Glucagon receptor signaling is essential for control of murine hepatocyte survival

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Glucagon receptor signaling is essential for control of murine hepatocyte survival


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Running title: Glucagon and hepatocyte survival

Abbreviations:

GLP-1 = glucagon-like peptide-1
GLP-2 = glucagon-like peptide-2
DPP-4 = dipeptidyl peptidase-4

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Abstract

Background & Aims: Glucagon action in the liver is essential for control of glucose homeostasis and the counterregulatory response to hypoglycemia. As receptors for the related peptides GLP-1 and GLP-2 regulate β-cell and enterocyte apoptosis, respectively, we examined whether glucagon receptor (Gcgr) signaling modulates hepatocyte survival.

Methods: The importance of the Gcgr for hepatocyte cell survival was examined using Gcgr+/+ and Gcgr-/- mice in vivo, and murine hepatocyte cultures in vitro.

Results: Gcgr-/- mice exhibited enhanced susceptibility to experimental liver injury induced by either FasL activation or a methionine and choline-deficient diet. Restoration of hepatic Gcgr expression in Gcgr-/- mice attenuated the development of hepatocellular injury. Furthermore, exogenous glucagon administration reduced Jo2-induced apoptosis in wild-type mice and decreased caspase activation in fibroblasts expressing a heterologous Gcgr and in primary murine hepatocyte cultures. The anti-apoptotic actions of glucagon were independent of PKA, PI3-K and MAPK and mimicked by the Epac agonist CPT-Me-cAMP.

Conclusions: These findings extend the essential actions of the Gcgr beyond the metabolic control of glucose homeostasis to encompass the regulation of hepatocyte survival.
Introduction

Glucagon is a 29 amino acid proglucagon-derived peptide (PGDP) released from pancreatic α-cells that regulates blood glucose via stimulation of hepatic gluconeogenesis and glycogenolysis. Glucagon also inhibits glycogen synthesis and glycolysis and is the primary counter-regulatory hormone to insulin. Loss of the α-cell glucagon response to hypoglycemia and dysregulation of glucagon secretion contribute to the pathophysiology of diabetes mellitus. As inappropriately elevated levels of plasma glucagon increase hepatic glucose production leading to hyperglycemia, there is considerable interest in determining whether diminution of glucagon action may be useful for the treatment of type 2 diabetes.

The biological importance of the glucagon receptor (Gcgr) has been analyzed via characterization of Gcgr−/− mice that exhibit modest fasting hypoglycemia, and improved glucose tolerance. Gcgr−/− mice also exhibit reduced adiposity, decreased circulating triglycerides, improved insulin sensitivity, and increased circulating levels of GLP-1. Moreover, following high fat feeding Gcgr−/− mice exhibit decreased body weight and food intake, reduced plasma glucose levels, and improved glucose tolerance.

The diabetes-resistant phenotype of Gcgr−/− mice, taken together with observations that the glucose-lowering actions of amylin, GLP-1R agonists and DPP-4 inhibitors are attributable in part to inhibition of inappropriate glucagon secretion has rekindled interest in attenuation of glucagon action for the treatment of diabetes. Indeed reduction of liver Gcgr expression using antisense oligonucleotides leads to reduced hepatic glucose production and amelioration of experimental diabetes and small molecule Gcgr antagonists attenuate glucagon action in human subjects. Hence, there is ongoing interest in exploring whether reduction of glucagon action may be useful for treatment of diabetes.

Although glucagon, GLP-1 and GLP-2 exert distinct biological actions through separate G protein coupled receptors, these peptides also share overlapping mechanisms of action. Whereas GLP-1 and GLP-2 regulate glucose homeostasis and nutrient absorption respectively, both peptides enhance cell survival via cAMP-dependent pathways. Intriguingly, glucagon also acts as a growth and/or survival factor for cultured hepatocytes in vitro. To determine the importance of Gcgr action for hepatocyte survival, we studied the consequences of enhanced or disrupted Gcgr signaling in murine hepatocytes. We show here that Gcgr signaling is essential for hepatocyte survival via regulation of cAMP-dependent pathways linked to attenuation of caspase activity.
**Materials and Methods**

*Materials*: Tissue culture reagents were from Invitrogen, Burlington, ON and chemicals were from Sigma-Aldrich. Human glucagon was purchased from California Peptides. H-89, LY294002 and U0126 were from Calbiochem. Primary tissue culture plates were from BD Biosciences. Anti-Fas antibody (Jo2) was from BD Pharmingen and the Epac (exchange protein directly activated by cyclic AMP) agonist 8-pCPT-Me-cAMP was from Biolog Life Sciences Institute.

