RESEARCH ARTICLE

Autophagy mediates hepatic GRK2 degradation to facilitate glucagon-induced metabolic adaptation to fasting

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Funding information

NIH, Grant/Award Number: R01 DK089883; MINECO, Grant/Award Number: SAF2017-84125-R

Abstract

The liver plays a key role during fasting to maintain energy homeostasis and euglycemia via metabolic processes mainly orchestrated by the insulin/glucagon ratio. We report here that fasting or calorie restriction protocols in C57BL6 mice promote a marked decrease in the hepatic protein levels of G protein-coupled receptor kinase 2 (GRK2), an important negative modulator of both G protein-coupled receptors (GPCRs) and insulin signaling. Such downregulation of GRK2 levels is liver-specific and can be rapidly reversed by refeeding. We find that autophagy, and not the proteasome, represents the main mechanism implicated in fasting-induced GRK2 degradation in the liver in vivo. Reducing GRK2 levels in murine primary hepatocytes facilitates glucagon-induced glucose production and enhances the expression of the key gluconeogenic enzyme *Pck1*. Conversely, preventing full downregulation of hepatic GRK2 during fasting using adenovirus-driven overexpression of this kinase in the liver leads to glycogen accumulation, decreased glycemia, and hampered glucagon-induced gluconeogenesis, thus preventing a proper and complete adaptation to nutrient deprivation. Overall, our data indicate that physiological fasting-induced downregulation of GRK2 in the liver is key for allowing complete glucagon-mediated responses and efficient metabolic adaptation to fasting in vivo.

KEYWORDS

autophagy, calorie restriction, fasting, glucagon signaling, gluconeogenesis, GPCR, GRK2, intermittent fasting

1 | INTRODUCTION

Metabolic homeostasis and adaptation to changing environmental conditions is a key feature of higher organisms. In particular, a continuous supply of glucose is maintained under

different conditions of nutrient availability to ensure proper function and survival of all organs. Plasma glucose levels must be kept within a narrow range that is controlled mainly by the liver but also by the skeletal muscle or the pancreas. The balance between the utilization and production of glucose

Abbreviations: GRK2, G protein-coupled receptor kinase 2; GPCR, G protein-coupled receptor; IR, insulin resistance GCGR glucagon receptor HFD high-fat diet.

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is primarily maintained by two opposing hormones acting in these and other tissues: insulin and glucagon. During fasting, glucagon plays a central role in the response to hypoglycemia increasing hepatic glucose output.¹⁻³ In the liver, glucagon acts through the glucagon receptor (GCGR), a member of the G protein-coupled receptor (GPCR) superfamily, to stimulate glycogen breakdown and de novo synthesis of glucose or gluconeogenesis from lactate, pyruvate, glycerol, and glucogenic amino acids. Glucagon regulates gluconeogenesis mainly by the upregulation of key enzymes such as phosphoenolpyruvate carboxykinase (PEPCK)^{4,5} and glycogenolysis by phosphorylating glycogen synthase and glycogen phosphorylase, leading to its inactivation and activation, respectively.⁶⁻⁸ The increase in circulating glucagon during starvation, together with the concomitant decrease in insulin and amino acids, also activates autophagy in the liver.⁹⁻¹² Autophagy helps maintain a positive energy balance in the liver during nutrient deprivation. This is achieved through the breakdown of intracellular stores of macromolecules that can be utilized as cellular fuel but also by autophagy-mediated control of proteins that are key regulators of cellular metabolism.^{12,13}

G protein-coupled receptor kinase 2 (GRK2) is a Ser/Thr kinase classically known for its role in the regulation of GPCRs. GRK2 phosphorylates the activated form of GPCRs, thus promoting the recruitment of β -arrestin and the uncoupling of G proteins what leads to internalization of the receptor and also to β -arrestin-dependent signaling.¹⁴⁻¹⁶ Besides such canonical role in GPCR modulation, GRK2 is also able to phosphorylate a variety of non-GPCR substrates and to dynamically interact with other important signal transduction partners in a kinase activity-independent manner.^{17,18} In this regard, GRK2 acts as a negative regulator of insulin signaling by different means downstream the insulin receptor.^{15,19} This latter role in insulin sensitivity is possibly the main explanation for the protection against insulin resistance found in GRK2-deficient animals.^{15,20,21}

Interestingly, GRK2 levels have been shown to be affected by different metabolic conditions. For instance, GRK2 protein is upregulated upon high-fat diet (HFD) feeding in different tissues such as the liver, muscle, adipose, or in the heart.^{15,16,20,22,23} GRK2 expression also increases during hepatic steatosis both in mice models and in humans by a mechanism involving increased transcription of the GRK2 gene (*adrbk1*).²⁴ Although the modulation of GRK2 dosage in obesity and insulin-resistance-related conditions is thus well established, the possible alteration of GRK2 levels during nutrient deprivation and its possible impact on the metabolic responses mediated by glucagon, as a key insulin-counter-regulatory hormone acting through a GPCR, has not been explored so far.

In this work, we uncover that hepatic GRK2 levels decrease abruptly upon food deprivation, and identify autophagy as a new in vivo mechanism involved in downregulation of this kinase. Moreover, results both in primary hepatocytes and in mice models indicate that this drop in GRK2 levels in the liver

represents an important step in the physiological response to fasting by facilitating glucagon-mediated effects such as glycogen breakdown and gluconeogenesis during starvation.

