MOLECULAR
METABOLISM

Glucagon receptor gene deletion in insulin knockout mice modestly reduces blood glucose and ketones but does not promote survival

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ABSTRACT

Objective: It has been thought that the depletion of insulin is responsible for the catabolic consequences of diabetes; however, evidence suggests that glucagon also plays a role in diabetes pathogenesis. Glucagon suppression by glucagon receptor (*Gcgr*) gene deletion, glucagon immunoneutralization, or Gcgr antagonist can reverse or prevent type 1 diabetes in rodents suggesting that dysregulated glucagon is also required for development of diabetic symptoms. However, the models used in these studies were rendered diabetic by chemical- or immune-mediated β-cell destruction, in which insulin depletion is incomplete. Therefore, it is unclear whether glucagon suppression could overcome the consequence of the complete lack of insulin.
Methods: To directly test this we characterized mice that lack the *Gcgr* and both insulin genes (*GcgrKO/InsKO*).
Results: In both P1 pups and mice that were kept alive to young adulthood using insulin therapy, blood glucose and plasma ketones were modestly normalized; however, mice survived for only up to 6 days, similar to *GcgrHet/InsKO* controls. In addition, *Gcgr* gene deletion was unable to normalize plasma leptin levels, triglycerides, fatty acids, or hepatic cholesterol accumulation compared to *GcgrHet/InsKO* controls.
Conclusion: Therefore, the metabolic manifestations associated with a complete lack of insulin cannot be overcome by glucagon receptor gene inactivation.

1. INTRODUCTION

Hyperglucagonemia is present in many forms of diabetes [1,2] and contributes to elevated blood glucose by promoting glycogenolysis, gluconeogenesis, and ketogenesis while inhibiting glycogen synthesis. Remarkably, mice with whole body *Gcgr* gene deletion (*GcgrKO*) are protected from streptozotocin (STZ) induced diabetes [3,4]. While *GcgrWT* mice developed severe hyperglycemia, hyperketonemia, and cachexia and had to be euthanized 6 weeks post-STZ treatment, *GcgrKO* mice remained healthy [3,4]. Moreover, other studies have shown that glucagon suppression by *Gcgr* gene deletion, glucagon immunoneutralizing antibody, or Gcgr antagonism can reduce fasting and fed blood glucose and improve glucose tolerance in healthy and diabetic models [5–8]. These studies support the idea that elevated glucagon action is required for hyperglycemia in insulin-deficient type 1 diabetes. However, those studies used immune- or chemical-mediated destruction of β-cells that reduce but do not eliminate insulin. In those models, animals retain islet insulin immunoreactivity and circulating insulin, and survive for weeks without treatment [3–6,8]. As mice with knockout of both insulin genes (*Ins1KO/Ins2KO*, herein referred to as *InsKO* mice) survive for <2 days after birth [9] and approximately 1 day in adulthood following cessation of insulin therapy [10], it is evident that these other models are not insulin-free. Therefore, we aimed to determine whether loss of glucagon action improves glucose metabolism and promotes survival in the complete absence of insulin. To achieve this, we characterized mice with *Gcgr*, *Ins1* and *Ins2* gene deletions.

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Brief communication

2. MATERIAL AND METHODS

2.1. Animals

Mice with global *Gcgr* knockout, originally described by Gelling et al. [7], were supplied by Dr. Daniel Drucker. Male *GcgrKO/Ins1KO/Ins2Het* and female *GcgrHet/Ins1KO/Ins2Het* mice were bred to generate male and female experimental and control mice. Mice were genotyped using primers found in Supplemental Table 1. For pup characterization, *InsKO* pups were not treated with insulin. For young adult characterization, *InsKO* mice were treated twice daily with subcutaneous injections of Lantus insulin glargine (Sanofi-Aventis Inc., Laval, Canada). On P13-15, *InsKO* mice underwent a transplantation of ∼130 islets into the anterior chamber of the eye from ∼20 week old $Ins1^{-/-}$ donors with 0—2 alleles of *Gcgr* and 1—2 alleles of *Ins2*. At 4 weeks of age mice underwent enucleation and were carefully monitored for rapid weight loss, hunching, piloerection, lethargy, and non-responsiveness in order to euthanize any animals that reached humane endpoint. Mice were housed on a 14:10 h light—dark cycle with *ad libitum* access to food (5053, PicoLab Rodent Diet 20, LabDiet) and water. All experiments were approved by the University of British Columbia Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines.

