



Cardiomyocyte glucagon receptor signaling modulates outcomes in mice with experimental myocardial infarction

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ABSTRACT

Objective: Glucagon is a hormone with metabolic actions that maintains normoglycemia during the fasting state. Strategies enabling either inhibition or activation of glucagon receptor (Gcgr) signaling are being explored for the treatment of diabetes or obesity. However, the cardiovascular consequences of manipulating glucagon action are poorly understood.

Methods: We assessed infarct size and the following outcomes following left anterior descending (LAD) coronary artery ligation; cardiac gene and protein expression, acylcarnitine profiles, and cardiomyocyte survival in normoglycemic non-obese wildtype mice, and in newly generated mice with selective inactivation of the cardiomyocyte Gcgr. Complementary experiments analyzed Gcgr signaling and cell survival in cardiomyocyte cultures and cell lines, in the presence or absence of exogenous glucagon.

Results: Exogenous glucagon administration directly impaired recovery of ventricular pressure in ischemic mouse hearts *ex vivo*, and increased mortality from myocardial infarction after LAD coronary artery ligation in mice in a p38 MAPK-dependent manner. In contrast, cardiomyocyte-specific reduction of glucagon action in adult *Gcgr*^{CM-/-} mice significantly improved survival, and reduced hypertrophy and infarct size following myocardial infarction. Metabolic profiling of hearts from *Gcgr*^{CM-/-} mice revealed a marked reduction in long chain acylcarnitines in both aerobic and ischemic hearts, and following high fat feeding, consistent with an essential role for Gcgr signaling in the control of cardiac fatty acid utilization.

Conclusions: Activation or reduction of cardiac Gcgr signaling in the ischemic heart produces substantial cardiac phenotypes, findings with implications for therapeutic strategies designed to augment or inhibit Gcgr signaling for the treatment of metabolic disorders.

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Keywords Glucagon; Myocardial infarction; Glucagon receptor; Cardiomyocytes; Heart; Diabetes; Fatty acid metabolism

1. INTRODUCTION

Glucagon is a 29 amino acid peptide hormone secreted from pancreatic islet α -cells that plays a critical role in maintenance of euglycemia, predominantly by increasing hepatic glucose output. Activation of glucagon receptor (Gcgr) signaling promotes glycogenolysis and enhanced gluconeogenesis, and regulates pathways controlling hepatic lipid oxidation and lipid secretion. Although the actions of glucagon are classically viewed as essential for prevention of hypoglycemia in the face of limited nutrient availability or excess insulin action [1], Gcgr signaling also controls cell survival pathways, as genetic interruption of Gcgr signaling increases the susceptibility to hepatic injury [2].

A single Gcgr is expressed not only in liver, but in extrahepatic tissues including the central and peripheral nervous system, pancreatic islets, adipose tissue, kidney, blood vessels and heart [3,4]. In the pancreas, glucagon potentiates glucose-dependent insulin secretion, whereas activation of Gcgr signaling in the brain regulates hepatic glucose production, control of appetite and body weight [5]. Glucagon actions in adipose tissue and kidney are less understood, but have been linked to control of fatty acid and glucose metabolism.

Although glucagon levels normally decrease during a meal, glucagon secretion is inappropriately increased in many subjects with type 2 diabetes (T2D) [1,6]. Over the last several decades, experimental studies attenuating glucagon action using glucagon immunoneutralizing antisera, Gcgr antagonists, antisense *Gcgr* oligonucleotides and

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Gcgr^{-/-} mice have demonstrated amelioration of hyperglycemia in experimental models of diabetes [1,7]. Collectively, these findings have raised enthusiasm for glucagon antagonism as a potential therapeutic strategy for T2D. Indeed, GCGR antagonists and antisense oligonucleotides targeting hepatic GCGR expression robustly lower glucose in clinical trials of human subjects with T2D. However, mechanism-based toxicities noted in preclinical studies, including dyslipidemia, and transaminase elevations [2,8], have also been reported in clinical studies. Hence, the risk:benefit proposition for partial attenuation of GCGR signaling in diabetic humans requires further evaluation.

Complementary efforts are exploring whether partial enhancement of glucagon action, together with agonism of the glucagon-like peptide-1 receptor (GLP-1R), may be useful for the treatment of diabetes and/or obesity [9,10]. Oxyntomodulin, a naturally occurring proglucagon-derived peptide, contains the 29 amino acid sequence of glucagon plus a carboxyterminal extension and exerts potent glucoregulatory and anorectic actions in rodents and humans through activation of the GLP-1 and glucagon receptors [11,12]. More recent studies have demonstrated that simultaneous activation of the glucagon and GLP-1 receptors using synthetic balanced co-agonists produces potent glucoregulatory activity and greater weight loss than observed with GLP-1R agonists alone [13]. Hence there is also considerable interest in understanding the metabolic consequences and therapeutic potential arising from partial selective activation of GCGR signaling.

The increasing interest in development of drugs that reduce or activate GCGR signaling for the treatment of metabolic disorders such as diabetes and obesity raises important questions about the cardiovascular actions and safety of such agents. Current understanding of glucagon action in the heart is limited, and activation of *Gcgr* signaling in this organ has been reported to be either beneficial or harmful, depending on the experimental or clinical context [14–17]. We have now examined the consequences of manipulating *Gcgr* signaling in the non-diabetic ischemic mouse heart. Our findings reveal that exogenous glucagon impairs survival following ligation of the left anterior descending (LAD) coronary artery, actions requiring p38 MAP kinase. In contrast, *Gcgr*^{CM-/-} mice with cardiac-specific inactivation of the *Gcgr* display a cardioprotective phenotype, associated with reduced accumulation of incompletely oxidized fatty acid metabolites in the heart. These findings have implications for pharmaceutical efforts directed at manipulating GCGR signaling for the treatment of human disease.

2. METHODS

2.1. Mice and reagents

Inducible α MHC^{Cre} (stock 005657) [18] and FLPe (stock 005703) transgenic mice in the C57BL/6 background were obtained from the Jackson Laboratory. *Gcgr*^{CM-/-} mice were generated by crossing α MHC^{Cre} mice with *Gcgr*^{Flox} mice [19] in the C57BL/6 background. LAD coronary artery ligation was used to induce myocardial infarction (MI) in 12–14-week-old male mice as described in Ref. [20]. All mice were housed (5 per cage) under a light/dark cycle of 12 h in the Toronto Centre for Phenogenomics (TCP) animal facility, with free access to food and water except where noted. All procedures were conducted according to protocols and guidelines approved by the TCP Animal Care Committee. Genotypes were determined through analysis of genomic DNA prepared from tail snips. Tamoxifen (Sigma Aldrich, 50 mg/kg) dissolved in corn oil was administered for 5 consecutive days to 6- or 7-week-old male α MHC^{Cre} or *Gcgr*^{CM-/-} mice to induce Cre expression. Before any cardiac assessment or

procedure, all mice were allowed 6 weeks to recover after the last tamoxifen injection, as Cre expression in the heart often induces a transient cardiomyopathy that dissipates 5 weeks after tamoxifen-induced Cre expression [21]. Glucagon (Sigma) 30 ng/g body weight or saline in 10% gelatin was administered to C57BL/6 mice as described in Ref. [8], 3 injections daily, with or without 2 injections daily of 1 μ mol/g body weight SB203580 (p38 MAPK inhibitor, Sigma) for 7 days. Blood pressure and heart rates were measured using a telemetry system (DSI technology) as described in Ref. [22]. The rat *Gcgr* adenovirus (Ad*Gcgr*) has been described previously in Ref. [2].

2.2. Ischemia/reperfusion

Global no-flow ischemia in Langendorff-perfused hearts was induced as described in Ref. [23]. Hearts underwent a 30 min aerobic perfusion with Krebs-Henseleit buffer, followed by a 30 min global no-flow ischemia, and either a 50 or 60 min reperfusion period during which left ventricular developed pressure (LVDP) was recorded (Biopac Systems Canada Inc.). In a separate set of hearts 1 μ g/mL glucagon was administered 20 min prior to ischemia.

2.3. Myocardium metabolic profiling

Mass spectrometry-based metabolic profiling was performed to determine myocardial levels of acylcarnitines and organic acids [24]. Triacylglycerol (TAG) was extracted from frozen myocardial tissue (~20 mg) with a 2:1 chloroform-methanol solution and quantified with a commercially available enzymatic assay kit (Wako Pure Chemical Industry) as described in Ref. [25].

2.4. Heart histology

Animals were anesthetized using avertin (250 mg/kg body weight ip injection). The chest cavity was opened to expose the heart and 1 M KCl was injected into the apex to arrest the heart in diastole. The heart was perfusion-fixed with 4% buffered formalin at physiological pressure, post-fixed in formalin, embedded in paraffin, and sectioned at 6 μ m, and stained with Masson's Trichrome or processed for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL staining was performed using the ApopTag peroxidase kit for apoptosis (EMD Millipore). Cardiac morphometry was performed on mid-ventricular cross-sections using Aperio ImageScope Viewer software (Aperio Technologies). The infarcted area was calculated as a % of total LV area. Cardiac hypertrophy was quantified as the heart weight-to-body weight ratio.

2.5. Glucose tolerance

12–14-week-old male mice were fasted overnight (16–18 h), and glucose (1.5 mg/g body weight) was administered orally (through a gavage tube) or via injection into the peritoneal cavity (intraperitoneal glucose tolerance test). Blood samples were drawn from the tail vein at 0, 15, 30, 60, 90, and 120 min post-glucose administration, and blood glucose and insulin levels were measured as described in Ref. [26].

2.6. Western blotting

Hearts were collected from fasted mice (5 h) 30 min following ischemia or sham surgery, washed in Krebs buffer containing 11 mM glucose and frozen. Frozen hearts were powdered and homogenized in buffer containing 50 mM Tris HCl, pH 8, 1 mM EDTA, 10% glycerol, 0.02% Brij-35. Western blotting was carried out as described in Refs. [27,28] and blots were visualized using an enhanced chemiluminescence Western blot detection kit (Perkin Elmer) and quantified with Carestream Molecular Imaging Software (Kodak).

