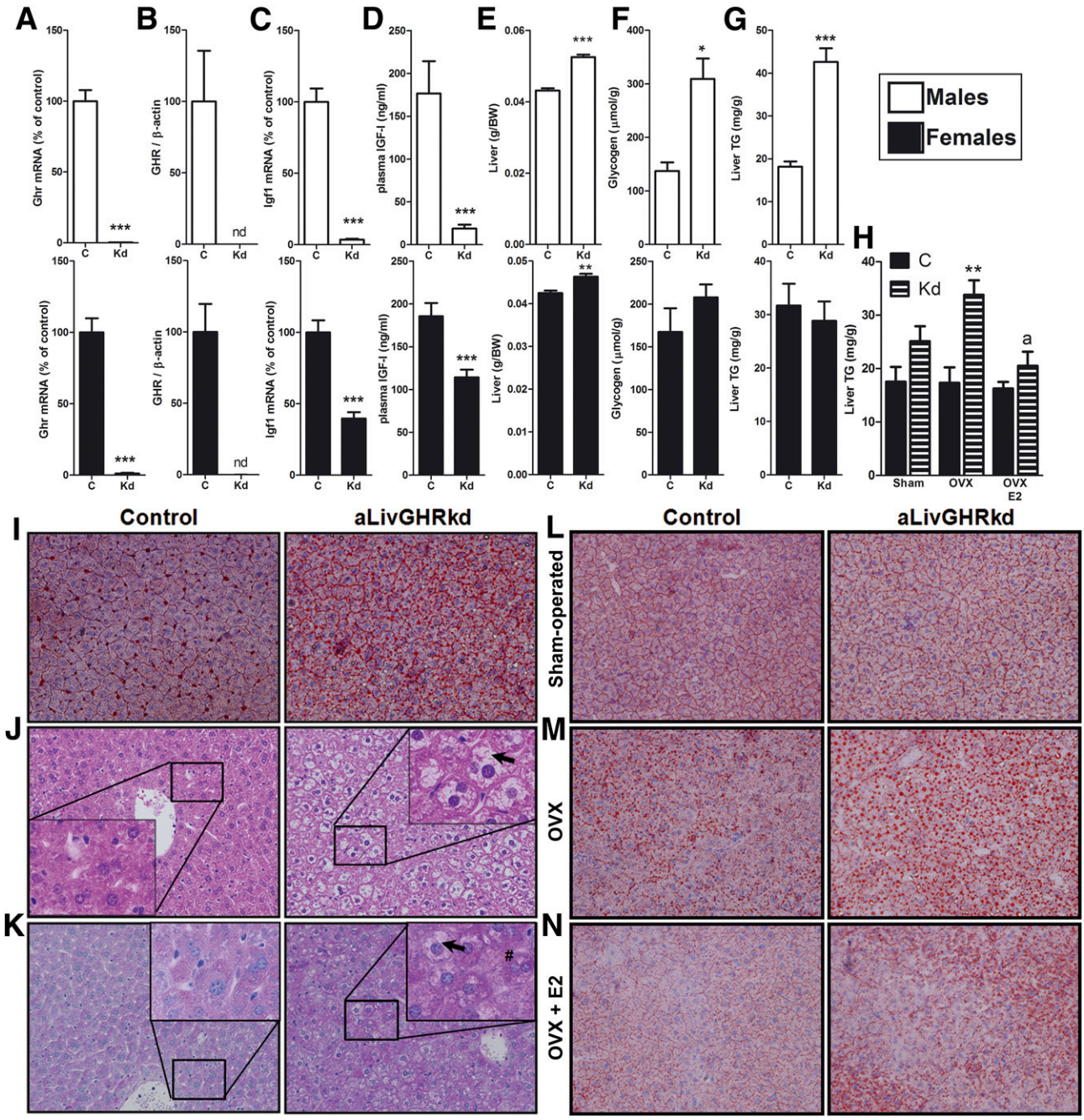

Figure 1—Phenotype of aLivGHRkd (Kd) mice. Hepatic Ghr mRNA (*A*), GHR protein (*B*), Igf1 mRNA (*C*), plasma IGF-I level (*D*), liver weight (*E*), glycogen (*F*), and TG content (*G* and *H*) in male and female mice. ×20 Oil Red O–stained (*I*), ×40 hematoxylin-eosin–stained (*J*), and ×40 periodic acid Schiff–stained (*K*) liver sections of male control and aLivGHRkd mice. *L–N*: Oil Red O–stained liver sections of female control and aLivGHRkd mice. *H* and *L–N*: Females were operated on at 8 weeks of age (sham surgery and OVX), a subset of OVX females received a subcutaneous pellet with 17β-estradiol (E2), and 2 weeks later females were injected with AAV-TBGp-Null (controls [C]) or AAV-TBGp-Cre (aLivGHRkd mice) and killed 7 days afterward. Asterisks indicate the difference between control and aLivGHRkd within group. *H*: a, indicates difference between OVX-Kd and OVX-E2-Kd females. *J* and *K*, insets: Original magnification ×100. Arrow, fat filling; #, glycogen deposition; nd, below the detection limit of the assay. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. *n* = 4–7/group (males), 4–8/group (females).

^{2}H-labeled fatty acids was analyzed by gas chromatography-MS. The ^{2}H-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 TG and palmitate was calculated using the following equation: % ^{2}H-labeled palmitate = [total ^{2}H-labeled palmitate × (^{2}H-labeled body water × *n*) − 1] × 100, where *n* is the number of exchangeable hydrogens, which is assumed to be 22 (37,38), and ^{2}H-labeled total body water enrichment was measured from the blood collected

from each mouse and determined using the acetone exchange method and measured by gas chromatography-MS (39).

Ovariectomy

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

Assessment of Other Metabolic End Points

TG clearance was assessed in mice fasted overnight (1700–0900 h) after an oral gavage of 200 μL of food-grade olive oil (Oleoestepa, Estepa, Spain). The rate of hepatic VLDL-TG secretion was assessed after tyloxapol injection (500 mg/kg i.p.; Sigma-Aldrich). Glucose tolerance tests were performed in mice fasted overnight that were injected with 2 mg