2.2. Metabolic measurements

All parameters were measured in the *ad libitum* fed state. Blood glucose was measured via the tail vein using an OneTouch Ultra Glucometer (LifeScan) with a detection limit of 1.1—33.3 mM. In Figure 3G & H, one glucometer reading was above the limit of detection and assigned a value of 33.3 mM and statistical analysis was not performed. Plasma β-hydroxybutyrate (β-Hydroxybutyrate LiquiColor Test, Stanbio, Boerne, TX), glucagon (Glucagon ELISA, Mercodia, Salem, NC), leptin (Mouse Leptin ELISA, Crystal Chem, Downers Grove, IL), triglycerides and glycerol (Serum Triglyceride Determination Kit, Sigma—Aldrich, Oakville, Canada), free fatty acids (HR Series NEFA HR [2] Kit, Wako Diagnostics, Richmond, VA), and cholesterol (Cholesterol E, Wako Diagnostics, Richmond, VA) were measured from trunk samples in pups or cardiac puncture samples in young adult mice which were collected as mice reached humane endpoint. Hepatic lipid extraction was performed as previously described [11].

2.3. Statistics

Data were analyzed using a 1-way or 2-way ANOVA with Bonferroni post-hoc testing for bar and line graphs respectively, or a Gehan—Breslow—Wilcoxon test for survival curves. Statistical analysis was performed using GraphPad Prism 6.05 (La Jolla, CA). Significance was set at $P < 0.05$.

3. RESULTS

3.1. Effect of *Gcgr* gene deletion on survival in *InsKO* pups

First, we aimed to determine whether *Gcgr* gene deletion would extend survival of *InsKO* pups. All male and female pups compared had 0 *Ins1* alleles, 0—1 *Gcgr* alleles and 0—2 *Ins2* alleles. *GcgrHet/InsWT*, *GcgrKO/InsWT*, *GcgrHet/InsHet*, and *GcgrKO/InsHet* pups were used as controls, while *GcgrHet/InsKO* and *GcgrKO/InsKO* pups tested the effect of *Gcgr* gene deletion on *InsKO* pups. All controls survived and gained weight throughout the first week of life, while both *GcgrHet/InsKO* and *GcgrKO/InsKO* failed to gain mass and died at a similar rate, surviving no longer than 6 days (Figure 1A & B). Therefore *Gcgr* gene deletion does not promote survival of *InsKO* pups.

3.2. Effect of *Gcgr* gene deletion on glucose and lipid metabolism in *InsKO* pups

We assessed if loss of glucagon action in *InsKO* pups improved metabolism by analyzing metabolic parameters associated with glucose metabolism in P1 pups. Results were variable, likely a reflection of litter size, when the animals last fed, and the time of day the samples were collected. *Gcgr* gene deletion with the *InsWT* or *Het* genotype did not affect any of the parameters measured. At P1 *GcgrHet/InsKO* pups weighed 30% less than controls, which was marginally increased in *GcgrKO/InsKO* pups but still 20% less than the control groups (Figure 2A). As expected, *GcgrHet/InsKO* pups were hyperglycemic compared to controls. While still hyperglycemic, this was modestly decreased by 24% in *GcgrKO/InsKO* pups (Figure 2B) relative to *GcgrHet/InsKO* pups. Similarly, plasma β-hydroxybutyrate levels were 9-fold higher in *GcgrHet/InsKO* pups compared to controls and reduced in *GcgrKO/InsKO* pups to 4-fold that of controls (Figure 2C). It has been published that adult *GcgrKO* mice exhibit elevated glucagon levels [3,7]. At P1, glucagon levels were significantly higher than controls in *GcgrKO/InsKO* pups (Figure 2D). Therefore *Gcgr* gene deletion modestly improved hyperglycemia and hyperketonemia in *InsKO* pups.

