GH directly inhibits steatosis and liver injury in a sex-dependent and IGF1-independent manner

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Abstract

A reduction in hepatocyte growth hormone (GH)-signaling promotes non-alcoholic fatty liver disease (NAFLD). However, debate remains as to the relative contribution of the direct effects of GH on hepatocyte function vs indirect effects, via alterations in insulin-like growth factor 1 (IGF1). To isolate the role of hepatocyte GH receptor (GHR) signaling, independent of changes in IGF1, mice with adult-onset, hepatocyte-specific GHR knockdown (aHepGHRkd) were treated with a vector expressing rat IGF1 targeted specifically to hepatocytes. Compared to GHR-intact mice, aHepGHRkd reduced circulating IGF1 and elevated GH. In male aHepGHRkd, the shift in IGF1/GH did not alter plasma glucose or non-esterified fatty acids (NEFA), but was associated with increased insulin, enhanced systemic lipid oxidation and reduced white adipose tissue (WAT) mass. Livers of male aHepGHRkd exhibited steatosis associated with increased de novo lipogenesis, hepatocyte ballooning and inflammation. In female aHepGHRkd, hepatic GHR protein levels were not detectable, but moderate levels of IGF1 were maintained, with minimal alterations in systemic metabolism and no evidence of steatosis. Reconstitution of hepatocyte IGF1 in male aHepGHRkd lowered GH and normalized insulin, whole body lipid utilization and WAT mass. However, IGF1 reconstitution did not reduce steatosis or eliminate liver injury. RNAseq analysis showed IGF1 reconstitution did not impact aHepGHRkd-induced changes in liver gene expression, despite changes in systemic metabolism. These results demonstrate the impact of aHepGHRkd is sexually dimorphic and the steatosis and liver injury observed in male aHepGHRkd mice is autonomous of IGF1, suggesting GH acts directly on the adult hepatocyte to control NAFLD progression.

Introduction

In the healthy liver, hepatocyte growth hormone receptor (GHR)-signaling activates multiple intracellular signal transduction pathways to modulate hepatic function (Dehkhoda et al. 2018). It is well accepted that GH signals through the GHR/janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 b (STAT5b) pathway to maintain hepatic expression of insulin-like growth factor 1 (IGF1; Rotwein 2012, Feigerlova et al. 2018). However, debate remains as to the relative contribution of the direct effects of GH on hepatocyte function vs indirect effects, via alterations in insulin-like growth factor 1 (IGF1). To isolate the role of hepatocyte GH receptor (GHR) signaling, independent of changes in IGF1, mice with adult-onset, hepatocyte-specific GHR knockdown (aHepGHRkd) were treated with a vector expressing rat IGF1 targeted specifically to hepatocytes. Compared to GHR-intact mice, aHepGHRkd reduced circulating IGF1 and elevated GH. In male aHepGHRkd, the shift in IGF1/GH did not alter plasma glucose or non-esterified fatty acids (NEFA), but was associated with increased insulin, enhanced systemic lipid oxidation and reduced white adipose tissue (WAT) mass. Livers of male aHepGHRkd exhibited steatosis associated with increased de novo lipogenesis, hepatocyte ballooning and inflammation. In female aHepGHRkd, hepatic GHR protein levels were not detectable, but moderate levels of IGF1 were maintained, with minimal alterations in systemic metabolism and no evidence of steatosis. Reconstitution of hepatocyte IGF1 in male aHepGHRkd lowered GH and normalized insulin, whole body lipid utilization and WAT mass. However, IGF1 reconstitution did not reduce steatosis or eliminate liver injury. RNAseq analysis showed IGF1 reconstitution did not impact aHepGHRkd-induced changes in liver gene expression, despite changes in systemic metabolism. These results demonstrate the impact of aHepGHRkd is sexually dimorphic and the steatosis and liver injury observed in male aHepGHRkd mice is autonomous of IGF1, suggesting GH acts directly on the adult hepatocyte to control NAFLD progression.
2013), where hepatic IGF1 represents >80% of the IGF1 found in the circulation (LeRoith 2008). In addition to regulating hepatic production of IGF1, GH regulates metabolic function in a tissue-specific fashion (List et al. 2019). A reduction in hepatic GH-signaling is associated with an increase in liver fat accumulation (steatosis) in mouse models (Cui et al. 2007, Fan et al. 2009, Sos et al. 2011, List et al. 2014, Cordoba-Chacon et al. 2015). Also, a reduction in circulating GH and IGF1 is associated with non-alcoholic fatty liver disease (NAFLD) in human subjects (Ichikawa et al. 2007, Völzke et al. 2009, Arturi et al. 2011, Fusco et al. 2012, Xu et al. 2012, Hazlehurst & Tomlinson 2013, Hribal et al. 2013, Sirbu et al. 2013, Cianfarani et al. 2014, Runchey et al. 2014, Sumida et al. 2015, Dichtel et al. 2017). However, it remains to be determined how much of the steatosis that is associated with a reduction in GH production and hepatic GH-signaling is due to the direct effects of GH on hepatocyte metabolism vs indirect effects of altered IGF1 and GH on systemic metabolic function.

