Original Article

MOLECULAR METABOLISM

Glucagon receptor antagonism impairs and glucagon receptor agonism enhances triglycerides metabolism in mice

Katrine D. Galsgaard [1,2,7], Emilie Elmelund [1,7], Christian D. Johansen [1,3], Anna B. Bomholt [1], Hüsün S. Kizilkaya [1], Frederik Ceutz [1], Jenna E. Hunt [1,2], Hannelouise Kissow [1], Marie Winther-Sørensen [1,3], Charlotte M. Sørensen [1], Thomas Kruse [4], Jesper F. Lau [4], Mette M. Rosenkilde [1], Cathrine Ørskov [1], Christina Christoffersen [1,5], Jens J. Holst [1,2,8], Nicolai J. Wewer Albrechtsen [1,3,6,*,8]

ABSTRACT

Objective: Treatment with glucagon receptor antagonists (GRAs) reduces blood glucose but causes dyslipidemia and accumulation of fat in the liver. We investigated the acute and chronic effects of glucagon on lipid metabolism in mice.

Methods: *Chronic* effects of glucagon receptor signaling on lipid metabolism were studied using oral lipid tolerance tests (OLTTs) in overnight fasted glucagon receptor knockout ($Gcgr^{-/-}$) mice, and in C57Bl/6JRj mice treated with a glucagon receptor antibody (GCGR Ab) or a long-acting glucagon analogue (GCGA) for eight weeks. Following treatment, liver tissue was harvested for RNA-sequencing and triglyceride measurements. *Acute* effects were studied in C57Bl/6JRj mice treated with a GRA or GCGA 1 h or immediately before OLTTs, respectively. Direct effects of glucagon on hepatic lipolysis were studied using isolated perfused mouse liver preparations. To investigate potential effects of GCGA and GRA on gastric emptying, paracetamol was, in separate experiments, administered immediately before OLTTs.

Results: Plasma triglyceride concentrations increased 2-fold in $Gcgr^{-/-}$ mice compared to their wild-type littermates during the OLTT ($P = 0.001$). Chronic treatment with GCGR Ab increased, whereas GCGA treatment decreased, plasma triglyceride concentrations during OLTTs ($P < 0.05$). Genes involved in lipid metabolism were upregulated upon GCGR Ab treatment while GCGA treatment had opposite effects. Acute GRA and GCGA treatment, respectively, increased ($P = 0.02$) and decreased ($P = 0.003$) plasma triglyceride concentrations during OLTTs. Glucagon stimulated hepatic lipolysis, evident by an increase in free fatty acid concentrations in the effluent from perfused mouse livers. In line with this, GCGR Ab treatment increased, while GCGA treatment decreased, liver triglyceride concentrations. The effects of glucagon appeared independent of changes in gastric emptying of paracetamol.

Conclusions: Glucagon receptor signaling regulates triglyceride metabolism, both chronically and acutely, in mice. These data expand glucagon's biological role and implicate that intact glucagon signaling is important for lipid metabolism. Glucagon agonism may have beneficial effects on hepatic and peripheral triglyceride metabolism.

Keywords Cholesterol; Glucagon; Non-esterified/ free fatty acids; Steatosis; Triglycerides

1. INTRODUCTION

Glucagon secreted from the pancreatic alpha cell is best known for its role in glucose homeostasis. Glucagon receptor antagonists (GRAs) are considered as glucose lowering therapy [1]. However, side effects including dyslipidemia [2] as well as accumulation of hepatic fat have been reported [3]. Hepatic fat content is also increased in other conditions with absent glucagon receptor signaling including pancreatectomy [4] and in db/db mice treated with glucagon receptor siRNA [5]. Conversely, glucagon administration decreases liver fat content in diet-induced obese rats and mice [6] and decreases plasma TG concentrations [7,8]. Detailed

[1]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark [2]Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark [3]Novo Nordisk Foundation Center for Protein Research, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark [4]Novo Nordisk A/S, Research Chemistry, Novo Nordisk Park, DK-2760 Måløv, Denmark [5]Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark [6]Department of Clinical Biochemistry, Bispebjerg & Frederiksberg Hospitals, University of Copenhagen, 2400 Bispebjerg, Denmark

[7] These authors contributed equally as first authors.
[8] These authors contributed equally as senior authors.

*Corresponding author. Department of Biomedical Sciences and Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, and Department of Clinical Biochemistry, Bispebjerg & Frederiksberg Hospitals, University of Copenhagen, 2400 Bispebjerg, Denmark. E-mail: nicolai.albrechtsen@sund.ku.dk (N.J. Wewer Albrechtsen).

Received October 5, 2022 • Revision received November 6, 2022 • Accepted November 9, 2022 • Available online 15 November 2022

https://doi.org/10.1016/j.molmet.2022.101639

information about glucagon's potential role in lipid metabolism is of both physiological and pharmacological importance considering that several compounds targeting the glucagon receptor (e.g. glucagon/GLP-1 (glucagon-like peptide 1) receptor co-agonists [9] and tri-agonists additionally targeting the GIP (glucose-dependent insulinotropic polypeptide) receptor [10–12]) are currently being developed as a treatment for non-alcoholic fatty liver disease (NAFLD) and obesity.

Here we investigate glucagon's role in lipid metabolism by activating and inhibiting the glucagon receptor in mice, both chronically and acutely. We hypothesize that inhibition of glucagon receptor signaling results in a dysregulated lipid metabolism, mainly in the liver, resulting in liver fat accumulation and dyslipidemia whereas glucagon receptor activation has opposite effects.

2. MATERIALS AND METHODS

2.1. Animal studies

Animal studies were conducted at the animal facilities at the Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, with permission from the Danish Animal Experiments Inspectorate, Ministry of Environment and Food of Denmark, permit 2018-15-0201-01397. All studies were approved by the local ethical committee. C57BL/6JRj mice were obtained from Janvier Laboratories (Saint-Berthevin Cedex, France). Glucagon receptor knockout (*Gcgr*$^{-/-}$) mice C57BL/6J^{Gcgrtm1Mjch} were bred in-house with permission from Dr. Maureen J. Charron as described previously [13]. Female mice were housed in groups of four to eight and male mice in groups of two to six in individually ventilated cages. All followed a light cycle of 12 h (lights on 6 AM to 6 PM) and had ad libitum access to standard chow and water unless otherwise stated. Mice were allowed a minimum of one week of acclimatization before being included in any experiment.

2.1.1. Oral lipid tolerance test upon genetic deletion of glucagon receptor signaling

Female and male *Gcgr*$^{-/-}$ mice and their wild-type littermates (*Gcgr*$^{+/+}$), 12–25 weeks of age, were fasted overnight, and the following morning blood glucose concentration was measured after tail tip puncture using a handheld glucometer (Accu-Chek® Mobile, catalog no. 05874149001; Roche Diagnostics, Mannheim, Germany); the mice were weighed, and blood collected (50–75 μL) from the retrobulbar plexus using ethylenediaminetetraacetic acid (EDTA) coated capillary tubes (Micro Haematocrit Tubes, ref. no. 167313; Vitrex Medical A/S, Herlev, Denmark), and subsequently stored on ice until spun (9,000 rpm, 4 °C, 10 min). Plasma was transferred to pre-chilled PCR tubes (Thermowell, Gold PCR; Corning, NY) and stored at −80 °C until further analysis. Immediately after, the mice were subjected to an oral lipid tolerance test (10 μL/g body weight of olive oil administered via oral gavage; OLTT). At times 30, 120, and 180 min after lipid administration, blood glucose concentrations were measured, and blood was collected as described. After blood collection the mice were killed by cervical dislocation, and the livers were excised and snap frozen in liquid nitrogen. Finally, the tip of the ear was cut with a scissor washed with ethanol for genotyping. To perform fast protein liquid chromatography (FPLC) analysis, aliquots (15 μL) of the plasma collected from a separate group of overnight fasted female *Gcgr*$^{-/-}$ and *Gcgr*$^{+/+}$ mice, 14 weeks of age, were pooled (according to the groups) in pre-chilled Eppendorf tubes and stored at −80 °C until analysis.

2.1.2. Oral lipid tolerance test upon chronic pharmacological inhibition and activation of glucagon receptor signaling

Female C57BL/6JRj mice (seven weeks old at the start of treatment) were randomized to treatment with: a long-acting glucagon receptor blocking antibody (GCGR Ab, REGN1193, 10 mg/kg body weight; Regeneron, Tarrytown, New York, USA [14]), a control antibody (Ctl Ab, REGN1945, 10 mg/kg body weight; Regeneron) dissolved in PBS + 1% BSA; a long-acting glucagon analogue (GCGA, NNC9204-0043, 1.5 nmol/kg body weight; Novo Nordisk A/S, Bagsværd, Denmark); or PBS + 1% BSA (PBS).

The antibodies (GCGR Ab and Ctl Ab) were injected once weekly, while GCGA and PBS were injected twice daily (at 8 AM and 8 PM), all subcutaneously. The dose of GCGA was initially 3 nmol/kg body weight, but after 16 days of treatment, one mouse had lost >20% of its body weight and had to be euthanized (this mouse was excluded from the study). Because of this, the GCGA dose was halved (1.5 nmol/kg body weight) for the remainder of the study. The mice were treated for a total of eight weeks and then fasted overnight (11 h) and subjected to an OLTT as described above. The last doses of GCGA and PBS were given 13 h prior to the OLTT, and the last doses of GCGR Ab and Ctl Ab were given one week prior to the OLTT. After the final blood collection, the mice were killed by cervical dislocation, and liver tissue and plasma for FLPC were obtained as described. Liver samples were also fixed in 4% paraformaldehyde for 24 h and then transferred to 70% ethanol and afterwards placed in a vacuum tissue processor (Shandon Exelsior) overnight and embedded in paraffin. The samples were cut in sections of 4 μM and dewaxed through Tissue Clear to tap water and then stained with hematoxylin and eosin.

To investigate glucagon's effect on hepatic fat independent of fasting and OLTT, we included a separate group of non-fasted mice, which had not been subjected to a previous OLTT, for measurements of liver triglyceride concentrations.

We previously used the mice included in this chronic experiment to investigate glucagon's effect on amino acid metabolism [15], and blood glucose, body weight, plasma insulin and glucagon measurements at baseline and after four weeks of treatment are shown in [15].

2.1.3. Oral lipid tolerance test upon acute pharmacological inhibition and activation of glucagon receptor signaling

Female and male C57BL/6JRj mice, 13 weeks of age, were fasted overnight (15 h) and the following morning blood glucose concentrations were measured, the mice were weighed and then treated with a glucagon receptor antagonist (GRA, 25-2648, a generous gift from Novo Nordisk A/S, 100 mg/kg body weight) or vehicle, both administered via oral gavage 1 h before the OLTT. GRA was dissolved in 5% ethanol, 20% propyleneglycol, 10% 2-hydroxypropyl-β-cyclodextrin (vol/vol) and phosphate buffer at pH 7.5–8.0 as described [16] to a concentration of 20 mg/mL. Separately, female C57BL/6JRj mice, 13 weeks of age, were fasted overnight (16 h), and the following morning blood glucose concentrations were measured, the mice were weighed, and immediately before the OLTT the mice were treated with GCGA (30 or 3 nmol/kg body weight) or an equal amount of PBS + 1% BSA, both administered as an intraperitoneal injection.

To investigate the effects of GRA on lipid tolerance in a non-fasted condition, non-fasted female and male C57BL/6JRj mice 13 weeks of age, were treated with GRA or vehicle at 9 AM and the food was removed. Three hours after, the mice received a second dose of GRA and 200 μL olive oil via oral gavage, and the procedure continued as described with the only exception that an additional blood sample was collected 240 min after lipid administration.