2.7. RNA analyses

RNA was isolated from cardiac extracts using TRI reagent (Sigma). First-strand cDNA was synthesized from total RNA using the Super-Script III reverse transcriptase synthesis system (Invitrogen). Real-time polymerase chain reaction was performed with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems). Relative levels of mRNA transcripts were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to levels of peptidyl-prolyl isomerase A (*Ppia*-cyclophilin) RNA.

2.8. Cell culture

Atrial cardiomyocytes were isolated as described in Ref. [22]. HL-1 atrial cardiac myocytes were provided by Dr. William Claycomb (Louisiana State University, New Orleans), and cultured in Claycomb Media (Sigma–Aldrich) with 10% FBS, 1% penicillin/streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. Cells were seeded onto 6-well plates (BD Falcon) coated with 0.02% gelatin/0.5% fibronectin. Confluent cells were serum-starved and supplemented with Claycomb Media without FBS and norepinephrine prior to infection with Ad β -Gal or Ad*Gcgr* at 10 \times multiplicity of infection. 24 h following infection, cells were treated with either PBS or glucagon for 3 h in supplemented Claycomb Media without FBS and norepinephrine. Cellular injury was induced by H₂O₂ [29].

2.9. Statistical analysis

Results are presented as mean \pm SEM. Statistical significance was determined using 1- or 2-way analysis of variance with Bonferroni post hoc tests (as appropriate) using GraphPad Prism 4.0 (GraphPad Software Inc). Statistical significance was noted when $p < 0.05$.

3. RESULTS

3.1. Glucagon impairs outcomes during experimental MI in a p38 MAPK-dependent manner

As recent studies of the related *Glp1r* revealed issues with receptor localization and surprising chamber-specific receptor expression [22,30,31], we first assessed *Gcgr* expression in the mouse heart using both regular PCR and primers corresponding to the full length open reading frame, as well as quantitative real-time PCR (Supplementary Figure 1A,B). Consistent with a previous report in Ref. [32], *Gcgr* expression was most abundant in RNA from the right atria but easily detectable in RNA from both right and left ventricle. In contrast, *Glp1r* expression was restricted to the atria as previously described in Refs. [22,32]. The expression of the *Gipr* was much less atrial-biased, relative to the *Glp1r*, whereas *Glp2r* mRNA transcripts were not detected in the mouse heart (Supplementary Figure 1A,B). To determine the effects of glucagon treatment on cardiovascular outcomes during MI, we treated mice with glucagon (3 \times daily, via subcutaneous injection, 30 ng/g body weight) prior to and following LAD coronary artery ligation (Figure 1A). No significant changes in random glycemia or body weight were observed with glucagon injections (Supplementary Figure 1C,D), however, glucagon significantly reduced survival following MI and was associated with a significant increase in TUNEL-positive apoptotic cardiac myocytes (48 h post-MI), although no effect on adverse LV remodeling or infarct scar formation was observed (Figure 1B–D). These negative cardiovascular outcomes required p38 MAPK; glucagon increased p38 MAPK phosphorylation in both the aerobic and ischemic heart (Figure 1E), and co-treatment with SB203580 (p38 MAPK inhibitor) prevented the glucagon-mediated reduction in MI survival

(Figure 1B), as well as the increase in TUNEL-positive cardiac myocytes (Figure 1D).

3.2. Glucagon activates PPAR α in a p38 MAPK-dependent manner in cardiac myocytes, and increases cardiac myocyte apoptosis *in vitro*

As glucagon increases hepatocyte PPAR α activity in a p38 MAPK-dependent manner [8], we examined whether similar regulation occurs in the heart. Treatment of HL-1 atrial cardiac myocytes (which do not express the endogenous *Gcgr*) with an adenovirus encoding the rat *Gcgr* (Ad*Gcgr*) followed by treatment with glucagon (20 nM) for 24 h increased the expression of PPAR α target genes (Figure 2A). In contrast, no such changes were observed in HL-1 cells treated with a control adenovirus (Ad β -Gal, Supplementary Figure 1E). Furthermore, glucagon (20 nM for 24 h) increased luciferase activity directed by a PPAR α response element in Ad*Gcgr*-infected HL-1 cells (Figure 2B), but not in Ad β -Gal-infected HL-1 cells (Supplementary Figure 1F). Furthermore, SB203580 abolished the glucagon-mediated increase in PPAR α luciferase activity in Ad*Gcgr*-infected HL-1 cells, illustrating the dependency on p38 MAPK activity (Figure 2C). We also observed an increase in nuclear PPAR α translocation in both glucagon-treated adult cardiac myocytes (Figure 2D) and Ad*Gcgr* infected HL-1 cells (Figure 2E), which was abolished by pre-treatment with SB203580 (Figure 2E).

We next determined whether pyruvate dehydrogenase (PDH, the rate-limiting enzyme of glucose oxidation) activity was altered in hearts and HL-1 cells treated with glucagon. Glucagon increased PDH phosphorylation (indicative of reduced PDH activity) in both the aerobic/ischemic heart (Figure 2F), and in HL-1 cells infected with Ad*Gcgr* (Figure 2G), but not in cells infected with Ad β -Gal (Supplementary Figure 1G). Glucagon also increased hydrogen peroxide (H₂O₂)-induced caspase-3 cleavage, an effect negated via pretreatment with the PDH kinase inhibitor, dichloroacetate (DCA) (Figure 2H). Pre-treatment with DCA also reduced expression of the pro-apoptotic protein, Bax, but had no effect on expression of the anti-apoptotic protein, Bcl-2, in Ad*Gcgr*-infected HL-1 cells treated with H₂O₂ and glucagon (Supplementary Figure 2).

3.3. Glucagon regulates acylcarnitine profiles in the heart

Although myocardial ischemia reduces fatty acid oxidation rates due to the reduction in oxygen supply, we observed a robust increase in long chain acylcarnitines in ischemic hearts 24-h post-glucagon and 30 min post-MI (Figure 3). The most likely interpretation of this profile is that glucagon triggered an increase in β -oxidation, but that flux through the TCA cycle, electron transport chain, and oxidative phosphorylation was limited by ischemia, resulting in accumulation of incompletely oxidized fats. Interestingly, Krebs cycle intermediates were not altered in glucagon-treated hearts (Supplementary Figure 3), suggesting that the increase in fatty acid oxidation may have provided acetyl CoA for the Krebs cycle to offset the apparent decrease in PDH activity.

3.4. Cardiomyocyte-specific deletion of the *Gcgr* produces a cardioprotective phenotype in response to ischemic injury

As increased glucagon action produces negative effects on the ischemic heart, we hypothesized that reducing glucagon action should protect against ischemic injury. Mice with global germline deletion of the *Gcgr* exhibit mild hypoglycemia, and increased levels of proglucagon-derived peptides, Fgf-21 and bile acids [26,33–35], complicating interpretation and mechanistic attribution of cardiac phenotypes arising from whole body loss of the *Gcgr*. Indeed,

