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

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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/6J 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/6J 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

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information about glucagon's potential role in lipid metabolism is of several physiological and pharmacological importance considering that several compounds targeting the glucagon receptor (e.g. glucagon/ GLP-1 (glucagon-like peptide 1) receptor co-agonists [5] and tri- agonists additionally targeting the GIP (glucose-dependent insulino- notropic 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/6J mice were obtained from Janvier Laborato- ries (Saint-Berthevin Cedex, France). Glucagon receptor knockout (Gcgr−/−) and wild-type littermates (Gcgr+/+) 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 mice 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.

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 weaned, 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/6J 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, Bagsvaerd, Denmark); or PBS + 1% BSA (PBS). The antibodies (GCGR Ab and Ctl Ab) were injected once weekly, while GCGB and PBS were injected twice daily (at 8 AM and 8 PM), all subcutaneously. The dose of GCGB 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 GCGB 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 GCGB 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 Exelior) 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/6J 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 admin- istered via oral gavage 1 h before the OLTT. GRA was dissolved in 5% ethanol, 20% propylene glycol, 10% 2-hydroxypropyl-[γ]-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/6J 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 GCGB (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/6J 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.

2.1.5. 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; Merckodia, Uppsala, 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; Merckodia, Uppsala, 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 EnzymeChrom Glycogen Assay Kit (catalog no. E2GN-100; BioAssay Systems, Hayward, CA); and plasma paracetamol concentrations with the Aetamiphen L3K kit (catalog no. 506-30; Sekisui Diagnostics, Burlington, Massachusetts, USA). Perfusion samples were analyzed for glucose using QuantiChrom Glucose assay kit (catalog no: DGL-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 637 and K3 689, 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. 1078970401; 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 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 Ctrl 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 stabilization 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 (totalAUC) baseline was Y = 0 and peaks above the baseline were considered. For net AUC (netAUC), 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 1A 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 1E 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), 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 1A).

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 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 2A), whereas GCGA treatment increased blood glucose concentrations compared to PBS (P < 0.0001) (Suppl. Figure 2B). 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 GCGR treated mice gained significantly less weight compared to PBS treated mice (P = 0.02) (body weights at week 0; GCGR: 18.1 ± 0.3 g, PBS: 18.0 ± 0.2 g and at week eight; GCGR: 19.6 ± 0.3 g, PBS: 20.7 ± 0.4 g) (the delta body weights are shown in Suppl. Figure 2C 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 compared to PBS (P = 0.045) (Suppl. Figure 2F). Fasting plasma glycerol concentrations were increased in GCGR Ab and GCGA treated mice compared to their respective controls (GCGR Ab vs. Ctl Ab P < 0.0001 and GCGR vs. PBS P = 0.0006) (Suppl. Figure 2G and H), and remained increased during the OLTT (GCGR Ab vs. Ctl Ab P < 0.0001 and GCGR vs. PBS P = 0.0002) (Suppl. Figure 2J–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 GCGR and PBS treated mice (P = 0.08, P = 0.8, and P = 0.4, respectively) (Suppl. Figure 2M–R).

During the OLTT, GCGR Ab treated mice had decreased blood glucose concentrations compared to Ctl Ab treated mice (P = 0.003) (Figure 2A and B), whereas the increases in blood glucose concentrations were similar in GCGR and PBS treated mice during the OLTT (P = 0.7) (Figure 2C and D). During the OLTT, insulin concentrations were similar in GCGR Ab and Ctl Ab treated mice (P = 0.3) (Figure 2E and F) and in GCGA and PBS treated mice (P = 0.6) (Figure 2G 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 2I and J). Plasma TG concentrations were increased in GCGR Ab treated mice after the OLTT compared to Ctl Ab (P = 0.04) (Figure 4M). Plasma lipid concentrations were higher in GCGR Ab treated mice compared to Ctl Ab (0.0222 μmol vs. 0.0009), but no difference was found for LDL and HDL cholesterol particles (Figure 2N). GCGA treatment decreased plasma TG concentrations after lipid administration compared to PBS (P = 0.049) (Figure 2O). LDL cholesterol peak concentration was lower in GCGR Ab treated mice compared to PBS (0.0024 vs. 0.0031 μmol), but VLDL and HDL cholesterol particles were similar (Figure 2P).