Since type 1 diabetes is associated with aberrant lipid metabolism, we characterized lipid homeostasis in P1 pups. As expected, due to the positive effect of insulin on adipogenesis and leptin production [12], *GcgrHet/InsKO* pups had undetectable plasma leptin levels which were unchanged due to *Gcgr* gene deletion (Figure 2E). Plasma triglycerides and free fatty acids were increased in *GcgrHet/InsKO* pups relative to
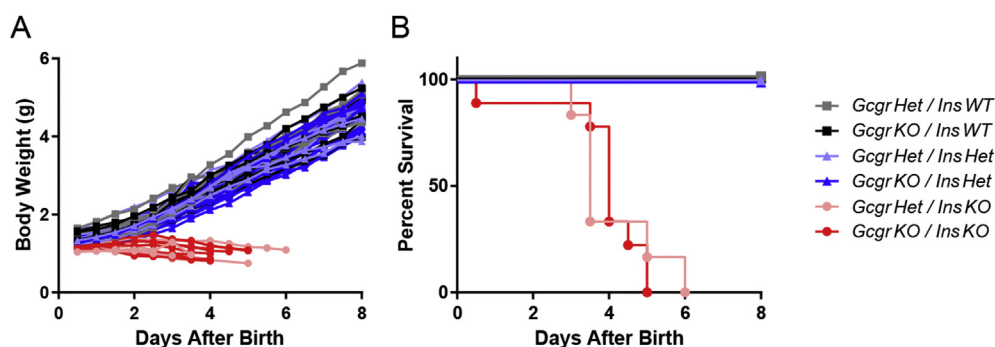

Figure 1: ***Gcgr* gene deletion does not promote survival in *InsKO* pups**. *Ins1KO* pups with 0—1 alleles of *Gcgr* and 0—2 alleles of *Ins2* were tracked twice daily for body weight (A) and survival (B) for 8 days after birth. For A statistical analysis was not performed, for B no statistical differences were observed between *GcgrHet/InsKO* and *GcgrKO/InsKO* pups. Data are graphed as individual pups (A) and % survival (B), n = 6—17.
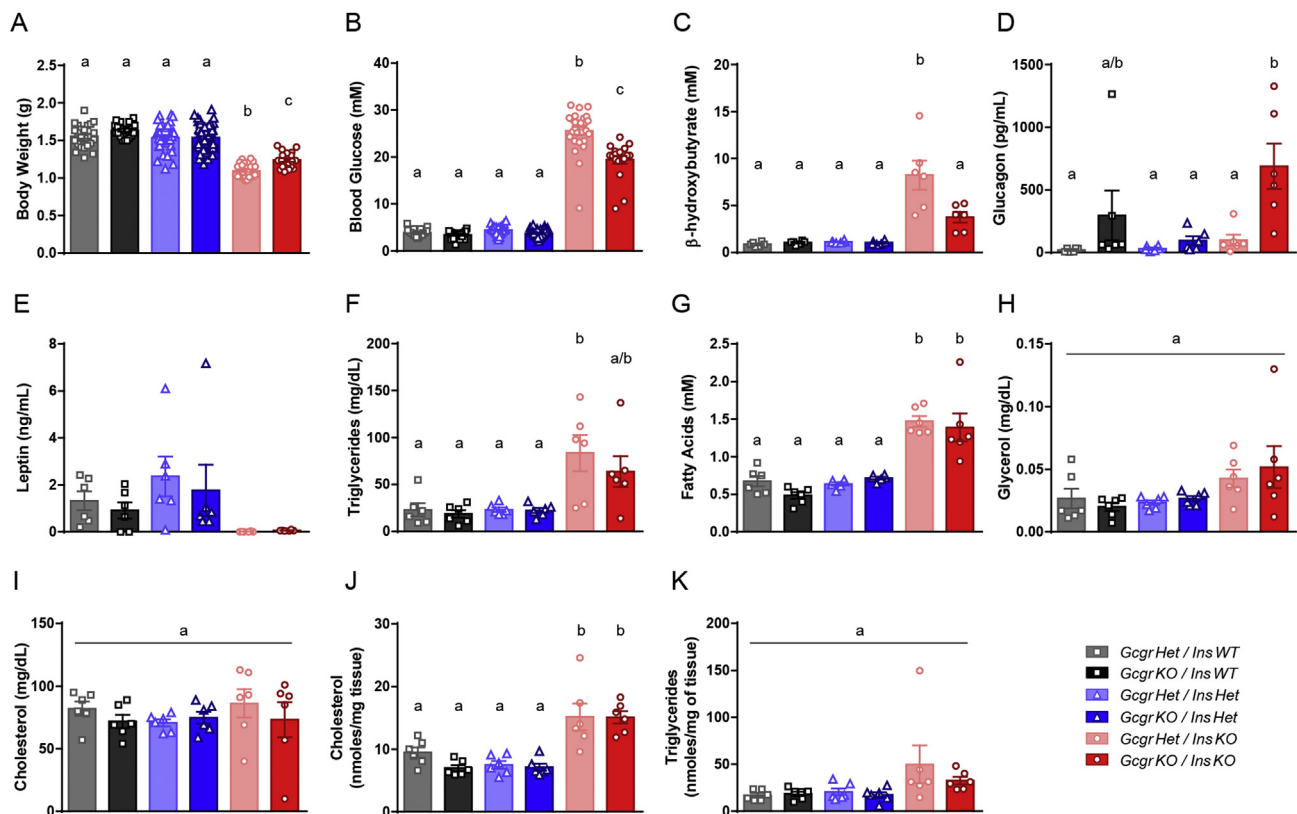
Figure 2: *Gcgr* gene deletion modestly improves body weight, blood glucose and plasma ketones but does not normalize plasma leptin, triglycerides, fatty acids or hepatic cholesterol accumulation in *InsKO* pups. On P1, *Ins1KO* pups with 0−1 alleles of *Gcgr* and 0−2 alleles of *Ins2* were harvested to measure body weight (A), trunk blood was collected to measure blood glucose (B) and plasma β-hydroxybutyrate (C), glucagon (D), leptin (E), triglycerides (F), fatty acids (G), glycerol (H), and cholesterol (I), and liver was collected to quantify hepatic cholesterol (J) and triglycerides (K). Different superscripts (a, b, c) are significantly different from each other within each graph. Statistical analysis was not performed on leptin measurements (D) as some samples were below the limit of detection. Data are mean ± SEM, n = 19−59 for A & B, n = 6 for C−K.