To investigate the effects of GCGA on plasma and liver triglycerides without an OLTT, male C57BL/6JRj mice, 13 weeks of age, were fasted from 9AM with free access to water, and at 12 PM, GCGA (30 nmol/kg body weight) or an equal amount of PBS + 1% BSA was administered as an intraperitoneal injection. Immediately prior to, and at times 2, 30, and 120 min after the injection, blood glucose was measured, and blood was collected. Finally, the mice were killed, and liver tissue collected as described.

2.1.4. Effects of GCGA and GRA on gastric emptying

The effects of GCGA and GRA on gastric emptying of paracetamol were determined in separate experiments. Female and male C57BL/6JRj mice were subjected to OLTT's as described for GRA and female C57BL/6JRj mice were subjected to OLTT as described for GCGA, but immediately before lipid administration, paracetamol (100 mg/kg body weight, catalog no. A7085; Sigma–Aldrich, St. Louis, Missouri, USA) dissolved in PBS was administered by oral gavage.

2.1.5. Liver perfusions

Livers from C57bl/6JRj male and female mice, 10–12 weeks of age, were perfused to investigate any direct effects of glucagon on hepatic lipolysis. Detailed description of the liver perfusion setup including the surgery can be found in [17].

2.2. Biochemical analysis

Plasma concentrations of glucagon were measured using a validated [18] low volume, two-site enzyme immunoassay (catalog no. 10-1281-01; Mercodia, Upsala, Sweden), also upon GCGA administration (using WHO calibrated glucagon as standards) as this assay also detects our GCGA. Plasma concentrations of insulin were measured using an enzyme immunoassay (catalog no. 10-1247-01; Mercodia, Upsala, Sweden). Triglyceride and glycerol concentrations were quantified using Serum Triglyceride Determination Kit (catalog no. TR0100-1 KT; Sigma–Aldrich), except when triglyceride concentrations were measured using the Triglyceride Assay Kit (catalog no. ab65336; Abcam, Cambridge, UK), indicated in the figure legends as "*measured by ab65336*". Additional measurements: Non-esterified fatty acid (NEFA) concentrations with NEFA-HR (R1 and R2) kit from Fujifilm Wako Chemicals; liver glycogen concentrations with EnzyChrom Glycogen Assay Kit (catalog no. E2GN-100; BioAssay Systems, Hayward, CA); and plasma paracetamol concentrations with the Acetaminophen L3K kit (catalog no. 506-30; Sekisui Diagnostics, Burlington, Massachusetts, USA). Perfusion samples were analyzed for glucose using QuantiChrom Glucose assay kit (catalog no: DIGL-100; BioAssay Systems). For FPLC analysis, a Superose 6 10/300 GL column was used and washed with PBS + EDTA pH 7.5 at a flow of 0.2 mL/min; subsequently the sample (consisting of 75 μL plasma diluted ×5 in PBS + EDTA pH 7.5) was added and run through the column at a flow rate of 0.4 mL/min. Effluent fractions were collected in a 96 well plate, cholesterol reagent (CHOD-PAP, ref no. 11491458-216; Roche Diagnostics) added, and absorbance read at 492 nM after 15 min. Two quality controls (K1 697 and K3 669, liquid unassayed multilingual; BioRad) were included. The standards used were CFas (ref. no. 10759350; Roche Diagnostics).

2.3. Lipid extraction

Snap frozen liver tissue (50–100 mg) was homogenized in 1.8 mL of extraction buffer (3% Triton™ X-100 (Catalog no. 10789704001; Sigma–Aldrich) (25% solution in ethanol) in sodium acetate buffer (0.15 mol/L, pH 4.9) and then placed in a 98 °C heat block for 2 min, and subsequently centrifuged at 9,000 g for 10 min. Triglyceride content was measured in the supernatant using the Serum Triglyceride Determination Kit (catalog no. TR0100-1 KT; Sigma–Aldrich). Lipids from the livers of $Gcgr^{-/-}$ and $Gcgr^{+/+}$ mice and non-fasted mice treated with GRA were extracted using chloroform/methanol extraction. Liver tissue (50–100 mg) was homogenized in methanol and left overnight for extraction in chloroform/methanol 2:1. After centrifugation, the supernatant was dried under nitrogen and re-suspended in 1% Triton X-100™ and assayed the using the Serum Triglyceride Determination Kit (catalog no. TR0100-1 KT; Sigma–Aldrich).

2.4. RNA-sequencing and bioinformatic analysis

The RNA-sequencing and bioinformatic analysis of the liver samples have been described in detail elsewhere [15]. In short, liver biopsies were taken from mice treated for eight weeks with GCGA, PBS, GCGR Ab, or Ctl Ab. Total DNA/RNA was extracted with an AllPrep DNA/RNA Minikit (catalog no. 80204; QIAgen, Hilden, Germany) and subsequently DNase treated. RNA sequencing libraries were paired-end sequenced (2 × 150 bp) on an Illumina Novaseq 6000 instrument. The read quality of the raw sequencing data (FASTQ) was evaluated using FastQC version 0.11.9. Reads were mapped to a decoy-aware transcriptome (M28/GRCm39) using Salmon version 1.6.0 and selective alignment. The data were normalized using the algorithm variance stabilizing transformation offered by the DESeq2 package [19] version 1.34.0 in R version 4.1.0. The same R package was used to identify differentially expressed genes (FDR<0.05). To minimize potential biases when comparing $Gcgr^{-/-}$ mice [20] and GCGR Ab mice, raw sequencing data for liver samples from male $Gcgr^{-/-}$ mice were re-analyzed using the bioinformatic analysis presented above. Gene ontology (GO) [21,22] enrichment analysis was performed at the level of biological processes (BP) using the list of all identified genes (excluding pseudo genes) as background [23]. Differentially expressed genes related to lipid metabolism were filtered using Gene Ontology (GO) [21,22] annotations for the umbrella terms *Lipid metabolic process*, *Lipid localization*, *Lipid homeostasis*, and all child terms [23,24].

2.5. Statistics

Except for RNA sequencing data, all statistical analyses were done in GraphPad Prism version 9.3.1 (La Jolla, California, USA). Area under the curve (AUC) was calculated using the trapezoid rule. For total AUC ($_{total}$AUC) baseline was Y = 0 and peaks above the baseline were considered. For net AUC ($_{net}$AUC), baseline was set to the first data point and peaks above and below the baseline were considered. If there were missing data at one or more time-points from a mouse, this mouse was excluded from the AUC analysis and the associated XY curve. Groups were compared by unpaired t-test or one-way ANOVA as indicated in the figure legend. In some cases, indicated in the figure legends, significance was analyzed by mixed effects analysis followed by Holm-Sidak post-hoc analysis to correct for multiple testing. $P < 0.05$ was considered significant.

3. RESULTS

3.1. Permanent genetic deletion of glucagon receptor signaling causes postprandial lipid intolerance

To investigate if glucagon receptor signaling is required for lipid metabolism in the postprandial state, we challenged overnight fasted $Gcgr^{-/-}$ and $Gcgr^{+/+}$ mice with oral lipids (10 μL/g body weight olive oil via oral gavage; OLTT). The mice were overnight fasted before the OLTT to ensure that potential differences in food consumption/gastric content did not influence lipid absorption. Before lipid administration (0 min time-point of the OLTT), female $Gcgr^{-/-}$ mice had lower blood

glucose concentrations ($P < 0.0001$), and higher plasma TG and glycerol concentrations ($P < 0.0001$ and $P = 0.047$, respectively) compared to *Gcgr*$^{+/+}$ mice, whereas fasting plasma NEFA concentrations did not differ ($P = 0.9$) (Figure 1).

Female *Gcgr*$^{-/-}$ and *Gcgr*$^{+/+}$ mice responded to the OLTT with a similar increase in blood glucose concentrations ($P = 0.5$) (Figure 1*A* and *B*), whereas plasma TG concentrations were markedly increased in female *Gcgr*$^{-/-}$ mice compared to *Gcgr*$^{+/+}$ mice ($P = 0.001$) (Figure 1C and D). No difference in plasma NEFA concentrations was observed ($P = 0.8$) (Figure 1*E* and *F*). Plasma glycerol concentrations were higher in female *Gcgr*$^{-/-}$ mice compared to *Gcgr*$^{+/+}$ ($P = 0.054$) (Figure 1G and H). Following the OLTT, liver TG concentrations were lower in female *Gcgr*$^{-/-}$ compared to *Gcgr*$^{+/+}$ mice ($P = 0.002$) (Figure 1*I*), possibly reflecting a decreased hepatic TG uptake in the *Gcgr*$^{-/-}$ during the OLTT. In overnight fasted female *Gcgr*$^{-/-}$ mice, which had not been subjected to an OLTT, plasma very-low density lipoprotein (VLDL), low density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol concentrations were increased compared to *Gcgr*$^{+/+}$ mice (Figure 1*J*).

Similar results were observed in male *Gcgr*$^{-/-}$ mice (Suppl. Figure 1).

3.2. Chronic pharmacological inhibition and activation of glucagon receptor signaling, respectively, impairs and enhances postprandial lipid tolerance in female mice

To further investigate glucagon's role in lipid metabolism, we randomized female C57BL/6JRj mice for an eight week treatment with either a glucagon receptor blocking antibody (GCGR Ab), control antibody (Ctl Ab), a long-acting glucagon analogue (GCGA), or PBS.

The eight weeks GCGR Ab treatment decreased blood glucose concentrations compared to Ctl Ab ($P = 0.01$) (Suppl. Figure 2*A*), whereas GCGA treatment increased blood glucose concentrations compared to PBS ($P < 0.0001$) (Suppl. Figure 2*B*). During the eight weeks of treatment GCGR Ab treated mice gained significantly more weight compared to Ctl Ab treated mice ($P = 0.02$) (body weights at week 0; GCGR Ab: 17.5 ± 0.3 and Ctl Ab: 17.3 ± 0.4 g and at week eight; GCGR Ab: 22.8 ± 0.4 and Ctl Ab: 21.7 ± 0.5 g), whereas GCGA treated mice gained significantly less weight compared to PBS treated mice ($P = 0.02$) (body weights at week 0; GCGA: 18.1 ± 0.3 g, PBS: 18.0 ± 0.2 g and at week eight; GCGA: 19.6 ± 0.3 g, PBS: 20.7 ± 0.4 g) (the delta body weights are shown in Suppl. Figure 2*C* and *D*).

After eight weeks of treatment the mice were overnight fasted and subjected to an OLTT. Following the overnight fast (0 min time-point of the OLTT), blood glucose concentrations were similar in GCGR Ab and Ctl Ab treated mice ($P = 0.9$) (Suppl. Figure 2*E*), whereas blood glucose concentrations were increased in GCGA treated mice compared to PBS ($P = 0.045$) (Suppl. Figure 2*F*). Fasting plasma glucagon concentrations were increased in GCGR Ab and GCGA treated mice compared to their respective controls (GCGR Ab vs. Ctl Ab $P < 0.0001$ and GCGA vs. PBS $P = 0.0006$) (Suppl. Figure 2*G* and *H*), and remained increased during the OLTT (GCGR Ab vs. Ctl Ab $P < 0.0001$ and GCGA vs. PBS $P = 0.002$) (Suppl. Figure 2*I*–*L*). Fasting plasma insulin, NEFA, and TG concentrations were similar in GCGR Ab and Ctl Ab treated mice ($P = 0.7$, $P = 0.8$, and $P = 0.5$, respectively) and in GCGA and PBS treated mice ($P = 0.08$, $P = 0.8$, and $P = 0.4$, respectively) (Suppl. Figure 2*M*–*R*).