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 3A) whereas GCGR had no effect (P = 0.5) (Figure 3B). 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 3C 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 3E and F). Liver histological examination of liver sections from the same groups revealed evident lipid accumulation in the cytoplasm but normal liver morphology (Figure 3G–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 3E and F), whereas GCGA had no effect (P = 0.02) (Figure 3K) 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 3L).

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 4A). 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 4B). Among these were patatin-like phospholipase domain containing 3 (Pnpla3), fatty acid binding protein 5 (Fabp5), fat storage-inducing transmembrane protein 1 (Fltm1), and monocyclonide lipase (Mgl) (Suppl. Table 2). A total of 19 genes were down-regulated in both groups (Figure 4C) (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) ∫_0^180[^{mM/L}]^2 glucose, (C) plasma triglyceride, (D) ∫_0^180[^{mM/L}]^2 triglyceride, (E) non-esterified fatty acid (NEFA), (F) ∫_0^180[^{mM/L}]^2 NEFA, (G) glycerol, (H) ∫_0^180[^{mM/L}]^2 glycerol concentrations during an oral lipid tolerance test (olive oil, 10 μL/g body weight via oral gavage) in female glucagon receptor knockout (Gcgr<sup>/−/−</sup>) (red circles and lines) and female wild-type littermates (Gcgr<sup>+/+</sup>) (black squares and lines). (I) Liver triglyceride concentrations. (J) Plasma cholesterol profiles in overnight fasted Gcgr<sup>/−/−</sup> (red circles) and Gcgr<sup>+/+</sup> (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 (J) are shown as mean ± SD, n = 15–19, mice 12–25 weeks of age. P-values by unpaired t-test.
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 4D). 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 4E. To understand the accumulation of glycogen in the livers of both GCGA and GCGR Ab treated mice, we investigated genes related to hepatic glycogen metabolism, including glycogen phosphorylase (Pygl) and glycogen synthase 2 (Gys2). Pygl was upregulated while 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 pharmacological inhibition and activation of glucagon receptor signaling, respectively, impairs and enhances postprandial lipid tolerance in female mice. (A, C) Blood glucose, (B, D) ΔH/AUC180-120 min blood glucose, (E, G) insulin, (F, H) ΔH/AUC120-180 min insulin, (I, K) non-esterified fatty acid (NEFA), (J, L) ΔH/AUC180 min NEFA, and (M, O) triglyceride (measured using ab65336) concentrations during an oral lipid tolerance test (olive oil, 10 μL/g body weight (BW) via oral gavage) in female C57BL/6J 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 ± 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 ΔH/AUC.
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 4A). 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 4B). GRA decreased blood glucose concentrations (P = 0.01) (Figure 5A and B) whereas GCGA (3 and 30 nmol/kg body weight) increased blood glucose concentrations (P < 0.0001 and P = 0.04, respectively) (Figure 5C and D). GRA treatment increased plasma TG (P = 0.02) (Figure 5E and F) while GCGA decreased plasma TG concentrations (both doses P = 0.003) (Figure 5G and H). GRA tended to increase plasma NEFA concentrations (P = 0.07) (Figure 5I and J), while GCGA administration (3 and 30 nmol/kg body weight) decreased plasma NEFA concentrations (both doses P = 0.0006) (Figure 5K and L). GRA increased plasma glycerol (P = 0.053) concentrations (Figure 5M and N). GRA (Figure 5O) and GCGA (Figure 5P) 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 of GCGA 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 perfused 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/6J 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.
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 postprandial lipid tolerance. 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}
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 coincided with a lower liver TG concentration, 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 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,

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/6J 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.

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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 GCGR treated 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 hypertriglycerideremia 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 glucagons 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 glucagons 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.

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**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.O., 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://wweralbrechtsenlab.shinyapps.io/GCGA/, https://wweralbrechtsenlab.shinyapps.io/GCGR_Ab/, and https://wweralbrechtsenlab.shinyapps.io/GcgrKO/.

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**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**

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