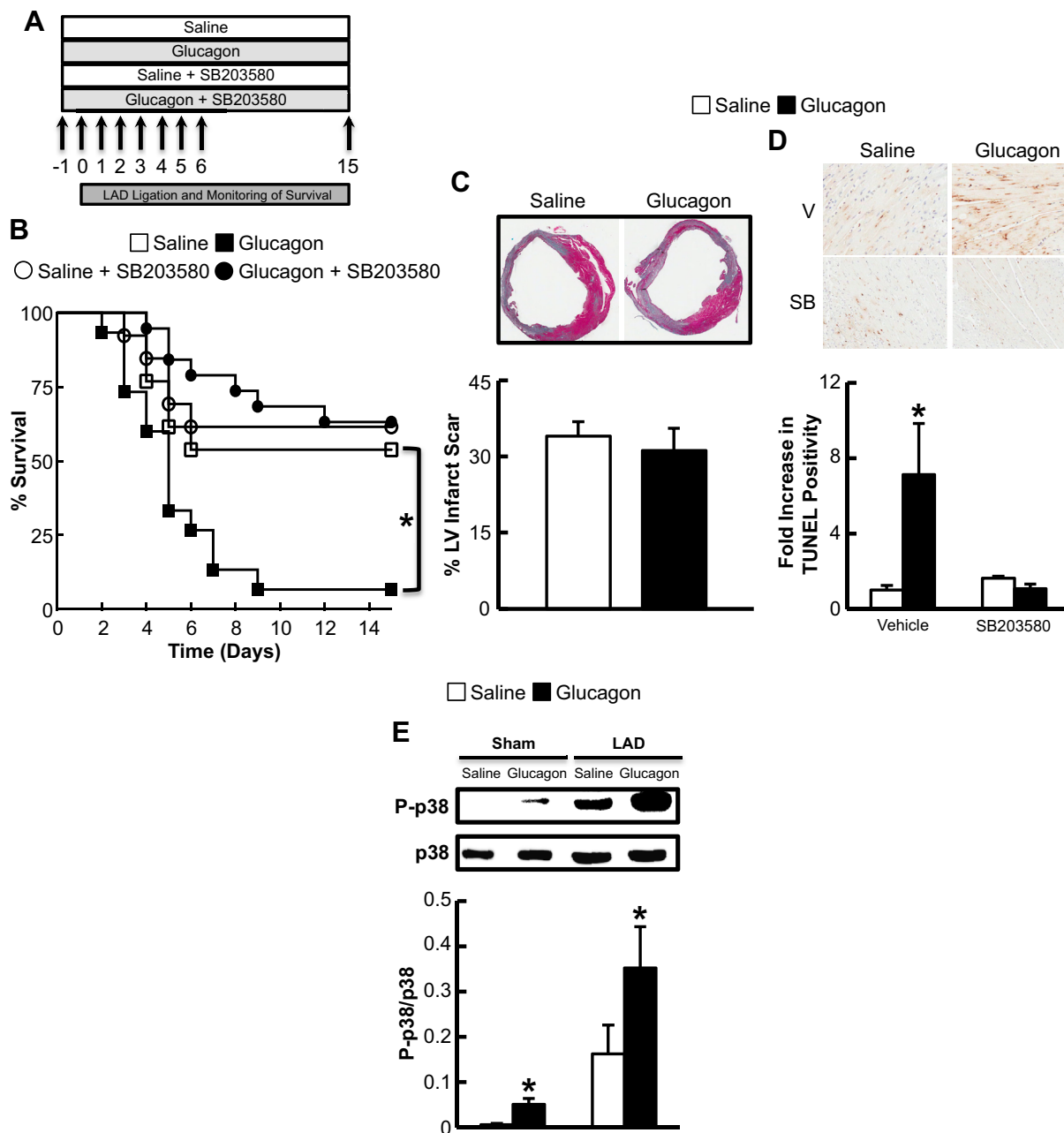


Figure 1: Glucagon impairs survival after MI in a p38 MAPK-dependent manner. (A) Schematic of overall study design. Mice were injected with vehicle/glucagon/SB203580 for 7 days starting 1 day prior to LAD coronary artery ligation and sacrificed 15 days later. (B) Survival following LAD coronary artery ligation in C57BL/6 mice treated with saline or glucagon (30 ng/g) with or without co-administration of the p38 MAPK inhibitor (SB203580 1 μ mol/kg) for 1 week. * $p < 0.05$ saline vs. glucagon. Data are mean \pm S.E.M (LAD $n = 13-15$ per treatment). (C) Infarct size assessed in mice described in (B) at day 15. Data are mean \pm S.E.M (LAD $n = 5-6$ per treatment). (D) C57BL/6 mice treated with saline or glucagon (30 ng/g) with or without co-administration of the p38 MAPK inhibitor (SB203580 1 μ mol/kg; SB) were subjected to LAD coronary artery ligation for 48 h to assess TUNEL positive cardiac myocytes. * $p < 0.05$ saline vs. glucagon. Data are mean \pm S.E.M (LAD $n = 7-8$ mice per treatment). (E) p38 MAPK phosphorylation in C57BL/6 mice treated with saline or glucagon (30 ng/g) with or without SB203580 (1 μ mol/kg) 48 h post-LAD coronary artery ligation or sham surgery. * $p < 0.05$ saline vs. glucagon group. Data are mean \pm S.E.M ($n = 3-5$).

secondary increases in GLP-1 and Fgf-21 in *Gcgr*^{-/-} mice may exert independent cardioprotective actions [36,37]. Accordingly, we generated a tamoxifen-inducible cardiomyocyte-specific *Gcgr* knock out mouse (*Gcgr*^{CM-/-}; Supplementary Figure 4A). Treatment with tamoxifen (50 mg/kg IP) for 5 consecutive days decreased cardiac *Gcgr* mRNA expression by $\sim 85\%$ without affecting *Gcgr* mRNA expression in the liver and kidney (Supplementary Figure 4B-D). *Gcgr*^{CM-/-} mice

appeared phenotypically normal, exhibiting normal weight gain and similar glucose tolerance relative to α myosin heavy chain-Cre (*α MHC*^{Cre}) littermate controls (Supplementary Figure 5). Following LAD coronary artery ligation, *Gcgr*^{CM-/-} mice exhibited a marked increase in survival, reduced cardiac hypertrophy, and substantially less adverse LV remodeling (Figure 4). A significant reduction in mRNA levels of (a) key PPAR α target genes and (b) mRNA transcripts

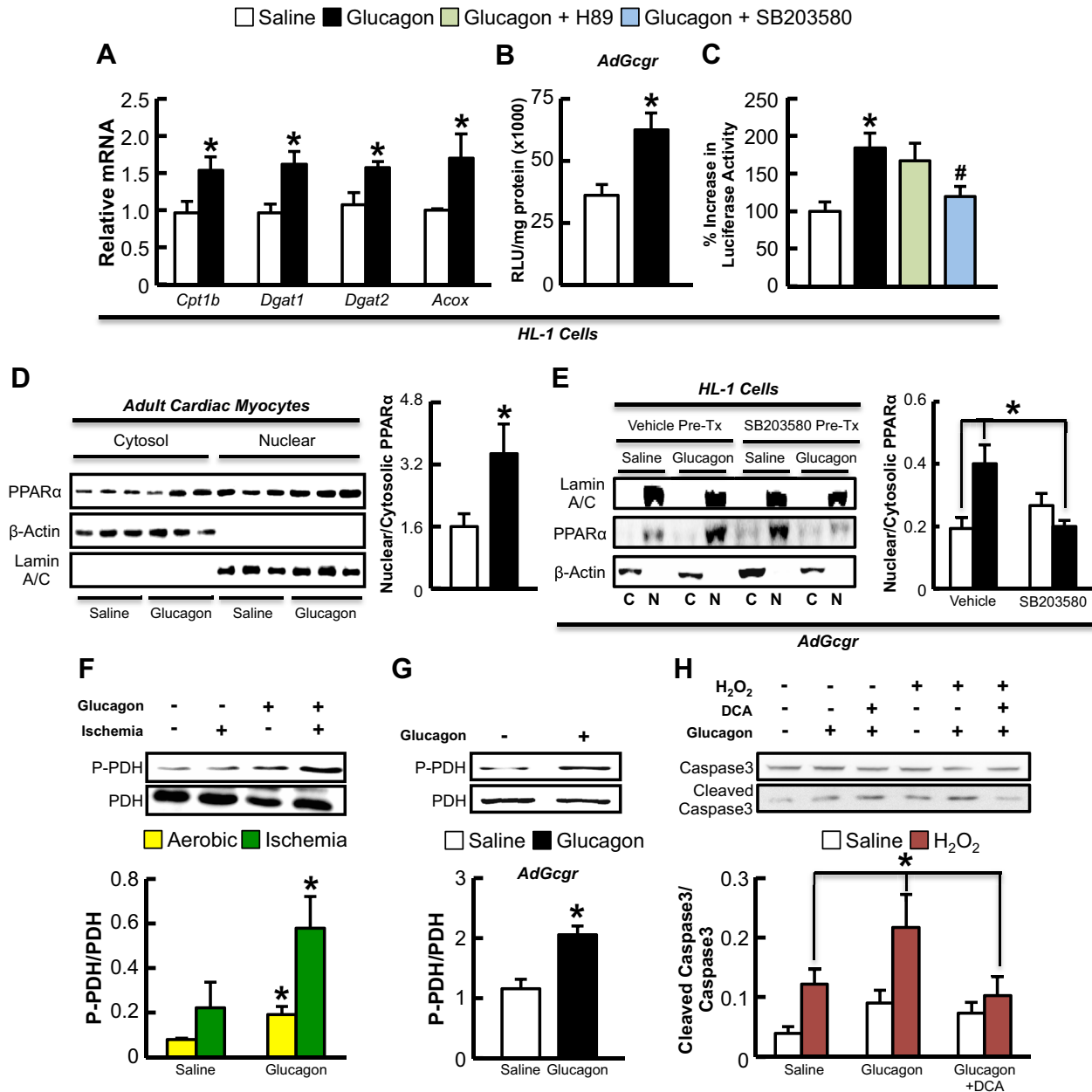


Figure 2: Glucagon increases PPAR α -dependent gene expression, PPAR α nuclear translocation, cleaved caspase 3 levels, and PDH phosphorylation. (A) HL-1 cells infected with adenovirus expressing the rat *Gcgr* cDNA were treated with saline or 20 nM glucagon for 3 h for assessment of *Cpt1b*, *Dgat1*, *Dgat2*, and *Acox* mRNA expression. (B,C) HL-1 cells were infected with Ad β -gal or Ad*Gcgr* for 24 h followed by transfection with a PPAR α gene promoter-luciferase construct. Cells were treated with saline or 20 nM glucagon for 3 h, with or without the PKA inhibitor (H89) or p38 MAPK inhibitor (SB203580) and luciferase expression was quantified as Relative Light Units (RLU). (D) Primary cultures of murine atrial cardiac myocytes were treated with saline or 20 nM glucagon for 3 h and cells were harvested for Western blot analysis of cytoplasmic and nuclear protein expression. (E) HL-1 cell lines infected with Ad*Gcgr* were treated with saline or 20 nM glucagon with and without SB203580 and cells were harvested for nuclear (N) and cytoplasmic (C) protein analysis. (F) C57BL/6 mice were treated with exogenous glucagon (30 ng/g) every 8 h for 24 h to assess PDH phosphorylation in hearts in mice that underwent 30 min occlusion of the LAD coronary artery or sham surgery ($n = 6$ per treatment). (G) PDH phosphorylation in HL-1 cells infected with Ad*Gcgr* for 24 h followed by a 3 h treated with glucagon. (H) Ad*Gcgr* infected HL-1 cell lines were treated for 24 h with 100 μ M H₂O₂ and/or 20 nM glucagon during the final 3 h of H₂O₂ treatment to detect cleaved caspase 3 levels. Separate groups of cells were treated with or without 1.5 mM DCA (PDH activator via inhibiting the PPAR α target gene, PDK4) for 24 h concomitantly with H₂O₂. * $p < 0.05$, saline vs. glucagon. # $p < 0.05$, glucagon + SB203580 vs. glucagon alone. All cell culture experiments were repeated at least twice, $n=4-6$ per condition.

encoding regulators of lipid metabolism was detected in *Gcgr*^{CM-/-} aerobic hearts, with similar trends detected in ischemic hearts (Figure 5A). PPAR α protein expression was also reduced in sham/aerobic but not in ischemic *Gcgr*^{CM-/-} hearts; ischemia alone reduced cardiac PPAR α protein expression (Figure 5B). In contrast, levels of Akt and GSK3 β phosphorylation were unaltered in ischemic hearts from

Gcgr^{CM-/-} mice, but Akt phosphorylation was increased in aerobic/sham hearts from *Gcgr*^{CM-/-} mice (Figure 5B). Furthermore, PDH phosphorylation showed a trend towards reduction in both sham/aerobic and ischemic hearts from *Gcgr*^{CM-/-} mice (Figure 5B), consistent with a role for glucagon action in the control of cardiac PDH activity and glucose oxidation.