*Baby hamster kidney (BHK) cell culture*: BHK:rGcgr cells were cultured in 4.5 g/L glucose, Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) containing G418 (0.8 mg/ml). Upon reaching 70-80 % confluence, cells were serum-deprived for 16-24 hours prior to induction of apoptosis with cycloheximide.

*Cell Viability and Proliferation Assays*: Cell viability was assessed by measuring bioreduction of a MTS tetrazolium salt at 490 nm using the Cell Titer 96 aqueous assay (Promega). Cell proliferation of BHK:rGcgr cells was determined using a BrdU proliferation ELISA kit (Roche).

*cAMP measurement*: Measurement of total cAMP was carried out using a radioimmunoassay kit from Biomedical Technologies, Inc.

*Primary hepatocyte isolation, culture and adenoviral infection*: Male C57BL/6 or Gcgr-/– mice (8-12 weeks old) were anesthetized with isoflurane/oxygen and hepatocytes were isolated by retrograde, non-recirculating *in situ* collagenase liver perfusion. Cell viability assessed with trypan blue was consistently greater than 90 %. Hepatocytes were plated at a density of 40,000-50,000 cells/cm$^2$ and allowed to attach for at least 3 hours before replacement of media with William’s E medium lacking serum and insulin and the indicated reagents for 4-5 hours.

*RNA preparation and quantitative real-time RT-PCR*: Total RNA was prepared using Tri-Reagent (Sigma-Aldrich). First-strand cDNA was synthesized using the Superscript II synthesis system (Invitrogen) and random hexamers. Real-time PCR analysis was performed using TaqMan® Gene Expression Assays and TaqMan® Universal PCR master mix (Applied Biosystems) using the ABI prism 7900 Sequence Detection System. The primers used were Mm00433546_m1 for the mouse glucagon receptor and Hs99999901_s1 for 18S (Applied Biosystems).

*Adenoviral transduction*: Adenoviruses carrying the rat glucagon receptor (rGcgr) or LacZ gene were constructed in the laboratory of Chris Rhodes. Transduction of hepatocyte cultures was performed in
William’s E media without serum or insulin at an MOI of 1,500 for 12-14 hours. Media was then removed and replaced with serum-free medium containing the indicated reagents for 5-6 hours.

**SDS-Polyacrylamide Gel Electrophoresis and Western blot analysis:** Following SDS-Polyacrylamide electrophoresis, proteins were electrotransferred onto Hybond-C nitrocellulose membrane (Amersham). Blots were incubated with primary antibody overnight at room temperature. Proteins were detected with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence kit (Amersham). Primary antibodies were utilized at the following dilutions: cleaved caspase-3, total Akt, phospho-Akt (Ser 473), Bcl-2, Bcl-xl, 

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<tr>
<th>Protein</th>
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<tr>
<td>cleaved caspase-3</td>
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<tr>
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<td>actin (Sigma-Aldrich)</td>
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**Quantification of apoptosis in mouse hepatocyte cultures:** Apoptosis was assessed using the Cell Death Detection ELISA PLUS (Roche) through determination of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes). Alternatively, cells were fixed in 4 % paraformaldehyde, nuclei stained with 20 ng/ml 4’-6-Diamidino-2-phenylindole (DAPI) in PBS and scored as apoptotic or healthy according to morphological criteria. A minimum of 100 nuclei was counted from 4 fields within each treatment. Images were recorded using a Leica DM 1RB microscope, DC 300F camera and Leica IM5000 software (version 1.2; Leica Microsystems).

**Caspase activity assay:** Caspase enzymatic activity was assessed by cleavage of fluorogenic substrates (7-amino-4-methylcoumarin) with the specificity of IETD (caspase-8&10) or DEVD (caspase-3&7) (Biomol International). Hepatocytes were lysed at 4 °C in 50 mM Hepes, pH 7.4, 75 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A and 100 KIU/ml aprotinin (Trasylol, Bayer), centrifuged at 4 °C and the supernatant recovered. Enzymatic assays were carried out at room temperature, using 50 µg of cell lysate and 100 µM of fluorogenic substrate in 100 mM Hepes, pH 7.4, 150 mM NaCl, 0.2 % CHAPS, 20 mM dithiothreitol and 20 % glycerol. Caspase catalyzed release of the fluorophore 7-amino-4-methylcoumarin was monitored by fluorometric analysis (SpectraMax Gemini) with an excitation of 380 nm and emission at 460 nm.