glucose/g BW i.p. Insulin tolerance tests were performed in mice 4 h after food removal, starting at 0700 h by injecting 1.5 mU insulin/g BW i.p. (Novolin; Novo Nordisk, Bagsvaerd, Denmark). Blood samples were taken from lateral tail vein, and blood glucose measured (AlphaTRAK 2; Abbott Laboratories, Abbott Park, IL). To assess ex vivo WAT lipolysis, 50–60 mg of tissue from urogenital fat pads was washed in cold PBS and minced into small pieces, then incubated in 500 μL Krebs-Ringer HEPES buffer without or with 1 μmol/L isoproterenol for 2 h at 37°C 5% CO_2. Media were collected to measure glycerol production (Free Glycerol Reagent; Sigma-Aldrich). Hepatic insulin sensitivity was assessed in mice fasted overnight that were injected with saline or 2 mU/g BW insulin i.p. Twenty minutes later, the liver was collected for Western blot analysis.

Gene Expression Analysis

Tissues were processed as previously described (35). The primers used for quantitative PCR are indicated in Supplementary 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), transferred to nitrocellulose membranes, and incubated with the primary and secondary antibodies shown in Supplementary Table 4.

Statistics

Two-tailed Student *t* 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 loading. P values <0.05 were considered to be significant. All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

Knockdown 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 (Supplementary Fig. 1*C*), leading to aLivGHRkd, as confirmed by a dramatic reduction in Ghr mRNA and GHR protein levels, compared with AAV-TBGp-Null–treated, GHR-floxed littermate controls (Fig. 1*A* and *B* and Supplementary Fig. 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 with controls (Fig. 1*C* and *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 (E2) on IGF-I expression (40). Since IGF-I inhibits pituitary GH production, we examined the expression of pituitary GH, GH-releasing hormone receptor, and ghrelin receptor, and all were increased in aLivGHRkd male mice, but not in female mice (Supplementary Fig. 4). Nonetheless, these pituitary changes did not lead to a significant increase in circulating GH levels (Supplementary Fig. 4*B*).