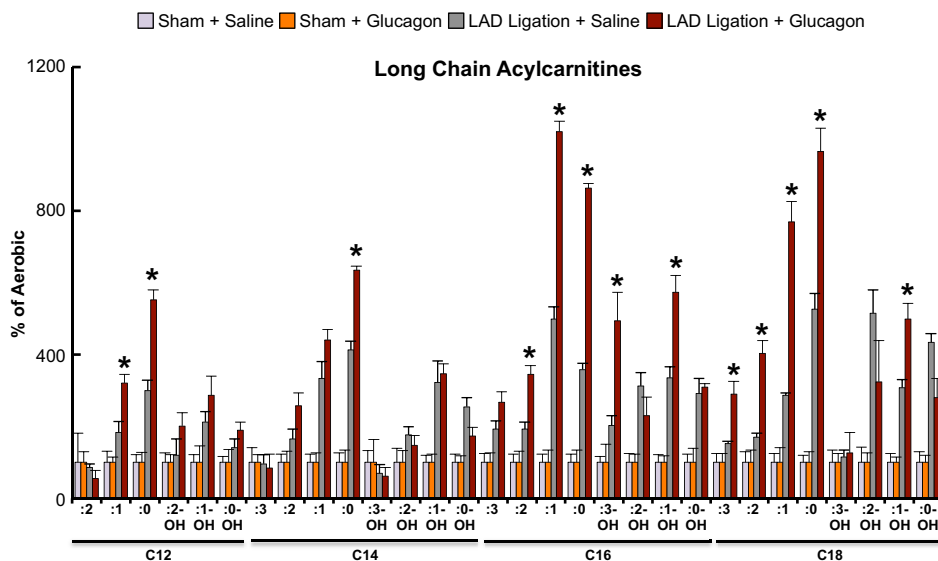


Figure 3: Glucagon increases long chain acylcarnitine levels in the ischemic heart. Glucagon (30 ng/g body weight) or saline injections were administered subcutaneously every 8 h over a 24 h period (4 total injections at 0, 8, 16, and 24 h) and hearts were harvested after a 5 h fast and subjected to metabolomics assessment to determine acylcarnitine content. Ischemic hearts were collected 30 min following LAD coronary artery ligation surgery (surgery took place 4 h into the 5 h fast). Data is expressed as percent of levels in aerobic mouse hearts for each respective group. * $p < 0.05$ saline vs. glucagon-treated LAD coronary artery ligation group. Data are mean \pm S.E.M ($n = 5$ per group).

3.5. Acylcarnitine profiling suggests reduced myocardial fatty acid oxidation in $Gcgr^{CM-/-}$ mice

We observed a significant reduction in several long chain acylcarnitine species in sham/aerobic hearts from $Gcgr^{CM-/-}$ mice (Figure 6A), suggesting that eliminating glucagon action in heart reduces substrate burden on the fatty acid oxidation pathway. Consistent with the likelihood that this metabolic effect was specific to fatty acid oxidation and not an overall decrease in oxidative metabolism, we observed a decrease in medium chain acylcarnitines in sham/aerobic hearts from $Gcgr^{CM-/-}$ mice, whereas myocardial C2 (acetylcarnitine) levels were similar between $Gcgr^{CM-/-}$ and αMHC^{Cre} mice (Figure 6B). In addition, TAG content and the majority of Krebs cycle intermediates were similar in $Gcgr^{CM-/-}$ and αMHC^{Cre} sham/aerobic hearts (Figure 6C–I). Similar results were observed in $Gcgr^{CM-/-}$ and αMHC^{Cre} ischemic hearts 30 min post-MI, with the exception of an increase in myocardial TAG content in $Gcgr^{CM-/-}$ hearts (Figure 6J–R). Ischemia itself reduces fatty acid oxidation and results in the mobilization of myocardial TAG stores (~ 2.2 vs. $1.1 \mu\text{mol/g}$ wet weight in αMHC^{Cre} aerobic and ischemic hearts, respectively), but the additional reduction in fatty acid oxidation in $Gcgr^{CM-/-}$ ischemic hearts likely explains their increase in myocardial TAG content relative to ischemic hearts from their αMHC^{Cre} littermates. Comparable results were observed in the hearts of $Gcgr^{CM-/-}$ and αMHC^{Cre} mice fed a high fat diet for 6 months, with the majority of long chain acylcarnitines trending lower in $Gcgr^{CM-/-}$ hearts (Supplementary Figure 6).

3.6. Glucagon attenuates recovery of left ventricular developed pressure during ischemia/reperfusion injury

We next determined whether reduced or enhanced cardiac Gcgr signaling modulates outcomes in response to *ex vivo* ischemia/reperfusion (I/R) injury. Isolated hearts from $Gcgr^{-/-}$ mice exhibited enhanced recovery of LV developed pressure (LVDP) following I/R injury, whereas glucagon treatment impaired recovery of LVDP in isolated hearts from WT mice (Figure 7A–F).

3.7. Glucagon fails to increase mortality or impair LV remodeling in ischemic $Gcgr^{CM-/-}$ mice

To determine whether the glucagon-induced increase in mortality and adverse LV remodeling following MI (Figure 1) was due to direct activation of the cardiac glucagon receptor, we treated $Gcgr^{CM-/-}$ mice and their αMHC^{Cre} littermates with glucagon (30 ng/g body weight; $3 \times$ daily, via subcutaneous injection) prior to and following LAD coronary artery ligation. Although glucagon increased mortality and infarct size in αMHC^{Cre} control mice, it had no effect on these parameters in $Gcgr^{CM-/-}$ mice (Figure 7G,H). Hence the deleterious effects of glucagon on the cardiac response to ischemic injury are not indirect and require a functional cardiomyocyte Gcgr.

4. DISCUSSION

Our findings demonstrate that exogenous glucagon administration has negative actions on the ischemic heart, whereas reduction in cardiac glucagon action in $Gcgr^{CM-/-}$ mice results in marked cardioprotection. In both situations, changes in fatty acid oxidation correlate with cardiac outcomes, as multiple long-chain acylcarnitines are increased by glucagon in the ischemic heart, whereas $Gcgr^{CM-/-}$ mice have reduced levels of these metabolites under ischemic conditions. Taken together these findings strongly and independently support the importance of both pharmacological and physiological Gcgr signaling in the cardiac response to ischemic injury.

Previous studies have provided conflicting data on the role of glucagon on the myocardium. Treatment with glucagon worsened the recovery of cardiac power in the isolated working rat heart during global no-flow ischemia/reperfusion [16] and cardiomyopathy developed in a patient with a glucagonoma that was completely reversed upon tumor resection [38]. In contrast, glucagon improved LV minute work and contractility in dogs with acute MI [39]. Furthermore, glucagon administration to 6 patients with AMI produced positive inotropic effects and temporarily improved cardiogenic shock [40], whereas glucagon increased cardiac performance and reduced LV failure

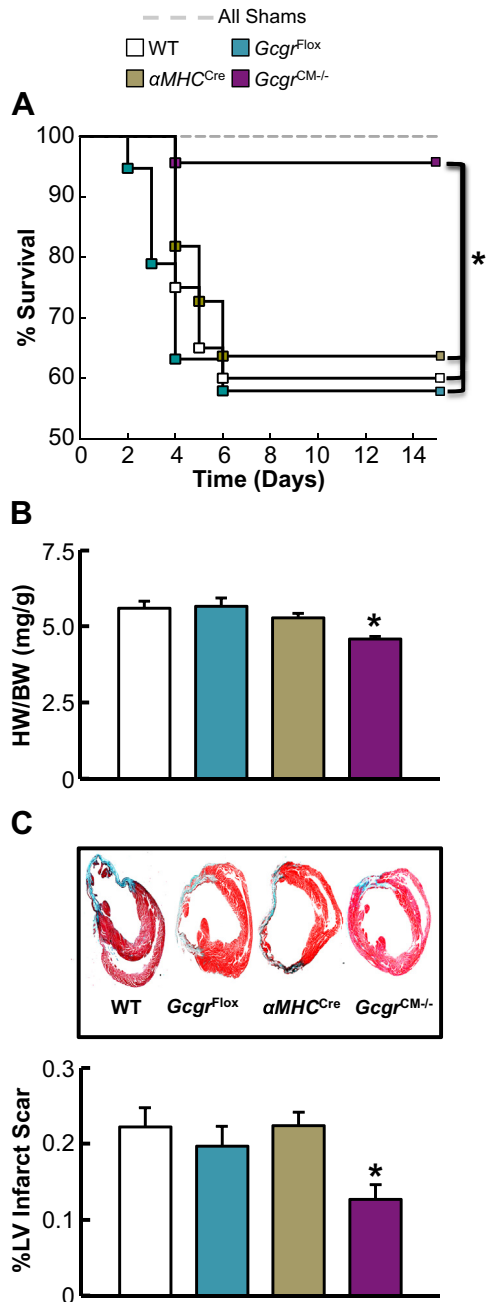


Figure 4: Loss of cardiac *Gcgr* signaling enhances survival following MI and attenuates adverse LV remodeling. (A) LAD coronary artery ligation was performed in 11–14-week-old *Gcgr^{CM-/-}* and littermate control mice and survival was monitored for 15 days following surgery. * $p < 0.05$ *Gcgr^{CM-/-}* vs. each control group. Data are mean \pm S.E.M (SHAM; $n = 10$ per genotype and LAD; $n = 11-23$ per genotype). (B) Heart weight:body weight (HW:BW) ratio 15 days following LAD coronary artery ligation. * $p < 0.05$ *Gcgr^{CM-/-}* vs. *Gcgr^{Flox}* and wild-type (WT) mice. Data are mean \pm S.E.M ($n = 8-11$ per genotype). (C) Masson's trichrome staining of infarcted heart and quantification of % left ventricular (LV) infarct scar area 15 days following LAD coronary artery ligation. * $p < 0.05$ *Gcgr^{CM-/-}* vs. littermate control mice. Data are mean \pm S.E.M ($n = 9-11$ per genotype).

without altering myocardial oxygen consumption in dogs subjected to MI [41]. Although heart rate and systolic blood pressure trended lower in non-ischemic *Gcgr^{CM-/-}* mice (data not shown), these differences were not statistically significant perhaps due to the small number of

animals analyzed. Our results support the contention that pharmacological glucagon agonism is deleterious to the ischemic heart, as glucagon significantly enhanced mortality following MI, whereas *Gcgr^{CM-/-}* mice exhibited a cardioprotective phenotype following LAD coronary artery ligation-induced myocardial injury. Thus, reduction of glucagon receptor signaling in the heart should be explored as a novel approach for the attenuation of ischemic myocardial injury. However, whether cardiac Gcgr signaling can be safely, selectively and effectively targeted pharmacologically in humans without incurring additional systemic liabilities is currently unknown.