In congenital mouse models with loss of hepatocyte-specific GH-signaling due to knockout of GHR, JAK2 or STAT5, it is thought that steatosis occurs secondary to the reduction in circulating IGF1, leading to a rise in GH due to loss of IGF1-negative feedback regulation. The low IGF1/high GH early in development alters metabolically relevant tissues, such as adipose, muscle and bone, which could indirectly contribute to the liver phenotype observed with congenital loss of hepatocyte GH signaling (Fan et al. 2009, List et al. 2014, Liu et al. 2018). In addition, the loss of the insulin-like effects of IGF1 (Clemmons 2012), coupled with the GH-mediated impairment of systemic insulin signaling (Moller & Jorgensen 2009) leads to hyperinsulinemia, and hyperglycemia, with some reports showing evidence of enhanced white adipose tissue (WAT) lipolysis (Cui et al. 2007, Fan et al. 2009, Nordstrom et al. 2013, List et al. 2014, Liu et al. 2016, Corbit et al. 2018). These changes in systemic metabolic function are thought to shift the flux of glucose and non-esterified fatty acids (NEFA) to the liver, thus providing substrates for triglyceride formation. The important role of the reciprocal shift in IGF1/GH and subsequent GH-mediated WAT lipolysis is based on studies using mice with congenital liver-specific knockout of JAK2 (JAK2L; Sos et al. 2011, Nordstrom et al. 2013), where JAK2 is required for a number of class I cytokine receptor-signaling cascades, including the GHR (Waters & Brooks 2015). When JAK2L mice are crossed with GH-deficient mice (lit/lit; Sos et al. 2011) or mice with adipose-tissue knockout of JAK2 (Nordstrom et al. 2013, Corbit et al. 2018)) steatosis was dramatically reduced.

In order to avoid the potential confounding effects of congenital loss of hepatocyte GH signaling, our laboratory developed a mouse model of adult-onset, hepatocyte-specific knockdown of the GHR (aHepGHRkd; 10–12 week-old, GHR\textsuperscript{fl/fl} mice treated with AAV8-TBGp-Cre). One week after aHepGHRkd, hepatic de novo lipogenesis (DNL) was increased in both male and female chow-fed mice. However, only males developed steatosis (Cordoba-Chacon et al. 2015). Enhanced DNL and steatosis were sustained with age and was associated with hepatocyte ballooning, inflammation and mild fibrosis in chow-fed mice, indicative of non-alcoholic steatohepatitis (NASH; (Cordoba-Chacon et al. 2018)). Despite the fact that aHepGHRkd mice exhibit low IGF1/high GH similar to that of congenital models, they did not exhibit overt signs of WAT lipolysis, suggesting the steatosis that develops in this model may be due to the direct actions of GH on hepatocyte metabolism. In order to extend these previous observations and specifically test the IGF1-independent role of hepatocyte GH signaling, adult male and female aHepGHRkd mice were injected with a vector expressing rat IGF1, targeted specifically to hepatocytes (AAV8-TBGp-rIGF1) and the impact on systemic metabolism, liver fat content and composition, liver injury and gene expression profile was determined.

Materials and methods

Animal care, aHepGHRkd induction and hepatocyte-specific IGF1 transgene expression

These studies were approved by the Institutional Animal Care and Use Committees of the Jesse Brown VA Medical Center and University of Illinois at Chicago. A breeding colony of Ghr\textsuperscript{fl/fl} mice, originally obtained from Dr John J Kopchick, Ohio University (List et al. 2013), were housed in a specific pathogen-free barrier facility maintained at 22–24°C, with a 12h light:12h darkness cycle (lights on 06:00 h). All mice were fed a standard chow diet (Teklad LM-485, Envigo, Madison, WI). As previously described (Cordoba-Chacon et al. 2015, Kineman et al. 2016), aHepGHRkd were generated by treating 10- to 12-week-old male (n = 8 mice/group) and female (n = 7 mice/group) Ghr\textsuperscript{fl/fl} littermates with a single lateral tail vein injection of 1.5 × 10\textsuperscript{11} genome copies (GC) of an adeno-associated virus serotype 8 (AAV8) vector bearing a hepatocyte-specific thyroxine-binding globulin promoter (TBGp) driving a Cre recombinase transgene.
(AAV8-TBGp-Cre; Cat # 107787-AAV8, AAV.TBG.PI.Cre. rBG (AAV8), Addgene, Watertown, MA, diluted in 100 μL sterile PBS). To reconstitute hepatocyte IGF1 expression in aHepGHRkd, a subset of mice were co-injected with AAV8-TBGp-Cre (1.5 × 10^{11} GC)+AAV8-TBGp-rIGF1 (2.0 × 10^{11} or 1.0 × 10^{11} GC AAV8.TBG.PI.ratIGF1.WPRE. bGH). The rIGF1 construct was originally used to generate transgenic mice with hepatocyte-specific expression of rat IGF1 (heterologous IGF1 transgenic mice – HIT (Wu et al. 2009, 2013). Specifically, the pBluescript KS (+) – rat IGF1 plasmid (clone IGF1AB2) was provided to us by Dr Shoshana Yakar (New York University) and sequenced. The plasmid and sequence map was provided to Penn Vector Core, University of Pennsylvania, that inserted the rIGF1 construct into the AAV8-TBGp vector, verified the sequence and performed quality control for titer and sterility. Injection of an AAV8-TBGp-Null (Cat # 105536-AAV8, pAAV8.TBG.PI.Null.bGH, Addgene) was used to generate control mice and to equalize the total amount of AAV8 injected in each mouse (3.5 × 10^{11} GC). Tissue (liver) and cell (hepatocyte) specificity of the AAV8-TBGp vectors in our hands, was previously reported (Cordoba-Chacon et al. 2015, Wolf Greenstein et al. 2017).