Seven days after the induction of aLivGHRkd, liver weight was increased in both male and female aLivGHRkd mice that were maintained on a standard chow diet (Fig. 1*E*). Hepatic glycogen and TG content increased in male, but not in female, aLivGHRkd mice (Fig. 1*F*, *G*, and *I–K*). Since it has been reported that premenopausal women are protected from NAFLD (41,42), and that ovary-intact mice, but not OVX mice, are protected from diet-induced fatty liver (43), we examined the impact of aLivGHRkd in OVX mice, with and without E2 replacement (Fig. 1*H* and *L–N*). Three weeks after the GHR-intact mice underwent OVX, hepatic TG content did not differ from that in sham-operated controls. However, the induction of aLivGHRkd 2 weeks after the OVX doubled hepatic TG content 1 week later, and this effect was blocked by estrogen replacement (Fig. 1*H* and *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 was conducted to identify the source of excess TG accumulation in the livers of male aLivGHRkd mice and to begin to explore why ovary-intact aLivGHRkd female mice are protected. The rate of hepatic VLDL-TG release, after the inhibition of systemic lipoprotein lipase activity by tyloxapol, did not differ between aLivGHRkd mice and controls (Fig. 2*A*). Also, the rate of TG clearance after an oral gavage of olive oil was not altered by aLivGHRkd (Fig. 2*B*). Although enhanced GH-mediated WAT lipolytic activity is thought to contribute to fatty liver in mouse models with congenital defects in hepatic