**Animal experiments:** All animal experiments were approved by the Toronto General Hospital Animal Care Committee. Male Gcgr−/− mice, 8-12 weeks of age in the C57BL/6 background and littermate controls (Gcgr+/+) were assessed for susceptibility to hepatocyte apoptosis using 10 µg of Jo2 administered by intraperitoneal injection. Mice were euthanized either after 4 hours or if they showed signs of clinical compromise according to Animal Care Guidelines. For analysis of glucagon action in vivo, C57BL/6 male mice (8-9 week old) were purchased from Charles River Laboratories, allowed to acclimatize for one week, then
injected subcutaneously with either 30 ng/g body weight glucagon in 10 % gelatin or gelatin alone. After 30 minutes mice were injected with either 20 µg Jo2 or PBS and euthanized after 6 hours. For experiments employing specific diets, male mice were fed a diet either deficient in methionine and choline (MCD) (A02082002B) or a control diet (A02082003B) from Research Diets Inc. For adenoviral transduction in mice, adenovirus (1 x 10⁹ plaque forming units) encoding the rGcgr or LacZ genes was administered by tail vein injection to 8-12 week old Gcgr-/- mice. Five days later mice were treated with either 10 µg of Jo2 or PBS and euthanized after 4 hours.

**Liver histology:** Histopathological evaluation of liver sections for quantification of apoptosis was performed as described in a blinded manner using the following scoring system; 0 – normal (no apoptosis), 1 - minimal apoptosis (rare/occasional apoptotic bodies < 5%), 2 -mild apoptosis (up to 25% positivity), 3 - moderate apoptosis (up to 75% positivity), 4 - severe apoptosis (75-100% positivity). For lipid oil red O staining, hepatic steatosis was scored as, 0: 0 %, 1: 0-33 %, 2: 33-67 %, 3: 67-100 %. Quantification of cleaved caspase-3 and steatosis in liver sections was carried out using the Scanscope® slide scanning system and Aperio Positive Pixel Count Algorithm (Aperio Technologies, Inc.).

**Statistical analysis:** The statistical significance of differences between Gcgr+/+ and Gcgr-/- mice administered Jo2 was analyzed by logrank or by Mann-Witney tests. Otherwise, data were analyzed by t-test or using analysis of variance where appropriate, with group comparisons done using Bonferroni multiple comparison post-test.
Results

Mouse hepatocytes exhibited a dose-dependent increase in levels of cAMP in response to glucagon with an EC₅₀ of between 0.13-0.8 nM (Fig. S1). Activation of the Fas pathway with the Jo2 antibody produced characteristic morphological signs of apoptosis, including blebbing, chromatin condensation and cell lysis (Fig. 1A, panel b); the morphological features of apoptosis were markedly attenuated following treatment with 2 nM glucagon or 20 µM forskolin (Fig. 1A, panels c-d, respectively). Similarly, glucagon significantly reduced (5-7-fold) the abundance of cytoplasmic mono and oligonucleosomes (Fig. 1B) and both glucagon and forskolin markedly reduced levels of cleaved caspase-3 (Figure 1C) and caspase-3-like DEVD hydrolase activity following Jo2-induced apoptosis (Fig. 1D). Glucagon also reduced hepatocyte apoptosis following exposure to TNF-α plus actinomycin D (Fig. S2).

The anti-apoptotic actions of Gcgr signaling were also detected in BHK fibroblasts transfected with the rat glucagon receptor (BHK:rGcgr) that exhibited a dose-dependent increase cAMP accumulation in response to glucagon (Fig. S3A). Cycloheximide (CHX) reduced BHK cell viability however 20 nM glucagon or 20 µM forskolin increased cell viability in CHX-treated cells (Fig. 2A). Furthermore, both glucagon and forskolin reduced levels of cleaved caspase-3, and attenuated reductions in levels of the executioner caspase substrates β-catenin and Akt (Fig. 2B). The preservation of cell viability was not attributable to increased cell proliferation (Fig. S3B). Although both glucagon and forskolin stimulate cAMP accumulation and activate protein kinase A (PKA)¹, the PKA inhibitor H-89 did not diminish the cytoprotective actions of glucagon or forskolin in BHK:rGcgr cells (Fig. 2C). Hence the Gcgr engages anti-apoptotic signaling pathways in a PKA-independent manner.