During the OLTT, GCGR Ab treated mice had decreased blood glucose concentrations compared to Ctl Ab treated mice ($P = 0.003$) (Figure 2*A* and *B*), whereas the increases in blood glucose concentrations were similar in GCGA and PBS treated mice during the OLTT ($P = 0.7$) (Figure 2*C* and *D*). During the OLTT, insulin concentrations were similar in GCGR Ab and Ctl Ab treated mice ($P = 0.3$) (Figure 2*E* and *F*) and in GCGA and PBS treated mice ($P = 0.6$) (Figure 2*G* and *H*). No significant difference between GCGR Ab and Ctl Ab treated mice was observed in plasma NEFA concentrations during the OLTT ($P = 0.3$) (Figure 2*I* and *J*). Plasma NEFA concentrations were also similar in GCGA and PBS treated mice ($P = 0.9$) (Figure 2*K* and *L*). Plasma TG concentrations were increased in GCGR Ab treated mice after the OLTT compared to Ctl Ab ($P = 0.04$) (Figure 4*M*). The VLDL cholesterol peak concentration was higher in GCGR Ab treated mice compared to Ctl Ab (0.0022 μmol vs .0.0009), but no difference was found for LDL and HDL cholesterol particles (Figure 2*N*). GCGA treatment decreased plasma TG concentrations after lipid administration compared to PBS ($P = 0.049$) (Figure 2*O*). LDL cholesterol peak concentration was lower in GCGA treated mice compared to PBS (0.0024 vs. 0.0031 μmol), but VLDL and HDL cholesterol particles were similar (Figure 2*P*).

Following the eight weeks of treatment and the OLTT, liver weights were increased in GCGR Ab treated mice compared to Ctl Ab ($P = 0.0004$) (Figure 3*A*) whereas GCGA had no effect ($P = 0.5$) (Figure 3*B*). Liver glycogen content was low after the overnight fast in Ctl Ab and PBS treated mice, whereas mice treated with GCGR Ab and GCGA had higher glycogen contents compared to their respective controls ($P < 0.001$) (Figure 3*C* and *D*). We found no significant effect of chronic GCGR Ab ($P = 0.2$) or GCGA ($P = 0.1$) treatment on hepatic TG concentrations following the overnight fast and the OLTT (Figure 3*E* and *F*). Histological examination of liver sections from the same groups revealed evident lipid accumulation in the cytoplasm but normal liver morphology (Figure 3*G*–*J*).

Overnight fast in mice has been reported to induce steatosis [25–27]. To investigate if the overnight fast might mask differences in liver TG concentrations after chronic GCGR Ab and GCGA treatment, we measured liver TG concentrations in mice treated with GCGR Ab and Ctl Ab, but without a preceding overnight fast and OLTT. In this condition, liver TG concentrations were significantly lower compared to the fasted state (Figure 3*E* and *F*), and higher in GCGR Ab treated mice compared to Ctl Ab ($P = 0.02$) (Figure 3*K*) indicating that overnight fasting and/or the OLTT may mask the effect of the GCGR Ab treatment. GCGA had no significant effect on liver TG levels in this condition ($P = 0.1$) (Figure 3*L*).

3.3. Glucagon receptor signaling may regulate lipid metabolism through changes in the hepatic transcriptome

To further explore the mechanisms by which increased and decreased glucagon receptor signaling could result in the observed alterations in TG metabolism, we performed RNA-sequencing of liver biopsies from the mice treated for eight weeks with GCGA and GCGR Ab and their respective controls; PBS and Ctl Ab. Workflow and data analysis are described in detail elsewhere [15]. An enrichment analysis of gene ontology biological processes (GOBP) [21,22] (Suppl. Table 1), revealed that several lipid metabolism processes were up-regulated in mice treated with GCGR Ab and down-regulated in GCGA treated mice (Figure 4*A*). We then compared the differentially expressed genes included in the GOBP umbrella terms; *lipid metabolic process*, *lipid localization*, and *lipid homeostasis* in our GCGR Ab treated mice to identify differentially expressed genes of livers of *Gcgr*$^{-/-}$ mice [28]. A total of 18 genes found under these umbrella terms were up-regulated in the livers of both GCGR Ab treated and *Gcgr*$^{-/-}$ mice (Figure 4*B*). Among these were patatin-like phospholipase domain containing 3 (Pnpla3), fatty acid binding protein 5 (Fabp5), fat storage-inducing transmembrane protein 1 (Fitm1), and monoglyceride lipase (Mgll) (Suppl. Table 2). A total of 19 genes were down-regulated in both groups (Figure 4*C*) (Suppl. Table 3). We then investigated whether certain lipid

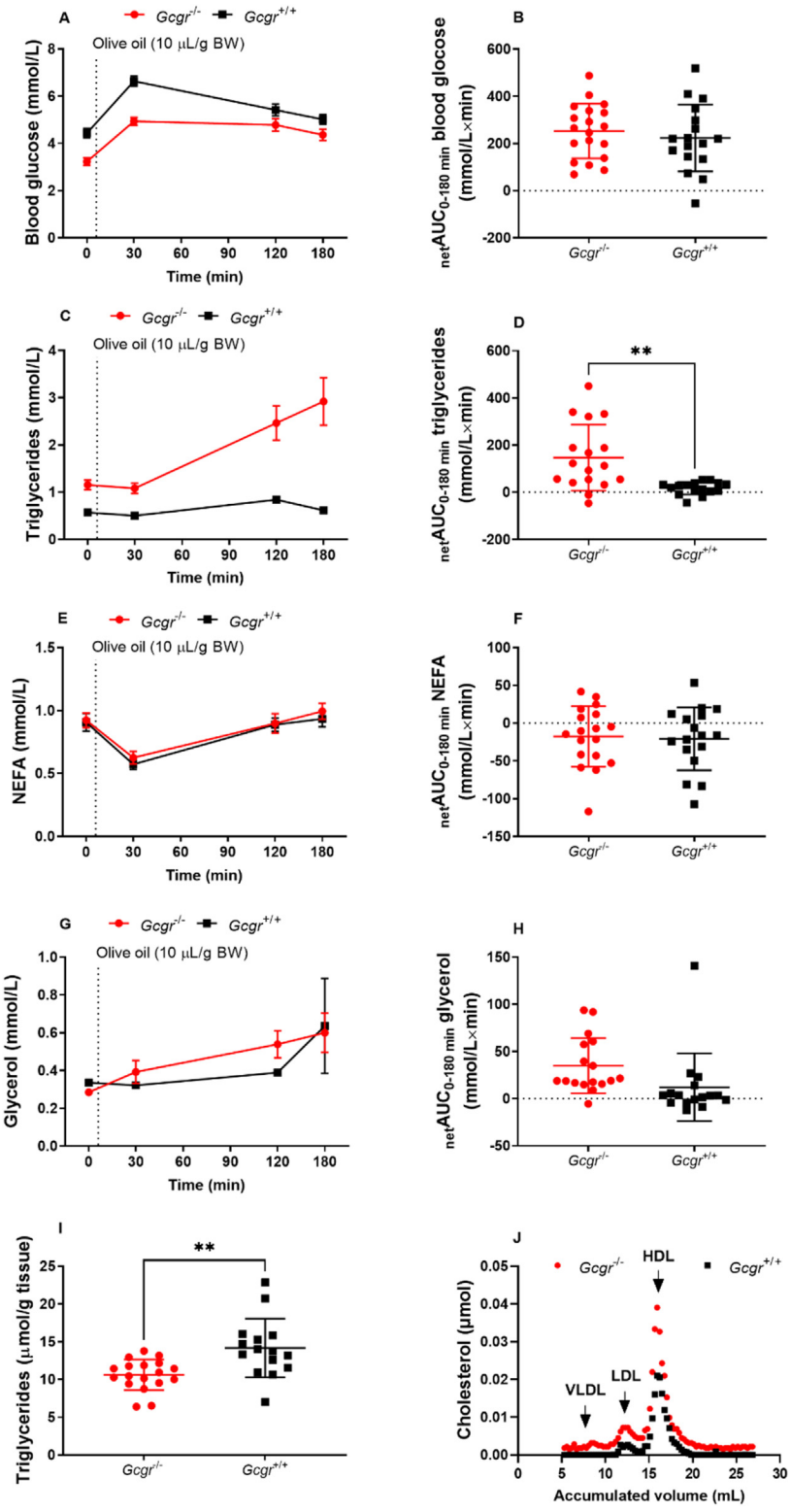

Figure 1: Fasted female mice with permanent genetic deletion of glucagon receptor signaling show postprandial lipid intolerance. (A) Blood glucose, (B) $_{net}AUC_{0-180\ min}$ blood glucose, (C) plasma triglyceride, (D) $_{net}AUC_{0-180\ min}$ triglyceride, (E) non-esterified fatty acid (NEFA), (F) $_{net}AUC_{0-180\ min}$ NEFA, (G) glycerol, (H) $_{net}AUC_{0-180\ min}$ glycerol concentrations during an oral lipid tolerance test (olive oil, 10 µL/g body weight via oral gavage) in female glucagon receptor knockout ($Gcgr^{-/-}$) (red circles and lines) and female wild-type littermates ($Gcgr^{+/+}$) (black squares and lines). (I) Liver triglyceride concentrations. (J) Plasma cholesterol profiles in overnight fasted $Gcgr^{-/-}$ (red circles) and $Gcgr^{+/+}$ (black squares) mice not subjected to an oral lipid tolerance test. VLDL (very-low density lipoprotein), LDL (low density lipoprotein), and HDL (high density lipoprotein). Data in XY graphs are shown as mean ± SEM, and data in AUC graphs and (*I*) are shown as mean ± SD, n = 15–19, mice 12–25 weeks of age. P-values by unpaired t-test.