controls and *Gcgr* gene deletion did not alter this (Figure 2F & G). Plasma glycerol and cholesterol in *GcgrHet/InsKO* pups were similar to controls and unchanged due to the loss of the *Gcgr* (Figure 2H & I). Finally, hepatic cholesterol accumulation was unaffected by *Gcgr* gene deletion, and hepatic triglyceride levels in *InsKO* pups were comparable to that of controls (Figure 2J & K). Therefore, *Gcgr* gene deletion does not normalize lipid metabolism in *InsKO* pups.

3.3. Effect of *Gcgr* gene deletion on survival of adult *InsKO* mice

We have shown that *GcgrKO/InsKO* pups do not have extended survival; however, because insulin is involved in growth and development [13], we sought to determine the effect of glucagon suppression in adult *InsKO* mice. As previously published, male and female *InsKO* mice were maintained to adulthood using insulin injections and islet transplantation into the eye [10]. *GcgrKO/InsKO* mice failed to grow normally following weaning despite post-transplant euglycemia (Figure 3A & B). Despite receiving the same number of islets, *GcgrKO/InsKO* mice had normal blood glucose levels compared to hyperglycemic *GcgrHet/InsKO* mice, suggesting that the loss of *Gcgr* aids in improving hyperglycemia when insulin is present. On day 0 of the study, the eye containing the exogenous islets was enucleated to render the *InsKO* mice insulin deficient; *InsHet* mice underwent enucleation to control for the effect of surgery. Following enucleation, all *InsKO* mice rapidly lost weight, were hunched, displayed piloerection, were lethargic or non-responsive, and reached humane

endpoint at a similar rate, by day 6 of the study (Figure 3C). As shown in Figure 3D−F, the kinetics of this weight loss was similar between *GcgrHet/InsKO* and *GcgrKO/InsKO* mice. Therefore in young adult mice, *Gcgr* gene deletion was unable to prevent body weight loss and death associated with a complete loss of insulin.

3.4. Effect of *Gcgr* gene deletion on glucose and lipid metabolism in adult *InsKO* mice

We next sought to determine whether *Gcgr* gene deletion improved blood glucose levels in young adult *InsKO* mice. *InsKO* mice rapidly became hyperglycemic upon removal of the graft. At day 0.75, 1.25, and 1.75, blood glucose of *GcgrKO/InsKO* mice was modestly lower than that of *GcgrHet/InsKO* mice (Figure 3G−K). Interestingly, at humane endpoint, 3 of the *GcgrKO/InsKO* mice were hypoglycemic (<1.3 mM), which was not observed in the *GcgrHet/InsKO* mice (Figure 3K and data not shown). In conclusion, *Gcgr* gene deletion modestly reduced blood glucose throughout the study and even resulted in hypoglycemia in some mice, but did not permit survival in young adult *InsKO* mice.