We next examined glucagon action in murine hepatocytes. Glucagon stimulated the phosphorylation of CREB and Akt, which was inhibited by the protein kinase inhibitors H-89 and LY294002, consistent with activation of the PKA and PI-3K pathways respectively. In contrast, glucagon reduced MAPK phosphorylation, which was further reduced by the MEK 1/2 inhibitor U0126 (Fig. 3A). Remarkably, inhibition of PKA, PI-3K or MEK 1/2 pathways did not interfere with the ability of glucagon or forskolin to attenuate Jo2-induced caspase-3 cleavage (Fig. 3B) or enhance cell survival (Fig. S4A). Furthermore, the ability of glucagon and forskolin to phosphorylate Akt and CREB was preserved in the presence of Jo2 (Fig. S4B).

As glucagon enhances cAMP formation yet exerts cytoprotective effects in a PKA-independent manner, we hypothesized that glucagon regulates apoptosis through activation of Epac, a family of cAMP-regulated guanine nucleotide exchange factors that act independently of PKA.⁵ Consistent with this possibility, the Epac agonist CPT-Me-cAMP mimicked the effects of glucagon, leading to reduced caspase-3 cleavage (Fig. 3C) and significantly (p < 0.001) decreased caspase-3-like DEVD hydrolase activity (Fig. 3D) in mouse hepatocytes following Jo2-induced apoptosis.

Activation of the Fas receptor leads to recruitment of an adaptor molecule FADD (Fas-associated protein with death domain) that aids in Fas receptor activation and recruits and binds caspase-8, forming the DISC (death-
inducing signaling complex)\textsuperscript{16}. As cAMP may inhibit DISC formation \textsuperscript{17} we assessed whether glucagon interferes with the ability of the DISC to promote caspase-8 activation. Caspase-8 activity was increased in hepatocytes treated with Jo2 and CHX however co-incubation with glucagon, forskolin or CPT-Me-cAMP attenuated caspase-8 activation (Fig. 3 E). In contrast glucagon had no effect on levels of the anti-apoptotic effectors Bcl-xL or Bcl-2 (Fig. S4C). Hence glucagon, likely acting via cAMP and Epac, interferes with Fas-induced apoptosis at a proximal level in Fas-DISC signaling.

To determine the importance of endogenous Gcgr signaling for hepatocyte survival we assessed susceptibility to Jo2-induced liver injury in Gcgr-/- mice. Jo2 produced more rapid morbidity and increased mortality in Gcgr-/- compared to Gcgr+/- mice ($p < 0.05$, Fig. 4A). Furthermore Gcgr-/- mice exhibited a significantly greater median liver apoptotic score (Fig. 4 B, $p < 0.05$). A greater proportion of Gcgr-/- mice had elevated serum levels of transaminases, enzymes released following liver injury, compared to Gcgr+/-, with 64% of Gcgr-/- vs. 33% of Gcgr+/- mice exhibiting AST levels greater than 600 U/L. Similarly, 73% of Gcgr-/- vs. 44% of Gcgr+/- mice had ALT levels greater than 100 U/L. Immunohistochemical analyses demonstrated more extensive cleaved caspase 3-immunopositivity in Gcgr-/- hepatocytes after Jo2 treatment (Fig. 4 C,D). These findings indicate that Gcgr-/- mice exhibit enhanced susceptibility to Jo2-induced liver injury.

To address whether the enhanced sensitivity of Gcgr-/- mice to liver injury reflects a direct role for the Gcgr in engagement of cell survival pathways, we re-introduced the Gcgr by viral transduction into Gcgr-/- hepatocytes. Gcgr-/- hepatocytes exhibited no cAMP accumulation in response to glucagon but retained responsiveness to forskolin (Fig. S5A). Despite increased basal levels of cAMP \textsuperscript{2}, Gcgr-/- hepatocytes were equally susceptible to Jo2-induced apoptosis (35-50% of both Gcgr+/- and Gcgr-/- hepatocytes exhibited morphological features of apoptosis) and forskolin, but not glucagon, attenuated features of apoptosis in Gcgr-/- hepatocytes (Fig. 5 A, panels a-d). Furthermore forskolin, but not glucagon, significantly reduced the abundance of cytoplasmic mono and oligonucleosomes and decreased levels of cleaved caspase-3 in Jo2-treated Gcgr-/- hepatocytes (Fig. 5 B,C). Adenoviral transduction of the rat Gcgr (rGcgr) restored glucagon-responsive cAMP production (EC\textsubscript{50} = 0.19 nM) and the anti-apoptotic actions of glucagon in Gcgr-/- hepatocytes (Fig. S5B and Fig. 5D).