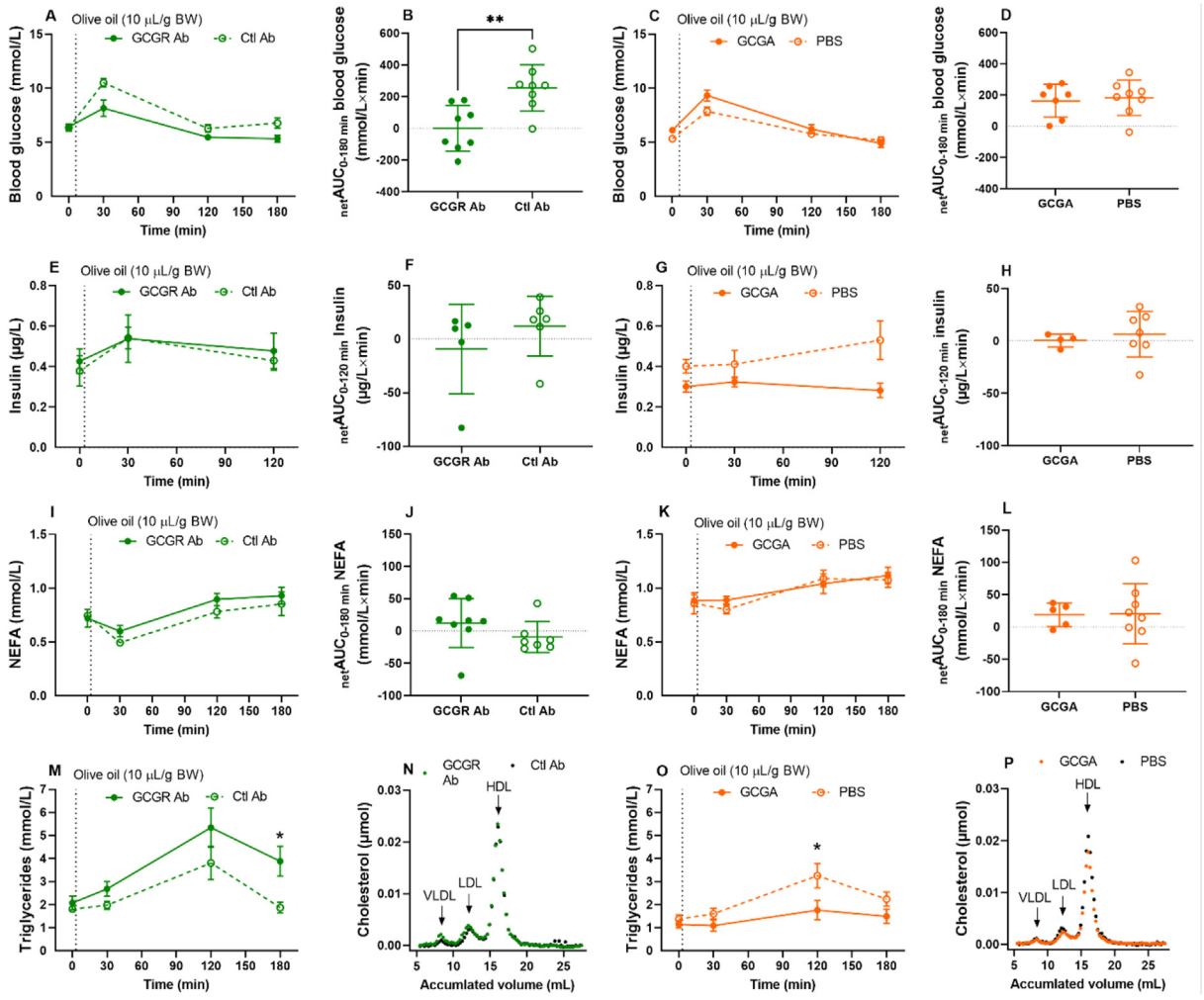

Figure 2: Chronic pharmacological inhibition and activation of glucagon receptor signaling, respectively, impairs and enhances postprandial lipid tolerance in female mice. (A, C) Blood glucose, (B, D) $_{net}AUC_{0-180\ min}$ blood glucose, (E, G) insulin, (F, H) $_{net}AUC_{0-120\ min}$ insulin, (I, K) non-esterified fatty acid (NEFA), (J, L) $_{net}AUC_{0-180\ min}$ NEFA, and (M, O) triglyceride (measured using ab65336) concentrations during an oral lipid tolerance test (olive oil, 10 uL/g body weight (BW) via oral gavage) in female C57BL/6JRj mice treated with a glucagon receptor antibody (GCGR Ab, REGN1193, Regeneron, 10 mg/kg BW) (green closed circles and solid lines), control antibody (Ctl Ab, REGN1945, Regeneron, 10 mg/kg BW) (green open circles and dotted lines) once weekly for eight weeks or a long-acting glucagon analogue (GCGA, NNC9204-0043, Novo Nordisk A/S, 1.5 nmol/kg BW) (orange closed circles and solid lines) or PBS + 1% BSA (PBS) (orange open circles and dotted lines) twice daily for eight weeks. The female mice were seven weeks old at the start of treatment. (N, P) Plasma cholesterol profiles in the GCGR Ab (green circles), Ctl Ab (black circles), GCGA (orange circles), and PBS (black circles) treated mice following the oral lipid tolerance test. VLDL (very-low density lipoprotein), LDL (low density lipoprotein), and HDL (high density lipoprotein). Data in XY graphs are shown as mean ± SEM, and data in AUC graphs are shown as mean ± SD, n = 4–8. *P < 0.05 by unpaired t-test of 180 or 120 min values and **P < 0.01 by unpaired t-test of $_{net}AUC$.

metabolism genes of interest were differentially regulated upon GCGR Ab and GCGA treatment. We found that in GCGR Ab treated mice the majority of the selected genes of interest were up-regulated (including acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), fatty acid synthase (Fasn), and acetyl-Coenzyme A carboxylase alpha (Acaca)) (Figure 4*D*). On the other hand, most of the selected genes of interest were down-regulated in GCGA treated mice (including Acox1, low-density lipoprotein receptor (Ldlr), and acetyl-Coenzyme A carboxylase b (Acacb)). The selected genes of interest are described in detail in Suppl. Table 4, and an overview of the differentially regulated genes is given in Figure 4*E*. To understand the accumulation of glycogen in the livers of both GCGA and GCGR Ab treated mice, we investigated genes related to hepatic glucose metabolism. Consistent with the decreased and increased blood glucose concentrations in GCGR Ab and GCGA treated mice, respectively, genes involved in glycolysis were upregulated in GCGR Ab treated mice while genes related to gluconeogenesis were downregulated, and the opposite was observed in GCGA treated mice (Suppl. Figure 3). In GCGR Ab treated mice, glycogen phosphorylase (Pygl) was upregulated while glycogen synthase 2 (Gys2) was downregulated, suggesting increased glycogen breakdown. In GCGA treated mice, phosphorylase kinase regulatory subunit alpha 2 (Pkha2) expression was downregulated, suggesting decreased glycogen breakdown. The entire RNA-sequencing dataset is accessible through three browsable apps: https://weweralbrechtsenlab.shinyapps.io/GCGA/, https://weweralbrechtsenlab.shinyapps.io/GCGR_Ab/, and https://weweralbrechtsenlab.shinyapps.io/GcgrKO/ [15].

3.4. Acute pharmacological inhibition and activation of glucagon receptor signaling, respectively, impairs and enhances postprandial lipid tolerance

After having found that chronic manipulation of glucagon receptor signaling affects lipid metabolism, we next investigated whether acute

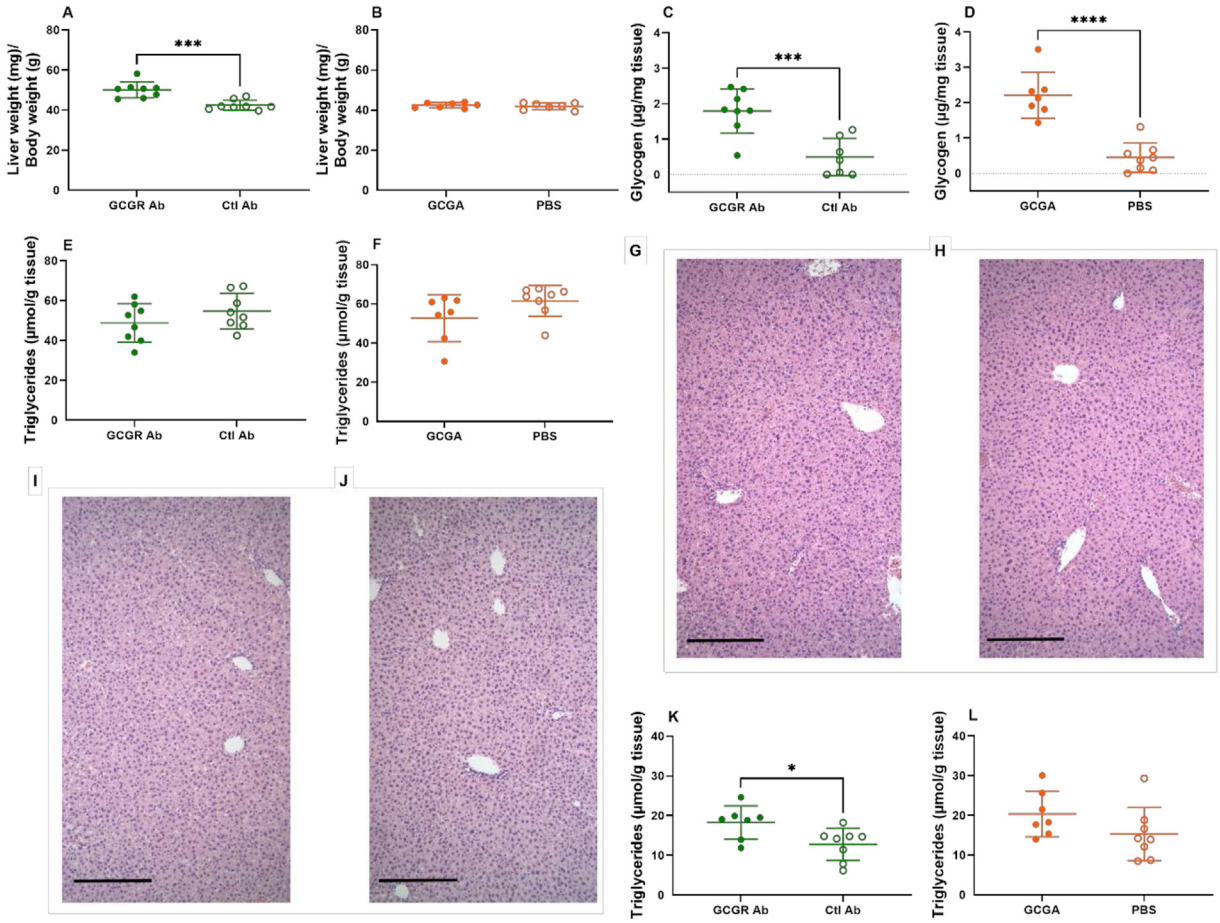

Figure 3: Chronic pharmacological inhibition of glucagon receptor signaling increases liver triglyceride concentrations in non-fasted female mice. (A, B) Liver weights, (C, D) liver glycogen (3 of the measurements were under the detection limit and are shown as 0 µg/mg), and (E, F) liver triglyceride concentrations following an overnight fast and oral lipid tolerance test (olive oil, 10 uL/g body weight (BW) via oral gavage) in female C57BL/6JRj mice treated with a glucagon receptor antibody (GCGR Ab, REGN1193, Regeneron, 10 mg/kg BW) (green closed circles), control antibody (Ctl Ab, REGN1945, Regeneron, 10 mg/kg BW) (green open circles) once weekly for eight weeks or a long-acting glucagon analogue (GCGA, NNC9204-0043, Novo Nordisk A/S, 1.5 nmol/kg BW) (orange closed circles) or PBS + 1% BSA (PBS) (orange open circles) twice daily for eight weeks. Hematoxylin- and eosin-stained liver sections following (G) GCGR Ab, (H) Ctl Ab, (I) PBS, and (J) GCGA treatment, overnight fasting, and oral lipid tolerance test shown using a ×10 magnification, scale bar 1 mm. (K, L) Liver triglyceride concentrations in GCGR Ab, Ctl Ab, GCGA, or PBS treated mice not subjected to an overnight fast or oral lipid tolerance test. The female mice were seven weeks old at the start of treatment. Data shown as mean ± SD, n = 7–8. P-values by unpaired t-test.

pharmacological inhibition or activation of glucagon receptor signaling would have effects on postprandial plasma TG concentrations. GRA was administered 1 h before lipid administration and was found to increase plasma glucagon concentrations during the OLTT in overnight fasted C57BL/6JRj female mice ($P < 0.0001$) (Suppl. Figure 4*A*). GCGA was administered immediately before OLTT at two doses (3 and 30 nmol/kg body weight), dose-dependently increasing plasma concentrations of glucagon (Suppl. Figure 4*B*). GRA decreased blood glucose concentrations ($P = 0.01$) (Figure 5*A* and *B*) whereas GCGA (3 and 30 nmol/kg body weight) increased blood glucose concentrations ($P < 0.0001$ and $P = 0.04$, respectively) (Figure 5*C* and *D*). GRA treatment increased plasma TG ($P = 0.02$) (Figure 5*E* and *F*) while GCGA decreased plasma TG concentrations (both doses $P = 0.003$) (Figure 5*G* and *H*). GRA tended to increase plasma NEFA concentrations ($P = 0.07$) (Figure 5*I* and *J*), while GCGA administration (3 and 30 nmol/kg body weight) decreased plasma NEFA concentrations (both doses $P = 0.0006$) (Figure 5*K* and *L*). GRA increased plasma glycerol ($P = 0.053$) concentrations (Figure 5*M* and *N*). GRA (Figure 5*O*) and GCGA (Figure 5*P*) did not affect liver TG concentrations after the OLTT ($P > 0.8$).

