Original article

MOLECULAR METABOLISM

LKB1 and AMPKα1 are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia

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ABSTRACT

Aims/Hypothesis: Glucagon release from pancreatic alpha cells is required for normal glucose homoeostasis and is dysregulated in both Type 1 and Type 2 diabetes. The tumour suppressor LKB1 (STK11) and the downstream kinase AMP-activated protein kinase (AMPK), modulate cellular metabolism and growth, and AMPK is an important target of the anti-hyperglycaemic agent metformin. While LKB1 and AMPK have emerged recently as regulators of beta cell mass and insulin secretion, the role of these enzymes in the control of glucagon production *in vivo* is unclear.
Methods: Here, we ablated LKB1 (αLKB1KO), or the catalytic alpha subunits of AMPK (αAMPKdKO, -α1KO, -α2KO), selectively in ~45% of alpha cells in mice by deleting the corresponding *flox*'d alleles with a preproglucagon promoter (*PPG*) *Cre*.
Results: Blood glucose levels in male αLKB1KO mice were lower during intraperitoneal glucose, aminoimidazole carboxamide ribonucleotide (AICAR) or arginine tolerance tests, and glucose infusion rates were increased in hypoglycemic clamps ($p < 0.01$). αLKB1KO mice also displayed impaired hypoglycemia-induced glucagon release. Glucose infusion rates were also elevated ($p < 0.001$) in αAMPKα1 null mice, and hypoglycemia-induced plasma glucagon increases tended to be lower ($p = 0.06$). Glucagon secretion from isolated islets was sensitized to the inhibitory action of glucose in αLKB1KO, αAMPKdKO, and -α1KO, but not -α2KO islets.
Conclusions/Interpretation: An LKB1-dependent signalling cassette, involving but not restricted to AMPKα1, is required in pancreatic alpha cells for the control of glucagon release by glucose.

Keywords LKB1; AMPK; Glucagon secretion; PPG; Knockout; Alpha cell

1. INTRODUCTION

Glucagon, secreted by the pancreatic islet alpha cell as blood glucose levels fall, is the key anti-hypoglycemic hormone in mammals, acting as a powerful stimulus for hepatic glucose production [1]. Impaired glucagon release is associated with the episodes of hypoglycemia frequently reported in Type 1 and advanced Type 2 diabetic (T2D) patients treated with insulin or sulphonylureas [2]. On the other hand, elevated circulating glucagon levels contribute to the chronic increase in blood glucose levels characteristic of T2D [1].

Lowered blood glucose levels stimulate glucagon release through multiple mechanisms including changes in parasympathetic [3] and sympathetic tone [4], increases in circulating adrenaline levels [5], as well as through effects of glucose on pancreatic alpha cells [6]. The exact nature of the latter are still debated. Thus, in the mouse, a direct effect of glucose [7,8] and indirect effects of γ-amino butyric acid (GABA) [9,10] and insulin [7,11] released from neighbouring beta cells have all been invoked as regulators of glucagon secretion. Roles for released Zn^{2+} ions have also been proposed [7,12–14].

AMP-activated protein kinase (AMPK) is a heterotrimeric complex comprising α, β and γ subunits that serves as a master energy sensor [15], highly sensitive to intracellular ATP:AMP [15] and ATP:ADP ratios [16]. Liver kinase B1 (LKB1), also called STK11, is a tumour suppressor whose inactivation leads to Peutz-Jegers syndrome [17], characterized by hamartomatous polyps and an increased risk of all cancers. LKB1 phosphorylates at least 13 different protein kinases including AMPK. LKB1 (and alternative protein kinases including calmodulin kinase kinase β [18,19] and transforming growth factor β-activated kinase-1, TAK1) [20], phosphorylate AMPK catalytic α-subunits at Thr172 in the "T-loop". A fall in intracellular energy, leading to

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Abbreviations: AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; T2D, Type 2 diabetes; PPG, preproglucagon promoter; AICAR, aminoimidazole carboxamide ribonucleotide

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altered occupancy by AMP and/or ADP of sites in the γ-subunit, leads to conformational changes that render T172 in the α-subunit less susceptible to dephosphorylation [16] by protein phosphatases [21]. Activated AMPK then phosphorylates a range of downstream metabolic targets to promote ATP synthesis and inhibit ATP consumption [15].

Through the use of pharmacological AMPK activators and inhibitors [22], we have previously provided evidence that AMPK is a negative regulator of insulin secretion from pancreatic beta cells [23,24]. Furthermore, activation of AMPK by metformin is associated with enhanced beta cell apoptosis [25]. Correspondingly, mice deleted selectively in the beta cell for LKB1 display beta cell hyperplasia and markedly enhanced insulin release [26–28]. By contrast, and unexpectedly, deletion of both AMPK catalytic subunits (α1 and α2) from beta cells causes impaired insulin secretion and glucose intolerance *in vivo* [29,30], possibly as a result of rat insulin promoter-2 (*RIP2*)-*Cre*-mediated recombination in the brain [31].

We have also recently provided evidence from *in vitro* studies that AMPK is involved in the regulation of glucagon release. Thus, activation of AMPK stimulated glucagon release from clonal αTC1-9 cells and mouse pancreatic islets, while a dominant-negative form of the kinase blocked the stimulatory effects of low glucose [22]. However, neither the role of LKB1 nor that of individual AMPK isoforms in controlling glucagon secretion has been examined *in vivo*.