2 | MATERIALS AND METHODS

2.1 | Animal protocols

Animals were housed on a 12-hour light/dark cycle, maintained at a temperature of $22 \pm 2^\circ\text{C}$ with a relative humidity of $50\% \pm 10\%$ and under pathogen-free conditions. Three- to four-month-old C57BL/6J male mice fed with a standard diet (Safe150, Safe-Diets) from weaning at 3 weeks of age. Mice were sacrificed after the indicated hours of food, but not water, deprivation (fasted animals). For the refeeding protocol, mice fasted for 24 hours were allowed to eat for 4 hours prior to sacrifice. To inhibit autophagy or the proteasome, mice fasted for 22 hours were injected intraperitoneally with leupeptin (Sigma-Aldrich, 40 mg/kg) or intravenously with bortezomib (ChemCruz, 1 mg/kg) 2 hours before sacrifice. For the intermittent fasting-calorie restriction protocol, animals were individualized and daily food intake was weighed for a week. The following week each mouse was allowed access to an amount of food pellet corresponding to a 40% in weight of the mean daily food intake of the previous week (equivalent to a 60% calorie restriction) and this feeding regime was maintained for 7 days. Ad libitum-fed mice were used as controls. For adenoviral overexpression experiments, 7-week-old C57BL/6J male animals (Charles River) were injected intravenously via the tail vein with 100 μL of 5×10^{10} pfu/mL Ad5 adenoviral constructs containing the GRK2 sequence²⁵ or with control Cre-expressing virus (Ad5CMVCre, VVC-U of Iowa-5, University of Iowa Viral Vector Core Facility). Mice were maintained with access to ad libitum food and water, monitored in the animal facility and sacrificed 23 days later. For the methionine- and choline-deficient diet (MCD) experimental model, 3- to 4-month-old C57BL/6J mice were fed either an MCD (TD.90262 E1553-94, ENVIGO) or the corresponding choline- and methionine-sufficient control diet (CD, TD.90262 E15654-04, ENVIGO) for 4 weeks.

Body weight and blood glucose levels were measured in tail blood samples using an automatic analyzer (One Touch Ultra, LifeScan). Glucagon challenge test (GCT) and pyruvate tolerance test (PTT) were performed to evaluate the gluconeogenic capacity of the mice. Glucagon (Sigma-Aldrich, 64 or 32 $\mu\text{g/kg}$ body weight) or sodium pyruvate (Sigma-Aldrich, 1.5 g/kg body weight) were administered intraperitoneally after an overnight fasting and glucose concentration was determined in blood samples before and at different time points after injection. After 14 or 24 hours of fasting, plasma concentrations of β -hydroxybutyrate or non-esterified fatty acids (NEFA) were measured using an automatic analyzer

(STAT STRIP Xpress, Nova biomedical) or enzymatic methods (NEFA-HR kit, Wako Chemicals), respectively.

All animal experimentation procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (Directive 86/609) and were approved by the Ethical Committees for Animal Experimentation of our University and the Comunidad Autónoma de Madrid, Spain.

2.2 | Intrahepatic glycogen and free glucose measurements

Liver glucose and glycogen content were assayed enzymatically as previously described.²⁶ Frozen liver (≈ 20 mg) from fed mice was lysed in deionized water (500 μ L) using metal beads in a Tissue Lyser (Qiagen) and the resulting lysates were centrifuged at 12 000 g for 20 minutes at 4°C. The supernatant (75 μ L) was boiled for 3 minutes and, once cooled, mixed with an amyloglucosidase solution (Sigma-Aldrich 25 μ L diluted 1:5 in KAc 0.1 M buffer pH 5.5) and left overnight at room temperature to hydrolyze glucose incorporated into glycogen. Samples were boiled for 3 minutes to stop the reaction and centrifuged for 3 minutes at 12 000 g and glucose was analyzed using the HK assay kit (Sigma-Aldrich) following manufacturer's instructions. A reaction without amyloglucosidase provided the amount of free glucose (expressed as μ g per mg of tissue). To obtain the amount of glucose derived from glycogen in each sample, free glucose value was subtracted from that of total glucose after amyloglucosidase digestion.

2.3 | Experiments in primary hepatocytes

For the glucose production assays, primary hepatocytes were isolated from 8- to 12-week-old male C57BL/6J mice as described.²⁷ Sixteen hours after isolation, cells were infected with 10 MOI of adenovirus carrying a silencing construct for murine GRK2 (shGRK2),²⁵ or human GRK2 as a non-silencing control (shC) for 4-5 hours. The medium was replaced every day and 48 hours post-infection plates were incubated with 200 nM glucagon (Sigma-Aldrich-Aldrich) for 4 hours in glucose-free medium (phenol-red/glucose-free DMEM, 0.2% BSA) with 2 mM sodium pyruvate and 20 mM sodium lactate as gluconeogenic substrates. The glucose level in the medium was measured using a glucose assay kit from Eton Bioscience following manufacturer's instructions.

2.4 | mRNA isolation and Real-Time PCR

Murine liver RNA was isolated from lysates from a Tissue Lyser (Qiagen) by purification using the RNeasy Mini Kit (Qiagen) following the instructions provided by the supplier.