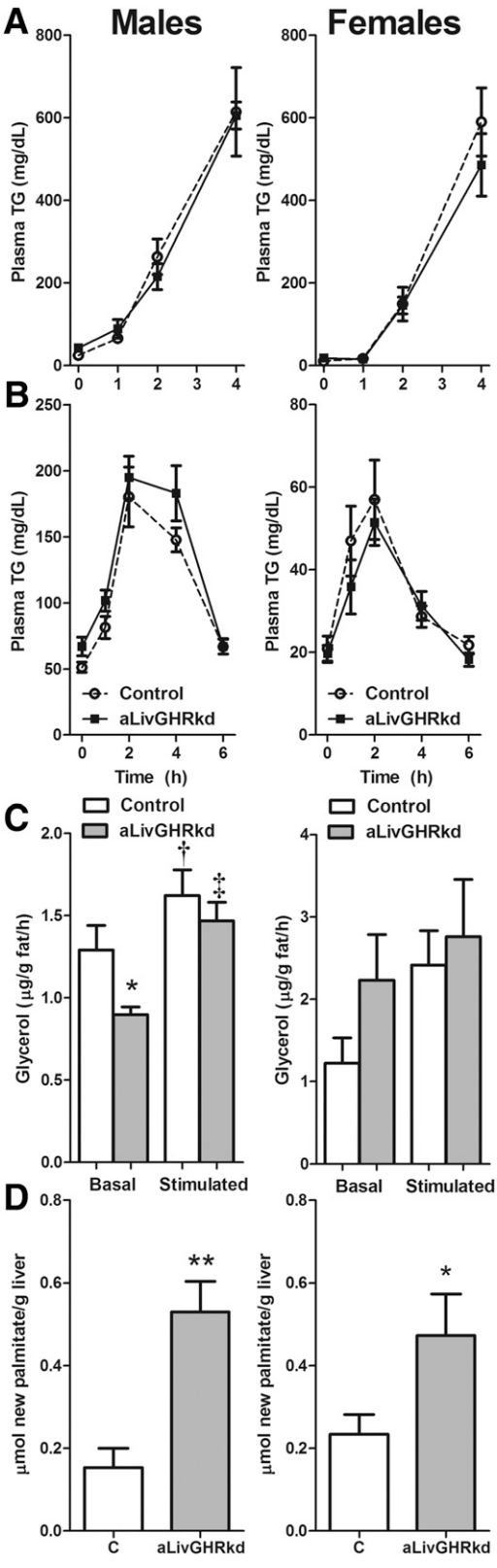

Figure 2—Potential sources of hepatic fat accumulation in aLivGHRkd mice. *A*: Rate of hepatic VLDL-TG secretion after tyloxapol injection (time 0, 500 mg/kg i.p.) in mice after 4-h food removal starting at 0800 h. Plasma TG clearance after an oral gavage of olive oil (200 μL) in mice fasted overnight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

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 2 and Supplementary Table 2). Also there was no reduction in WAT subdepot 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 (Fig. 2*C* and Supplementary Table 1 and 2). Taken together, these results demonstrate that the increase in hepatic TG observed in male aLivGHRkd mice is unlikely to be due to a decrease in hepatic lipoprotein 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 (Fig. 2*D* and Supplementary Fig. 5). 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 (46,47) suggest that estrogen promotes the flux of lipids from the liver into adipose tissue stores. This would be consistent with the observation that urogenital fat pad weight increased in female aLivGHRkd mice, but not in male aLivGHRkd mice (Table 1). In addition, circulating TG levels increase in male aLivGHRkd mice, but not in female aLivGHRkd mice (Supplementary Table 1 and 2). 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 SREBP-1c (6), we examined the impact of short-term hepatic GH resistance on circulating insulin levels, as well as on systemic and hepatic insulin sensitivity. Insulin levels did not differ from those of controls under conditions used to study DNL (10 h after food removal starting at 0600 h; Table 2) or after an overnight fast (16 h after food removal starting at 1700 h; Supplementary Table 2).

Table 1—aLivGHRkd does not increase WAT lypolysis

	Males		Females	
	Control	aLivGHRkd	Control	aLivGHRkd
Plasma NEFA (mEq/L)	1.06 ± 0.12	1.15 ± 0.11	1.07 ± 0.12	1.13 ± 0.09
Fat depot weight/BW				
UG-fat	6.89 ± 0.74	7.58 ± 0.28	5.21 ± 0.22	7.40 ± 0.69*
RP-fat	1.81 ± 0.36	2.01 ± 0.19	3.53 ± 0.24	4.58 ± 0.80
SC-fat	4.55 ± 0.56	4.24 ± 0.30	6.44 ± 0.28	7.03 ± 0.67
Lipolytic genes mRNA				
Hsl	100.00 ± 2.56	111.31 ± 4.30	100.00 ± 19.57	109.06 ± 13.70
Atgl	100.00 ± 16.76	100.67 ± 8.63	100.00 ± 24.92	89.52 ± 12.30
Adrb3	100.00 ± 26.14	101.64 ± 8.83	100.00 ± 29.09	83.65 ± 10.34
Lipogenic genes mRNA				
SREBP-1c	100.00 ± 12.19	97.50 ± 7.36	100.00 ± 23.13	101.58 ± 19.13
Pparγ	100.00 ± 22.70	132.69 ± 4.66	100.00 ± 15.06	95.82 ± 10.38
Fasn	100.00 ± 18.55	118.11 ± 26.93	100.00 ± 21.69	92.76 ± 27.34
Scd-1	100.00 ± 18.06	103.36 ± 8.20	100.00 ± 18.22	92.62 ± 22.21
Gpat	100.00 ± 10.72	88.28 ± 8.18	100.00 ± 15.02	119.08 ± 14.34
Dgat2	100.00 ± 38.35	131.35 ± 13.96	100.00 ± 21.14	91.15 ± 20.29