Similar acute effects of GRA on lipid tolerance were observed in overnight fasted male mice (Suppl. Figure 5). GRA also increased plasma TG concentrations in non-fasted female mice during an OLTT, but this effect was not evident in non-fasted male mice (Suppl. Figure 6). Similar acute effects GGCA on plasma TG concentrations were observed in non-fasted male mice (Suppl. Figure 7).

As the acute effects of glucagon on lipid tolerance could be due to altered intestinal lipid uptake, we investigated whether GCGA and GRA treatment affect gastric emptying of paracetamol and found that GCGA and GRA did not affect this parameter ($P > 0.09$) (Suppl. Figure 8).

3.5. Glucagon acutely enhances hepatic lipolysis

We finally investigated if the observed effect of glucagon on lipid tolerance was due to non-transcriptional enhancement of hepatic lipolysis by measuring NEFA concentrations in the effluent from isolated perfused male C57BL/6JRj mouse livers stimulated with glucagon, and found that NEFA and glucose concentrations increased in the effluent upon glucagon infusion ($P = 0.005$ and $P = 0.007$, respectively) (Figure 6). NEFA concentrations also increased in effluent from female C57BL/6JRj mouse livers perfused with glucagon (mean

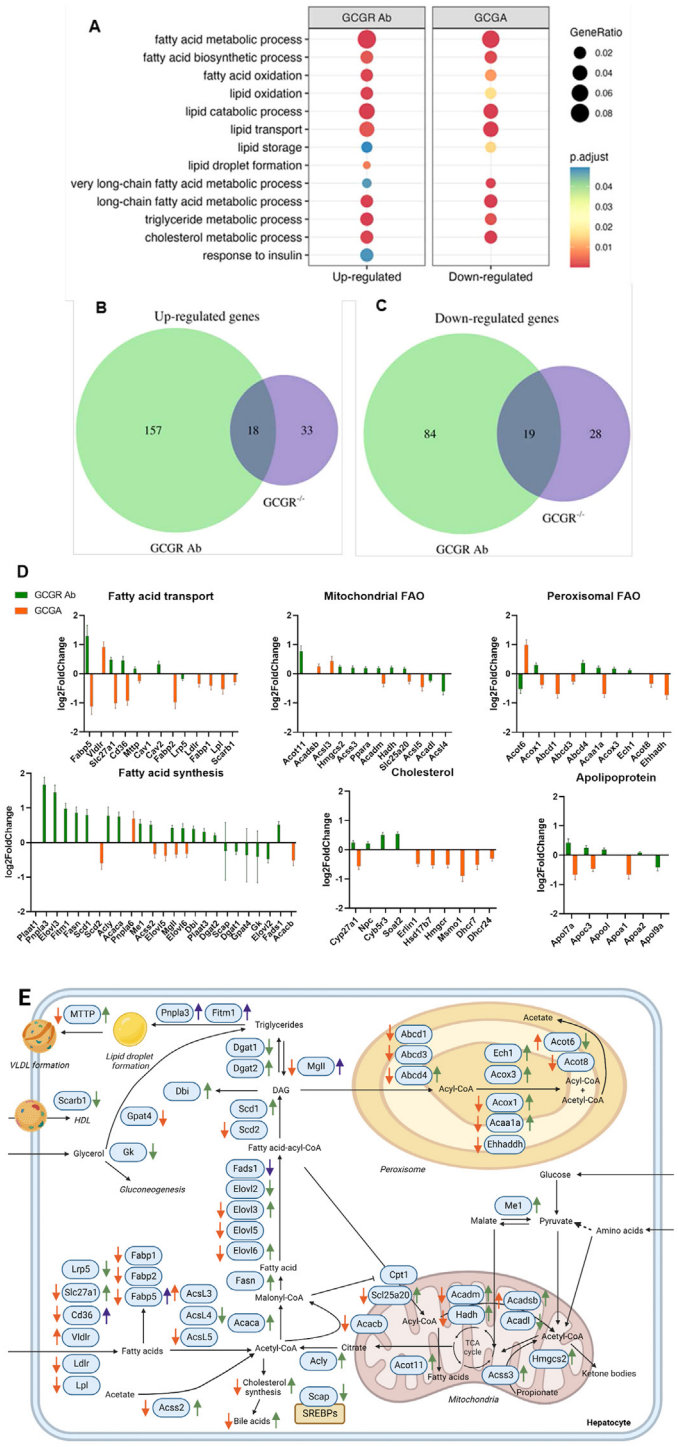

Figure 4: Chronic pharmacological inhibition and activation of glucagon receptor signaling cause up- and down-regulation, respectively, of genes regulating lipid metabolism in female mice. (A) Gene ontology (GOBP) biological processes enriched for up- and down-regulated genes in the livers of female C57BL/6JRj mice treated with glucagon receptor antibody (GCGR Ab, REGN1193, Regeneron, 10 mg/kg body weight (BW), once weekly) or a long-acting glucagon analogue (GCGA, NNC9204-0043, Novo Nordisk A/S, 1.5 nmol/kg BW, twice daily) for eight weeks compared to their respective controls (control antibody (REGN1945, Regeneron) or PBS + 1% BSA), n = 6–8. Venn diagrams showing the number of significantly up-regulated (B) and down-regulated (C) genes in GCGR Ab mice (green) and *Gcgr*$^{-/-}$ mice (purple) and overlapping genes. FDR<0.05 was applied to all analyses to correct for multiple testing. (D) Log2fold changes of selected genes of interest in GCGR Ab (green) and GCGA (orange) treated mice presented as mean ± SEM. (E) Differentially regulated selected genes of interest are shown in blue rectangles. The orange arrows indicate differential mRNA expression in the livers GCGA treated mice compared to PBS, green arrows indicate differential mRNA expression in the livers of GCGR Ab treated mice compared to Ctl Ab, and purple arrows indicate differential mRNA expression in livers of both GCGR Ab treated and *Gcgr*$^{-/-}$ mice. Arrows pointing upwards indicate increased expression compared to the respective control and downward arrows indicate decreased expression. Created with BioRender.com.

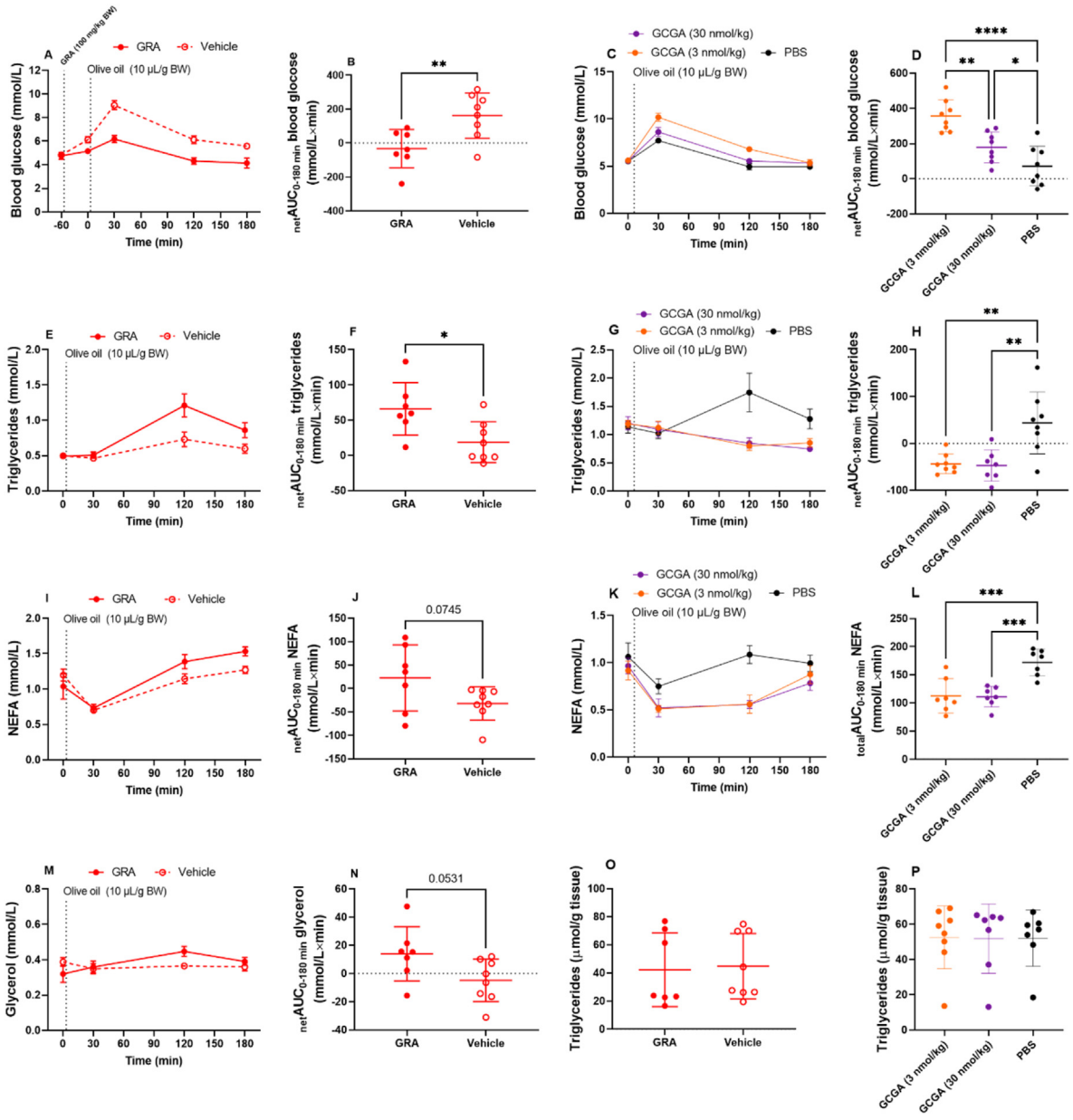

Figure 5: Acute pharmacological inhibition and activation of glucagon receptor signaling, respectively, impairs and enhances postprandial lipid tolerance. (A, C) Blood glucose, (B, D) $_{net}AUC_{0-180\ min}$ blood glucose, (E, G) plasma triglyceride, (F, H) $_{net}AUC_{0-180\ min}$ triglyceride, (I, K) non-esterified fatty acid (NEFA), (J) $_{net}AUC_{0-180\ min}$ NEFA, (L) $_{total}AUC_{0-180\ min}$ NEFA, (M) glycerol, and (N) $_{net}AUC_{0-180\ min}$ glycerol concentrations during an oral lipid tolerance test (olive oil, 10 µL/g body weight (BW) via oral gavage) in overnight fasted C57BL/6JRj female mice treated with a glucagon receptor antagonist (GRA, 25-2648, Novo Nordisk A/S, 100 mg/kg BW) (closed circles and solid lines), vehicle (open circles and dotted lines), a long-acting glucagon analogue (GCGA, NNC9204-0043, Novo Nordisk A/S, 3 nmol/kg BW, orange circles and lines or 30 nmol/kg BW, purple circles and lines), or PBS + 1% BSA (PBS) (black circles and lines). Liver triglyceride concentrations in (O) GRA and vehicle and (P) GCGA and PBS treated mice. (G) Measured using ab65336. Data in XY graphs are shown as mean ± SEM, and data in AUC graphs are shown as mean ± SD, n = 6–8, mice 13 weeks of age. (*B, F, J,* and *N*) P-values by unpaired t-test and (D, H, and L) P-values by one-way ANOVA.