Although the mechanism(s) via which glucagon receptor signaling modulates cardiovascular outcomes following MI remain incompletely identified, changes in myocardial fatty acid oxidation appear to be implicated. Acylcarnitine profiling indicated that glucagon caused accumulation of incompletely oxidized lipids in the heart, as has also been seen in experimental models of heart failure [42]. In skeletal muscle, accumulation of acylcarnitines has been described in obesity and type 2 diabetes. Relief of acylcarnitine accumulation by reducing entry of fatty acids into the mitochondria relieves substrate overload and enhances insulin action [24] and conversely, increasing acyl CoA: acylcarnitine accumulation by transgenic knockout of carnitine acyl transferase (CrAT) impairs insulin sensitivity [43]. Here, we observed increased acylcarnitines in response to glucagon administration in the ischemic heart, consistent with activation of the early, but not later phases of fatty acid oxidation, whereas heart-specific deletion of *Gcgr* appeared to reduce substrate pressure on the fatty acid oxidation pathway. Consistent with the metabolic profile, glucagon treatment of cardiac myocytes increased PPAR α activity, a known activator of the β -oxidative machinery, whereas PPAR α downstream target gene mRNA expression was reduced in hearts from *Gcgr^{CM-/-}* mice.

PPAR α is a key regulator of fatty acid oxidation in the heart [44,45] and cardiac-specific PPAR α overexpression (a) worsens the recovery of cardiac function during *ex vivo* I/R injury in the isolated working heart [46] and (b) induces a diabetic-like cardiomyopathy [45]. Furthermore, genetic elimination of PPAR α in dominant-negative NADPH oxidase transgenic mice reversed their increased infarct size and cardiac myocyte apoptosis in response to *in vivo* I/R injury [47] and elimination of PPAR α in cardiac-specific aryl hydrocarbon nuclear translocator deficient mice reversed their associated cardiomyopathy and lipotoxicity [48]. Likewise, PPAR α deficient mice are protected against both *ex vivo* I/R injury and streptozotocin-induced diabetic cardiomyopathy [46,49]. In contrast, treatment with the PPAR α agonist, GW7647, reduced infarct size in CD1 mice following temporary occlusion of the LAD coronary artery [50], whereas the PPAR α agonist, fenofibrate, improved recovery following *ex vivo* I/R injury in hearts from mice with diet-induced obesity [51]. While direct PPAR α agonism increases fatty acid oxidation, chronic peripheral PPAR α agonism actually increases hepatic fatty acid oxidation rates, which decreases circulating lipids without changes in cardiac PPAR α activity [51]. Acknowledging the ongoing controversy regarding PPAR α agonism, fatty acid oxidation and ventricular function, our findings with glucagon are consistent with a negative role for Gcgr-dependent PPAR α activity in the ischemic heart.

Consistent with previous studies of glucagon action in the liver [8] we demonstrated that glucagon activates PPAR α in the heart in a p38 MAPK dependent manner. However, p38 MAPK has also been demonstrated to increase cardiac injury through mechanisms independent of changes in fatty acid oxidation, such as an increase in intracellular acidosis which decreases the efficiency of contractile function [52], or via interaction with TAK1-binding protein 1 to enhance cardiac myocyte apoptosis [53]. Thus, other mechanisms may also

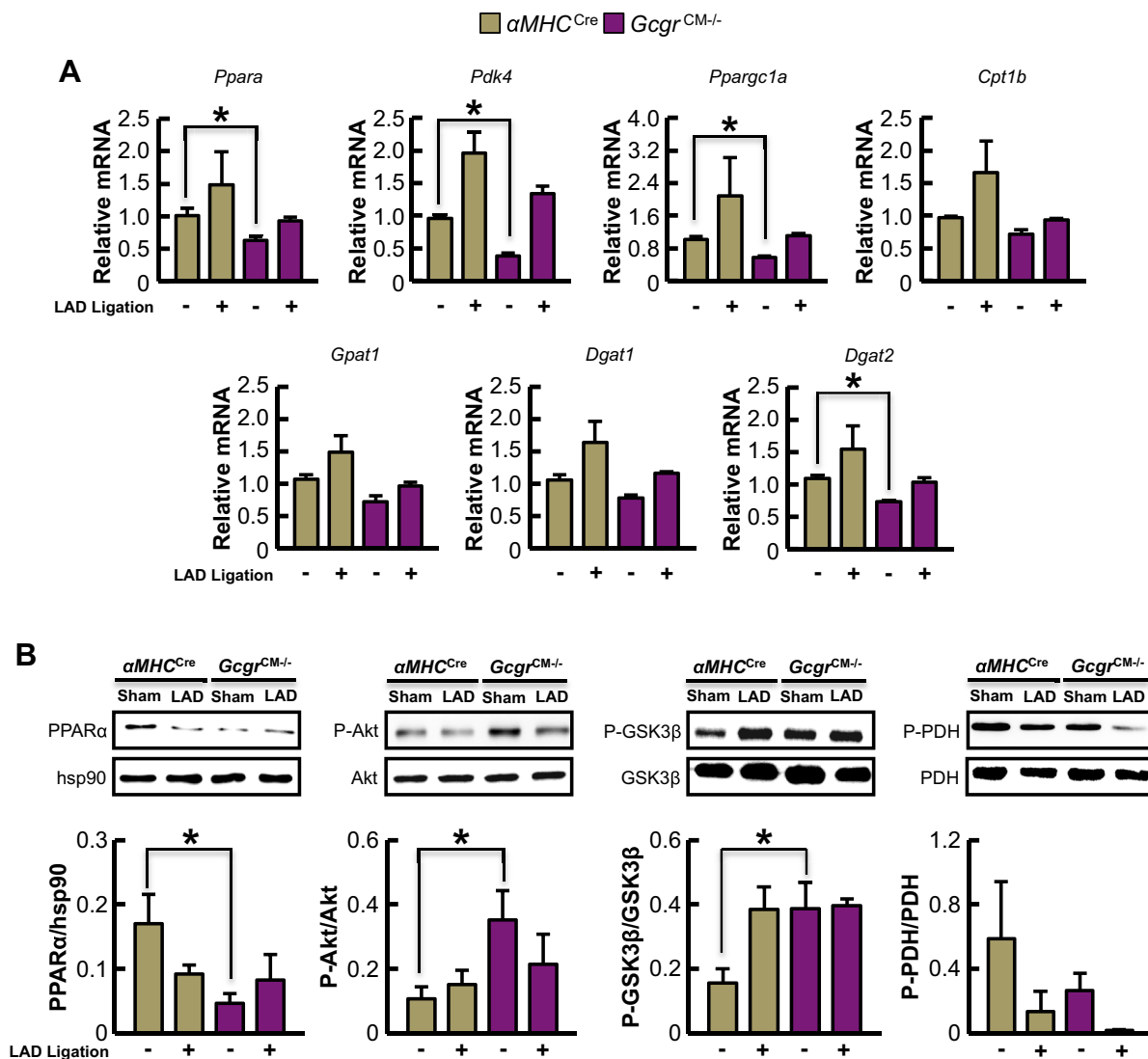


Figure 5: Selective loss of Gcgr signaling in cardiomyocytes leads to reduced expression of genes and proteins regulating fatty acid metabolism. (A) Quantification of mRNA transcript levels from sham/aerobic and ischemic hearts (30 min post-LAD coronary artery ligation) from 12-week-old αMHC^{Cre} and $Gcgr^{CM-/-}$ mice fasted for 5 h ($n = 4$). $*p < 0.05$ $Gcgr^{CM-/-}$ vs. αMHC^{Cre} . Data are mean \pm S.E.M. (B) Protein expression/phosphorylation in fasted (5 h) hearts from 12-week-old αMHC^{Cre} and $Gcgr^{CM-/-}$ mice subjected to sham surgery or LAD coronary artery occlusion for 30 min $*p < 0.05$ for αMHC^{Cre} vs. $Gcgr^{CM-/-}$ mice. Data are mean \pm S.E.M. ($n = 4$ mice in each group).

contribute to our observed phenotypes via activation of p38 MAPK activity.

Our current studies have several important limitations. First, the majority of our experiments were performed in relatively young, healthy, non-diabetic, non-obese mice. Perturbations of the normal metabolic environment may greatly influence myocardial energy uptake, patterns of fuel utilization, and potentially, cardiovascular outcomes. Moreover, the role(s) of and pathways activated by enhanced or disrupted cardiac Gcgr signaling in the ischemic myocardium of older mice with established cardiovascular disease may be different. Furthermore, it cannot be assumed that gain and loss of Gcgr signaling in the normal, diabetic, or obese human heart will produce similar deleterious or beneficial outcomes. Notably, we have not ascertained whether the acute negative actions of glucagon on the mouse heart reflect achievement of a critical Cmax or total exposure to glucagon. Moreover, whether sustained exposure to glucagon would result in partial desensitization of the cardiac Gcgr signaling pathway has not been examined. Of potential relevance to future studies of the Gcgr and

related cyclic AMP-linked cardiac receptors, a recent report described cardioprotective actions of secreted cyclic AMP metabolized to adenosine, which differs substantially from the classical cardiotoxic effects of intracellular cyclic AMP [54]. Hence understanding the effects of glucagon and GLP-1 on intracellular vs. secreted cyclic AMP in the heart may be important. Finally, our experiments focused on the consequences of ischemic cardiac injury, and the importance of Gcgr signaling in experimental models of heart failure under normoglycemic and diabetic conditions requires further elucidation.

