## Supplemental Information

Circulating Glucagon 1-61 Regulates

Blood Glucose by Increasing Insulin Secretion

## and Hepatic Glucose Production

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Supplementary Figures

Supplementary Figure 1: Shows the relative abundancies of glucagon and N-terminally elongated glucagon

isoforms. The raw data is uploaded to the Pride database. Related to Figure 1.

Supplementary Figure 2: A: Db/db and Ob/Ob mice were obtained from Janvier (db/db strain: BKS(D)Leprdb/JorlRJ) (ob/ob strain:B6.V-LepOb/JRj). Administration (time zero) of 1pmol PG 1-61 (n=5) (black, square) or saline (n=5) (red circle) to db/db mice (12 weeks of age), and administration (time zero) of 1pmol PG 1-61 (n=3) (black, triangle) or saline (n=5) (red, triangle) to ob/ob mice (14-15 weeks of age). Mean±SD. Related to Figure 4. **B:** Glucagon receptor expression data by qPCR related to the experiments shown in Figure 5.
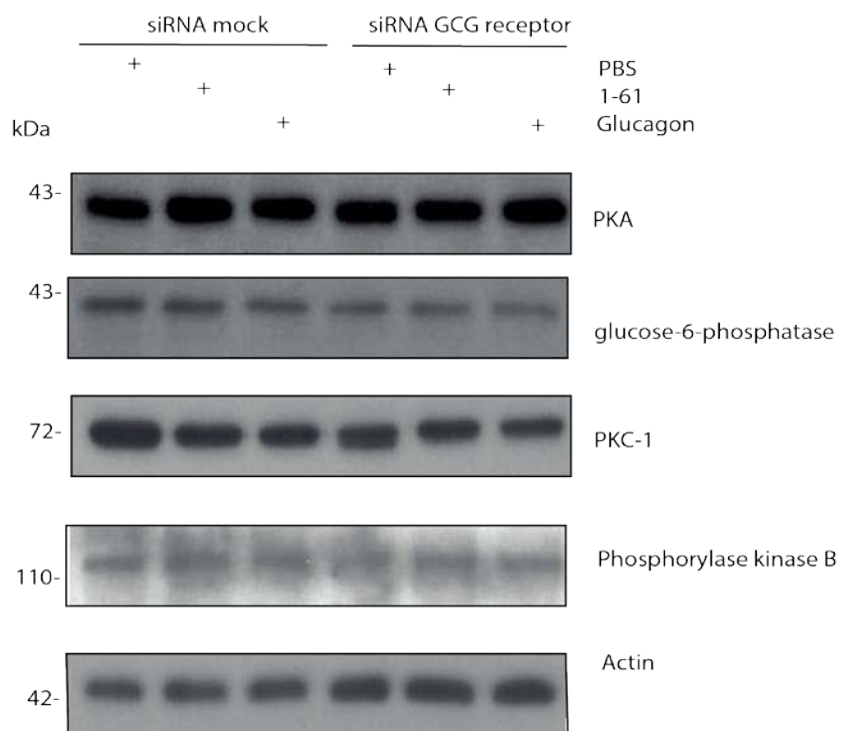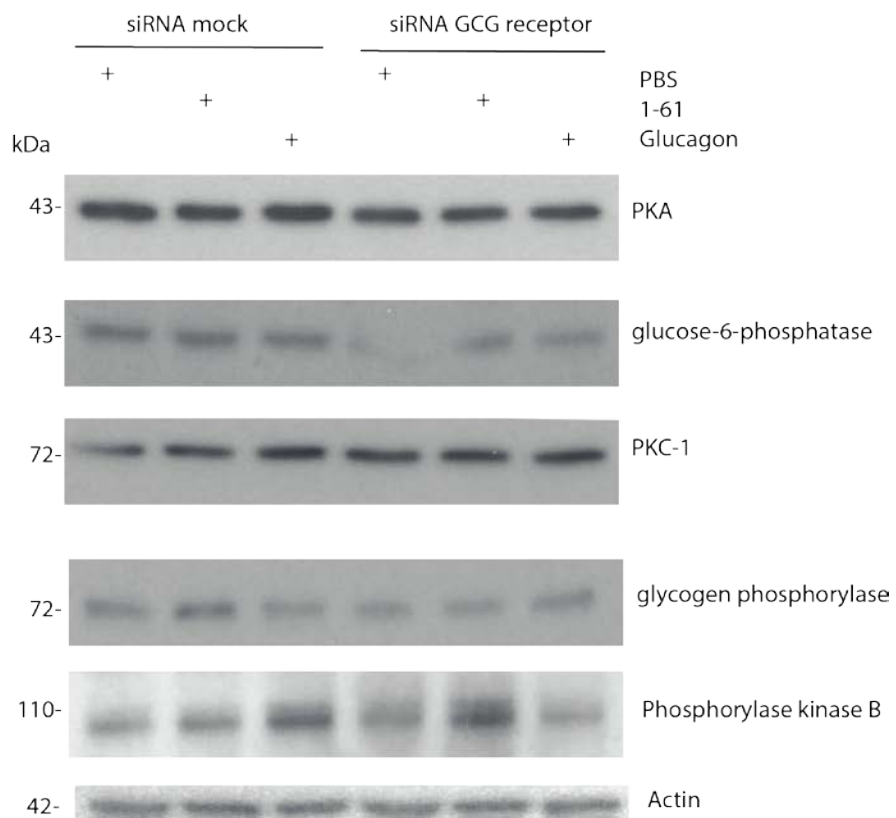
A
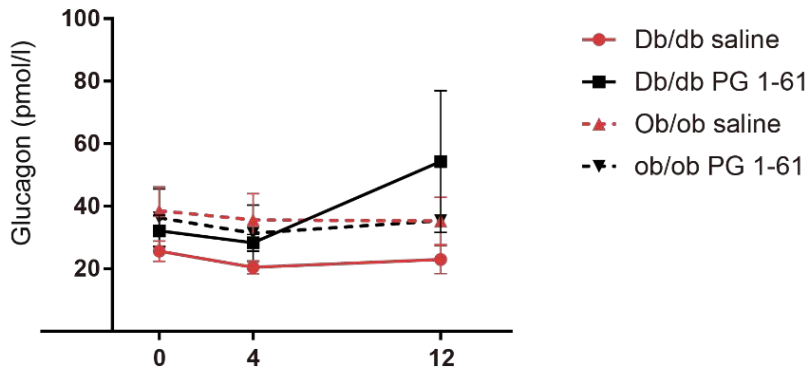
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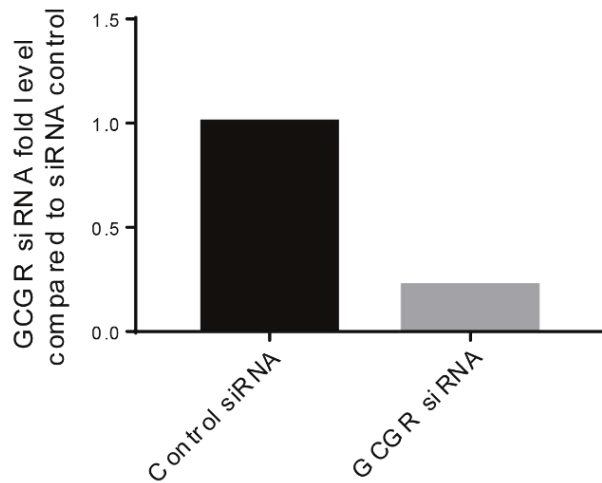
Supplementary Figure 3: A illustrates 30min, and **B** 3 hours' incubation, with PG 1-61 or glucagon in siRNA mock treated or siRNA GCG receptor treated cells. Protein levels of several enzymes are shown: Protein kinase A (PKA), Glucose-6-phosphate (G6P), Protein Kinase C, glycogen phosphorylase, Phosphorylase B. Actin is shown as control. Related to Figure 5.
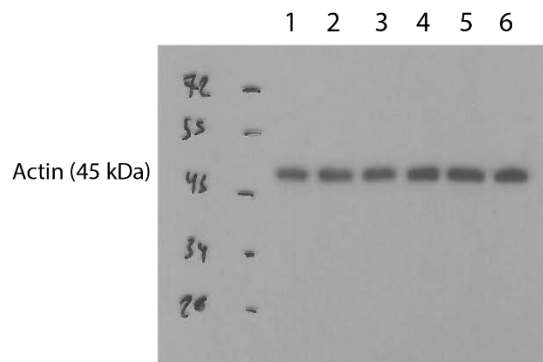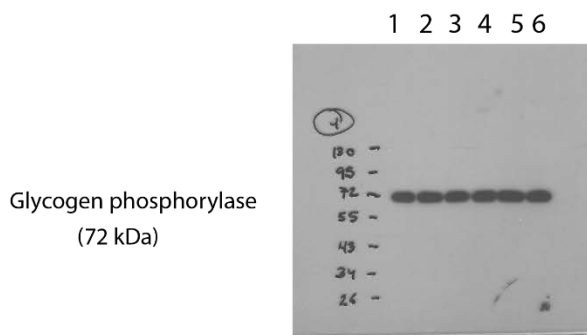
A