To ascertain whether partial restoration of hepatic Gcgr expression would mitigate the extent of experimental hepatic injury \textit{in vivo}, we administered Ad-rGcgr to Gcgr-/- mice via intravenous infusion. Hepatic Gcgr expression in Gcgr-/- mice (Fig. S6A) was associated with a significant reduction in plasma glucagon levels and a significant increase in ambient plasma glucose compared to Ad-LacZ transduced mice ($p < 0.001$, Fig. 6 A and $p < 0.01$, Fig. S6B respectively). Furthermore, Gcgr-/- mice transduced with the Ad-rGcgr exhibited significantly reduced hepatic injury following Jo2 administration \textit{in vivo} ($p < 0.01$) (Fig. 6 B). Moreover, a significant reduction in the number of hepatocytes exhibiting immunopositivity for cleaved caspase-3 after Jo2 administration was observed in Ad-rGcgr vs. Ad-LacZ-transduced Gcgr-/- mice (Fig. 6 C). Exogenous glucagon also increased hepatocyte survival following liver injury in wild-type mice. Glucagon administration significantly ($p < 0.05$) lowered the median hepatic apoptosis score (Fig. 6 D) and fewer glucagon-treated mice exhibited elevated levels (>300 U/L) of serum AST (12.5% vs. 50%) and ALT (25% vs. 50%) compared to
vehicle-treated mice. Furthermore, glucagon markedly reduced the extent of cleaved caspase-3 immunopositivity in liver (Fig. 6 E p< 0.05).

We next examined the susceptibility of Gcgr-/- mice to diet-induced liver injury. Gcgr+/+ and Gcgr-/- mice were fed a methionine and choline-deficient (MCD) diet known to produce experimental liver injury with histopathological abnormalities characteristic of non-alcoholic steatohepatitis 18. Control Gcgr+/+ and Gcgr-/− mice received the identical diet supplemented with methionine and choline. Histological examination of liver tissue showed a significantly greater accumulation of lipid in Gcgr-/- mice (Fig. 7A, panel a & b and Fig. 7B, p < 0.05) despite similar levels of food intake, and comparable changes in body weight in both groups of mice (Fig. S7A, B). Furthermore, significantly greater levels of lipid accumulation were observed in Gcgr-/- liver (Fig. 7B, C). Consistent with the greater degree of liver injury (Fig 7 A panels c-e), plasma glucose was lower in Gcgr-/- mice (Fig. S7C). Furthermore, histological features of apoptosis were more evident in Gcgr-/- mice following 6 weeks on the MCD diet (Fig. 7 A, panel c & d, quantified in Fig. 7 D, p < 0.05). Taken together, these findings demonstrate that loss of Gcgr signaling increases hepatocyte susceptibility to liver injury.
Discussion

Signaling through class B G protein-coupled receptors (GPCRs) is known to promote cell survival. For example, GLP-2 directly reduces apoptosis in cells and in rodents with experimental intestinal injury. Similarly, GLP-1 receptor activation reduced cell death in rodent insulinomas and in rodent and human pancreatic islet β-cells. Conversely, Glp1r-/β-cells display enhanced susceptibility to apoptotic injury. Our observations extend these concepts by establishing that endogenous Gcgr signaling plays an essential role in the control of hepatocyte survival.

A potential pathway linking PGDP receptor activation to control of cell survival is via cAMP as cAMP levels influence cell survival in diverse cell types including hepatocytes. The observation that glucagon, CPT-Me-cAMP and forskolin enhance cell survival in murine hepatocytes is consistent with accumulating evidence invoking a critical role for cAMP as a key determinant of hepatocyte viability. Although activation of PKA, PI-3K, and Erk1/2 MAPK pathways has been linked to enhanced hepatocyte survival, the effects of glucagon to reduce Jo-2-induced hepatocyte apoptosis were independent of these signaling pathways. Accordingly, our data are consistent with a role for Epac as a potential downstream mediator of cAMP-dependent, PKA-independent regulation of hepatocyte survival.