For the isolation of RNA from primary hepatocytes, cells were lysed with QIAzol reagent (Qiagen). Quantity and quality of RNA were analyzed using Nanodrop ND-1000 (Thermo Scientific) and Bioanalyzer 2100 (Agilent). RT-PCRs were performed with the aid of the Genomic Facility of the Centro de Biología Molecular Severo Ochoa using Light Cycler equipment (Roche) with SyBr Green technology and self-designed murine probes purchased from Sigma-Aldrich. DNase-treated RNA was reverse transcribed into cDNA with d(N)6 random hexamer primers and qRT-PCR was performed with an ABI 7900HT sequence detector (Applied Biosystems) using the SyBr Green method. A single or a geometric mean of several stably expressed and commonly used reference genes was used depending on the recommendations of GenNorm and NormFinder algorithms. qPCRs and statistical analysis of the data were performed using GenEx software. The sequences of the probes are listed below:

actb: 5'-CTAAGGCCAACCGTGAAAAG-3' and 5'-AC CAGAGGCATACAGGGACA-3'; *adrbk1*: 5'-CATGCACA ATCGCTTTGTAGTC-3' and 5'-GGTCCGAGATTCTCACA TGG-3'; *b2m*: 5'-TACATACGCTGCAGAGTTAAGCA-3' and 5'-TGATCACATGTCTCGATCCCAG-3'; *hbms*: 5'-AT GAGGGTGATTTCGAGTGGG-3' and 5'-TTGTCTCCCGTG GTGGACATA-3'; *pck1*: 5'-GGAGTACCCATTGAGGGTA TCAT-3' and 5'-GCTGAGGGCTTCATAGACAAG-3'; *thp*: 5'-CCACAGGGCGCCATGA-3' and 5'-GCTGTGGAGTAA GTCCTGTGCC-3'.

2.5 | Western blot analysis

Murine tissues were homogenized using metal beads in a Tissue Lyser (Qiagen) as previously described²² and cells were lysed in RIPA lysis buffer. 30-50 μ g of total protein was resolved per lane by SDS-PAGE and transferred to a 0.45 mm nitrocellulose membrane. Blots were probed with antibodies against β -actin or anti-Ubiquitin (Sigma-Aldrich), p62 (Progen), phosphorylated (Ser 240/244) and total S6 (Cell Signaling), LC3 I/II (Novus Biologicals), GRK2, GAPDH, and α -tubulin (Santa Cruz Biotechnology). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK) or the Odyssey Infrared Imaging System (Li-Cor Biosciences). Films were scanned with a GS-700 Imaging Densitometer and analyzed with Quantity One Software (Bio-Rad), or using an Odyssey Classic reader and the Odyssey software package 3.0 (Li-Cor Biosciences).

2.6 | Data analysis

All data are expressed as mean values $\pm$ SEM and n represents the number of animals. Statistical significance was

analyzed using unpaired Student's *t* test or one- or two-way repeated measures ANOVA followed by Bonferroni's post hoc test. Differences were considered statistically significant when *P* value < .05.

3 | RESULTS

C57BL6 mice were deprived of food for 14 or 24 hours, what caused a concomitant decrease in body weight and in glycemia (Figure 1A,B). In the liver, an overnight fasting (14 hours) or a 24-hour fasting markedly reduced GRK2 protein levels to $30\% \pm 4\%$ and $18\% \pm 2\%$, respectively, of the levels observed in fed mice (Figure 1C). Interestingly, this downregulation of GRK2 protein was not observed in other organs such as the epididymal white adipose tissue (eWAT) or the heart (Figure 1D,E). GRK2 decrease appears to be an early event, being detected already 5 hours after fasting was initiated, before a decline in circulating glucose levels was observed, although GRK2 down-modulation did not reach statistical significance until later time points (14 hours, Figure S1 and Figure 1C). In a different experimental setting of food deprivation, animals subjected to a 7-day-long protocol of intermittent fasting with calorie restriction also displayed a $\approx 75\%$ reduction in liver GRK2 protein compared to mice fed ad libitum along with the expected reduction in body weight and in blood glucose levels (Figure 1F-H). Regarding the mechanism of GRK2 downregulation upon fasting, the mRNA levels of the GRK2 gene (*adrbk1*) did not decrease and were even enhanced in 24-hour-fasted mice compared with fed controls (Figure S2). These data suggested that a post-transcriptional mechanism was implicated in the reduction of liver GRK2 protein levels. One of the main mechanisms for regulating protein levels in the liver upon fasting is autophagy.¹² To interfere with this process, we blocked the ongoing autophagy in 24-hour-fasted mice by refeeding for 4 hours. Autophagy impairment in such conditions was confirmed by the accumulation of LC3 II and p62 autophagy markers, as well as by the enhanced phosphorylation status of S6 as readout of the reactivation of the mTORC1 autophagy-inhibitory pathway (Figure 2A). Interestingly, GRK2 levels were increased concurrently with autophagy impairment in refed animals compared to fasted mice (Figure 2A). Consistently, when autophagy was pharmacologically blocked in fasted animals by injection of the lysosomal proteolysis inhibitor leupeptin, a well-established inhibitor of autophagy in animal models at doses and times similar to those utilized in our study (see references^{28,29}), we detected a marked (more than sixfold) accumulation of GRK2 protein, along with the expected increase in the LC3 II marker (Figure 2B). LC3II also accumulated in other tissues upon leupeptin treatment (Figure S3) indicating that autophagy is effectively blocked in our experimental setting.