siRNA mock   siRNA GCG receptor

+			+			PBS
	+			+		1-61
		+			+	Glucagon

kDa

43- — PKA

43- — glucose-6-phosphatase

72- — PKC-1

110- — Phosphorylase kinase B

42- — Actin

B

siRNA mock   siRNA GCG receptor

+			+			PBS
	+			+		1-61
		+			+	Glucagon

kDa

43- — PKA

43- — glucose-6-phosphatase

72- — PKC-1

72- — glycogen phosphorylase

110- — Phosphorylase kinase B

42- — Actin

Supplementary Figure 4: illustrates 30min incubation, with PG 1-61 or glucagon in siRNA mock treated or siRNA GCG receptor treated cells. Compared to Supplementary Figure 3, these are the uncropped blots. They are numbered 1-6. 1-3 is mock treated control, 4-6 is siRNA GCG receptor. 1 and 4 is PBS, 2 and 5 is 1-61, and 3 and 6 is glucagon. Protein levels of several enzymes are shown: Protein kinase A (PKA), Glucose-6-phosphate (G6P), Protein Kinase C, glycogen phosphorylase, Phosphorylase B. Actin is shown as control. Related to Figure 5.
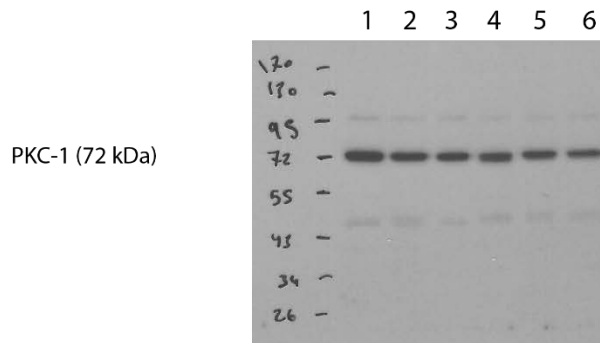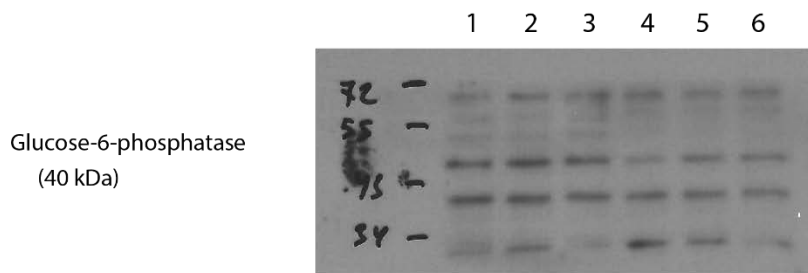
30 min stimulation (TF-627)

1 2 3 4 5 6

PKA (43 kDa)

Glucose-6-phosphatase
(40 kDa)

1 2 3 4 5 6

PKC-1 (72 kDa)

1 2 3 4 5 6

Glycogen phosphorylase
(72 kDa)

1 2 3 4 5 6

Actin (45 kDa)