Nuclear magnetic resonance (NMR) and indirect calorimetry

Age-dependent changes in whole body fat, lean and water were assessed by NMR (Minispec benchtop LF50 Body Composition mice analyzer; Bruker, Billerica, MA). After 6 months post AAV injection, whole-body metabolic rate was determined by indirect calorimetry (based on lean mass) assessed by NMR, as well as activity level, and food/water intake using the PHYSIOCAGE system (Harvard, Holliston, MA) and METABOLISM analysis software (Panlab Harvard Apparatus, Barcelona, Spain), as previously reported (Gahete et al. 2011, Luque et al. 2011).

Tissue collection and blood analysis

Eight months post-AAV injection, mice were killed by decapitation between 12:00 and 14:00 h (food was withdrawn at 08:00 h). Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes (BD 365974, Franklin Lakes, NJ) and plasma stored at −20°C. Livers and adipose tissue depots (retroperitoneal, urogenital and subcutaneous posterior) were weighed and pituitary glands collected and snap-frozen in liquid nitrogen and stored at −80°C. Specifically for livers, aliquots were weighed and frozen for RNA, lipid or protein analysis and the left lobe was fixed in 10% formalin and stored in 70% ethanol for histologic assessment.

Commercial kits were used to determine plasma GH (EZRMGH-45K, Millipore Sigma, Burlington, MA), insulin (10-1247-01, Mercodia, Uppsala, Sweden) and IGF binding protein 3 (IGFBP3, EMIGFBP3, Thermo Fisher, Waltham, MA). For total IGF1 (22-IG1MS-E01, ALPCO, Salem, NH) and free IGF1 (AL-136, ANSH Labs LLC, Webster, TX), a blood sample was collected from a lateral tail vein nick (at 4 months of age, 4 h after food removal at 12:00 h) using a capillary tube with EDTA. Plasma IGF binding protein acid-lable subunit (IGFALS) levels were determined by Western immunoblotting as previously described (Liu et al. 2017).

To evaluate signs of liver damage, plasma alanine aminotransferase (ALT) levels were assessed (Pointe Scientific-Fisher Scientific, Hampton, NH). NEFA were measured as an indicator of VAT lipolysis (999-34691, 995-34791, 991-34891, 993-35191, Fujifilm Wako Diagnostics USA Corporation, Richmond, VA). In addition, plasma triglycerides (TG) were determined (994-02891, 990-02991, Fujifilm Wako Diagnostics USA Corporation).

Hepatic lipid analysis

Hepatic neutral lipids were extracted with isopropanol, as previously described (Cordoba-Chacon et al. 2015, 2018), for determination of hepatic TG content (Fujifilm Wako Diagnostics USA Corporation). Total hepatic lipids were extracted by Bligh and Dyer method and transmethylated with boron trifluoride (BF3)-methanol (Sigma-Aldrich Inc) to determine amount of specific fatty acyl methyl esters using gas chromatography/mass spectrometry (GC/MS), with heptadecenoic acid as an internal standard, as previously described (Kineman et al. 2016).

Histology

Formalin fixed liver specimens were paraffin-embedded and sectioned (5-μm), then stained with Mayer’s hematoxylin/eosin Y (H&E) or Sirius Red Fast Green (SRFG) (Cordoba-Chacon 2020) to detect collagen deposition indicative of fibrosis. All H&E stained sections were evaluated for steatosis, ballooning, and inflammatory foci (in blinded manner) by a clinical pathologist (Dr Grace Guzman). The level of fibrosis was assessed by Image J analysis (https://imagej.nih.gov/ij/) of SRFG stained slides, by averaging the % of red staining in four separate (20x) images per
slide (taken in a blinded fashion), excluding blood vessels and bile ducts.

Liver and pituitary gene expression analysis

Liver mRNA was extracted using TRIzol™ Reagent (Cat# 15596018, Invitrogen, Thermo Fisher), and pituitary mRNA was extracted using the Mini Total Tissue RNA Sample Kit (Cat# IB47302, IBI Scientific, Dubuque, IA). Gene expression was assessed by quantitative PCR (qPCR) after RT of RNA to cDNA, where the expression of peptidylprolyl isomerase A (Ppia), actin beta (Actb) and hypoxanthine-guanine phosphoribosyltransferase (Hprt) were used as references to calculate normalization factor, as previously reported (Cordova-Chacon et al. 2014). The specific sequences of qPCR primers are provided in Supplementary Table 1 (see section on supplementary materials given at the end of this article). It should be noted that primers for IGF1 amplified both the mouse and rat sequences.