Since the proteasome has been described to degrade GRK2 in cultured cell lines,³¹ we also analyzed the possible implication of the proteasomal machinery in GRK2-mediated degradation in our in vivo setting after fasting. As shown in Figure 2C, bortezomib (a very potent and selective pharmacological inhibitor of the activity of the proteasome^{32,33}) does not alter the amount of GRK2 protein in the liver after fasting in conditions where it efficiently increases the levels of ubiquitinated proteins in this organ and under which leupeptin completely restores and even increases GRK2 protein levels compared to those of fed controls. Altogether, these data suggested that GRK2 is a substrate of autophagy in mouse liver during fasting and that this process seems to predominate over proteasomal degradation for the regulation of GRK2 levels, at least in these conditions.

We next sought to investigate whether the notable drop in GRK2 protein observed in the liver upon starvation could have a physiological impact in key fasting-induced responses. In particular, we studied gluconeogenesis as a hepatocyte-specific response to food deprivation that is essential to maintain whole-body euglycemia. We silenced GRK2 in primary murine hepatocytes to mimic fasting-induced GRK2 degradation using an adenovirus carrying a shRNA for GRK2.²⁵ The efficacy of this in situ silencing approach was corroborated by Western Blot and qPCR (Figure 3A). When hepatocytes were treated with glucagon in the presence of gluconeogenic substrates (pyruvate and lactate), we found a significant enhancement of glucose output in hepatocytes with silenced GRK2 with respect to control-infected cells (Figure 3B). This enhanced gluconeogenic response detected upon GRK2 downregulation correlated with an increased expression of the *Pck1* gene that encodes for PEPCK, a key enzyme in the regulation of gluconeogenesis, in GRK2-silenced cells (Figure 3D).

To investigate the role of GRK2 in gluconeogenesis modulation in vivo, we injected adult mice through the tail vein with adenovirus carrying the sequence of GRK2 (AdGRK2)²⁵ or a control protein (AdC) to achieve hepatic GRK2 overexpression and thus attempt to counteract the downregulation of GRK2 observed in this tissue upon a 24-hour fasting. Of note, we did not observe overexpression of GRK2 in other organs such as pancreas or heart upon adenovirus injection (Figure S4). As shown in Figure 4A, fasting induced a marked reduction in hepatic GRK2 protein in control AdC-infected animals and also in GRK2-overexpressing mice (AdGRK2). However, the resulting hepatic levels of GRK2 after 24 hours of food deprivation were significantly higher in AdGRK2 compared to AdC mice (Figure 4A), and not significantly different from those of fed controls. Therefore, this model allowed us to investigate the effects of preventing GRK2 downregulation normally taking place during fasting. The AdGRK2 and AdC animals displayed a similar response to a 24-h fasting in terms of weight loss (Figure 4B). However, glucose

FIGURE 1 GRK2 levels are specifically reduced in the liver upon fasting. C57BL6WT animals were euthanized in either fed or fasted conditions. Body weight (A) and glycemia (B) of fed mice, or of animals fasted for 14 and 24 hours are shown. Western Blot (WB) analysis of lysates of liver (C), eWAT (D) and heart (E) from animals either fed or fasted for the time indicated in the figure were performed using antibodies against GRK2 and β-tubulin, actin and GAPDH as loading controls. (F-H) WT mice were fed for 7 days following an intermittent fasting (Int. Fast.) feeding protocol with a 60% calorie restriction (CR). Body weight (F) and glycemia (G) of ad libitum fed and intermittently fasted calorie-restricted mice are shown. Representative WB and densitometric analysis of liver lysates from these mice are depicted (H). Data are means ± SEM of 4-5 individuals per group. Statistical significance was analyzed by one-way ANOVA followed by Bonferroni's post-test (A,B,C) or unpaired *t* test. **P* < .05, ***P* < .01, ****P* < .005

FIGURE 2 Hepatic GRK2 is degraded by fasting-induced autophagy. Mice were fasted for 24 hours and either euthanized or allowed to eat for 4 hours (refed). Liver lysates from these mice were analyzed by WB with antibodies against GRK2, the loading control β -actin, LC3 I/II, p62 and phosphorylated and total S6 (A). To block autophagy or the proteasome pharmacologically, mice fasted for 22 hours were injected with the lysosomal proteolysis inhibitor leupeptin (B) or with bortezomib (C) or vehicle 2 hours before sacrifice. Representative WB of liver lysates from these mice using GRK2, β -actin, anti-Ubiquitin and LC3 I/II antibodies are shown (for anti-Ub blots, lower panel quantifications were used). Data are means $\pm$ SEM of 4-5 individuals per group in (A) and (B) and 3-4 in (C). Statistical significance was analyzed by unpaired *t* test (**P* < .05, ***P* < .01, ****P* < .005) in (A) and (B) and by one-way ANOVA (**P* < .05, ***P* < .01, ****P* < .005 vs. Fasted+vehic; ###*P* < .01, ####*P* < .005 vs Fasted+bort; and \$\$\$*P* < .005 vs. Fasted+leup) in (C)