Several studies have identified a role for cAMP in modulating FasL/CD95 signaling in hepatocytes, resulting in the attenuation of cell death. Following FasL binding to CD95, an adaptor molecule FADD (Fas-associated protein with death domain) is recruited and activates the Fas receptor, leading to subsequent recruitment of caspase-8 to form the DISC. The assembly of the DISC is critical for further downstream apoptotic signaling. Our experiments provide new mechanistic understanding of how the Gcgr enhances hepatocyte viability by demonstrating that glucagon and the Epac agonist (CPT-Me-cAMP) inhibit caspase-3 and caspase-8 activity in injured hepatocytes. These findings suggest that attenuation of Fas-induced apoptosis by glucagon and the Epac agonist likely occurs at the level of the formation of the DISC.

Our data demonstrating that Gcgr−/− mice exhibit enhanced susceptibility to diet-induced liver injury is consistent with observations linking diet-induced hepatic steatosis with reduced Gcgr expression and decreased sensitivity to glucagon in vivo. Moreover glucagon directly reduces hepatocyte and fibroblast apoptosis in vitro, strongly implicating the Gcgr as a direct modulator of apoptosis. The demonstration that Gcgr−/− mice are more sensitive to the development of steatohepatitis and FasL-induced hepatocyte apoptosis may have implications for strategies directed at interruption of glucagon action for the treatment of type 2 diabetes. The improvement in glucose control mediated by reduced glucagon action may theoretically be accompanied, in vulnerable diabetic subjects, by an increased susceptibility to liver injury. Our studies imply that a threshold level of hepatocyte Gcgr signaling may be optimal for hepatocellular survival. Hence a more detailed understanding of the relationship between Gcgr signaling and hepatocyte survival under diverse metabolic circumstances seems warranted.


Figure Legends:

Figure 1 Glucagon and forskolin protect primary mouse hepatocytes from apoptosis (A) assessment of nuclear morphology in DAPI-stained mouse hepatocytes following treatment for 4-5 hours with 10 µM CHX (a) or with 10 µM CHX plus 100 ng/ml Jo2 in the absence (b) or presence of 2 nM glucagon (c) or 20 µM forskolin (d). Arrowheads in panel b indicate cells undergoing apoptosis. Magnification in a-d x 400 (B) mouse hepatocytes were exposed to 10 µM CHX plus 100 ng/ml Jo2 in the absence or presence of glucagon. After 4-5 hours apoptosis was quantified using the Cell Death ELISA kit. Data are expressed as fold-increase relative to CHX-alone-treated cultures and are mean ± SD from 2 independent experiments. ** p < 0.01, *** p < 0.001, CHX and Jo2 plus glucagon vs. CHX plus Jo2 alone. Cell extracts from primary mouse hepatocytes treated with CHX alone or CHX plus Jo2 with or without glucagon or forskolin for 4-5 hours were analyzed by Western blotting for cleaved caspase-3 (C) or for DEVD hydrolase activity (D). Results are representative of four independent experiments. DEVDase activity data are mean ± SD and represent three independent experiments each performed in duplicate. *** p < 0.001, CHX plus Jo2 and glucagon or forskolin vs. CHX and Jo2 alone.

Figure 2 Glucagon and forskolin protect BHK-rGcgr cells from CHX-induced cytotoxicity. (A) cells were treated with 80 µM CHX in the presence or absence of 20 nM glucagon or 20 µM forskolin and cell viability was measured by a tetrazolium salt bioreduction assay and expressed as a percentage of the cell viability at time zero. Data are the means ± SEM from four independent experiments * p < 0.05, ** p < 0.01, CHX vs. CHX plus glucagon or forskolin. (B) cultures were treated with vehicle alone or with 80 µM CHX with or without 20 nM glucagon or 20 µM forskolin for 16 hours. Whole cell extracts were analyzed by immunoblotting for cleaved caspase-3, intact β-catenin and total Akt and results are representative of 2 independent experiments. (C) cells were treated with 10 µM H-89 or vehicle 30 minutes prior to and during a 16 hour incubation with 80 µM CHX with or without 20 nM glucagon or 20 µM forskolin. Cell viability was determined as in A. Data are the means ± SD of four independent experiments, ** p < 0.01, *** p < 0.001, CHX plus glucagon or forskolin vs. CHX alone, # p<0.05, vs. H-89.