1 2 3 4 5 6
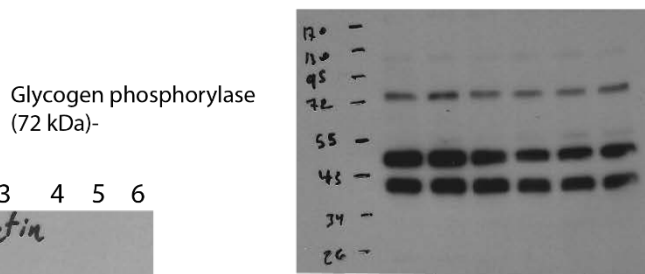
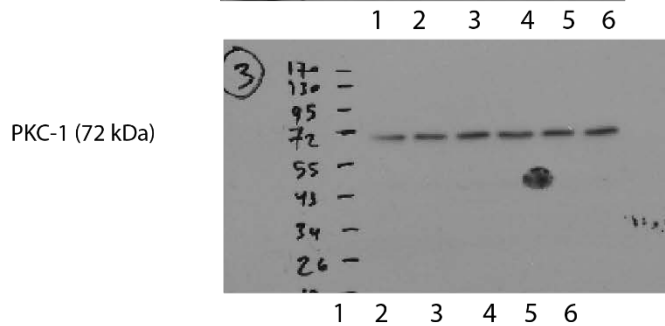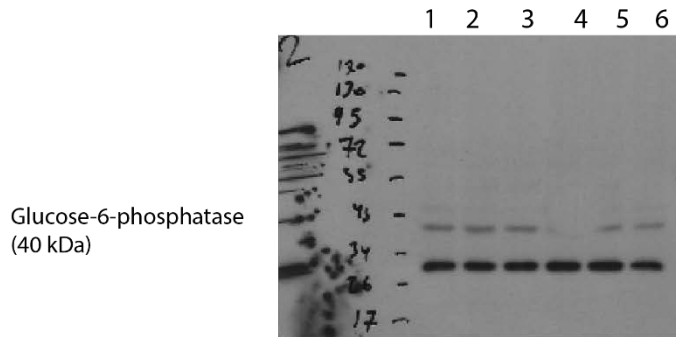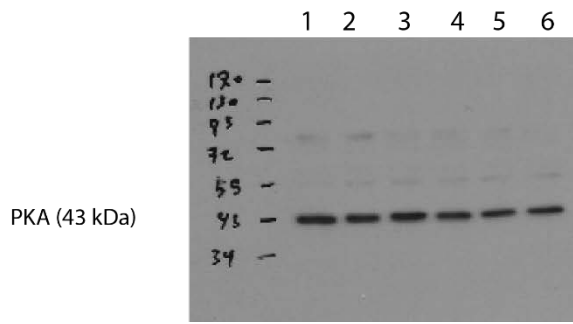Phosphorylase kinase B (102kDa)

1 2 3 4 5 6
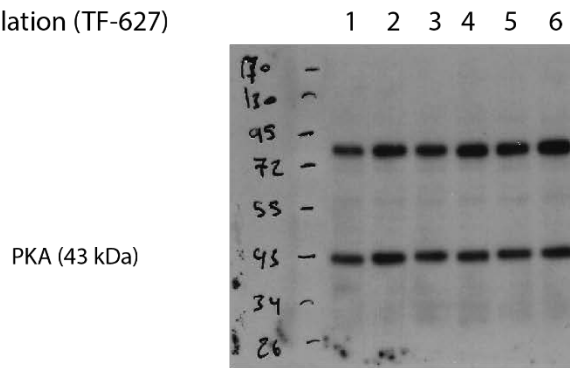
Supplementary Figure 5: illustrates 3 hours' incubation, with PG 1-61 or glucagon in siRNA mock treated or siRNA GCG receptor treated cells. Compared to Supplementary Figure 3, these are the uncropped blots. They are numbered 1-6. 1-3 is mock treated control, 4-6 is siRNA GCG receptor. 1 and 4 is PBS, 2 and 5 is 1-61, and 3 and 6 is glucagon. Protein levels of several enzymes are shown: Protein kinase A (PKA), Glucose-6-phosphate (G6P), Protein Kinase C, glycogen phosphorylase, Phosphorylase B. Actin is shown as control. Related to Figure 5.
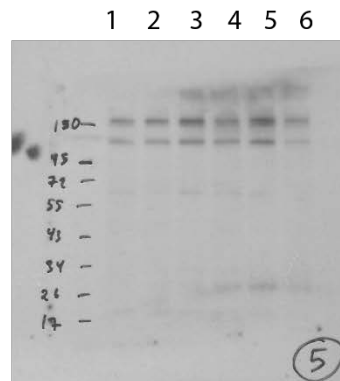
3 hrs stimulation (TF-620)

PKA (43 kDa)

Glucose-6-phosphatase
(40 kDa)

PKC-1 (72 kDa)

Glycogen phosphorylase
(72 kDa)-
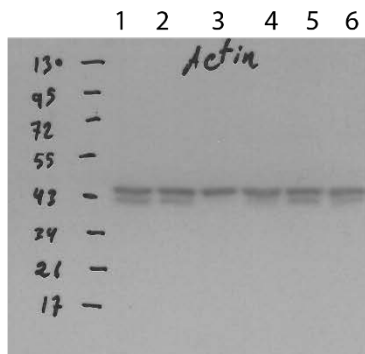
Actin (45 kDa)