FIGURE 3 Silencing GRK2 in primary hepatocytes increases glucagon-induced glucose production and PEPCK (*pck1*) expression. Primary hepatocytes from C57BL6 mice were infected with adenoviruses carrying a silencing construct for murine GRK2 (shGRK2) or human GRK2 as a non-silencing control (shC). qPCR of GRK2 mRNA (*adrbk1* gene normalized to *rlp13*) and WB of GRK2 protein levels relative to β -actin are shown (A). Glucose production was quantified in these cells following glucagon stimulation in glucose-free medium supplemented with pyruvate and lactate as gluconeogenic substrates (B). qPCR expression was measured in shC- and shGRK2-infected hepatocytes treated with glucagon or PBS for *pck1* (normalized to *rlp13*) (C). Data are means $\pm$ SEM of three individual experiments with triplicates. Statistical significance was analyzed by unpaired *t* test (A) or one-way ANOVA followed by Bonferroni's post-test (B,C). **P* < .05, ***P* < .01, ****P* < .005

levels upon fasting were significantly lower in AdGRK2 mice compared to controls (Figure 4C). This correlated with decreased mRNA levels of *Pck1* (Figure 4D) in Ad-GRK2 animals, suggesting a blunted fasting-induced hepatic glucose output was taking place in these mice. Consistent with this notion, GRK2-overexpressing mice showed a decreased capability to obtain glucose from pyruvate compared to controls as put forward by a pyruvate tolerance test (Figure S5).

These results suggested that a blunted response to glucagon might be taking place in mice when preventing efficient GRK2 downregulation by fasting in the liver. To assess this possibility, we directly analyzed glucagon-induced gluconeogenesis in these animals by measuring blood glucose levels after an intraperitoneal glucagon injection. As observed in Figure 4E, AdGRK2 animals had lower glucose levels during this glucagon challenge with a significant decrease in the area under the curve (AUC) compared to controls, this consequence of GRK2 overexpression being higher at larger glucagon concentrations compared to lower doses (Figure S6). This effect in gluconeogenesis is also coherent with the

decreased intrahepatic glucose observed in AdGRK2-infected animals after 24 hours of fasting, a glycogen-depleted state (Figure S7). However, glycogenolysis, a process mainly regulated by glucagon in the liver and that occurs at earlier time points during fasting, was also impaired in AdGRK2 mice since the amount of hepatic glycogen was significantly higher in these animals compared to controls (Figure 4F). Of note, fasted AdGRK2 animals showed increased circulating fatty acids and β -hydroxybutyrate (Figure S8), possibly as a compensatory response to provide an alternative source of energy in these mice. Together, these data indicated that downregulation of hepatic GRK2 dosage upon fasting is necessary to achieve a complete response to glucagon in vivo in such physiological setting.

4 | DISCUSSION

We unveil in this report that autophagy-mediated hepatic GRK2 degradation during fasting is key to facilitate

FIGURE 4 Impairing fasting-induced reduction in GRK2 hepatic levels lowers glycemia and hampers glucose production by different gluconeogenic stimuli in vivo. C57BL/6 mice were injected intravenously with an adenovirus carrying the sequence of GRK2 (AdGRK2) or a control adenovirus (AdC) and were sacrificed 3 weeks after injection. Analysis by WB of liver lysates from AdGRK2 and AdC either fed or fasted for 24 hours using antibodies against GRK2 and β -actin is shown (A). Body weight (B) or plasma glucose levels (C) of AdC and AdGRK2 mice either fed or fasted for 24 hours were quantified. Expression levels of mRNAs encoding PEPCK (*pck1*) were measured by qPCR and normalized with stably expressed genes (*hbms*, *tbp* and *b2m*) in liver samples from AdC and AdGRK2 fasted for 24 hours (D). Glucagon challenge tests (E) were performed in AdC and AdGRK2 and the area under the curve (AUC) is represented next to the graph. Glycogen content was measured in AdC and AdGRK2 livers and is represented as glucose produced following amyloglucosidase treatment (F). Data are means $\pm$ SEM of $n = 7-10$ animals per group. Statistical significance was analyzed by unpaired *t* test (B,C,D and AUC in E,F), one-way ANOVA followed by Bonferroni's post-test (A) or two-way ANOVA followed by Bonferroni's post-test (E and F). * $P < .05$, ** $P < .01$, *** $P < .005$

glucagon-induced adaptive metabolic responses. Our data uncover a novel mechanism of regulation of GRK2 levels in physio-pathological conditions. GRK2 expression has been shown to be regulated by both transcriptional and/or post-translational mechanisms at the level of protein stability, depending on the tissue, cell type, and condition.^{16,34,35} Notably, we observe a marked reduction in GRK2 protein despite a concurrent increase in its mRNA levels, indicating that a GRK2 degradation process is actively taking place. GRK2 can be degraded by the proteasome upon GPCR stimulation^{31,36} or by calpains in situations such as oxidative stress.³⁷ However, autophagy appears to be the main mechanism by which GRK2 levels are reduced in the murine liver upon food deprivation. This notion is supported by the fact that fasting-induced hepatic GRK2 downregulation is rapidly restored upon refeeding (a physiological inhibitor of autophagy) and prevented upon pharmacological (leupeptin injection) inhibition of autophagy in vivo, while proteasomal inhibition with bortezomib does not prevent the decrease in the amount of hepatic GRK2 levels after fasting, even in conditions where it efficiently increases the levels of ubiquitinated proteins in this organ.