Finally, we measured various metabolic parameters in young adult *InsKO* mice with and without *Gcgr* gene deletion. Similar to P1 pups, *GcgrKO/InsKO* mice had substantially elevated plasma glucagon levels compared to all other groups, while the *GcgrKO/InsHet* group trended towards increased levels of glucagon compared to *GcgrHet* groups (Figure 4A). As expected β-hydroxybutyrate levels were substantially
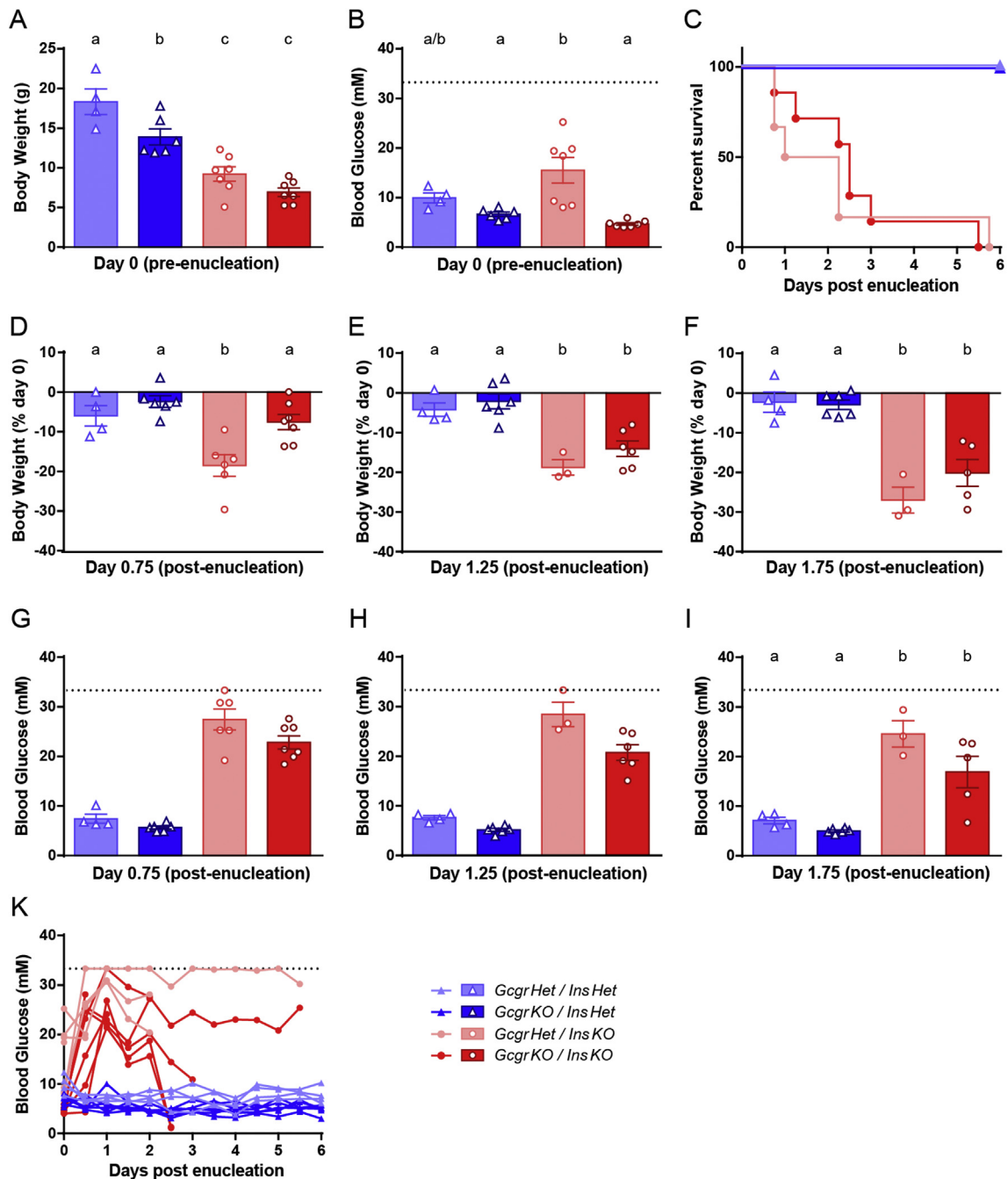
Figure 3: *Gcgr* gene deletion modestly lowers blood glucose but does not promote survival in adult *InsKO* mice. *InsKO* mice with and without *Gcgr* gene deletion were kept alive to 4 weeks of age using insulin injections and an islet transplant into the eye. On day 0 of the study, the eye containing islets was enucleated rendering the mice completely insulin deficient. Day 0 absolute body weight (A) and blood glucose levels (B) prior to enucleation. Following enucleation, survival (C) and day 0.75 (D), 1.25 (E) and 1.75 (F) body weight loss were measured. Blood glucose at day 0.75 (G), 1.25 (H), 1.75 (I) and throughout the study (H) are graphed. Different superscripts (a, b, c) are significantly different from each other within each graph. Statistical analysis was not performed on G and H as one blood glucose value fell above the limit of detection and was assigned a value of 33.3 mM, or in K because the n value decreased over time. Data are graphed as mean $\pm$ SEM (A, B, D−I), % survival (C), or individual mice (K), n = 4−7, throughout study *InsKO* mice reached humane endpoint therefore n is reduced in E, F, H, and I.