Data are reported as mean ± SEM. Plasma NEFA and WAT (unilateral subdepot mg/g BW × 10^3) weight and UG-WAT lipolytic/lipogenic gene expression. Adrb3, adrenergic receptor β3; Atgl, adipose TG lipase; Dgat2, diacylglycerol *O*-acyltransferase 2; Gpat, glycerol-3-phosphate acyltransferase; Hsl, hormone-sensitive lipase; Pparγ, peroxisome proliferator–activated receptor γ; RP, retroperitoneal; SC, subcutaneous; UG, urogenital. *$P < 0.05$, differences between control and aLivGHRkd mice. Mice were killed 4 h after food removal starting at 0800 h. Males and females were analyzed at separated times and they were not littermates; therefore, differences between values for males and females should not be directly compared.

However, insulin was elevated under basal conditions (4 h food removal starting at 0800 h; Supplementary Table 1) but was not associated with systemic insulin resistance, as measured by insulin or glucose tolerance tests (Fig. 3*A* and *B*). In addition, hepatic levels of mature SREBP-1c (mSREBP-1c) were not elevated and in fact were reduced in the same conditions used to assess DNL (Fig. 3*C*). This disconnect between insulin and mSREBP-1c could be explained in part by the observation that insulin-mediated phosphorylation of AKT was impaired in the livers of aLivGHRkd mice (Fig. 3*D*). These results suggest that the increased hepatic DNL rate in aLivGHRkd mice is not due to hyperactivation of the canonical insulin signaling associated with DNL.

Hepatic Gene Profile, Cytosolic Glucokinase, 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 Fig. 4*A* (6), was 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 levels of mSREBP-1c were not increased, the expression of SREBP-1c lipogenic target genes (acetyl-CoA cocarboxylase-1 [Acc-1], fatty acid synthase [Fasn], and fatty acid elongase 6 [Elovl6]) was also not elevated above control levels (Fig. 4*B*). Interestingly, stearoyl-CoA desaturase-1 (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 carnitine palmitoyl-transferase 1a (Cpt1a) mRNA (Fig. 4*B*) and BHB-CoA levels and a tendency ($P = 0.06$) for lower plasma ketone levels (Fig. 4*C* and *D*) indicate that 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 (Fig. 4*E* and *F*), where malonyl-CoA may serve to suppress CPT1a activity (6).

Table 2—Plasma NEFA and insulin levels in male and female control and aLivGHRkd mice

	Males		Females	
	Control	aLivGHRkd	Control	aLivGHRkd
NEFA (mEq/L)	1.05 ± 0.07	0.91 ± 0.07	0.62 ± 0.06	0.66 ± 0.05
Insulin (ng/mL)	1.04 ± 0.21	1.37 ± 0.14	0.65 ± 0.07	0.78 ± 0.08

Data are reported as mean ± SEM. Mice were killed 10 h after food removal at 0600 h. Blood was taken from the 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 = 7–8/group.