Phosphorylase kinase B (102kDa)

Supplemental Experimental Procedures

Animal studies

Rats were anesthetized with Hypnorm®/midazolam (1.25 mg/mL midazolam, 2.5 mg/mL fluanisone and 0.079 mg/mL fentanyl citrate); the abdominal cavity was opened, and a needle inserted into the inferior vena cava. Basal samples were collected 5 min after insertion (-5min) and immediately prior to administration of test substance (0min). At time zero (0min), glucagon (1 pmol), PG 1-61 (1 pmol) or D-mannitol (1g/kg; negative control/osmolality control; cat no. M4125, Sigma Aldrich, Brondby, Denmark) was administered through the needle in the vena cava, and the needle was immediately flushed with 100µL saline. Next, blood (200 µL/time point) for hormone and glucose analysis was collected at times 2, 4, 6, 8, 12, and 20min, through the same needle, and transferred into EDTA-coated tubes (cat no. 200 K3E, Microvette; Sarstedt, Nümbrecht, Germany). Blood glucose was measured immediately after collection using a handheld glucometer (Accu-chek Compact plus device; Roche, Mannheim, Germany). The remaining sample was centrifuged (1,650 x g, 4°C, 10min), and plasma was transferred to fresh Eppendorf tubes and immediately frozen. Samples were stored at -20°C.

Isolated perfused rat pancreas

Male Wistar rats (~250g) were obtained from Taconic and housed two per cage under standard conditions with ad libitum access to chow and water. They were allowed to acclimatize for at least one week before being anesthetized as described above. The entire large intestine and the small intestine were removed after tying off the supplying vasculature, leaving only the proximal duodenum connected to the pancreas *in situ*. The spleen and stomach were removed and the kidneys were excluded from the circulation by tying off the renal vessels. The abdominal aorta was ligated just below the diaphragm and, immediately after, a catheter was inserted into the abdominal aorta just proximal to the renal arteries (tip pointing towards the diaphragm) to restrict perfusion to the pancreas only (through both the coeliac and the superior mesenteric artery). The pancreas was perfused (5.0 mL/min) with a modified Krebs-Ringer buffer (perfusion buffer) which had been oxygenated prior to perfusion. Venous effluent samples were collected each minute from a catheter inserted into the portal vein. Immediately after placement of catheters, the rat was euthanized by perforation of the diaphragm and the preparation allowed to stabilize for approximately 30 min before the experiment was started. Perfusion pressure and effluent flow were closely monitored as indicators of the wellbeing of the pancreas. Test stimulants were administered intravascularly through a three-way valve and consisted of PG 1-61, glucagon and L-

arginine (positive control), administered so the final concentration in the organ was 1nM (PG 1-61 and glucagon) or 10 mM (L-arginine). All stimulants were diluted in perfusion buffer without addition of solvents.

Cell culture experiments

INS-1E cells were kindly provided by Professor Jens Højriis Nielsen (University of Copenhagen, Denmark). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Cat. No. 31966-021, Gibco, Grand Island, NY 14072 USA) containing 5000U/ml Pen-Strep (Cat. No. 15140-122, Gibco) and 10 % fetal bovine serum (FBS) (Cat. No. Sv3016003, Thermo, Roskilde, Denmark) before being seeded in 24-well plates (Nunc™, Thermo Scientific) at a cell density of $4x10^4$ per well.

Primary human hepatocytes were obtained using CellStream Isolation Technology (Cat. No. HMCS10, Lot. No. HUE50-F, ThermoScientific, Naerum, Denmark) and contained a pool of single-cell isolated hepatocytes derived from 50 individual donors (25 females and 25 males). Cells were thawed and plated in Cryopreserved Hepatocyte Thawing and Plating Medium (ThermoScientific Cat.No: CM3000). Cells were allowed to plate for 5 hours (37°C, 5% CO_2), medium was removed and cells were incubated for 10 min (37°C, 5% CO_2) in William's E medium supplemented with either PBS, PG 1-61 (10-1000nM) or glucagon (100nM). Supernatants were isolated and cells were lysed using M-per Mammalian Protein Extraction Reagent (ThermoScientific cat.no: 87503) supplemented with Protease Inhibitor Cocktail, EDTA-free (ThermoScientific cat.no: 877859).