Liver GHR protein levels

Livers were homogenized using radioimmunoprecipitation assay buffer (RIPA) (Cat# 9806S, Cell Signaling Technology) containing protease and phosphatase inhibitors (Cat# 5892970001 and 4906837001, respectively, Sigma-Aldrich). Fifty micrograms of protein extract from each sample were mixed with Laemmli Buffer (Cat# 1610747, BioRad) and denatured samples were separated by SDS-PAGE gels (Cat# 5671095 Bio-Rad), transferred to nitrocellulose membranes and stained with Ponceau to assess protein loading and transfer. Membranes were incubated with GHR primary antibody (diluted 1:2000; AL47, provided by Dr Stuart J Frank, University of Birmingham, AB) and Goat anti-Rabbit IgG horseradish peroxidase (HRP) conjugate secondary antibody (diluted 1:2000; Cat# 7074, Cell Signaling). Finally, blots were developed by ECL (Cat# 1705061, Clarity TM ECL, Bio-Rad).

Liver RNAseq: library preparation, sequencing and analysis

Total RNA from male and female livers (6 mice/sex), from GHR-intact controls, aHepGHRkd and aHepGHRkd+2.0 × 10¹¹ AAV8-TBGp-rIGF1 mice were extracted using RNeasy Plus Mini Kit (Cat# 74134, Qiagen, Germantown, MD) according to manufacturer’s instructions. Total RNA was quality checked (RIN > 7) using the Agilent Bioanalyzer 2100 system. Library preparation, sequencing and bioinformatic analysis was performed by Novogene Corporation Inc (Sacramento, CA). Briefly, RNA sequencing used Illumina platforms, based on mechanism of sequencing by synthesis. RNAseq reads were mapped to the mouse reference genome sequence (GRCm38/mm10) using STAR v2.5 (Dobin et al. 2013). HTSeq v0.6.1 to count the read numbers mapped of each gene and then, FPKM (reads per kilobase of exon model per Million mapped reads) of each gene was calculated based on the length of the gene and reads mapped to the gene (Mortazavi et al. 2008). Differential expression analysis between groups was done using the DESeq2 R package (2_1.6.3) (Anders & Huber 2010). The resulting p-values were adjusted using the Benjamini and Hochberg’s method to control the False Discovery Rate (FDR). Genes with an adjusted p-value <0.05 were considered as differentially expressed. It should be noted that rat IGF1 transgene expression was excluded for this analysis due to non-homology with the mouse reference. ‘Pathway enrichment analyses’ (Gene Ontology (GO), Reactome, and KEGG enrichment analysis) were implemented using the clusterProfiler R package. Principal component analysis (PCA) and Volcano plots were implemented using Galaxy software (Afgan et al. 2018). RNAseq data including raw data files (fastq format) and processed data files (matrix table in xls format) is available in the Gene Expression Omnibus (GEO) repository, accession number GSE154217.

Statistical analysis

With the exception of RNAseq data values are represented as mean ± S.E.M., with individual data points. For some endpoints, there was insufficient sample, or technical errors due to sample processing or loading resulting in undetectable values. The remaining points were evaluated for outliers using Grubbs’ test, and outliers were omitted from the analysis and graphs. Due to variability in plasma GH levels, values were log-transformed prior to analysis. For the indirect calorimetry measurements, n = 3–4 mice/group within sex were compared during the same run, where data was analyzed by two-way ANOVA followed by a Bonferroni post hoc test. All other data were analyzed by one-way ANOVA followed by Bonferroni post hoc test or Kruskal–Wallis followed by a Dunn’s multiple comparison test if the data set did not pass the normality test. Statistical analysis was performed using GraphPad Prism 7. P-values less than 0.05 were considered significant.
GH directly inhibits steatosis and liver injury

Figure 1

Hepatocyte IGF1 reconstitution increases expression and circulating IGF1 levels in both male and female aHepGHRkd mice. Ghr+/mice (10–12 weeks of age) were treated with Null (control = Ctrl), Cre (aHepGHRkd = Kd), or Cre + IGF1 AAV vectors (Kd+IGF1 1.0 (1.0 × 10^{11} GC) or 2.0 (2.0 × 10^{11} GC)) and 8 months later, tissues were collected for analysis. Liver GHR (A, mRNA; B, representative Western blot) and Igf1 (C), Igfals (D) and Igfbp3 (E) mRNA levels. Plasma total IGF1 (F), free IGF1 (G), and IGFBP3 (J) assessed by ELISA and IGFALS assessed by Western blot (H, quantification of all samples; I, representative blot). Values are represented as mean ± s.e.m., with all data points included (n = 7–8 mice per group). Values that do not share a common letter (a, b, c) are statistically different (P < 0.05). Absence of letters means no differences between any group.
Results

Reconstitution of IGF1 in hepatocytes of aHepGHRkd mice raises circulating IGF1 and restores negative feedback to the pituitary somatotrope

A single dose of AAV8-TBGp-Cre resulted in the persistent suppression of hepatic GHR mRNA (Fig. 1A) and protein levels (Fig. 1B), in both male and female mice. This was associated with a reduction in hepatic expression and plasma levels of IGF1 (Fig. 1C, F and G), and IGFALS (Fig. 1D, H and I). Although Igfbp3 hepatic mRNA levels were not reduced by aHepGHRkd in males (Fig. 1E), plasma IGFBP3 levels were dramatically reduced (Fig. 1I), this may be attributed to increased degradation due to loss of the stabilizing actions of IGFALS (Liu et al. 2017). Of note, despite the fact that GHR protein levels were undetectable in both male and female aHepGHRkd mice (Fig. 1B), the reduction in IGF1, IGFBP3 and IGFALS was greater in males compared to females. In both males and females, AAV-vector delivery of IGF1 increased Igf1 mRNA (Fig. 1C, using qPCR primers that amplified both rat and mouse transcripts), as well as plasma levels of total and free IGF1 (Fig. 1F and G).