There is currently great interest in the therapeutic potential of glucagon/GLP-1 co-agonists and glucagon-containing tri-agonists for the treatment of obesity and/or diabetes [7,10,55]. Our findings demonstrate that pure unopposed acute glucagon agonism has negative effects on the myocardium during ischemic injury, whereas cardiomyocyte-specific elimination of glucagon receptor activity results in robust cardioprotection against MI-induced mortality and adverse LV remodeling. Nevertheless, we did not assess a full range of glucagon doses in our gain of function studies, hence it may be possible to

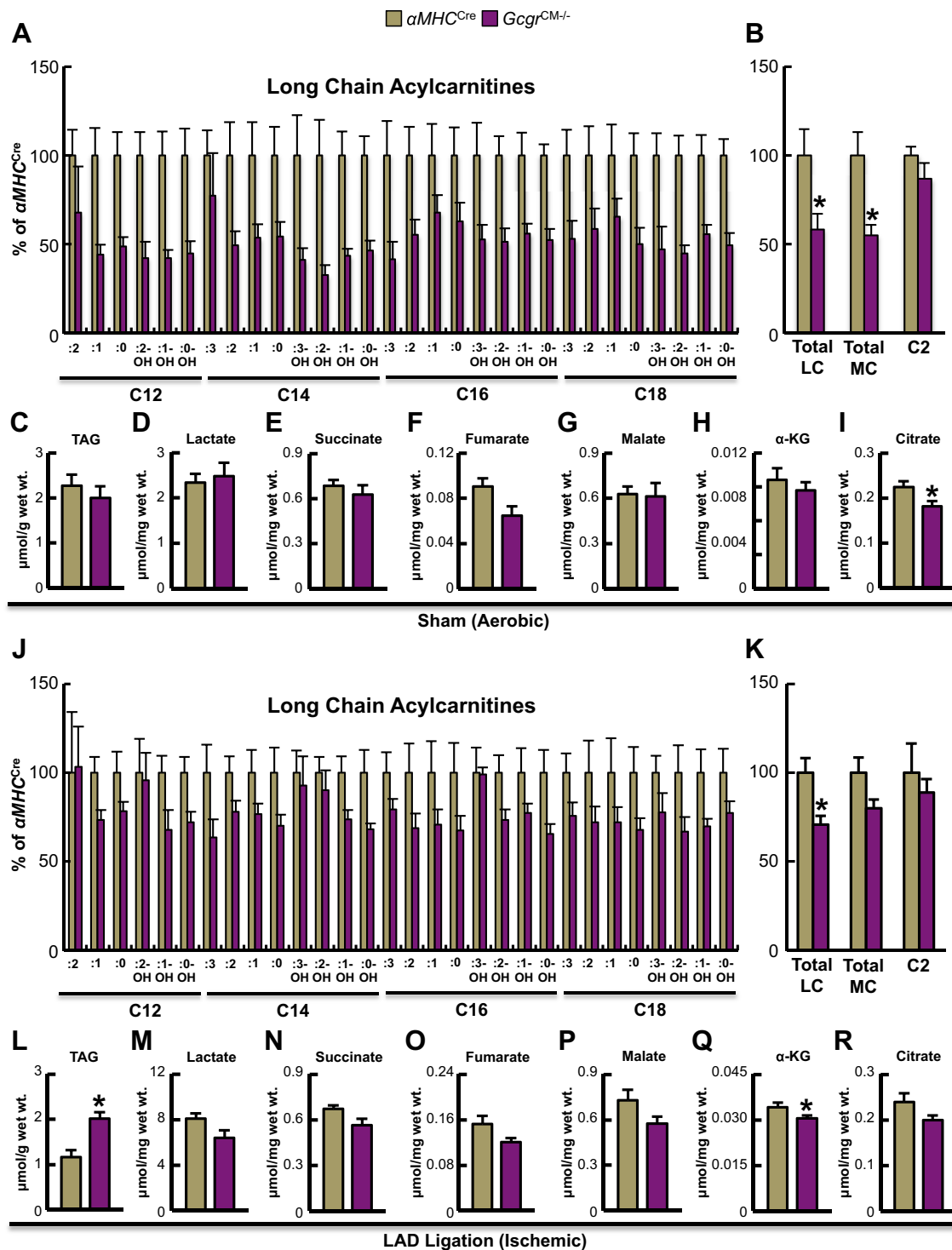


Figure 6: Targeted metabolomics reveals reduced fatty acid oxidation in $Gcgr^{CM-/-}$ hearts. (A) Acylcarnitine levels in aerobic hearts harvested from fasted (5 h) αMHC^{Cre} and $Gcgr^{CM-/-}$ mice ($n = 5$ per genotype). Values are expressed as percent of levels in hearts from αMHC^{Cre} mice. (B) Aerobic myocardial total long chain (LC), medium chain (MC) and acetyl (C2) acylcarnitines. $*p < 0.05$ αMHC^{Cre} vs. $Gcgr^{CM-/-}$. Levels of triacylglycerol (TAG) (C), lactate (D) and Krebs cycle intermediates (E–I) in 5 h fasted sham/aerobic hearts. $*p < 0.05$ αMHC^{Cre} vs. $Gcgr^{CM-/-}$. (J) Acylcarnitine levels in hearts harvested 30 min following cardiac ischemia from 5 h fasted αMHC^{Cre} and $Gcgr^{CM-/-}$ mice ($n = 5$ per genotype). Values are expressed as percent of αMHC^{Cre} mice values. (K) Levels of total LC, MC and acetyl (C2) acylcarnitines in ischemic hearts. $*p < 0.05$ αMHC^{Cre} vs. $Gcgr^{CM-/-}$. TAG (L) and lactate (M) content in ischemic hearts. $*p < 0.05$ αMHC^{Cre} vs. $Gcgr^{CM-/-}$. (N–R) Levels of Krebs cycle intermediates in hearts harvested 30 min following cardiac ischemia from 5 h fasted αMHC^{Cre} and $Gcgr^{CM-/-}$ mice. $*p < 0.05$ αMHC^{Cre} vs. $Gcgr^{CM-/-}$.

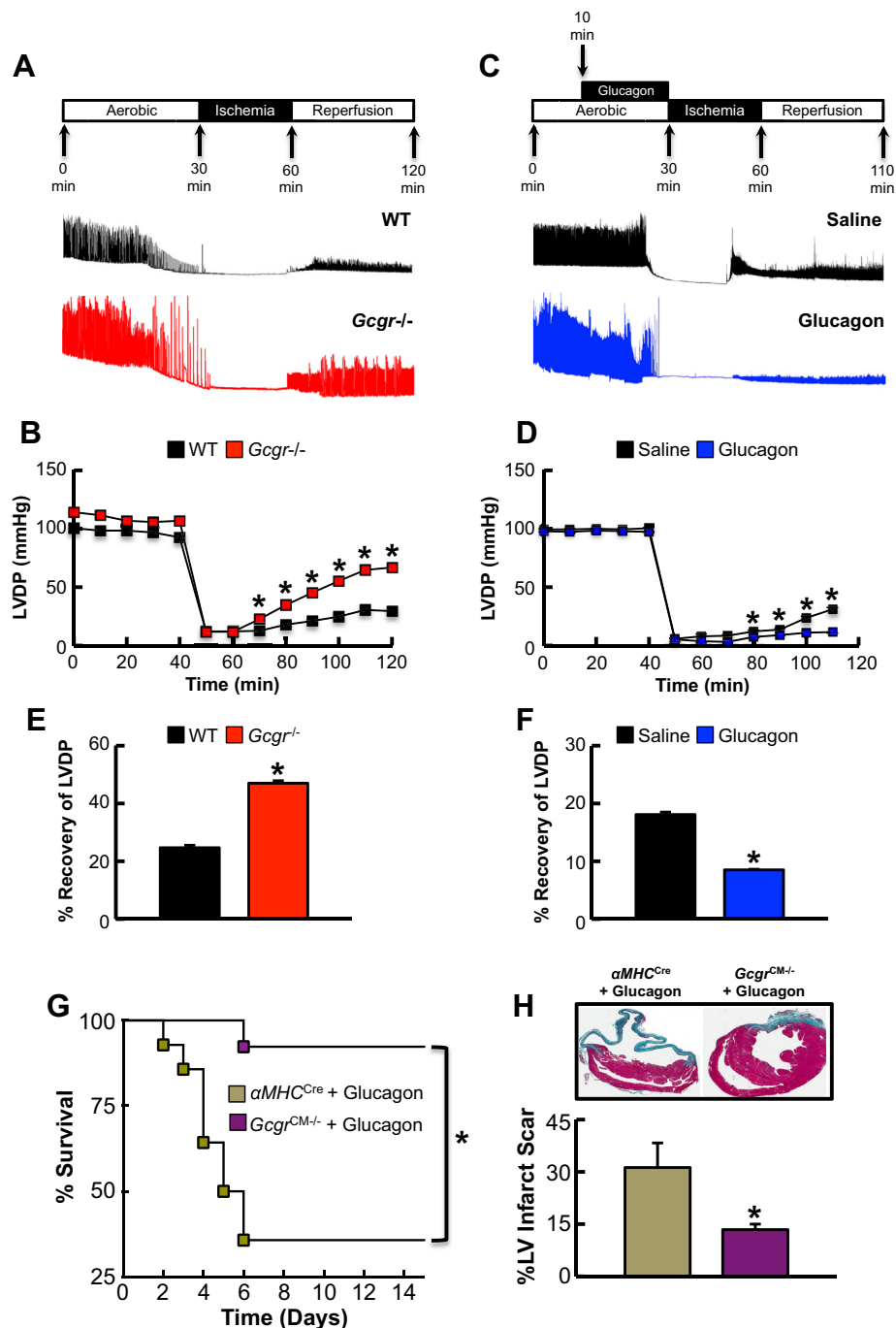


Figure 7: Loss of *Gcgr* signaling protects whereas glucagon impairs recovery of LV developed pressure (LVDP) after I/R injury in the isolated heart *ex vivo*. (A–F) Schematic depiction of peptide infusions, ischemia and reperfusion times, and representative LVDP recordings and data from isolated perfused hearts. LVDP measurements in isolated perfused *Gcgr*^{-/-} (B,E) and WT (D,F) hearts subjected to I/R injury with and without glucagon (1 μ g/mL for 20 min) (D,F). For E&F, graph depicts the percentage recovery rate of LVDP following ischemia. Data shown are means \pm S.E.M. ($n = 3-6$ per genotype). * $p < 0.05$ compared with the control group. (G,H) Glucagon impairs survival after myocardial infarction in a cardiac *Gcgr*-dependent manner. (G) Left anterior descending coronary artery ligation (LAD) surgeries were performed in 11–14-week-old α MHC^{Cre} and *Gcgr*^{CM-/-} mice treated with 30 ng/g body weight glucagon given by subcutaneous injection (every 8 h for 7 days). Survival was monitored for 15 days following surgery. (H) Infarct size was assessed 15 days following LAD coronary artery ligation. * $p < 0.05$ α MHC^{Cre} WT vs. *Gcgr*^{CM-/-} mice. Data are mean \pm S.E.M ($n = 12-13$ per genotype).

administer glucagon at doses that prevent hypoglycemia (type 1 diabetes), or achieve some degree of weight loss (diabetes and/or obesity), without cardiotoxicity. Whether the combination of glucagon with GLP-1 agonism in the same molecule or as a mixture of two different agonists, will similarly mitigate the potential adverse consequences of unopposed *Gcgr* activation on the ischemic diabetic heart is

an important question [13]. Indeed, observations using the isolated rat perfused heart model suggest that glucagon alone compromised the energetic state of ischemic hearts, whereas a glucagon-GLP-1 dual agonist exerted preferential actions on cardiac energetics without increasing levels of cyclic AMP accumulation, thereby mitigating the adverse effects of glucagon [56]. Furthermore, it cannot be assumed

that administration of GCGR antagonists to diabetic subjects, at doses resulting in partial attenuation of GCGR signaling in multiple tissues, will produce a cardiovascular phenotype that mirrors or overlaps our findings in non-diabetic mice with marked selective genetic reduction of Gcgr signaling in cardiomyocytes.