HepG2 cells are human liver carcinoma cells, derived from liver tissue of a 15-year-old male Caucasian who had a well-differentiated hepatocellular carcinoma, and were kindly provided by dr. Hanne Cathrine Bisgaard, (Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. LipofectamineTM2000 reagent (Invitrogen) was used for siRNA-induced knockdown of the human glucagon receptor (GCGR (ID 2642); Trilencer-27, OriGene Technologies, Inc, Rockville, MD 20850, USA). The siRNA universal negative control was purchased from Sigma-Aldrich, Saint Louis, MO 63103, USA. A mixture of three different siRNA against the glucagon receptor was used (final concentration of 25nM). The HepG2 cells were siRNA treated for 2 days before glucagon or PG 1-61 was added (final concentration of 10nM) to the cell cultures for 10 min. Cells were then immediately washed in cold PBS and proteins were extracted with Riba buffer (Sigma Aldrich). COS-7 fibroblast cells (CV-1 Origin with SV40 genetic material) were grown in 10% CO_2 and at 37°C in DMEM 1885 (with 10% FBS, 2mM glutamine, 180U/mL penicillin,

and 45g/mL streptomycin). The phosphate precipitation method with chloroquine addition was used for transfection of the cells as previously described (Kissow et al., 2012).

For acute stimulation protocols, we used ~80% confluent cells from different batch numbers (*n*=3). Cells were stimulated with varying concentrations of hormones ranging from 1 pM to 10 nM. After the end of the stimulation period, cell media or cell lysate were obtained and centrifuged (1,500 x g, 4°C, 5 min) to remove any cells or debris and kept at -80°C until analysis.

Receptor studies

COS-7 cells (35.000 cells/well) were seeded in 96-well plates one day after transfection with either the human glucagon receptor (hGCGR) or the glucagon-like peptide-1 receptor (hGLP-1R) cDNA. Two days after transfection with the calcium phosphate precipitation(Kissow et al., 2012), the cells were washed once with HEPES buffered saline (HBS) and incubated with HBS and 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37°C. Glucagon, PG 1-61, or GLP-1 7-36NH$_2$ were added to the cells and incubated for 30 minutes at 37°C in order to test for intrinsic receptor activity through G protein activation. The HitHunter^{TM} cAMP XS assay (an enzyme fragment complementation-based assay; DiscoveRx, Birmingham, United Kingdom) was subsequently carried out as described previously (Hansen et al., 2016). All experiments were carried out in triplicate, and repeated at least three times. Luminescence was measured using a Perkin Elmer^{TM} EnVision 2104 Multilabled reader (Skovlunde, Denmark).

Quantitative polymerase chain reaction (qPCR) and small interfering RNAs

Total RNA was extracted and isolated from cell lines and qPCR was performed with human and rat glucagon receptor primers (GeneCopoeia, Inc. Rockville, MD 20850 USA). Small interfering RNAs (siRNAs) against the human (Hep2g cell line) and the rat (INS1 cell line) glucagon receptor were obtained as SMARTpool reagents from Thermo Scientific Dharmacon® (Lafayette, LA 70605 USA), and siRNA universal negative control was from Sigma-Aldrich (Brondby, Denmark). siRNA transfection was performed using OPTI-MEM® I and Lipofectamine™ 2000 (Invitrogen). The cultured cells were transiently transfected with the plasmids or with vector control using X-treme gene 6 Transfection Reagent (Cat. No. 063365787001, Roche Applied Science, Indianapolis, IN 46250, USA) according to the manufacturer's instructions. Relative mRNA was calculated using the ΔΔCq method.

Western blot analysis

Equal amounts of protein from cell extracts were separated by reducing SDS-PAGE and transferred to polyvinylidene difluoride (Immobilon-P Membrane, Millipore, Darmstadt, Germany) or nitrocellulose membranes (Hybond ECL, GE Healthcare, Brondby, Denmark) using standard procedures. Membranes were blocked in 5% non-fat milk or bovine serum albumin (BSA, Sigma-Aldrich), incubated overnight at 4°C with the indicated primary antibodies at dilutions of 1:500–1:1000, and subsequently with the appropriate horseradish peroxidase-conjugated secondary antibodies. Blots were visualized with LAS3000 Imager (Fujifilm).