The reduction in IGF1 negative feedback in aHepGHRkd mice, increased circulating GH levels (Fig. 2A) associated with an increase in pituitary expression of Gh, GH secretagogue receptor (Ghsr) and GH releasing hormone receptor (Ghrhr) (Fig. 2B, C and D, respectively). The restoration of IGF1 levels reduced circulating GH levels and pituitary expression of Gh, Ghsr and Ghrhr consistent with the direct inhibitory actions of IGF1/IGF1 receptor (IGF1R) on somatotrope function (Gahete et al. 2013).

Reconstitution of IGF1 in hepatocytes of aHepGHRkd mice normalizes systemic metabolism, but does not prevent hyperlipidemia, steatosis or liver injury in male mice

The reciprocal shift in IGF1 and GH levels observed in male aHepGHRkd mice was associated with an age-dependent decrease in fat mass as assessed by NMR (Fig. 3A and Supplementary Figs 1, 2) compared to GHR-intact controls. In addition, circulating insulin levels were elevated in male aHepGHRkd mice, without alterations in glucose levels, suggesting mild systemic insulin resistance (Fig. 3B). However, circulating NEFA levels were not altered (Fig. 3B bottom panel). Interestingly, whole body lipid oxidation was increased by aHepGHRkd as indicated by a reduction in the respiratory exchange ratio (RER, assessed
GH directly inhibits steatosis and liver injury

A Sarmento-Cabral et al.

GH directly inhibits steatosis and liver injury by indirect calorimetry; Fig. 3C), independent of changes in energy expenditure and activity (Supplementary Fig. 3). Importantly, reconstitution of IGF1 reversed the impact of aHepGHRkd on whole body lipid oxidation, fat mass and insulin levels in male mice. Systemic metabolic endpoints were only modestly impacted or not changed in female aHepGHRkd mice without and with IGF1 reconstitution (Fig. 3A, B, C and Supplementary Figs 1, 2, 3).

There was a trend for aHepGHRkd to increase circulating TG levels ($P < 0.1$, Fig. 4A), with hepatic TG levels significantly greater than that of GHR-intact controls (Fig. 4B). The increase in TG was associated with increases in hepatic fatty acid (FA) ratios indicative of enhanced DNL (Fig. 4C, D and E; stearoyl-CoA desaturase (SCD)-indexes (16:1(n-7)/16:0 and 18:1(n-9)/18:0) and the DNL-index (16:0/18:2 (n-6))). Supplementary Tables 2 and 3 provide information on the relative levels of major FAs for each treatment group, as assessed by GC/MS. Interestingly, despite the fact that reconstitution of IGF1 in male aHepGHRkd mice normalized metabolic function, it did not reduce circulating or hepatic TG levels, or alter FA composition indicative of enhanced DNL.

The sustained increase in hepatic lipid content in aHepGHRkd male livers was associated with an increase in markers of liver injury including, circulating ALT levels (Fig. 5A), steatosis (Fig. 5B), hepatocyte ballooning (Fig. 5C) and inflammatory foci (Fig. 5D). Fibrosis, assessed by % Sirius Red staining, did not reach significance (Fig. 5E and Supplementary Fig. 4 for representative images of HE and SRFG staining). Reconstitution of IGF1 in male aHepGHRkd mice modestly reduced, but did not eliminate endpoints of liver injury. Consistent with other endpoints studied, females were relatively protected from steatosis and liver injury induced by aHepGHRkd.

**Hepatocyte reconstitution of IGF1 in aHepGHRkd mice does not alter hepatic gene expression due to aHepGHRkd**

Principle component analysis (PCA) of RNAseq data revealed a dramatic shift in overall gene expression between control and aHepGHRkd male hepatic transcriptomes (Fig. 6A, top panel), with $n = 259$ genes downregulated and $n = 212$ genes upregulated by aHepGHRkd, as illustrated by the Volcano plot shown in Fig. 6B. Interestingly, reconstitution of IGF1 did not alter hepatic gene expression in aHepGHRkd mice, as illustrated by the flat line in the Volcano plot shown in Fig. 6B (right panels). Consistent with the modest impact of aHepGHRkd on specific endpoints studied in female
GH directly inhibits steatosis and liver injury

A Sarmento-Cabral et al.