NEFA concentration at baseline 0.024 ± 0.003 mmol/L and mean NEFA concentration during glucagon infusion 0.045 ± 0.008 mmol/L).

4. DISCUSSION

We here show that glucagon receptor inhibition and activation, respectively, impairs and enhances lipid tolerance, chronically as well as acutely. Additionally, chronic glucagon receptor inhibition and activation change the hepatic expression of genes related to lipid metabolism in opposite directions and seem to, respectively, increase and decrease liver TG concentrations. These data collectively support that glucagon has powerful metabolic effects besides regulation of glucose and amino acid metabolism and that glucagon receptor agonism may have beneficial effects on dyslipidemia and reduce hepatic fat content.

In humans, intravenous injections of supra-physiological doses of glucagon decrease plasma TG concentrations within minutes [29]. Likewise, glucagon administration (10 µg of Zn-protamine glucagon

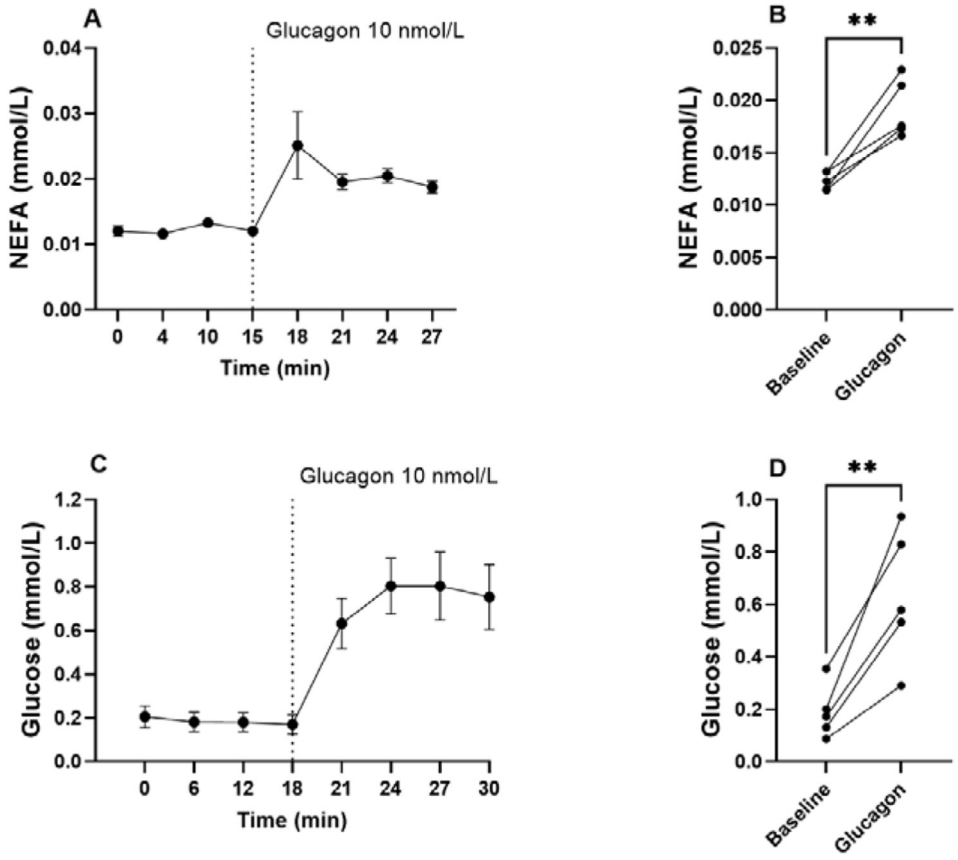

Figure 6: Glucagon increases hepatic lipolysis in perfused livers of male mice. (A) Non-esterified fatty acids (NEFA) and (C) glucose concentrations in effluent from the perfused liver of male C57BL/6JRj mice, 10–12 weeks of age, perfused with 10 nmol/L glucagon. The vertical dotted line indicates the start of glucagon stimulation. Data shown as mean ± SEM. (B) Comparison of the average NEFA and (D) glucose concentrations during the baseline (Baseline) stimulation with the average concentration during glucagon stimulation (Glucagon). ** indicates a significant increase in the average NEFA output during glucagon stimulation compared with the baseline using a paired t-test. n = 5.

for 21 and 8 days) reduced plasma TG concentrations [30,31], possibly by accelerating the rate of TG removal from plasma by either increasing lipoprotein lipase (LPL) activity or increasing hepatic lipoprotein receptor and lipase activity [32]. Using an acylated glucagon peptide (GCGA, extending the short half-life (~2 min) of native glucagon [18] to 5–6 h [15]), we confirmed that both chronic and acute glucagon receptor activation decreased plasma TG concentrations during an OLTT. This is consistent with the observation that rats treated with glucagon for 21 days showed a 49% increase in clearance of an intravenous lipid administration [32]. Conversely, we observed that female mice chronically treated with a glucagon receptor antibody (GCGR Ab) and *Gcgr*$^{-/-}$ mice (both males and females) showed hypertriglyceridemia during an OLTT. In the *Gcgr*$^{-/-}$ mice the hypertriglyceridemia conincided with a lower liver TG concent, compared to their wild-type littermates, possibly reflecting a decreased hepatic TG uptake in the *Gcgr*$^{-/-}$ mice during the OLTT. In contrast to GCGR Ab treated mice, *Gcgr*$^{-/-}$ mice showed fasting hypertriglyceridemia. A possible explanation may be that the permanent deletion of the glucagon receptor in *Gcgr*$^{-/-}$ mice may impair lipid metabolism to a greater extent than the eight weeks of GCGR Ab treatment. Additionally, glucagon receptor signaling has been shown to be required for the adaptive response to fasting [8], and the *Gcgr*$^{-/-}$ mice were fasted from 5 PM until the lipid administration the following morning, whereas the GCGR Ab treated mice were fasted from 10 PM. Thus the GCGR Ab treated mice most likely consumed food for the first 4 h of the dark phase (from 6PM to 6AM) in which mice consume >60% of their total food intake [33], while the *Gcgr*$^{-/-}$ mice did not. The GCGR Ab treated mice may thus not have been fasted sufficiently enough to show increased fasting plasma TG concentrations. Hypertriglyceridemia was also observed in female mice upon acute pharmacological inhibition of glucagon receptor signaling using a GRA. Glucagon may increase LPL activity in humans [34] and rats [35], thus making decreased LPL activity a potential explanation of the hypertriglyceridemia and hypotriglyceridemia observed in conditions of impaired and enhanced glucagon receptor signaling, respectively. Importantly, the studies reporting glucagon-mediated LPL activity were conducted using stimulations with grossly pharmacological dose (1 mg) of glucagon with which the sympathetic nervous system is probably activated in humans [36] which, in turn, is likely to result in peripheral lipolysis [37]. We cannot exclude activation of the sympatho-adrenal system in our studies although our doses are considerably lower (~0.01 ng per mouse twice daily in the chronic study). An increased hydrolysis of circulating TGs would be reflected by an increase in glycerol concentrations during the OLTT. However, glycerol is rapidly taken up and metabolized by the liver which further complicates the matter, and in the basal state glycerol only contributes to a small extent (~3%) to the glucose produced by the liver [38], but the increase in plasma glycerol concentrations following the OLTT may nevertheless increase hepatic glucose production. Further suggesting that impaired glucagon receptor signaling may result in dyslipidemia,

we observed a tendency to increased VLDL cholesterol concentrations in GCGR Ab treated mice and a tendency towards decreased LDL cholesterol in GCGA treated mice. This is in line with reports of impaired and increased glucagon receptor signaling, respectively, increasing [2,5,39] and decreasing [40,41] plasma cholesterol concentrations.

In the liver, glucagon reduces TG accumulation [42,43] and VLDL secretion [44] by stimulating lipolysis and β-oxidation [6,8,45]. In addition, glucagon inhibits hepatic lipogenesis [46–48]. The inhibition of TG synthesis has been suggested to be indirect and mediated by increased β-oxidation (stimulated in a peroxisome proliferator activated receptor-α dependent manner) decreasing the availability of fatty acids for TG synthesis and subsequent secretion [8]. Glucagon-stimulated hepatic lipolysis has been reported to result in NEFA accumulation in perfused livers [49], and increased NEFA concentrations in isolated hepatocyte media [6]. In line with this, we observed that glucagon stimulation increased NEFA concentrations in the effluent from perfused mouse livers, and acute (2 h) GCGA treatment decreased liver TG concentrations whereas liver TG concentrations were increased in GCGR Ab treated mice. Interestingly, the expression of phospholipase A and acyltransferase 1 (Plaat1) was 10-fold increased in the livers chronically treated with GCGA, suggesting increased lipid catabolism. Furthermore, we found hepatic expression of genes related to fatty acid transportation, fatty acid synthesis, and fatty acid oxidation (FAO) in both mitochondria and peroxisomes to be downregulated in chronically GCGA treated mice and upregulated in chronically GCGR Ab treated mice. The simultaneous upregulation of fatty acid transport, fatty acid synthesis, lipid storage, lipid droplet formation, and FAO related genes upon GCGR Ab treatment might indicate increased lipid uptake and synthesis resulting in lipid accumulation (possibly a consequence of dominating hepatic insulin signaling, as genes involved in insulin response were up-regulated in GCGR Ab treated mice) causing an adaptive increase in FAO by increasing substrate push. Patatin-like phospholipase domain-containing protein 3 (Pnpla3) expression was upregulated in livers of both GCGR Ab treated mice and *Gcgr*$^{-/-}$ mice. Genetic variation in Pnpla3 (I148 M) is strongly associated with increased liver fat content in humans and is thus a risk factor for NAFLD [50]. It has been reported that carriers of the risk allele of Pnpla3 are particularly susceptible for hepatic fat accumulation when treated with a glucagon receptor antagonist (LY2409021) [51]. The function of PNPLA3 is complex, as it has both TG hydrolase activity [52] and acyltransferase activity [53]. In a study showing that liver TG content correlated with Pnpla3 mRNA levels in mice fed a high-carbohydrate diet, but not in mice with high fat diet induced steatosis, increased hepatic Pnpla3 expression was suggested to represent increased lipogenesis rather than increased liver fat *per se* [54]. This is in line with our observation of increased Pnpla3 expression occurring simultaneously with increased expression of two key lipogenic genes (Acaca and Fasn) in GCGR Ab treated mice. Glucagon has been shown to stimulate lipolysis in rat [55,56] and human adipocytes [57,58]. However, *in vivo*, an effect of glucagon on adipocytes, as well as other extrahepatic effects, may be mediated mainly by activation of the sympathetic nervous system [35,59,60] or other indirect mechanisms [61,62]. Supporting this, glucagon receptor expression was not detected in mouse or human adipocytes [63,64], and glucagon was found not to regulate white adipose tissue lipolysis, either directly or indirectly [65]. In general, we observed no consistent indications of a differential adipose tissue lipolysis in our models of enhanced and impaired glucagon receptor signaling, which would have been indicated by differences in plasma concentrations of NEFA *and* glycerol in the basal state.