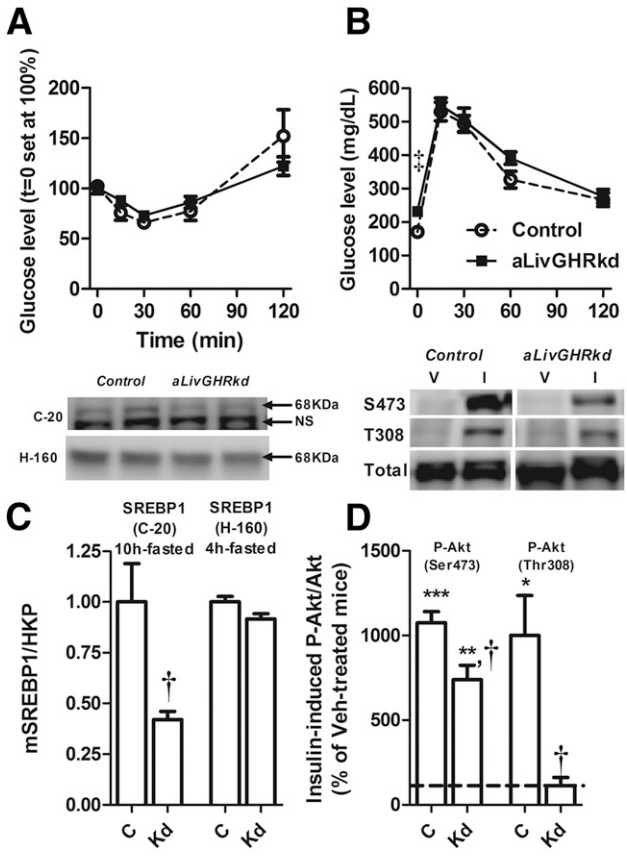

Figure 3—Peripheral glucose homeostasis and hepatic insulin sensitivity in male control and aLivGHRkd mice. Insulin tolerance tests (1.5 mU insulin/g BW i.p. in mice after 4 h of food removal) (*A*) and glucose tolerance tests (2 mg glucose/g BW i.p. in mice after overnight food removal) (*B*) of control (dotted lines) and aLivGHRkd (solid lines) mice. *C*: Hepatic mSREBP-1c protein levels in mice after 10 or 4 h of food removal of control (C) and aLivGHRkd (Kd) livers measured by Western blot analysis using housekeeping proteins: β-tubulin for SREBP-1c antibody C-20 and Sypro staining for SREBP-1c antibody H-160. *D*: Insulin-mediated hepatic AKT phosphorylation (S473 and T308) in mice fasted overnight 20 min after a bolus of 2 mU insulin/g BW i.p. measured by Western blot analysis. Asterisks indicate differences between vehicle- and insulin-mediated AKT phosphorylation. † and ‡ indicate difference between control and aLivGHRkd within experiment or within insulin treatment. NS: nonspecific band. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; †$P < 0.05$; ‡$P < 0.001$. n = 7–10/group (*A* and *B*); 5–6/group (*C*); 3/group (*D*). I, insulin; V, vehicle.

In addition, the expression of the gluconeogenic gene phosphoenolpyruvate carboxykinase 1 (Pck-1) was suppressed (Fig. 4*B*) and is consistent with previous reports (49) showing that GH/JAK2/STAT5b directly stimulates hepatic Pck-1 expression.

Normal acetyl-CoA levels in aLivGHRkd livers, in the 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 (Glut-2 and glucokinase gene [Gck]; Fig. 4*B*) and total GCK protein levels (Fig. 4*G*) 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 after a meal, the same gene panel measured in Fig. 4*B* was measured in a different set of mice that were fasted overnight and then fed with a standard chow diet for 6 h (Fig. 5*A*). The expression of Glut-2; Gck; pyruvate kinase liver and red blood cell (Pklr); glucose-6-phosphatase, catalytic (G6pc); Fasn; and Scd-1 was greater in refed aLivGHRkd mice compared with refed controls. Of note, cytosolic GCK protein levels remained elevated in aLivGHRkd in both fasted and refed conditions (Fig. 5*B*). Since GCK is active in the cytosol, it can be concluded that GCK activity is sustained in the GH-resistant liver. Importantly, levels of F2,6BP (Fig. 5*C*), the most potent activator of glycolysis and inhibitor of gluconeogenesis (50), were increased in aLivGHRkd livers. Taken together, these results suggest that hepatic GH resistance enhances hepatic glucose uptake (GCK) and glycolysis (F2,6BP), thereby supplying a 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 (44,45,51–53) that mouse models with congenital, liver-specific knockout of the GHR or its downstream effectors (JAK2 and Stat5) develop hepatosteatosis as adults. In these mice, hepatosteatosis is thought to be mediated by the indirect actions of GH since circulating levels of IGF-I were 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 alter 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 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.