Hepatic autophagy fluctuates with the fasting-feeding cycle and has a fundamental role in adaptation to starvation through the induction of breakdown of macromolecules and via specific regulation of particular sets of proteins and enzymes that are able to switch the metabolic status of the cell.^{12,13,38} Upon starvation, the switch in the insulin/glucagon ratio and particularly the reduced levels of circulating amino acids taking place upon more prolonged fasting induce autophagy in the liver, which is rich in lysosomes and is characterized by its ability to reach high levels of metabolic stress-related autophagy compared to other organs.^{12,39,40} Calorie restriction also results in upregulation of this process in different tissues,⁴¹ with extensive autophagy in the liver.⁴² This background is consistent with the pattern and time frame of GRK2 downregulation observed in these experimental conditions, and with the apparently liver-specific reduction of GRK2 levels detected upon fasting. Our data also suggest that refeeding-mediated inhibition of autophagy would rapidly stop further degradation of GRK2, which would be restored to homeostatic levels. However, the detailed molecular mechanisms linking GRK2 to the autophagic machinery during nutrient deprivation remain to be determined.

Our results suggest that the targeted degradation of hepatic GRK2 by autophagy is physiologically relevant to facilitate glucagon signaling during nutrient deprivation conditions, thus contributing to the global adaptive response to fasting. Glucagon is the major hormone orchestrating glucose metabolism in the fasted state via promotion of glycogenolysis and gluconeogenesis.^{2,12} We demonstrate that GRK2 silencing in primary hepatocytes results in increased glucagon-dependent glucose production and *Pck1* mRNA expression. On the other

hand, we observe that preventing hepatic GRK2 downregulation below a certain threshold during fasting (by means of adenoviral-mediated delivery of a GRK2 construct) impairs a proper and full metabolic adaptation to fasting. We find that AdGRK2-expressing mice retain more glycogen and display lower glycemia and decreased *Pck1* expression after fasting compared to controls, along with a blunted gluconeogenic response to pyruvate. The hampered gluconeogenesis observed in AdGRK2 mice is more evident upon direct administration of glucagon. Moreover, in the face of impaired gluconeogenesis, a likely compensatory increase in circulating levels of fatty acids and β -hydroxybutyrate to serve as alternative energy substrates is detected in these animals.

Taken together, these results suggest that a physiological GRK2 downregulation achieved by autophagy upon fasting is key to facilitate glucagon effects in the liver. Since GRK2 levels overtly decrease after an overnight fasting and rapidly recover upon refeeding, it is tempting to postulate the occurrence of a physiological fasting/feeding oscillation in liver GRK2 dosage to help maintain metabolic homeostasis. Our data further establish a role of GRK2 as a central modulator of hepatic metabolism, given its unique ability to control different cascades, including adrenergic, glucagon, and insulin receptor signaling.^{15,19,20} Therefore, the functional impact of changes in GRK2 expression reported in different physiological and pathological situations would depend on its integrated effects in such cascades.

Mechanistically, the observed effects of GRK2 levels in glucagon actions are consistent with the canonical negative role of GRK2 in GPCR signaling, leading to receptor phosphorylation and β -arrestin-dependent desensitization and trafficking. GRK2 has been shown to desensitize the GCGR in HEK293 cells, where GRK2 overexpression triggers enhanced glucagon receptor internalization via β -arrestin recruitment.⁴³ Consistently, silencing β -arrestin expression in primary hepatocytes enhances glucagon-triggered cAMP production, glucose output, and the expression of gluconeogenic enzymes, along with reduced agonist-induced GCGR internalization. Moreover, hepatocyte-specific β -arrestin2 deficiency increases glucagon receptor signaling in vivo.⁴⁴ Therefore, GRK2 downregulation during fasting would likely decrease agonist-dependent glucagon receptor phosphorylation, β -arrestin recruitment, and receptor internalization, thus enhancing glucagon-mediated metabolic cascades.

Of note, GRK2 levels are upregulated in hepatic tissue obtained from patients with NAFLD and NASH as well as in mice with hepatic steatosis induced by feeding a HFD or an MCD diet.^{20,24,45,46} This increase has been related to hepatic damage and insulin resistance. Interestingly, glucagon infusion in cows⁴⁷⁻⁴⁹ or the treatment with a glucagon/GLP1 dual receptor agonists in mice has been shown to reduce NASH⁵⁰ and might represent a promising therapeutic strategy in humans.⁵¹ Furthermore, reducing GRK2 levels

in mice (as in GRK2+/- animals) confers protection from MCD-induced NASH,²⁴ along with increased liver PEPCK expression (Figure S9). In light of our newly described role of GRK2 in GCGR signaling, it is tempting to postulate that improved responses to endogenous glucagon could also be contributing to the protection against NASH in conditions of reduced GRK2 levels, and that fasting and calorie restriction protocols leading to reduced GRK2 levels may enhance the response to GLP1/glucagon pharmacological agents.

In sum, our results put forward that the cell-type specific control of GRK2 levels by the metabolic status (concentration of hormones and nutrients) would be key to facilitate the switch in the liver metabolic programs required to adapt to the changing energetic demands in different physiopathological contexts.

ACKNOWLEDGMENTS

We acknowledge support by Ministerio de Economía y Competitividad (MINECO/FEDER), Spain (grant SAF2017-84125-R to F.M. and C. M.); CIBER de Enfermedades Cardiovasculares (CIBERCv). Instituto de Salud Carlos III, Spain (grant CB16/11/00278 to FM, co-funded with European FEDER contribution); European Foundation for the Study of Diabetes (EFSD) Novo Nordisk Partnership for Diabetes Research in Europe Grant (to FM); NIH R01 DK089883 grant to P.P. and Programa de Actividades en Biomedicina de la Comunidad de Madrid-B2017/BMD-3671-INFLAMUNE to F.M. We appreciate the help of the CBMSO Facilities, in particular Genomics and Animal Care. K.S. is funded by the Charles King Postdoctoral Fellowship. We also acknowledge the support of Contratos Predoctorales para Formación de Personal Investigador 2017 (FPI-UAM) from Universidad Autónoma de Madrid to A. C. A. and institutional support to the CBMSO from **Fundación Ramón Areces** and Fundación Banco de Santander.