Acute GCGA and GRA treatment did not affect gastric emptying of the liquid phase (as determined by paracetamol absorption profiles), however this does not conclusively rule out GCGA and GRA affecting emptying of lipids and solids. Additional experiments investigating the potential influence of glucagon receptor mediated differences on intestinal lipid absorption, and whether increased secretion of intestinal and/or hepatic lipids caused the hypertriglyceridemia upon impaired glucagon receptor are warranted. The conclusions of this study are, furthermore, limited by only female mice being used in the chronic study. This was done to avoid potential injuries due to fighting between male mice or, alternatively, single housing of male mice. However, the effects of permanent (*Gcgr*$^{-/-}$ mice) and acute (GRA) inhibition of glucagon receptor signaling were investigated in both male and female mice, and no major sex differences were observed with the exception that GRA did not significantly increase plasma TG concentrations during the OLTT in male mice, as it did in female mice, most likely due to lack of power. Furthermore, GCGA was administered only to female mice during an OLTT and to male mice not subjected to an OLTT, and in both conditions GCGA lowered plasma TG concentrations. Insulin is a powerful regulator of adipocyte lipolysis, but due to the constraints of blood sampling we were unable to measure insulin concentrations in the majority of the OLTT studies. It is however likely that the insulin concentrations would have been very low or below the detection limit and the potential increase undetectable, as previously described in overnight fasted mice [66].

Dual- (GLP-1 in combination with glucagon or GIP) and tri-agonists (GLP-1, glucagon, and GIP) are currently being pursued as new therapeutic drugs in the treatment of type 2 diabetes and recently also obesity and, potentially, NAFLD [9,12]. Glucagon receptor agonism increases blood glucose, but this is counteracted by incretin action [67,68], allowing exploitation of the other actions of glucagon receptor agonism. Using a unique combination of ways to impair and enhance glucagon receptor signaling, without relying on intravenous infusion or injections of large doses of glucagon, our study accurately assesses glucagon's actions *in vivo* (which until recently has been challenging) and shows that glucagon is a physiological regulator of lipid metabolism. By showing that glucagon agonism lowers plasma and liver TGs, our study supports glucagon receptor agonism as a strategy to treat NAFLD and dyslipidemia. Finally, our study supports that inhibiting glucagon receptor signaling may result in dyslipidemia and increased hepatic lipid content, thus limiting the applicability of GRAs in the treatment of NAFLD. However, studies investigating lipid metabolism upon glucagon receptor antagonism and agonism in models of obesity and type 2 diabetes are warranted as glucagon's suppressive effect on hepatic VLDL-TG secretion was recently shown to be reduced in subjects with metabolic dysfunction–associated fatty liver disease [69], implying that glucagon's effect might be altered in conditions of metabolic disease.

5. CONCLUSION

We here show that chronic and acute glucagon receptor inhibition and activation, respectively, increases and decreases plasma TG concentrations during an OLTT in mice. Moreover, glucagon receptor antagonism tends to increase, whereas agonism lowers, liver TG concentrations. Finally, pharmacological and genetic glucagon receptor inhibition increases hepatic Pnpla3 expression consistent with increased lipogenesis. By investigating models of both acute and chronic glucagon receptor inhibition and activation, we provide novel and detailed information regarding glucagon's regulation of lipid metabolism, adding to the accumulating evidence that glucagon

agonism may be beneficial in the treatment of NAFLD and dyslipidemia.

FUNDING

The project is supported by grants from the The A.P. Møller Foundation; NNF Tandem Programme (NNF Application Number: 31526); NNF Project Support in Endocrinology and Metabolism—Nordic Region (NNF Application Number: 34250). Associate Prof. Nicolai J. Wewer Albrechtsen is supported by NNF Excellence Emerging Investigator Grant — Endocrinology and Metabolism (Application No. NNF19OC0055001), EFSD Future Leader Award (NNF21SA0072746) and DFF Sapere Aude (1052-00003B). NNF Center for Protein Research is supported financially by the NNF (grant agreement NNF14CC0001). Prof. Jens Juul Holst is supported by the Novo Nordisk Foundation (NNF) Center for Basic Metabolic Research University of Copenhagen (NNF Application Number: 13563). Emilie Elmelund is supported by the Novo Scholarship Program (2022). Some of the data were presented at American Diabetes Association's 82nd Scientific Session, June 3rd—7th, New Orleans, LA, USA.

AUTHOR CONTRIBUTIONS

Conceptualization; K.D.G., E.E., J.J.H., and N.J.W.A. Data curation; K.D.G., E.E., C.D.J., A.B.B., H.S.K., F.C., and J.E.H. Formal analysis; K.D.G., E.E., C.D.J., H.S.K., C.Ø., M.M.R., J.J.H., and N.J.W.A. Funding acquisition; K.D.G., J.J.H., and N.J.W.A. Project administration/supervision; J.J.H. and N.J.W.A. Writing - original draft; K.D.G. Writing - review & editing; E.E., H.K., M.W-S., C.M.S., C.C., T.K., J.F.L., J.J.H., and N.J.W.A. All authors revised and approved the final version of the manuscript.

DATA AVAILABILITY

RNA-sequencing data is accessible through apps: https://weweralbrechtsenlab.shinyapps.io/GCGA/, https://weweralbrechtsenlab.shinyapps.io/GCGR_Ab/, and https://weweralbrechtsenlab.shinyapps.io/GcgrKO/.

ACKNOWLEGDEMENTS

We thank Maureen J. Charron, Departments of Biochemistry, Obstetrics and Gynecology and Women's Health, and Medicine, Albert Einstein College of Medicine, New York, for providing glucagon receptor knockout mice. We thank Jesper Lau, Novo Nordisk A/S, Måløv, Denmark, for providing the glucagon receptor antagonist 26-2548. We thank Regeneron for providing the antibodies REGN1193 and REGN1945. We thank laboratory technician Heidi Marie Paulsen for preparation and staining of liver slices and Associate Professor Jens Brings Jacobsen for access to his microscope. We thank laboratory technician Christine Rasmussen for assistance at the Department of Clinical Biochemistry, Rigshospitalet. The graphical abstract is created with BioRender.com.

CONFLICT OF INTEREST

Thomas Kruse and Jesper F. Lau are employed by Novo Nordisk A/S. The remaining authors have no declarations of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101639.

REFERENCES

[1] Kazda, C.M., Ding, Y., Kelly, R.P., Garhyan, P., Shi, C., Lim, C.N., et al., 2016. Evaluation of efficacy and safety of the glucagon receptor antagonist LY2409021 in patients with type 2 diabetes: 12- and 24-week phase 2 studies. Diabetes Care 39(7):1241—1249.

[2] Kazda, C.M., Frias, J., Foga, I., Cui, X., Guzman, C.B., Garhyan, P., et al., 2017. Treatment with the glucagon receptor antagonist LY2409021 increases ambulatory blood pressure in patients with type 2 diabetes. Diabetes, Obesity and Metabolism 19(8):1071—1077.

[3] Guzman, C.B., Zhang, X.M., Liu, R., Regev, A., Shankar, S., Garhyan, P., et al., 2017. Treatment with LY2409021, a glucagon receptor antagonist, increases liver fat in patients with type 2 diabetes. Diabetes, Obesity and Metabolism 19(11):1521—1528.

[4] Dresler, C.M., Fortner, J.G., McDermott, K., Bajorunas, D.R., 1991. Metabolic consequences of (regional) total pancreatectomy. Annals of Surgery 214(2): 131—140.

[5] Han, S., Akiyama, T.E., Previs, S.F., Herath, K., Roddy, T.P., Jensen, K.K., et al., 2013. Effects of small interfering RNA-mediated hepatic glucagon receptor inhibition on lipid metabolism in db/db mice. The Journal of Lipid Research 54(10):2615—2622.

[6] Perry, R.J., Zhang, D., Guerra, M.T., Brill, A.L., Goedeke, L., Nasiri, A.R., et al., 2020. Glucagon stimulates gluconeogenesis by INSP3R1-mediated hepatic lipolysis. Nature 579(7798):279—283.

[7] Amatuzio, D.S., Grande, F., Wada, S., 1962. Effect of glucagon on the serum lipids in essential hyperlipemia and in hypercholesterolemia. Metabolism 11: 1240—1249.

[8] Longuet, C., Sinclair, E.M., Maida, A., Baggio, L.L., Maziarz, M., Charron, M.J., et al., 2008. The glucagon receptor is required for the adaptive metabolic response to fasting. Cell MetabolismolismCell Metabolism 8(5):359—371.

[9] Alba, M., Yee, J., Frustaci, M.E., Samtani, M.N., Fleck, P., 2021. Efficacy and safety of glucagon-like peptide-1/glucagon receptor co-agonist JNJ-64565111 in individuals with obesity without type 2 diabetes mellitus: a randomized dose-ranging study. Clinical Obesity 11(2):e12432.

[10] Finan, B., Yang, B., Ottaway, N., Smiley, D.L., Ma, T., Clemmensen, C., et al., 2015. A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. Nature Medicine 21(1):27—36.

[11] Bossart, M., Wagner, M., Elvert, R., Evers, A., Hübschle, T., Kloeckener, T., et al., 2022. Effects on weight loss and glycemic control with SAR441255, a potent unimolecular peptide GLP-1/GIP/GCG receptor triagonist. Cell Metabolismolism 34(1):59—74.

[12] Coskun, T., Urva, S., Roell, W.C., Qu, H., Loghin, C., Moyers, J.S., et al., 2022. LY3437943, a novel triple glucagon, GIP, and GLP-1 receptor agonist for glycemic control and weight loss: from discovery to clinical proof of concept. Cell Metabolism.

[13] Gelling, R.W., Du, X.Q., Dichmann, D.S., Romer, J., Huang, H., Cui, L., et al., 2003. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. Proceedings of the National Academy of Sciences of the United States of America 100(3):1438—1443.

[14] Okamoto, H., Kim, J., Aglione, J., Lee, J., Cavino, K., Na, E., et al., 2015. Glucagon receptor blockade with a human antibody normalizes blood glucose in diabetic mice and monkeys. Endocrinology 156(8):2781—2794.

[15] Elmelund, E., Galsgaard, K.D., Johansen, C.D., Trammell, S.A.J., Bomholt, A.B., Winther-Sørensen, M., et al., 2022. Opposing effects of chronic glucagon receptor agonism and antagonism on amino acids, hepatic gene expression, and alpha cells. iScience 25(11):105296.

[16] Kodra, J.T., Jorgensen, A.S., Andersen, B., Behrens, C., Brand, C.L., Christensen, I.T., et al., 2008. Novel glucagon receptor antagonists with improved selectivity over the glucose-dependent insulinotropic polypeptide receptor. Journal of Medicinal Chemistry 51(17):5387—5396.

[17] Maruszczak, K., Rasmussen, C., Ceutz, F.R., Ørgaard, A., Elmelund, E., Richter, M.M., et al., 2022. Arginine-induced glucagon secretion and glucagon-induced enhancement of amino acid catabolism are not influenced by ambient glucose levels in mice. American Journal of Physiology. Endocrinology and Metabolism.

[18] Wewer Albrechtsen, N.J., Kuhre, R.E., Windelov, J.A., Orgaard, A., Deacon, C.F., Kissow, H., et al., 2016. Dynamics of glucagon secretion in mice and rats revealed using a validated sandwich ELISA for small sample volumes. American Journal of Physiology. Endocrinology and Metabolism 311(2):E302–E309.

[19] Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biology 11(10):R106.

[20] Winther-Sorensen, M., Galsgaard, K.D., Santos, A., Trammell, S.A.J., Sulek, K., Kuhre, R.E., et al., 2020. Glucagon acutely regulates hepatic amino acid catabolism and the effect may be disturbed by steatosis. Molecular Metabolism 42:101080.

[21] Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., et al., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nature Genetics 25(1):25–29.

[22] Gene Ontology, C., 2021. The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Research 49(D1):D325–D334.

[23] Yu, G., Wang, L.G., Han, Y., He, Q.Y., 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16(5):284–287.