elevated in *GcgrHet/InsKO* mice. The *GcgrKO/InsKO* mice trended (P = 0.09) to have lower β-hydroxybutyrate levels, which were similar to that of controls, however there was large variability in ketones levels in the *GcgrHet/InsKO* mice (Figure 4B). Plasma cholesterol levels were similar between controls and *InsKO* mice, and fatty acids levels were increased in *GcgrKO/InsKO* compared to controls (Figure 4C & D).

4. DISCUSSION

Recent evidence suggests that glucagon itself causes the lethal catabolic consequences of insulin deficiency [14]. Remarkably, despite a 90% reduction in insulin, *GcgrKO* mice remained euglycemic and healthy compared to controls, which required euthanization 6 weeks
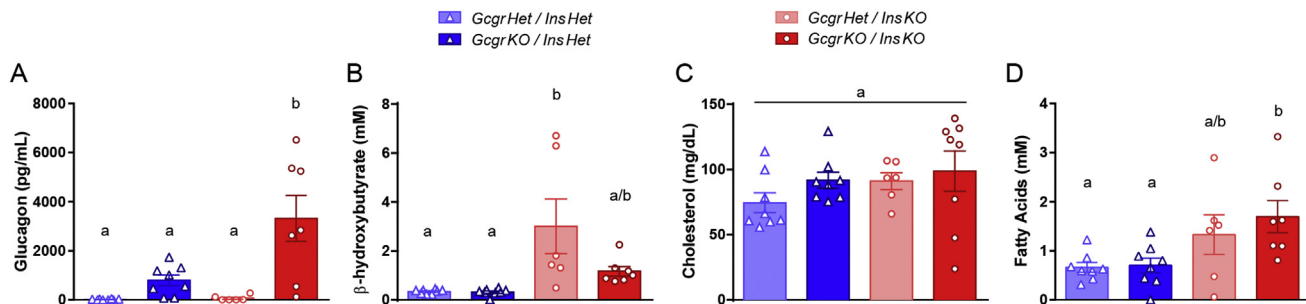
Figure 4: *Gcgr* gene deletion increases plasma glucagon, normalizes plasma ketones, and does not affect plasma cholesterol or fatty acids in adult *InsKO* mice. At humane endpoint (*InsKO* mice) or the end of the study (controls), cardiac blood was collected to measure plasma glucagon (A), β-hydroxybutyrate (B), cholesterol (C), and fatty acids (D). Different superscripts (a, b, c) are significantly different from each other within each graph. Data are mean ± SEM, n = 6—8.

post-STZ [3,6]. However, we found that with 100% elimination of insulin, *Gcgr* gene deletion in mice does not extend survival. At both P1 and in early adulthood, *GcgrKO/InsKO* mice failed to gain mass or lost a substantial amount of weight and survived no longer than *GcgrHet/InsKO* controls. In a previous study, we generated *InsKO* mice on a *GcgrWT* background and found that these mice survived for ~1 day following islet graft removal, less than the 6 days observed in the current study. While it may appear that the reduction of one *Gcgr* allele may have had a slight protective effect on *InsKO* mice, we believe this is unlikely, because it has been shown that *GcgrWT* and *GcgrHet* mice display similar *Gcgr* function and metabolic phenotypes. Indeed, liver membranes prepared from *GcgrWT* and *GcgrHet* mice exhibit identical responses to glucagon stimulation as measured by cAMP production and these mice exhibit similar blood glucose elevations in response to i.p. injection of glucagon, suggesting that the loss of 1 allele does not significantly alter the function of glucagon receptor in the mouse [7]. In addition, in another strain of *GcgrKO* mice, it has been shown that gross appearance, body weight, visceral fat, liver and pancreas weight, and random glucose are not different between *GcgrWT* and *GcgrHet* mice [15]. Therefore, we believe that the difference in days of survival between *GcgrWT* and *GcgrHet* mice between studies may be due to differences between background strain, age, diet, and facility. Nonetheless, it is clear that deletion of both *Gcgr* alleles cannot promote long-term survival in mice completely lacking insulin as all reached humane endpoint within 1 week of birth.