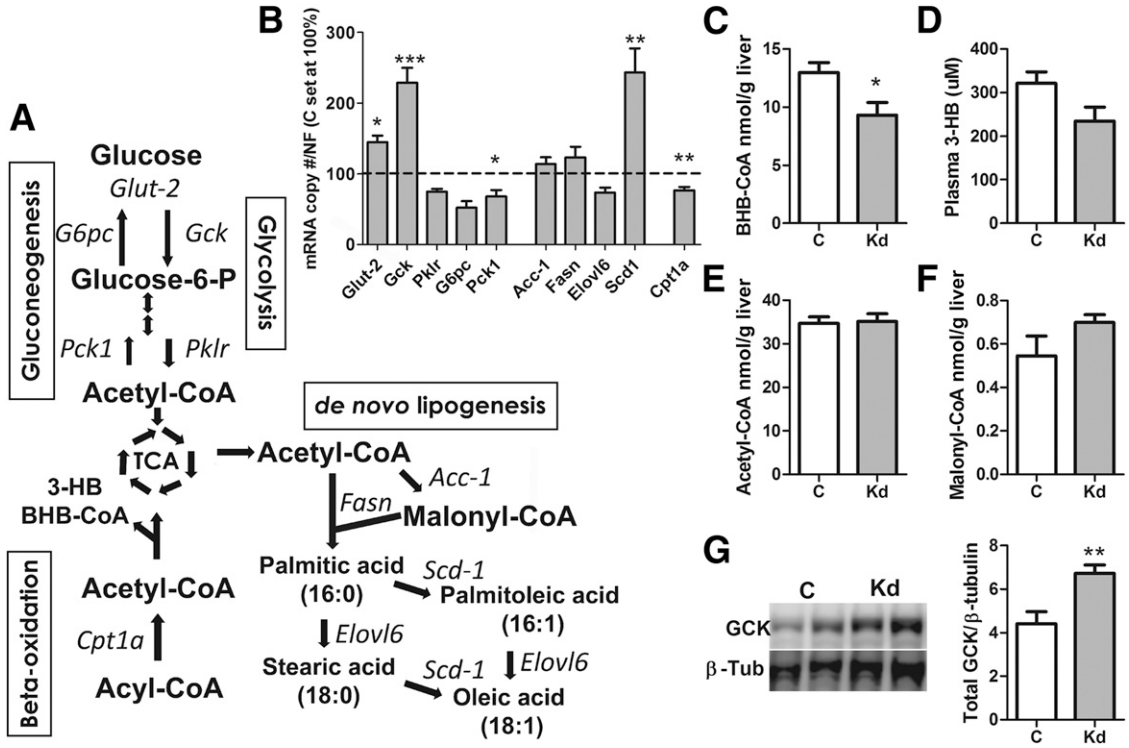

Figure 4—Hepatic gene expression profile and acyl-CoA, GCK, and plasma ketone levels in male control and aLivGHRkd mice, measured in the same mice used to assess DNL. *A*: Diagram of key hepatic metabolic pathways. *B*: Hepatic gene expression in aLivGHRkd mice (closed columns) relative to control mice (set at 100%, dotted line). Hepatic BHB-CoA (*C*), plasma ketones (3-HB or β-hydroxybutyrate) (*D*), hepatic acetyl-CoA (*E*), malonyl-CoA (*F*), and GCK protein (*G*) levels from control and aLivGHRkd male mice. Mice were killed 10 h after food removal, starting at 0600 h. Asterisks indicate the differences between control and aLivGHRkd mice within the experiment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$. n = 7–8 mice/group. C, control; Kd, aLivGHRkd; NF, normalization factor.

Both male and female aLivGHRkd mice exhibited an increase in hepatic DNL, but only males rapidly accumulated 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 6 months of age, which is consistent