Here, we have generated mice deleted for LKB1 (αLKB1KO), and for one (AMPKα1KO, -α2KO) or both (αAMPKdKO) AMPKα catalytic subunits selectively in pancreatic alpha cells using preproglucagon (*PPG*)-*Cre* driven recombination. We show that LKB1 signalling, at least partly mediated via AMPKα1, is essential for the normal stimulation of glucagon secretion at low glucose levels both *in vitro* and *in vivo*. Moreover, and in marked contrast to its action in the beta cell, we show that LKB1 plays a limited if any role in the control of alpha cell size or total alpha cell mass [26–28].

2. METHODS

2.1. Generation of mutant mice lacking LKB1 selectively in pancreatic alpha cells

Mice heterozygous for *flox*'d alleles of the *Lkb1/Stk11* gene (mixed FVB/129S6 and C57BL/6 background) [32] were obtained from the Mouse Models of Human Cancer Consortium (MMHCC) (www.nih.gov/science/models/mouse/resources/hcc.html) and backcrossed with C57/B6 mice four times. These animals were then bred against *PPG-Cre* expressing mice [33] and the resulting heterozygous offspring were inter-crossed as siblings to generate αLKB1KO mice ($Lkb1^{fl/fl}$, *Cre* positive). αLKB1KO mice were further bred with $Lkb1^{fl/fl}$ mice to generate littermate controls ($Lkb1^{fl/fl}$). It should be noted that the *PPG-Cre* transgene is not reported to exert effects on glucagon secretion or glucose homoeostasis [34] or to lead to significant recombination in extra-pancreatic tissues [11]. αLKB1KO mice and littermate controls were born at the expected Mendelian ratios.

2.2. Generation of mutant mice selectively lacking AMPK α1 and α2 in pancreatic alpha cells

Mice homozygous for $Ampk\alpha1^{fl/fl}$ were crossed with mice heterozygous for $Ampk\alpha2^{fl/+}$. The resulting double heterozygotes ($Ampk\alpha1^{fl/+}$, $\alpha2^{fl/+}$) were crossed with *PPG-Cre*-expressing animals [33] to generate triple heterozygous mice ($Ampk\alpha1^{fl/+}$, $\alpha2^{fl/+}$, *Cre* positive). The latter were then bred with mice homozygous for both *flox*'d *Ampkα1* and *α2* alleles ($Ampk\alpha1^{fl/fl}$, $\alpha2^{fl/fl}$) to produce αAMPKdKO mice ($Ampk\alpha1^{fl/fl}$, $\alpha2^{fl/fl}$, *Cre* positive). αAMPKdKO mice were further crossed with $Ampk\alpha1^{fl/fl}$, $\alpha2^{fl/fl}$ mice to generate littermate controls ($Ampk\alpha1^{fl/fl}$, $\alpha2^{fl/fl}$). All mice were kept on a C57/B6 background.

2.3. Generation of mice selectively expressing RFP in pancreatic alpha cells

Mice heterozygous for Rosa26tdRFP [35] were crossed with mice heterozygous for *PPG-Cre* to generate double heterozygous mice.

2.4. Mouse maintenance and diet

Animals were housed two to five per individually ventilated cage in a pathogen-free facility with 12 h light/dark cycle and had free access to standard mouse chow diet. All *in vivo* procedures described were performed at the Imperial College Central Biomedical Service and approved by the UK Home Office according to the Animals (Scientific Procedures) Act 1986 of the United Kingdom (PPL 70/7349).

2.5. Glucose, insulin, AICAR and arginine tolerance tests

Intraperitoneal glucose and insulin tolerance tests were performed as previously described [28,30]. For AICAR tolerance, mice fasted for 16 h (water allowed) were intraperitoneally injected with 1.75 g AICAR/kg (Toronto Research Chemicals, North York, Canada). Blood (2–4 μl) from the tail vein was obtained at 0, 20, 40, 60, 90 and 120 min after injection [36]. Blood glucose levels were measured with an automatic glucometer (Accuchek; Roche, Burgess Hill, UK). For arginine tolerance, mice fasted for 16 h (water allowed) were intraperitoneally injected with 3 g/kg L-arginine (pH 7.4; Arginine hydrochloride from Sigma) [11]. Blood (50 μl) from the tail vein was collected at 0, 10 and 30 min after injection into EDTA coated tubes containing 1 μl DPP IV inhibitor (final concentration: 100 μmol/l; Millipore, Watford, UK). Plasma insulin and glucagon levels were measured with an ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden) [37] and a glucagon radioimmunoassay kit with a competitive ^{125}I labelled glucagon (Millipore, Watford, UK) [7] respectively. Experiments were performed on mice aged 8–12 weeks.

2.6. Hyperinsulinemic-hypoglycemic clamp

Nine week-old male mice were implanted with catheters in the right jugular vein under general ketamine (100 mg/kg)/xylazine (10 mg/kg) anaesthesia. Catheters were flushed daily with saline containing heparin (0.4U/100 ml). Mice were housed individually in separate cages for post-surgery recovery. On the day of the clamp study, mice were starved for five hours before catheters were connected onto infusion tubing. Mice were infused with bolus insulin (0.033U) at an infusion rate of 30 μl/min for 5 min. To maintain blood glucose levels close to 2.7–3.3 mmol/l, insulin (0.6 U/kg/h) were infused for 120 min, during which 20% glucose was co-infused with adjustable infusion rates. Blood was collected from tail veins for measurement of glucose levels every 10 or 20 min.

2.7. Other methods

Please see Supplementary Materials and Methods.

2.8. Statistical analysis

Significance was tested using unpaired or paired Student's two-tailed *t*-tests with Bonferroni post-tests for multiple comparisons, or two-way ANOVA with Sidak post-doc tests as required. Analysis was performed using Excel (Microsoft) and Graphpad Prism 4.0 (Graphpad Software). $p < 0.05$ was considered significant and values represent mean ± SEM.