AUTHOR CONTRIBUTIONS

M. Cruces-Sande, A.C. Arcones, R. Vila-Bedmar, A. Val-Blasco, and D. Díaz-Rodríguez performed research; M. Cruces-Sande, A.C. Arcones, R. Vila-Bedmar, F. Mayor, and C. Murga designed research and analyzed data; K. Sharabi and P. Puigserver contributed new reagents and analytic tools, designed research, analyzed data, and revised the manuscript; M. Cruces-Sande, R. Vila-Bedmar, F. Mayor, and C. Murga wrote and revised the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Quesada I, Tuduri E, Ripoll C, Nadal A. Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol*. 2008;199:5-19.
- Rines AK, Sharabi K, Tavares CD, Puigserver P. Targeting hepatic glucose metabolism in the treatment of type 2 diabetes. *Nat Rev Drug Discov*. 2016;15:786-804.
- Miller RA, Birnbaum MJ. Glucagon: acute actions on hepatic metabolism. *Diabetologia*. 2016;59:1376-1381.
- Herzig S, Long F, Jhala US, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature*. 2001;413:179-183.
- Yoon JC, Puigserver P, Chen G, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature*. 2001;413:131-138.
- Band GC, Jones CT. Functional activation by glucagon of glucose 6-phosphatase and gluconeogenesis in the perfused liver of the fetal guinea pig. *FEBS Lett*. 1980;119:190-194.
- Ciudad C, Camici M, Ahmad Z, Wang Y, DePaoli-Roach AA, Roach PJ. Control of glycogen synthase phosphorylation in isolated rat hepatocytes by epinephrine, vasopressin and glucagon. *Eur J Biochem*. 1984;142:511-520.
- Westergaard N, Brand CL, Lewinsky RH, et al. Peroxyvanadium compounds inhibit glucose-6-phosphatase activity and glucagon-stimulated hepatic glucose output in the rat in vivo. *Arch Biochem Biophys*. 1999;366:55-60.
- Deter RL, Baudhuin P, De Duve C. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. *J Cell Biol*. 1967;35:C11-C16.
- Mortimore GE, Mondon CE. Inhibition by insulin of valine turnover in liver. Evidence for a general control of proteolysis. *J Biol Chem*. 1970;245:2375-2383.
- Mortimore GE, Schworer CM. Induction of autophagy by amino-acid deprivation in perfused rat liver. *Nature*. 1977;270:174-176.
- Ueno T, Komatsu M. Autophagy in the liver: functions in health and disease. *Nat Rev Gastroenterol Hepatol*. 2017;14:170-184.
- Schneider JL, Cuervo AM. Liver autophagy: much more than just taking out the trash. *Nat Rev Gastroenterol Hepatol*. 2014;11:187-200.
- Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther*. 2012;133:40-69.
- Mayor F Jr, Cruces-Sande M, Arcones AC, et al. G protein-coupled receptor kinase 2 (GRK2) as an integrative signalling node in the regulation of cardiovascular function and metabolic homeostasis. *Cell Signal*. 2018;41:7.
- Murga C, Arcones AC, Cruces-Sande M, Briones AM, Salaices M, Mayor F Jr. G protein-coupled receptor kinase 2 (GRK2) as a potential therapeutic target in cardiovascular and metabolic diseases. *Front Pharmacol*. 2019;10:112.
- Penela P, Murga C, Ribas C, Lafarga V, Mayor F Jr. The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol*. 2010;160:821-832.
- Watari K, Nakaya M, Kurose H. Multiple functions of G protein-coupled receptor kinases. *J Mol Signal*. 2014;9:1.
- Ciccarelli M, Chuprun JK, Rengo G, et al. G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation*. 2011;123:1953-1962.
- Vila-Bedmar R, Cruces-Sande M, Lucas E, et al. Reversal of diet-induced obesity and insulin resistance by inducible genetic ablation of GRK2. *Sci Signal*. 2015;8:ra73.
- Cipolletta E, Gambardella J, Fiordelisi A, et al. (2019) Antidiabetic and cardioprotective effects of pharmacological inhibition of GRK2 in db/db Mice. *Int J Mol Sci*. 2019;20(6):1492.