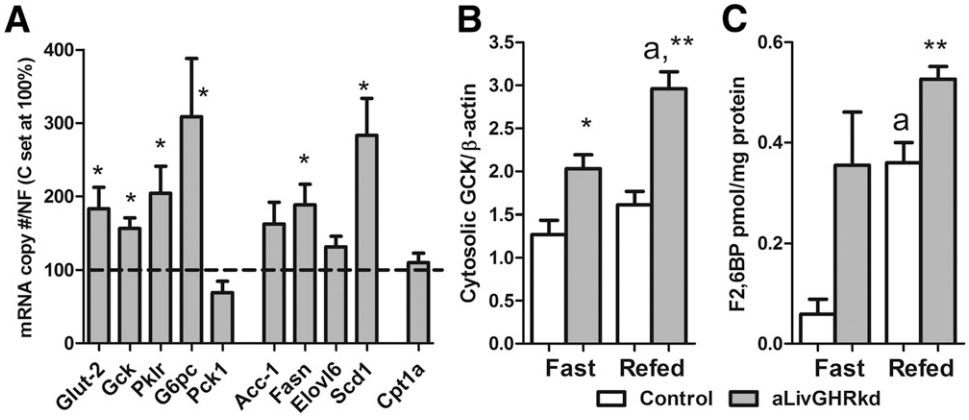

Figure 5—Hepatic gene expression profile and cytosolic GCK and F2,6BP levels in fasted and refed male control and aLivGHRkd mice. *A*: Hepatic gene expression in aLivGHRkd (closed columns) mice relative to control mice 6 h after refeeding a standard chow diet, following an overnight fast (1700–0800 h) (refed controls set at 100% as a dotted line). Hepatic cytosolic GCK protein (*B*) and F2,6BP (*C*) levels in mice after an overnight fast or in 6-h refed mice following an overnight fast. Asterisks indicate the differences between control and aLivGHRkd within experiment. a, indicates differences between fasted and refed mice within the group. $^{*}P < 0.05$; $^{**}P < 0.01$; a, $P < 0.05$. n = 4–6 mice/group. NF, normalization factor.

with our observations in aLivGHRkd mice. In the current study, we found that intact ovary function and estrogen protect female aLivGHRkd mice from excess hepatic fat accumulation. These results are consistent with reports indicating that premenopausal women show a lower prevalence of NAFLD, compared with 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 (46) suggest that estrogen, acting through hepatic ERα, blocks insulin-mediated suppression of hepatic TG secretion, thereby reducing hepatic TG accumulation. 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, SREBP-1c maturation, and expression of SREBP-1c target genes (Acc-1, Fasn, Elovl6) were not increased in association with enhanced DNL that was measured in the postabsorptive state. Since SREBP-1c is considered the canonical target of insulin-mediated increases in hepatic TG production, the normal/low levels of mSREBP-1c in aLivGHRkd mice suggest that 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 by 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 2 days of GH treatment (intraperitoneally twice daily) suppressed Gck mRNA in diet-induced obese male mice (E.O. List, personal communication), where GH treatment ultimately led to a reduction in hepatic TG content (33). Second, the level of F2,6BP was increased in fasted and refed aLivGHRkd mice. F2,6BP is a potent inhibitor of fructose-1,6-bisphosphatase and an 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 carbohydrate response element–binding protein, where the carbohydrate response element–binding protein requires glucose metabolites produced during glycolysis for its activation (6). These results, coupled with experimental and clinical reports showing that overexpression or pharmacologic activation of GCK can increase DNL and hepatic TG accumulation (59,60) and that patients with NAFLD exhibit an increase in Gck expression (61) and DNL (8), raise the possibility that hepatic GH resistance 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 in understanding how GH therapy reverses NAFLD in individuals with GH deficiency and may provide insight into an ongoing clinical trial (Clinical trial reg. no. NCT02217345, clinicaltrials.gov) examining whether low-dose GH therapy can reverse NAFLD in the general population.

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Author Contributions. J.C.-C. and R.D.K. designed the study, performed the experiments, analyzed the data, and wrote the article. N.M. performed the experiments. E.O.L. and J.J.K. provided the GHR-floxed mice. A.D.-R. and M.P. performed the experiments and provided key technical assistance. S.J.F. provided the growth hormone receptor antibody. A.M. and R.B. measured fructose 2,6-bisphosphate. All authors reviewed and approved the final version of the article. R.D.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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