Brief communication

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.

Keywords Mice; Type 1 diabetes; Insulin; Glucagon; Glucose metabolism; Lipid metabolism

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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Abbreviations: Gcgr, glucagon receptor; Het, heterozygous; *Ins1*, insulin 1; *Ins2*, insulin 2; *InsKO*, insulin knockout; KO, knockout; P, post-natal day; STZ, streptozotocin; WT, wildtype

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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

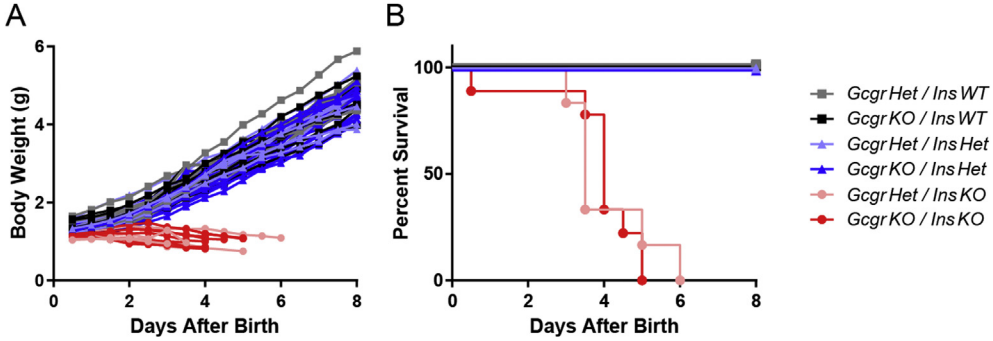

Figure 1: *Gcgr* gene deletion does not promote survival in *InsKO* pups. *Ins1KO* pups with 0–1 alleles of *Gcgr* and 0–2 alleles of *Ins2* were tracked twice daily for body weight (A) and survival (B) for 8 days after birth. For A statistical analysis was not performed, for B no statistical differences were observed between *GcgrHet/InsKO* and *GcgrKO/InsKO* pups. Data are graphed as individual pups (A) and % survival (B), n = 6–17.