22. Lucas E, Jurado-Pueyo M, Fortuno MA, et al. Downregulation of G protein-coupled receptor kinase 2 levels enhances cardiac insulin sensitivity and switches on cardioprotective gene expression patterns. *Biochim Biophys Acta*. 2014;1842:2448-2456.
23. Lucas E, Vila-Bedmar R, Arcones AC, et al. Obesity-induced cardiac lipid accumulation in adult mice is modulated by G protein-coupled receptor kinase 2 levels. *Cardiovasc Diabetol*. 2016;15:155.
24. Cruces-Sande M, Vila-Bedmar R, Arcones AC, et al. Involvement of G protein-coupled receptor kinase 2 (GRK2) in the development of non-alcoholic steatosis and steatohepatitis in mice and humans. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864:3655-3667.
25. Nogue L, Reglero C, Rivas V, et al. G protein-coupled receptor kinase 2 (GRK2) promotes breast tumorigenesis through a HDAC6-Pin1 axis. *EBioMedicine*. 2016;13:132-145.
26. Yi H, Thurberg BL, Curtis S, et al. Characterization of a canine model of glycogen storage disease type IIIa. *Dis Model Mech*. 2012;5:804-811.
27. Sharabi K, Lin H, Tavares CDJ, et al. Selective chemical inhibition of PGC-1 α gluconeogenic activity ameliorates type 2 diabetes. *Cell*. 2017;169:148-160.e115.
28. Moulis M, Vindis C. Methods for measuring autophagy in mice. *Cells*. 2017;6:1-14.
29. Esteban-Martinez L, Boya P. Autophagic flux determination in vivo and ex vivo. *Methods*. 2015;75:79-86.
30. Haspel J, Shaik RS, Ifedigbo E, et al. Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy*. 2011;7:629-642.
31. Penela P, Ruiz-Gomez A, Castano JG, Mayor F Jr. Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. *J Biol Chem*. 1998;273:35238-35244.
32. Neubert K, Meister S, Moser K, et al. The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. *Nat Med*. 2008;14:748-755.
33. Zhang L, Mager DE. Physiologically-based pharmacokinetic modeling of target-mediated drug disposition of bortezomib in mice. *J Pharmacokinet Pharmacodyn*. 2015;42:541-552.
34. Penela P, Ribas C, Mayor F Jr. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal*. 2003;15:973-981.
35. Penela P, Rivas V, Salcedo A, Mayor F Jr. G protein-coupled receptor kinase 2 (GRK2) modulation and cell cycle progression. *Proc Natl Acad Sci U S A*. 2010;107:1118-1123.
36. Penela P, Elorza A, Sarnago S, Mayor F Jr. Beta-arrestin- and c-Src-dependent degradation of G-protein-coupled receptor kinase 2. *EMBO J*. 2001;20:5129-5138.
37. Lombardi MS, Kavelaars A, Penela P, et al. Oxidative stress decreases G protein-coupled receptor kinase 2 in lymphocytes via a calpain-dependent mechanism. *Mol Pharmacol*. 2002;62:379-388.
38. Madrigal-Matute J, Cuervo AM. Regulation of liver metabolism by autophagy. *Gastroenterology*. 2016;150:328-339.
39. Ezaki J, Matsumoto N, Takeda-Ezaki M, et al. Liver autophagy contributes to the maintenance of blood glucose and amino acid levels. *Autophagy*. 2011;7:727-736.
40. Naito T, Kuma A, Mizushima N. Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle. *J Biol Chem*. 2013;288:21074-21081.
41. Escobar KA, Cole NH, Mermier CM, VanDusseldorp TA. Autophagy and aging: maintaining the proteome through exercise and caloric restriction. *Aging Cell*. 2019;18:e12876.
42. Fang F, Shi X, Brown MS, Goldstein JL, Liang G. Growth hormone acts on liver to stimulate autophagy, support glucose production, and preserve blood glucose in chronically starved mice. *Proc Natl Acad Sci U S A*. 2019;116:7449-7454.
43. Krilov L, Nguyen A, Miyazaki T, et al. Dual mode of glucagon receptor internalization: role of PKC α , GRKs and beta-arrestins. *Exp Cell Res*. 2011;317:2981-2994.
44. Zhu L, Rossi M, Cui Y, et al. Hepatic beta-arrestin 2 is essential for maintaining euglycemia. *J Clin Invest*. 2017;127:2941-2945.
45. Taguchi K, Hida M, Hasegawa M, Narimatsu H, Matsumoto T, Kobayashi T. Suppression of GRK2 expression reduces endothelial dysfunction by restoring glucose homeostasis. *Sci Rep*. 2017;7:8436.
46. Charbonneau A, Unson CG, Lavoie JM. High-fat diet-induced hepatic steatosis reduces glucagon receptor content in rat hepatocytes: potential interaction with acute exercise. *J Physiol*. 2007;579:255-267.
47. Nafikov RA, Ametaj BN, Bobe G, Koehler KJ, Young JW, Beitz DC. Prevention of fatty liver in transition dairy cows by subcutaneous injections of glucagon. *J Dairy Sci*. 2006;89:1533-1545.
48. Bobe G, Ametaj BN, Young JW, Beitz DC. Effects of exogenous glucagon on lipids in lipoproteins and liver of lactating dairy cows. *J Dairy Sci*. 2003;86:2895-2903.
49. Bobe G, Ametaj BN, Young JW, Anderson LL, Beitz DC. Exogenous glucagon effects on health and reproductive performance of lactating dairy cows with mild fatty liver. *Anim Reprod Sci*. 2007;102:194-207.
50. Valdecantos MP, Pardo V, Ruiz L, et al. A novel glucagon-like peptide 1/glucagon receptor dual agonist improves steatohepatitis and liver regeneration in mice. *Hepatology*. 2017;65:950-968.
51. Quinones M, Ferno J, Dieguez C, Nogueiras R, Al-Massadi O. Exciting advances in GPCR-based drugs discovery for treating metabolic disease and future perspectives. *Expert Opin Drug Discov*. 2019;14(5):421-431.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Cruces-Sande M, Arcones AC, Vila-Bedmar R, et al. Autophagy mediates hepatic GRK2 degradation to facilitate glucagon-induced metabolic adaptation to fasting. *The FASEB Journal*. 2020;34:399–409. <https://doi.org/10.1096/fj.201901444R>