Peptides

The concentrations of synthetic glucagon 1-29 (Bachem, Bubendorf, Switzerland, Cat. No.: H-6790) and N-terminally elongated glucagon 1-61 (Caslo, Lyngby, Denmark, custom made service no. P160915-01-01) were verified by quantitative amino acid analysis (QAAA; duplicate determination) at the Department of Systems Biology, Enzyme and Protein Chemistry (Soltofts Plads, Danish Technical University, building 224, Kgs. Lyngby, Denmark), as well as by an in-house mid-region specific glucagon RIA, employing antiserum code number 4304(Orskov et al., 1991). Peptides were dissolved in phosphate buffer containing 1% human serum albumin (Calbiochem, affiliate of Merck KGaA, Cat. No. 12666, Darmstadt, Germany), and 1 μM stock solutions were prepared for each peptide.

Antibodies

Antibodies against Protein kinase A (Cat. No. ab26322), glucose-6-phosphatase (Cat. No. ab83690), Phosphorylase kinase (Cat. No. ab55620), glycogen phosphorylase (Cat. No. ab103419) and phosphoenolpyruvate carboxykinase (to estimate gluconeogenesis) (Cat. No. ab70358) were from Abcam (San Francisco, CA 94010, USA).

Gel filtration of human plasma

Pooled plasma samples (n = 8) from subjects with kidney failure were centrifuged (4°C, 4 min, 4,500g) and the supernatants fractionated by gel filtration on a Sephadex 164 G50SF-packed K16-100 column (Pharmacia, Uppsala Sweden), equilibrated and eluted with sodium phosphate buffer at 4°C. Gel filtration effluents were collected

automatically in fractions corresponding to approximately 1/50 of the fractionation volume of the column. The column was precalibrated using ^{125}I-labeled albumin (V0), ^{22}Na (Vi) and unlabeled glucagon and PG 1-61.

Mass-spectrometry-based detection of glucagon

In short, plasma fractions were digested in a time course (0,30 min, 60 min and overnight) with either LysC, GluC or Chymotrypsin. Afterwards, peptides were desalted and separated on a Thermo Scientific EASY-nLC 1000 HPLC system (Thermo Fisher Scientific, Odense, Denmark). Columns (75 µm inner diameter, 20 cm length) were packed in-house with 1.9 µm C18 particles (Dr. Maisch GmbH, Germany). Peptides were loaded in buffer A (0.5% formic acid) and separated with a gradient from 5% to 30% buffer B (80% acetonitrile, 0.5% formic acid) over 90 min at a flow rate of 200 nl/min. The column temperature was set to 50ºC. The liquid chromatography was directly coupled to a quadrupole Orbitrap mass spectrometer (Olsen et al., 2005) (LTQ Orbitrap, Thermo Fisher Scientific) via a nano electrospray source. The survey scan range was set to 300 to 1,650 m/z, with a resolution of 60,000. Up to 5 of the most abundant isotope patterns with a charge ≥ 2 were subjected to linear ion trap fragmentation (Olsen et al., 2005) at a normalized collision energy of 35. Dynamic exclusion of sequenced peptides was set to 30 s. Thresholds for ion injection time and ion target values were set to 1s and 1*E6 for the survey scans and 250 ms and a minimum signal of 500 required for the MS/MS scans, respectively. Data were acquired using the Xcalibur software (Thermo Scientific) and processed with MaxQuant software (1.5.3.14) (Tyanova et al., 2016). We employed the Andromeda search engine (Cox and Mann, 2008), which is integrated into MaxQuant, to search MS/MS spectra against the human UniProtKB FASTA database (91,649 forward entries; version of 2015). We employed MaxQuant "semi specific" search option in order to discover endogenous cleavage products. A false discovery rate (FDR) cut-off of 1% was applied at the peptide level. The cut-off score (delta score) for accepting individual MS/MS was 17. For bioinformatic analysis as well as visualization, we used the open PERSEUS environment, which is part of MaxQuant. For several calculations and plots we also used the R framework (Team, 2015). Identified peptides were mapped to the GCG gene. In order to display quantitative evidence for overlapping peptides, intensities of identified peptides were summed and plotted per amino acid residue.

Calculations and statistics

To assess distribution and homoscedasticity in datasets, the Shapiro–Wilk test (swilk command) was applied and residual plots were drafted. Areas under the curves (AUC) were calculated using the trapezoidal rule and for net areas

under the curve (nAUC) 'baseline subtracted data', which included both increases (increments) and decreases (decrements).

Supplemental References

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