Similar questions surround the dose—response relationships for the cardiovascular actions of glucagon in subjects with type 1 diabetes and pre-existing coronary artery disease. Indeed, the investigational use of combined glucagon-insulin delivery systems for the optimized treatment of type 1 diabetes [57] emphasizes the need to explore the safety of a range of glucagon concentrations in humans at risk for cardiovascular events. Our data highlights the importance of understanding the cardiovascular actions of novel peptide therapies being evaluated for the treatment of patients with metabolic disorders associated with a substantial concomitant risk of developing ischemic heart disease.

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CONFLICT OF INTEREST

Dr. Drucker has served as an advisor or consultant within the past 12 months to Arisaph Pharmaceuticals Inc., Intarcia Therapeutics, Merck Research Laboratories, MedImmune, Novo Nordisk Inc., NPS Pharmaceuticals Inc., Receptos, Sanofi, and Transition Pharmaceuticals Inc.

APPENDIX A. SUPPLEMENTARY DATA

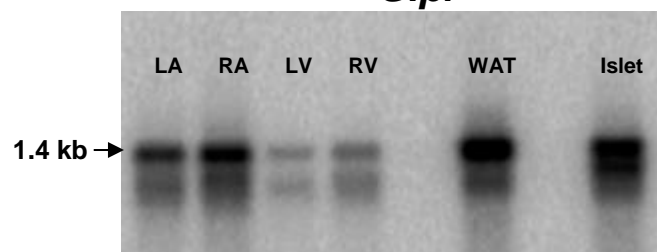
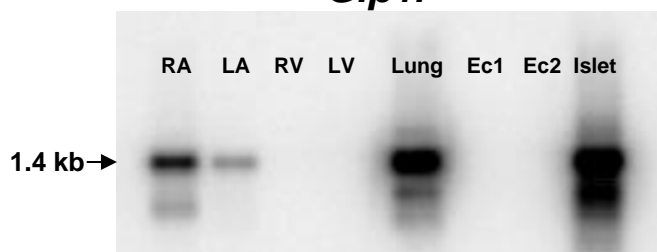
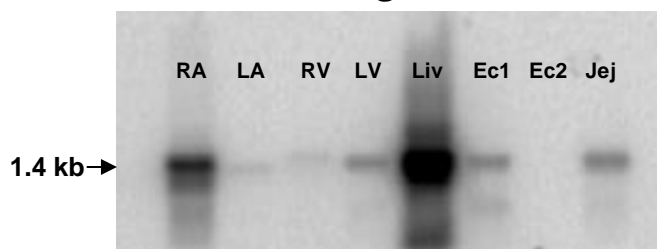
Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2014.11.005>.

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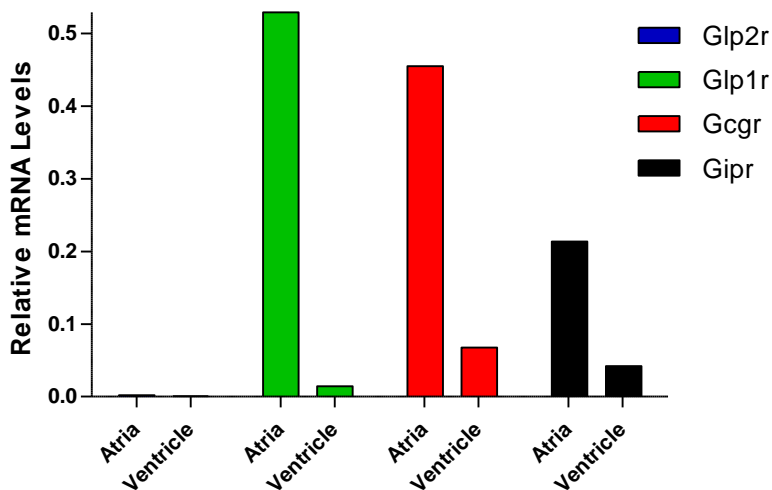
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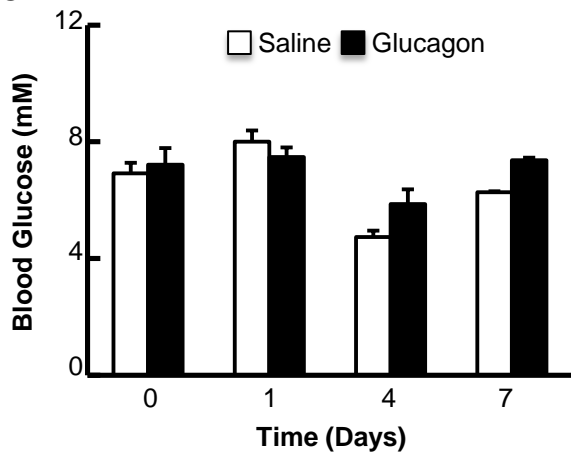
*Glp1r**Gipr**Gcgr**Glp2r*