Figure 3 Glucagon, forskolin and the Epac agonist CPT-Me-cAMP protect primary mouse hepatocytes from Jo2-induced apoptosis. (A) hepatocyte cultures were serum starved for 4-5 hours before pre-incubating with 10 µM H-89, 50 µM LY294002, 20 µM U0126, or vehicle alone for 30 min. Cells were then stimulated for 10 minutes with either vehicle or 20 nM glucagon, whereupon cell extracts were analyzed by immunoblotting with phospho-specific antibodies for CREB (H-89-treated cultures), Akt (LY294002-treated cultures), and Erk1/2 MAPK (U0126-treated cultures). Results are representative of 2-3 independent experiments. (B&C) Hepatocytes were pre-incubated with protein kinase inhibitors for 30 min prior to and during a 4-5 hour
treatment with 10 µM CHX or 10 µM CHX plus 100 ng/ml Jo2 with or without 2 nM glucagon or 20 µM forskolin. Apoptosis was quantified using the Cell Death ELISA kit (B) or immunoblotting for cleaved caspase-3. (C) Data are expressed as fold increase relative to CHX-alone treated cultures and are mean ±SEM from 3 independent experiments, each performed in duplicate, * p<0.05, **p<0.01, ***p<0.001. Results are representative of 3 independent experiments. DEVDase (D) and IETDase (E) enzymatic activity was assessed in hepatocytes treated for 4-5 hours with 10 µM CHX or 10 µM CHX plus 100 ng/ml Jo2 alone or with µM CPT- Me-cAMP (D&E) or glucagon or forskolin (E). Data are means ± SD and are representative of 2-3 independent experiments carried out in duplicate, *** p < 0.001, CHX plus Jo2 vs. CHX plus Jo2 plus either glucagon, forskolin or CPT-Me-cAMP.

Figure 4 Gcgr-/- mice exhibit enhanced susceptibility to Jo2-induced liver apoptosis. (A) Gcgr-/- and Gcgr+/+ male mice were injected with 10 µg Jo2 I.P and closely monitored for signs of clinical compromise whereupon they were euthanized, p < 0.05, Gcgr/-/- vs. Gcgr+/+, n=17 per group. (B-C) mice were injected I.P with 10 µg Jo2 for 4 hours whereupon liver tissue samples were taken for analysis, n = 11 per genotype. (B) individual hepatic apoptosis scores assessed following H&E staining of liver tissue sections. In B, the horizontal line indicates the median. (C) immunohistochemical detection of cleaved caspase-3 in liver sections from mice following Jo2 challenge (magnification x 100). (D) quantification of cleaved caspase-3 staining from liver sections of Gcgr+/+ and Gcgr-/- mice.

Figure 5 Expression of the rat glucagon receptor (rGcgr) in Gcgr-/- hepatocytes restores the cytoprotective effect of glucagon. (A) primary hepatocytes from Gcgr-/- mice were incubated for 4-5 hours with 10 µM CHX (a), 10 µM CHX plus 100 ng/ml Jo2, in the absence (b) or presence of 2 nM glucagon (c) or 20 µM forskolin (d) and stained with DAPI. Arrowheads indicate cells undergoing apoptosis. (B) apoptosis was quantified by Cell Death ELISA in cells treated for 4-5 hours as indicated in panel A, except that the glucagon concentration was 20 nM. Data are mean ± SD and are representative of 2 independent experiments carried out in duplicate, * p < 0.05, CHX-alone vs. CHX plus Jo2 in the presence or absence of glucagon or forskolin. (C) Western blot analysis of cell extracts from Gcgr-/- hepatocytes treated with 10 µM CHX or 10 µM CHX plus Jo2 in the absence or presence of either glucagon or forskolin as indicated. Blots are representative of 3 independent experiments. (D) Isolated Gcgr-/- mouse hepatocytes were transduced with a rGcgr adenovirus (1,500 MOI) for 12-14 hours in serum-free conditions. Cells were then treated with 10 µM CHX and Jo2 in the absence or presence of glucagon. Cell extracts were prepared after 5-6 hours for immunoblot analysis. Blots are representative of 2 independent experiments.