Figure 4
Hepatic IGF1 reconstitution did not prevent hyperlipidemia or steatosis in male aHepGHRkd mice. Ghr^fl/fl^ mice (10–12 weeks of age) were treated with Null (control = Ctrl), Cre (aHepGHRkd = Kd), or Cre + IGF1 AAV vectors (Kd+IGF1 1.0 (1.0 × 10^11 GC) or 2.0 (2.0 × 10^11 GC)) and 8 months later, tissues were collected for analysis. Plasma triglycerides (A, TG) and liver TG content (B). Hepatic fatty acid ratios, assessed by GC/MS, indicative of stearoyl-CoA desaturase-1 (SCD1) conversion of 16:0 to 16:1 (n-7) (C) and from 18:0 to 18:1 (n-9) (D), and de novo lipogenesis (DNL)-index (16:0/18:2 (n-6)) (E). Values are represented as mean ± s.e.m., with all data points included (n = 7–8 mice per group). Values that do not share a common letter (a, b) are statistically different (P < 0.05), while absence of letters means no differences between any group.

Figure 5
Hepatic IGF1 reconstitution did not prevent liver injury in male aHepGHRkd mice. Ghr^fl/fl^ mice (10–12 weeks of age) were treated with Null (control = Ctrl), Cre (aHepGHRkd = Kd), or Cre + IGF1 AAV vectors (Kd+IGF1 1.0 (1.0 × 10^11 GC) or 2.0 (2.0 × 10^11 GC)) and 8 months later, tissues were collected for analysis. Plasma alanine aminotransferase (ALT) levels (A), histological assessment of % steatotic area (B), grade of hepatocyte ballooning (C), number of inflammatory foci (D) and % fibrotic area (E). n = 7–8 mice per group. Values are represented as mean ± s.e.m., with all data points included, and values that do not share a common letter (a, b) are statistically different (P < 0.05). Absence of letters means no differences between groups.
mice (Figs 2, 3, 4 and 5), PCA analysis revealed a clear overlap in gene expression between the three groups analyzed (Fig. 6A, bottom panel). As compared with males, Volcano plots reveal fewer genes were differentially regulated by aHepGHRkd in females (Fig. 6B, bottom panel; n = 208 downregulated and n = 137 upregulated), with IGF1 reconstitution having minimal effect on differentially expressed genes (DEGs) in aHepGHRkd livers (Fig. 6C, bottom panels). Significantly regulated GO, Reactome and KEGG pathways between aHepGHRkd and GHR-intact livers are provided in Supplementary Figs 5 and 6.

Impact of aHepGHRkd on genes related to hepatic carbohydrate/lipid metabolism, inflammation and fibrosis

Heat-map representation of RNAseq data (Fig. 6C), confirmed qPCR data (Fig. 1), showing aHepGHRkd reduced Ghr, Igf1 and Igfals, as well as suppressor of cytokine signaling 2 (Socs2) expression. In male aHepGHRkd mice, consistent with increased steatosis, there was an overall increase in expression of carbohydrate processing (glucokinase (Gck), ketohexokinase (Khk)) and FA synthesis (acetyl-CoA carboxylase 1 (Acc1), fatty acid

Figure 6
Hepatic IGF1 reconstitution did not significantly altered the liver gene transcriptome of aHepGHRkd mice. Principal Component Analysis (PCA) of hepatic transcriptomes in control (Ctrl), aHepGHRkd (Kd) and Kd + IGF1 2.0 (2.0 × 10¹¹ GC) mice (A). Volcano plots (B) showing Log₂ Fold Change (logFC) expression vs −Log₁₀ (P-value) of differentially expressed genes (DEG). Heat-maps showing relative expression levels (Log2FC) of select genes in the GHR-signaling and hepatic carbohydrate/fat metabolism (C) and liver injury (D). Kupffer cell (KC) marker, infiltrating macrophage (Infilt. Mac.) markers. *p-adj < 0.05; n = 6 mice/group.
synthase (*Fasn*), stearoyl-CoA desaturase-1 (*Scd1*) genes, with significant elevations in genes related to FA uptake (cluster of differentiation 36/fatty acid translocase (*Cd36*)), and esterification (monoacylglycerol O-acyltransferase 1 (*Mogat1*)). The expression of sterol regulatory element-binding transcription factor 1 (*Srebf1*), a major downstream regulator of insulin-mediated induction of lipogenesis, was not altered by aHepGHRkd. In female aHepGHRkd mice, there was a significant increase in *Gck*, with more modest effects on the expression of genes related to FA synthesis/processing/uptake. Consistent with the increase in biochemical and histopathologic endpoints of liver injury (Fig. 5), heat-map representation of hepatic expression of genes related to extracellular matrix (ECM), inflammation, Kupffer cell activation and macrophage infiltration (Fig. 6D) show an overall increase in male aHepGHRkd livers, compared to GHR-intact controls. Of note, there was significant upregulation of expression of genes important in ECM proteins (collagen type I alpha 2 chain (*Col1a2*), vimentin (*Vim*)), ECM remodeling (matrix metallopeptidase 12 (*Mmp12*)) and macrophage infiltration (triggering receptor expressed on myeloid cells 2 (*Trem2*)). Interestingly, in livers of female aHepGHRkd mice, there was an overall trend for the expression of ECM and inflammatory genes to be reduced.