B Atria and Ventricle mRNA Levels

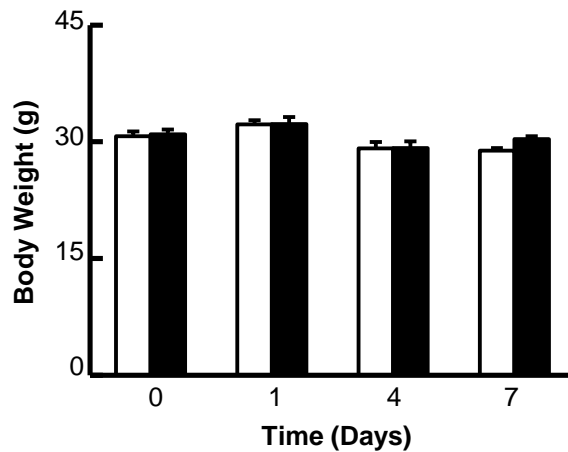
B

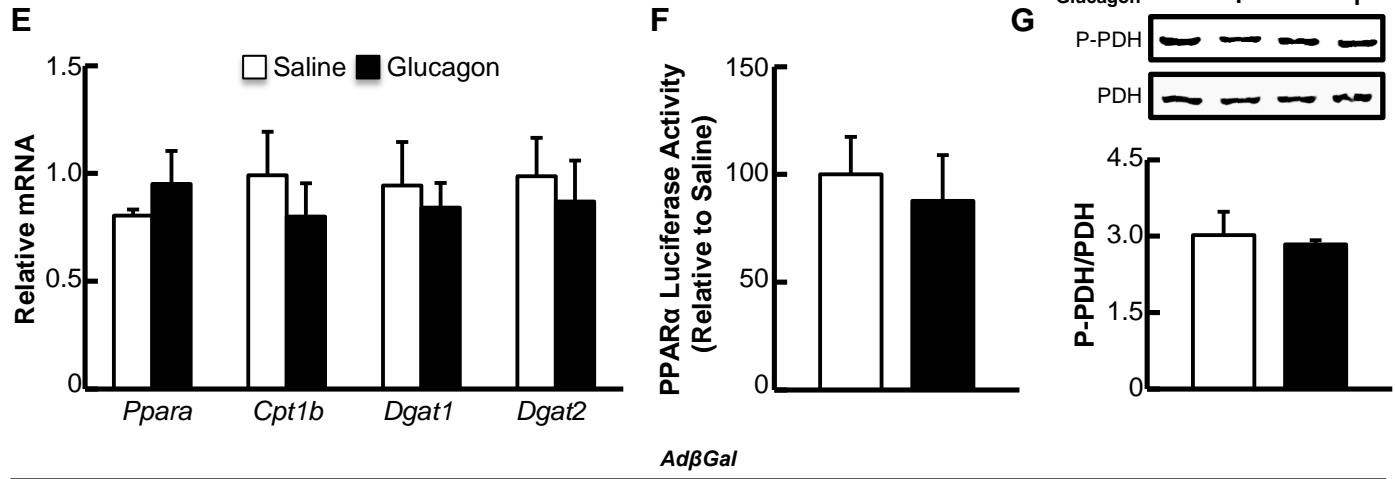


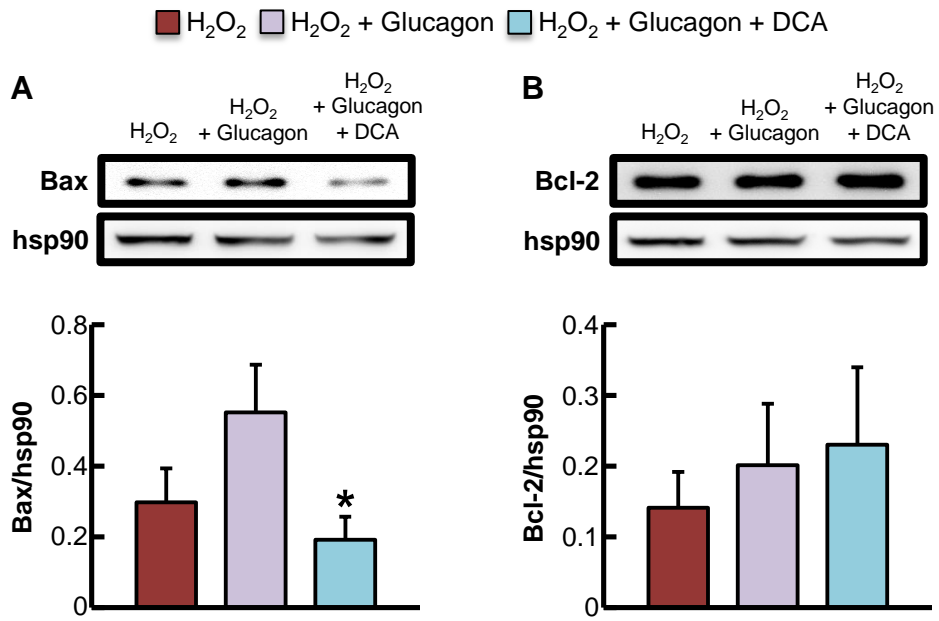
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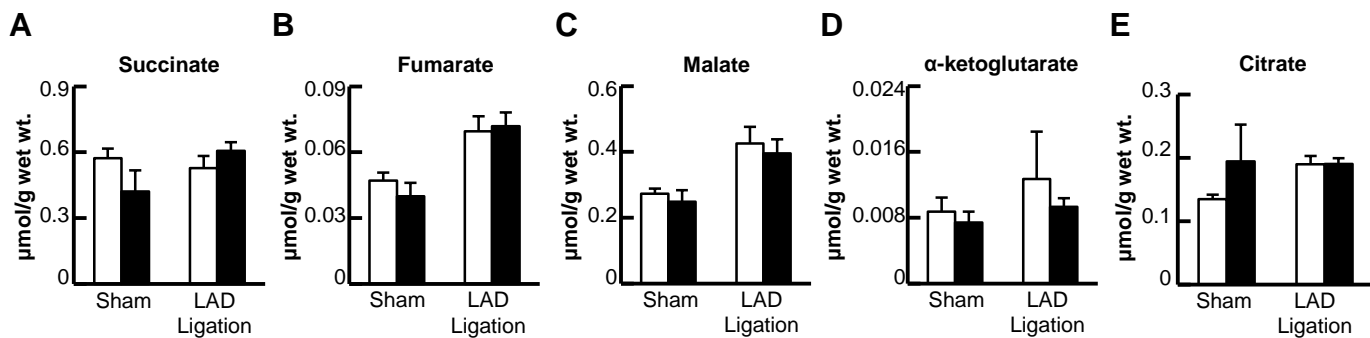
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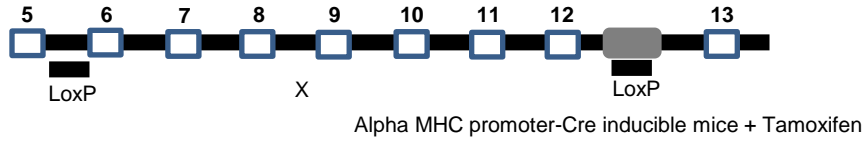
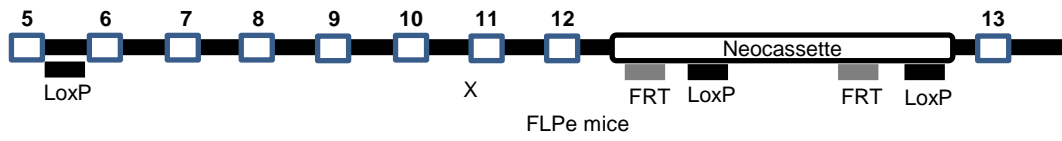




□ Saline ■ Glucagon

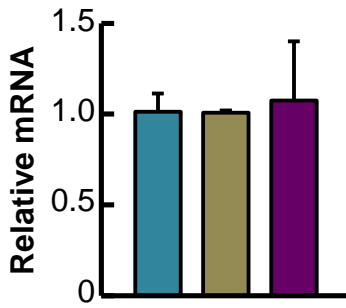


A

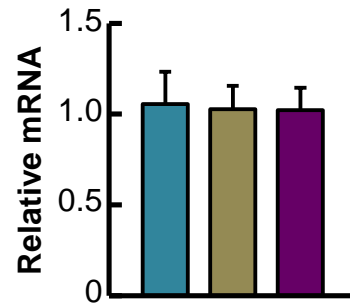


■ *Gcgr*^{Flox} ■ *αMHC*^{Cre} ■ *Gcgr*^{CM-/-}

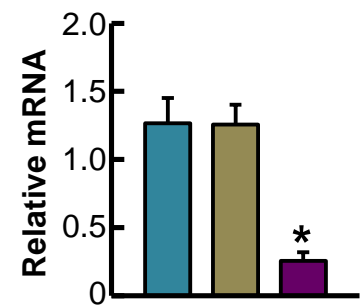
B

Liver Gcgr

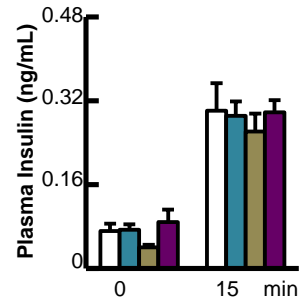
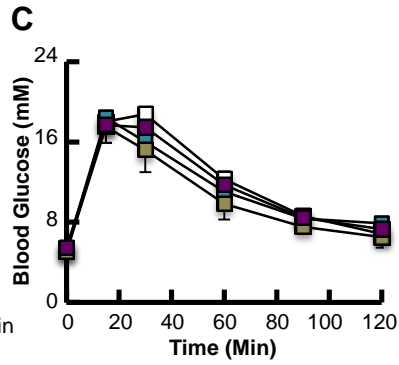
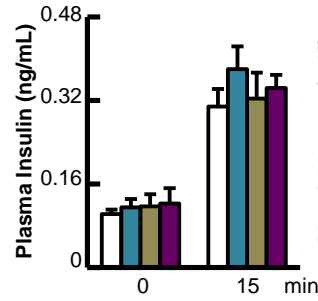
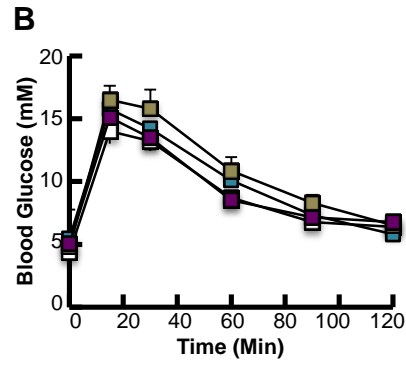
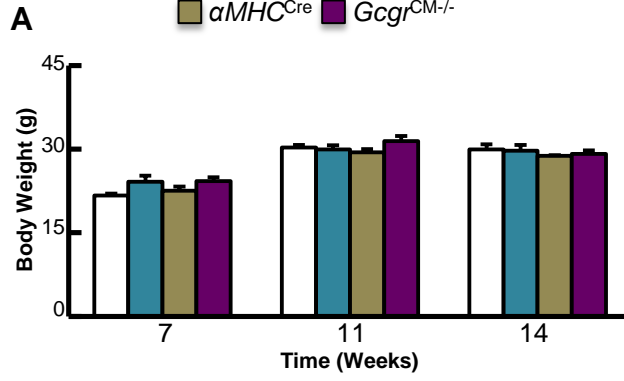
C

Kidney Gcgr

D

Heart Gcgr

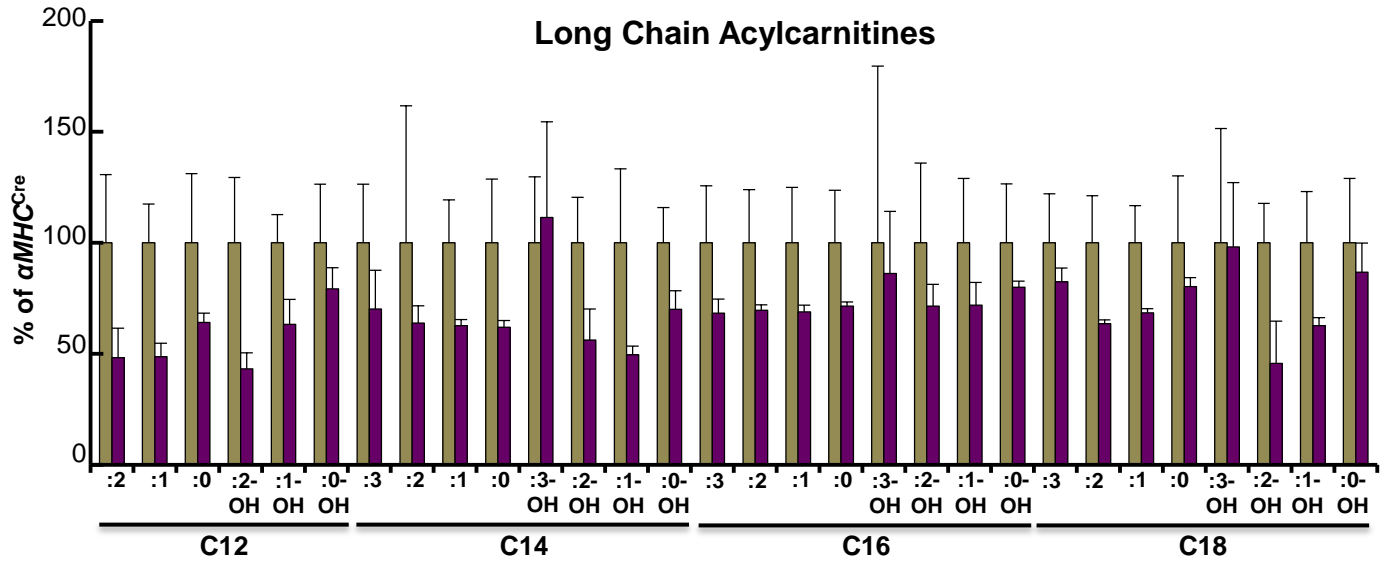
WT $Gcgr^{Flx}$
 αMHC^{Cre} $Gcgr^{CM-/-}$



Oral Glucose Tolerance

Intraperitoneal Glucose Tolerance

α MHC^{Cre} $Gcgr^{CM-/-}$



Sham (Aerobic) Hearts from Mice Fed a High Fat Diet (45% Kcal) for 6 Months