[24] Carlson, M., 2022. GO.db: a set of annotation maps describing the entire Gene Ontology. R package version 3.8.2.

[25] Heijboer, A.C., Donga, E., Voshol, P.J., Dang, Z.-C., Havekes, L.M., Romijn, J.A., et al., 2005. Sixteen hours of fasting differentially affects hepatic and muscle insulin sensitivity in mice. Journal of Lipid Research 46(3):582–588.

[26] Matsuda, M., Korn, B.S., Hammer, R.E., Moon, Y.A., Komuro, R., Horton, J.D., et al., 2001. SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. Genes & Development 15(10):1206–1216.

[27] Hodge, H.C., Maclachlan, P.L., Bloor, W.R., Stoneburg, C.A., Oleson, M.C., R.T.J.J.o.B.C. Whitehead, 1941. Lipoids of the fasting mouse. 1. The relation between carcass lipids and liver lipids 139. p. 897–916.

[28] Winther-Sørensen, M., Galsgaard, K.D., Santos, A., Trammell, S.A.J., Sulek, K., Kuhre, R.E., et al., 2020. Glucagon acutely regulates hepatic amino acid catabolism and the effect may be disturbed by steatosis. Molecular Metabolism 42:101080.

[29] Schade, D.S., Eaton, R.P., 1975. Modulation of fatty acid metabolism by glucagon in man. I. Effects in normal subjects. Diabetes 24(5):502–509.

[30] Guettet, C., Mathe, D., Riottot, M., Lutton, C., 1988. Effects of chronic glucagon administration on cholesterol and bile acid metabolism. Biochimica et Biophysica Acta 963(2):215–223.

[31] Guettet, C., Rostaqui, N., Mathé, D., Lecuyer, B., Navarro, N., Jacotot, B., 1991. Effect of chronic glucagon administration on lipoprotein composition in normally fed, fasted and cholesterol-fed rats. Lipids 26(6):451–458.

[32] Guettet, C., Rostaqui, N., Navarro, N., Lecuyer, B., Mathe, D., 1991. Effect of chronic glucagon administration on the metabolism of triacylglycerol-rich lipoproteins in rats fed a high sucrose diet. Journal of Nutrition 121(1):24–30.

[33] Fonken, L., Workman, J., Walton, J., Weil, Z., Morris, J., Haim, A., et al., 2010. Light at night increases body mass by shifting the time of food intake. Proceedings of the National Academy of Sciences of the United States of America 107:18664–18669.

[34] Caren, R., Corbo, L., 1974. Glucagon and plasma lipoprotein lipase. Proceedings of the Society for Experimental Biology and Medicine 146(4):1106–1110.

[35] Billington, C.J., Bartness, T.J., Briggs, J., Levine, A.S., Morley, J.E., 1987. Glucagon stimulation of brown adipose tissue growth and thermogenesis. American Journal of Physiology 252(1 Pt 2):R160–R165.

[36] Takayama, S., Nakajima, Y., Toma, S., Sakamoto, T., 1995. Increased muscle sympathetic nerve activity after glucagon administration in man. Journal of the Autonomic Nervous System 54(2):171–175.

[37] Bartness, T.J., Shrestha, Y.B., Vaughan, C.H., Schwartz, G.J., Song, C.K., 2010. Sensory and sympathetic nervous system control of white adipose tissue lipolysis. Molecular and Cellular Endocrinology 318(1–2):34–43.

[38] Terrettaz, J., Jeanrenaud, B., 1990. Contribution of glycerol and alanine to basal hepatic glucose production in the genetically obese (fa/fa) rat. Biochemical Journal 270(3):803–807.

[39] Guan, H.P., Yang, X., Lu, K., Wang, S.P., Castro-Perez, J.M., Previs, S., et al., 2015. Glucagon receptor antagonism induces increased cholesterol absorption. The Journal of Lipid Research 56(11):2183–2195.

[40] Spolitu, S., Okamoto, H., Dai, W., Zadroga, J.A., Wittchen, E.S., Gromada, J., et al., 2019. Hepatic glucagon signaling regulates PCSK9 and low-density lipoprotein cholesterol. Circulation Research 124(1):38–51.

[41] Caren, R., Corbo, L., 1960. Glucagon and cholesterol metabolism. Metabolism 9:938–945.

[42] Bobe, G., Ametaj, B.N., Young, J.W., Beitz, D.C., 2003. Effects of exogenous glucagon on lipids in lipoproteins and liver of lactating dairy cows. Journal of Dairy Science 86(9):2895–2903.

[43] Bobe, G., Ametaj, B.N., Young, J.W., Beitz, D.C., 2003. Potential treatment of fatty liver with 14-day subcutaneous injections of glucagon. Journal of Dairy Science 86(10):3138–3147.

[44] Pullinger, C.R., Gibbons, G.F., 1985. Effects of hormones and pyruvate on the rates of secretion of very-low-density lipoprotein triacylglycerol and cholesterol by rat hepatocytes. Biochimica et Biophysica Acta 833(1):44–51.

[45] Pegorier, J.P., Garcia-Garcia, M.V., Prip-Buus, C., Duee, P.H., Kohl, C., Girard, J., 1989. Induction of ketogenesis and fatty acid oxidation by glucagon and cyclic AMP in cultured hepatocytes from rabbit fetuses. Evidence for a decreased sensitivity of carnitine palmitoyltransferase I to malonyl-CoA inhibition after glucagon or cyclic AMP treatment. Biochemical Journal 264(1):93–100.

[46] Haugaard, E.S., Haugaard, N., 1954. The effect of hyperglycemic-glycogenolytic factor on fat metabolism of liver. Journal of Biological Chemistry 206(2):641–645.

[47] Prip-Buus, C., Pegorier, J.P., Duee, P.H., Kohl, C., Girard, J., 1990. Evidence that the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA is an important site of regulation of hepatic fatty acid oxidation in the fetal and newborn rabbit. Perinatal development and effects of pancreatic hormones in cultured rabbit hepatocytes. Biochemical Journal 269(2):409–415.

[48] Wang, H., Zhao, M., Sud, N., Christian, P., Shen, J., Song, Y., et al., 2016. Glucagon regulates hepatic lipid metabolism via cAMP and Insig-2 signaling: implication for the pathogenesis of hypertriglyceridemia and hepatic steatosis. Scientific Reports 6:32246.

[49] Divakaran, P., Friedmann, N., 1976. A fast in vitro effect of glucocorticoids on hepatic lipolysis. Endocrinology 98(6):1550–1553.

[50] Romeo, S., Kozlitina, J., Xing, C., Pertsemlidis, A., Cox, D., Pennacchio, L.A., et al., 2008. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nature Genetics 40(12):1461–1465.

[51] Guzman, C.B., Duvvuru, S., Akkari, A., Bhatnagar, P., Battioui, C., Foster, W., et al., 2018. Coding variants in PNPLA3 and TM6SF2 are risk factors for hepatic steatosis and elevated serum alanine aminotransferases caused by a glucagon receptor antagonist. Hepatology Communications 2(5):561–570.

[52] He, S., McPhaul, C., Li, J.Z., Garuti, R., Kinch, L., Grishin, N.V., et al., 2010. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. Journal of Biological Chemistry 285(9):6706–6715.

[53] Kumari, M., Schoiswohl, G., Chitraju, C., Paar, M., Cornaciu, I., Rangrez, Ashraf Y., et al., 2012. Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase. Cell Metabolismolism 15(5):691–702.

[54] Hao, L., Ito, K., Huang, K.-H., Sae-tan, S., Lambert, J.D., Ross, A.C., 2014. Shifts in dietary carbohydrate-lipid exposure regulate expression of the non-alcoholic fatty liver disease-associated gene PNPLA3/adiponutrin in mouse liver and HepG2 human liver cells. Metabolism 63(10):1352—1362.

[55] Lefebvre, P.J., Luyckx, A.S., 1969. Effect of insulin on glucagon enhanced lipolysis in vitro. Diabetologia 5(3):195—197.

[56] Heckemeyer, C.M., Barker, J., Duckworth, W.C., Solomon, S.S., 1983. Studies of the biological effect and degradation of glucagon in the rat perifused isolated adipose cell. Endocrinology 113(1):270—276.

[57] Pereira, M.J., Thombare, K., Sarsenbayeva, A., Kamble, P.G., Almby, K., Lundqvist, M., et al., 2020. Direct effects of glucagon on glucose uptake and lipolysis in human adipocytes. Molecular and Cellular Endocrinology 503: 110696.

[58] Tripathy, D., Fourcaudot, M., Norton, L., Defronzo, R.A., Chen, X., 2020. 1914-P: prolonged (12-hour) glucagon infusion stimulates adipocyte lipolysis and inflammation in vivo in humans. Diabetes 69(Supplement 1):1914.

[59] Heim, T., Hull, D., 1966. The effect of propranalol on the calorigenic response in brown adipose tissue of new-born rabbits to catecholamines, glucagon, corticotrophin and cold exposure. Journal of Physiology 187(2):271—283.

[60] Dicker, A., Zhao, J., Cannon, B., Nedergaard, J., 1998. Apparent thermogenic effect of injected glucagon is not due to a direct effect on brown fat cells. American Journal of Physiology 275(5):R1674—R1682.

[61] Beaudry, J.L., Kaur, K.D., Varin, E.M., Baggio, L.L., Cao, X., Mulvihill, E.E., et al., 2019. The brown adipose tissue glucagon receptor is functional but not essential for control of energy homeostasis in mice. Molecular Metabolism 22: 37—48.

[62] Nason, S.R., Antipenko, J., Presedo, N., Cunningham, S.E., Pierre, T.H., Kim, T., et al., 2021. Glucagon receptor signaling regulates weight loss via central KLB receptor complexes. JCI Insight 6(4):e141323.

[63] Kjeldsen, S., Galsgaard, K.D., Winther-Sørensen, M., Serizawa, R., Pedersen, J., Orskov, C., et al., 2020. 1908-P: glucagon receptor expression evaluated by antibody-dependent and -independent approaches. Diabetes 69(Supplement 1):1908.

[64] Campbell, J.E., Beaudry, J.L., Svendsen, B., Baggio, L.L., Gordon, A.N., Ussher, J.R., et al., 2022. The GIPR is predominantly localized to non-adipocyte cell types within white adipose tissue. Diabetes.

[65] Vasileva, A., Marx, T., Beaudry, J.L., Stern, J.H., 2022. Glucagon receptor signaling at white adipose tissue does not regulate lipolysis, 2022.03.20.485051.

[66] Capozzi, M.E., Coch, R.W., Koech, J., Astapova II, , Wait, J.B., Encisco, S.E., et al., 2020. The limited role of glucagon for ketogenesis during fasting or in response to SGLT2 inhibition. Diabetes 69(5):882—892.

[67] Tan, T.M., Field, B.C., McCullough, K.A., Troke, R.C., Chambers, E.S., Salem, V., et al., 2013. Coadministration of glucagon-like peptide-1 during glucagon infusion in humans results in increased energy expenditure and amelioration of hyperglycemia. Diabetes 62(4):1131—1138.

[68] Cegla, J., Troke, R.C., Jones, B., Tharakan, G., Kenkre, J., McCullough, K.A., et al., 2014. Coinfusion of low-dose GLP-1 and glucagon in man results in a reduction in food intake. Diabetes 63(11):3711—3720.

[69] Heebøll, S., Risikesan, J., Ringgaard, S., Kumarathas, I., Sandahl, T.D., Grønbæk, H., et al., 2022. Impaired glucagon-mediated suppression of VLDL-triglyceride secretion in individuals with metabolic dysfunction-associated fatty liver disease (MAFLD). Diabetes 71(11):2402—2411.