**Discussion**

Shifts in IGF1/GH alter systemic metabolism and play a major role in the development of steatosis driven by mobilization of lipids from adipose tissue to the liver, as described with congenital loss of hepatocyte JAK2 (*Sos* et al. 2011, Nordstrom et al. 2013, Corbit et al. 2018). However, several pieces of evidence suggest GH also has a direct role in regulating hepatocyte fat accumulation. First, two-week infusion of IGF1, in adult congenital liver-specific GHR knockout mice, reduced circulating GH but did not rescue hepatic steatosis (*Fan et al. 2009*). Also, when congenital liver-specific GHR knockout mice are crossbred to mice expressing a hepatocyte-specific, rat IGF1 transgene (HIT mice), the rise in circulating GH levels was prevented and steatosis was reduced but not normalized (*Liu et al. 2016*). In addition, although the steatosis observed after aHepGHRkd is associated with a reciprocal shift in IGF1/GH and age-associated reduction in WAT mass, we previously reported no change in circulating NEFA levels, *ex vivo* basal and stimulated WAT glycerol release or WAT expression of active hormone sensitive lipase, indicating enhanced WAT lipolysis is not a major contributor to steatosis in the aHepGHRkd model (*Cordoba-Chacon et al. 2018*). However, in the current and previous study (*Cordoba-Chacon et al. 2018*) whole body lipid oxidation is increased, perhaps in part due to GH-mediated changes in muscle metabolism to favor FA utilization (*Vijayakumar et al. 2012*) which may contribute to the reduction in fat mass with prolonged aHepGHRkd. Finally, in the current study, reconstitution of hepatocyte IGF1 in male aHepGHRkd mice normalized plasma GH and insulin levels, as well as whole body lipid oxidation and WAT mass, without preventing the development of steatosis. Since mature hepatocytes do not express the IGF1 receptor (*Waraky et al. 2016*), these studies support the conclusion that GH acts directly on the hepatocyte, autonomous of IGF1, to control hepatic fat accumulation.

We have previously reported hepatic DNL, as measured by deuterated water labeling of TG-bound FA, is increased 7 days after aHepGHRkd and is associated with steatosis in male, but not female, mice (*Cordoba-Chacon et al. 2015*). In addition, consistent with our previous report (*Cordoba-Chacon et al. 2018*), in the current study hepatic DNL remained elevated with age, as suggested by the increase in FA ratios indicative of DNL. Since insulin was elevated in male aHepGHRkd mice and insulin promotes hepatic lipogenesis (*Wang et al. 2015*), it is possible that elevated insulin may be driving lipogenesis in the absence of hepatic GHR. However, RNAseq revealed hepatic expression of *Srebf1*, a key insulin-induced transcription factor that promotes lipogenic gene expression (*Horton et al. 2002*), was not altered by aHepGHRkd. In addition, reconstitution of hepatocyte IGF1 in aHepGHRkd mice normalized insulin levels, without altering hepatic lipid content or composition. Although the exact mechanism by which loss of hepatocyte GHR directly promotes hepatic DNL remains to be determined, this and our previous studies, examining both short- and long-term effects of aHepGHRkd (*Cordoba-Chacon et al. 2015, 2018, Kineman et al. 2016*), consistently show enhanced hepatic expression of glycolytic (*Gck, Khk*) and lipogenic (*Acc1, Fasn, Scd1*) genes. We previously demonstrated short-term aHepGHRkd increases cytosolic GCK and fructose 2,6-bisphosphate, suggesting enhanced glycolysis provides substrate to drive DNL and promote steatosis (*Cordoba-Chacon et al. 2015*). However, we also observe that aHepGHRkd increased the expression peroxisome proliferator-activated receptor gamma (PPARγ) and downstream targets, *Cd36* and *Mogat1*. Although expression of PPARγ has been shown to increase...
GH directly inhibits steatosis and liver injury

A Sarmento-Cabral et al.

DNL, it was not required for enhanced DNL and steatosis observed with short-term aHepGHRkd (Kineman et al. 2016). However, the FA derived from enhanced DNL may activate PPARγ to enhance expression of Cd36 and Mogat1, to promote FA uptake and esterification respectively, thus sustaining steatosis in aHepGHRkd mice with age. In fact, congenital loss of hepatic CD36 reduced FA uptake in JAK2L mice but did not completely prevent steatosis (Wilson et al. 2016).

In both congenital and adult-onset loss of the hepatic GHR, steatosis is associated with age-dependent signs of liver injury (current study and Fan et al. 2014, Liu et al. 2016, Cordoba-Chacon et al. 2018). However, the extent to which steatosis vs loss of other actions of GHR-signaling contributes to liver injury remains to be determined. It has been shown that the steatosis and associated liver injury and tumor formation are not evident with congenital loss of both liver and adipose JAK2, suggesting GH-mediated metabolic dysfunction of adipose tissue contributes to liver injury in this model (Corbit et al. 2019). However, it is becoming evident that hepatic DNL is a major driver of steatosis in mice and humans (Lambert et al. 2014, Smith et al. 2020) and suppression of DNL improves NASH in a murine diet-induced NASH model (Gapp et al. 2020). In fact, DNL is one of the mechanisms being targeted in clinical trials to reverse NASH (Alkhouri et al. 2020, Gao et al. 2020, Ross et al. 2020, Syed-Abdul et al. 2020).