The loss of glucagon action in the complete absence of insulin does moderately improve aspects of metabolism. Glucagon plays a role not only in the fasting state but also in the fed state, which is evident as *Gcgr* gene deletion can normalize fed blood glucose levels and glucose tolerance [3,4,6]. In our study, *GcgrKO/InsKO* mice only experienced a partial improvement in the fed blood glucose levels in both P1 pups and young adults. Furthermore, although we saw modest blood glucose reduction throughout the study in the fed state, at euthanasia, some of the *GcgrKO/InsKO* were hypoglycemic, a state not observed in the *GcgrHet/InsKO* mice. The feeding state of the mice at the time of euthanization is unclear. Therefore, we speculate that if we had fasted the *GcgrKO/InsKO* mice, they may have experienced a larger decrease in blood glucose due to insufficient endogenous glucose production. Furthermore, it has been shown that *Gcgr* gene deletion normalizes ketones [3]. Since *GcgrHet/InsKO* mice had highly variable ketones, levels only trended towards improvement in *GcgrKO/InsKO* adult mice, and although we observed a significant improvement in ketones in P1 pups, levels in *GcgrKO/InsKO* mice still remained 4× higher than controls. Interestingly, in both pups and adults, loss of the *Gcgr* gene did not improve aberrant lipid metabolism in the *InsKO* mice. Thus, while loss of glucagon action can improve some aspects of

metabolism, metabolism is not normalized in the absence of both insulin and glucagon action.

While several reports indicate that loss of glucagon action can protect rodents from diabetes [3—6,8], some studies suggest a less substantial role for glucagon in diabetes pathogenesis [16,17]. Meek et al. suppressed glucagon action in STZ-diabetic rats using a glucagon-like peptide 1 receptor agonist or a glucagon neutralizing antibody, both of which did not change blood glucose levels, but lowered ketones levels, suggesting that hyperglucagonemia is required for ketosis but not hyperglycemia [16]. Their report only partially agrees with our data as we observed minor improvements in both blood glucose and ketones. Furthermore, Steenberg et al. report that ablation of glucagon expressing cells using GCG-DTR mice does not improve glucose tolerance but modestly prevents further deterioration in STZ-diabetic mice [17]. In addition, a recent study by Damond et al. attempted to maximize insulin blockage in *GcgrKO* mice through combined STZ treatment and insulin receptor antagonism or diphtheria toxin induced β-cell loss [18]. Interestingly, although insulin-depleted *GcgrKO* mice were hyperglycemic compared to untreated *GcgrKO* mice, these mice had modestly improved blood glucose levels compared to *GcgrHet* mice treated with the same insulin-blocking regimen [18]. This effect is similar to the results of our paper, strengthening the conclusion that *Gcgr* gene deletion can only modestly reduce blood glucose levels in the absence of insulin but cannot prevent diabetes. Furthermore, in the report by Damond et al., although *GcgrKO* treated mice had decreased survival compared to untreated *GcgrKO* mice, they survived 3 times longer than *GcgrHet* mice with the same insulin-blocking regimen [18]. This is in contrast to our results in which both *GcgrKO/InsKO* and *GcgrHet/InsKO* mice survived for only 6 days. This difference may reflect the incomplete depletion of insulin by Damond et al., compared to the complete lack of insulin in our model.

Collectively, we have shown that the absence of glucagon signaling can modestly improve diabetic symptoms in insulin knockout mice, but this improvement was insufficient in promoting survival. Therefore, we have provided unequivocal evidence that it is not possible to protect from the catabolic consequences due to the complete loss of insulin by eliminating glucagon action.

AUTHOR CONTRIBUTIONS

Brief communication

takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.05.014.

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