Figure 6 Adenoviral rescue of the glucagon receptor in Gcgr-/- mice and exogenous glucagon administration in wild-type mice attenuates Jo2-induced apoptosis. (A) fed-state levels of plasma glucagon in Gcgr-/- mice 5 days after i.v. administration of 1 x10⁹ pfu of either Ad-LacZ, or Ad-rGcgr; glucagon levels in non-transduced
Gcgr+/+ mice are also shown. Data are mean ± S.E.M., n = 6-8, *** p < 0.001, Ad-LacZ vs. Ad-Gcgr-transduced Gcgr-/- mice or Gcgr+/+ mice. (B-D) 5 days following administration of adenovirus as indicated in A, Gcgr-/- mice were injected with 10 µg Jo2 (n = 6-8 mice per group). After 4 hours liver samples were taken for analysis. (B) individual hepatic apoptosis scores were assessed as per criteria stated in Materials and Methods. The horizontal line indicates the median. (C) immunohistochemical and quantitative detection of cleaved caspase-3 in liver sections from either Ad-LacZ- or Ad-rGcgr-infected Gcgr-/- mice after Jo2 administration (magnification x 50). (D) wild-type male mice were injected s.c. with 30 ng/g body weight of glucagon or vehicle 30 min prior to i.p. injection of 20 µg Jo2 (n = 8 mice per group). After 6 hours liver samples were taken for analysis. Individual hepatic apoptosis scores were assessed following H&E staining of liver tissue sections. The horizontal line indicates the median. (E) Immunohistochemical and quantitative detection of cleaved caspase-3 in liver sections from vehicle or glucagon-treated C57BL/6 mice (magnification x 100).

Figure 7  Gcgr-/- mice are more susceptible to the development of steatohepatitis. (A) oil red O (a and b, magnification x 100) and H&E (c and d, magnification x 200) staining of liver from Gcgr-/- (a and c) and Gcgr+/+ (b and d) mice following 3 weeks (oil red O staining) or 6 weeks (H&E) on a MCD diet. The thick arrow in panel c identifies an apoptotic cell and the thin arrow designates an area of inflammation with an apoptotic cell in the centre. A higher power image of the apoptotic cell in panel c is shown in panel e. Individual hepatic steatosis score (B), quantification of steatosis (C) and individual hepatic apoptosis scores (D) in Gcgr-/- and Gcgr+/+ mice after 6 weeks on the MCD diet. The horizontal line in (B) and (D) indicates the median.
Figure 1

A

B

C

D

Jo2 (100 ng/ml) Glucagon (2 nM) Forskolin (20 µM)

Cleaved Caspase-3

Actin

DEVDase Activity (Fold Increase)
Figure 2

A

[Graph showing cell viability (% control) over time (hours) with different treatments: CHX (80 µM), Glucagon (20 nM), Forskolin (20 µM), CHX + GLU, CHX + FSK.]

B

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[Western blot images for Cleaved Caspase-3, Intact β-Catenin, Akt, and Actin.

C

[Bar graph showing cell viability (% control) with different treatments: Vehicle, H-89.]

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Figure 3

A

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B

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C

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D

Graph showing the effect of various conditions on DEK cleavage activity.

E

Graph showing the effect of various conditions on IET cleavage activity.
Figure 5

A

B

C

D

Jo2 (100 ng/ml) Glucagon (2 nM) Glucagon (5 nM) Glucagon (10 nM)

Cleaved caspase-3

HSP90

Jo2 (100 ng/ml) Glucagon (2 nM) Forskolin (20 µM) Glucagon (20 nM)

Cleaved caspase-3

HSP90
Figure 6

A. Plasma Glucagon (pM) comparison between Ad-LacZ, Ad-rGcgr, and Gcgr+/+.

B. Liver Score comparison between Ad-LacZ and Ad-rGcgr.

C. Immunohistochemical staining for cleaved caspase-3 positivity in Ad-LacZ and Ad-rGcgr groups.

D. Liver Score comparison between Vehicle and Glucagon treatment.

E. Immunohistochemical staining for cleaved caspase-3 positivity in Vehicle and Glucagon treatment.
Figure 7

(A) 3 weeks MCDD

Gcgr -/-  Gcgr +/+  

6 weeks MCDD

(B) Liver Steatosis Score

(Gcgr +/+  Gcgr -/-)

(C) Steatosis Positivity

(Gcgr +/+  Gcgr -/-)

(D) Liver Apoptosis Score

(Gcgr +/+  Gcgr -/-)