Although systemic dysregulation of metabolic function can contribute to enhanced hepatic DNL by increasing insulin and available substrates, the current observations show DNL, steatosis and the NASH phenotype is sustained in aHepGHRkd mice with IGFl reconstitution, despite normalization of systemic metabolic function. These observations strongly suggest that GH directly regulates hepatocyte DNL to prevent steatosis and subsequent liver injury. In addition, GH may act independent of steatosis to prevent liver injury. Specifically, GH-mediated STAT5 signaling regulates multiple genes critical in xenobiotic clearance, oxidative stress and cell cycle control that maintain liver health (Clodfelter et al. 2006, Oshida et al. 2016). However, congenital hepatocyte-specific STAT5 knockout increases signal transducer and activator of transcription 3 (STAT3) and c-Jun N-terminal kinases (JNK) signaling in hepatocytes, which favors oxidative stress, inflammation and proliferation (Yu et al. 2012). Of note, using RNaseq we did not observe an increase in STAT3 gene expression after aHepGHRkd, but it remains to be determined whether enhanced STAT3/JNK activity or the direct loss of STAT5 signaling contributes to liver injury.

Furthermore, it should also be noted that, although mature hepatocytes do not express the IGF1R (Waraky et al. 2016), IGFl may play a protective role against liver injury by inactivating hepatic stellate cells, thereby reducing fibrosis (Takahashi 2017), or by promoting an anti-inflammatory response in macrophages (Spadaro et al. 2017). However, in the current study restoration of IGFl did not prevent liver injury or alter the hepatic transcriptome, further supporting a direct role of GH-signaling in preventing liver injury.

Majority of studies have focused on the impact of congenital loss of hepatocyte GH-signaling in male mice. However, List and colleagues (List et al. 2014) reported female mice with congenital hepatocyte-specific GHR knockout, exhibited a more modest alteration in systemic metabolic function and did not develop steatosis, consistent with our current observation in aHepGHRkd mice. In this study, we showed undetectable levels of GHR protein in the livers of both male and female aHepGHRkd mice. However, the relative reduction of IGFl and IGFALS mRNA and plasma levels in female aHepGHRkd was less than that observed in male aHepGHRkd mice. Similar sex-dependent differences were observed after only 7 days of aHepGHRkd (Cordoba-Chacon et al. 2015). Maintenance of IGFl levels in female aHepGHRkd livers, may be due to positive effects of estrogen, since estrogen treatment was shown to enhance hepatic STAT5 phosphorylation and IGFl expression in mice with whole body GHRKO (Venken et al. 2005). These actions may be direct, since it has been demonstrated that estrogen can stimulate STAT5 activity in cell lines (Björnström & Sjöberg 2002). It is unlikely that the sustained production of IGFl per se is responsible for protecting female aHepGHRkd mice against steatosis, since reconstitution of IGFl in males did not reduce hepatic triglyceride levels or FA composition. However, estrogens may play a role independent of IGFl regulation, since ovariectomized aHepGHRkd mice develop steatosis similar to male aHepGHRkd mice and estrogen treatment of ovariectomized aHepGHRkd mice could prevent steatosis (Cordoba-Chacon et al. 2015). It remains to be determined if the protective role of estrogen in the aHepGHRkd model is through direct actions on hepatocyte function, perhaps by sustaining hepatic STAT5 activity, or indirect by modulating systemic metabolism and immune function (Mauvais-Jarvis 2017). Nonetheless, our current findings are consistent with other mouse models and humans (Lonardo et al. 2019), showing relative to males, cycling females are protected from NAFLD/NASH development.
In summary, our results clearly demonstrate that under standard diet and housing conditions, adult-onset loss of hepatocyte GHR-signaling leads to steatosis and liver injury in male, but not female mice. In males, steatosis develops independent of IGF-I-mediated changes in GH secretion and systemic metabolism. In males, loss of the hepatocyte GHR leads to liver injury that is slightly reduced, but not prevented by IGF-I-mediated improvement in systemic function. Taken together, these results further support the hypothesis that GH acts directly on the adult hepatocyte to prevent excess fat accumulation and liver injury. Although the intracellular pathways mediating these protective effects remains to be determined, these findings suggest selectively enhancing hepatocyte GH-signaling may prevent NAFLD progression.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0326.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was funded by NIH R01DK114326 and VA Merit BX001114 (to R D K), K01DK115525 (to J C C) and VA Merit BX004315 (to P V S).

Author contribution statement
A S C, J C C and R D K designed the study, performed the experiments, analyzed the data and wrote the manuscript. M d R M, M C V B, M M, E G C, N P performed experiments and analyzed the data related to protein, GC/MS, image analysis and RNAseq analysis. G G analyzed and scored the liver histological characteristics. P V S provided technical assistance on the use of GC/MS, as well as data interpretation. S Y provided the rIGF1 construct used to develop the AAV8-TBGp-rIGF1 vector and performed analysis for circulating IGFBP3 and IGFLALS. All authors have discussed the results, revised and approved the final version of the manuscript. R D K and A S C are 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.

Acknowledgements
The authors would wish to thank Dr John J Kopchick (Edison Biotechnology Institute and Department of Biomedical Sciences, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH) for providing the Ghrnull mouse model, and Dr Stuart J Frank (Department of Medicine, Section of Endocrinology, Diabetes and Metabolism, University of Alabama at Birmingham) for providing the mouse GHR antibody.

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Received in final form 25 September 2020
Accepted 20 October 2020
Accepted Manuscript published online 20 October 2020