Loss of LAMTOR1 in pancreatic β-cells increases glucose-stimulated insulin secretion in mice

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Abstract. Insulin secretion from pancreatic β-cells regulates glucose metabolism and is related to various diseases including diabetes. The late endosomal/lysosomal adaptor MAPK and mTOR activator 1 (LAMTOR1) is one of the subunits of the 'Ragulator' complex and plays an important role in energy metabolism including glucose metabolism. The present study was designed to explore the role of LAMTOR1 in murine pancreatic β-cell function. A murine model with β cell-specific deficiency (*βLamtor1-KO*) was generated to assess β-cell function (insulin sensitivity paired with β-cell responses) by hyperglycemic clamp *in vivo*. Islet perfusion and mitochondrial functional analyses were performed to investigate the individual steps in the insulin secretion pathway. Results showed that glucose tolerance *in vivo* as well as glucose-stimulated insulin secretion in the hyperglycemic clamp and islet perfusion were higher in *βLamtor1*-KO mice compared to the control models. Although mitochondrial dysfunction was present, the deletion of *Lamtor1* increased glutamate content in the mouse insulin granules as well as acetyl-CoA carboxylase 1 (ACC1) activity thus enhancing insulin secretion. Together, our data indicate that LAMTOR1 is important for maintaining mitochondrial function in mouse pancreatic β-cells, however deletion of *Lamtor1* increases the amplification pathway induced by glutamate and ACC1, ultimately leading to increased insulin secretion. These findings suggest that knockout of *Lamtor1* is a potential technique for improving pancreatic β-cell function and treating diabetes.

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Introduction

Glucose is the most widely distributed monosaccharide in nature. It is an important component of the human body and the main source of energy for various tissues and organs. The interaction between insulin and glucose plays a crucial role in the stabilization of blood glucose, which is closely related to various diseases including diabetes. It is well known that insulin secreted by pancreatic β-cells plays an important role in the development of diabetes. Glucose uptake causes a series of responses in pancreatic β-cells ultimately leading to insulin release (1). In pancreatic β-cells, glucose is transported by the glucose transporter 2 (GLUT2) and phosphorylated by glucokinase to produce glucose 6-phosphate, which is then oxidatively phosphorylated by the mitochondria, resulting in an increase in the adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio in the cytoplasm (2). This in turn leads to the closure of ATP-sensitive K^+ channels (K_{ATP} channels) and results in cell membrane depolarization and opening of voltage-dependent calcium channels (VDCCs). Ca^+ influx triggers the release of insulin from β-cell insulin granules (3-5).

Adenosine monophosphate-activated protein kinase (AMPK) is one of the most important regulators of metabolic homeostasis and energy metabolism, maintaining the balance of energy supply and demand, as well as improving insulin resistance (6). When cellular nutrition and energy are deficient, AMPK is activated by an allosteric mechanism. Once activated, AMPK increases catabolism and inhibits anabolism in order to respond to stress signals induced by nutrient or energy deficiency and increase intracellular ATP stores (7). The tumor suppressor liver kinase B1 (LKB1) complex, comprising of STRAD α/β and MO25 α/β, is the major upstream kinase for AMPK. It activates AMPK by phosphorylating Thr172 (8).

The LKB1/AMPK pathway plays a crucial role in regulating the polarity, size and total mass of β-cells, thus limiting glucose-stimulated insulin secretion (GSIS) (9). Moreover, it has been reported that an increase of insulin secretion occurs in specific *Lkb1*-knockout (KO) mice, partly as a result of improved glutamine levels and acetyl-CoA carboxylase 1 (ACC1) activity (10). All of these observations point to the essential effects of LKB1 on mitochondrial homeostasis in pancreatic β-cells and its strong negative regulatory function with regards to insulin secretion.

Late endosomal/lysosomal adaptor MAPK and mTOR activator 1 (LAMTOR1) is a membrane protein specifically localized to the surface of endosomes/lysosomes. It serves as an anchor for the 'Ragulator' complex with LAMTOR2 (P14), LAMTOR3 (MP1), LAMTOR4 (C7ORF59) and LAMTOR5 (HBXIP) (11). LAMTOR1 promotes the activation of AMPK and inhibits mTORC1 activity, thereby turning off the anabolic pathway and switching on the catabolic pathway when there is a need for increased energy production (12). As mentioned previously, insulin secretion is increased in the pancreatic β-cells of specific *Lkb1* knockout mice. However, the role of LAMTOR1 in the regulation of β-cell function remains unknown. Herein, the role of LAMTOR1 in pancreatic β-cell function is explored in order to elucidate its molecular mechanisms both *in vivo* and *in vitro*.

Materials and methods

Animals. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. F1 generation mice (age, 4-6 weeks; weight, 20-25 g) were purchased from Model Animal Research Center Of Nanjing University and kept in an air-conditioned room (25˚C; relative humidity, 50±20%; 12-h light/dark cycle) with free access to food and water. The control group included 36 male Loxp/Loxp mice while the experimental group included 36 male *βLamtor1*-KO mice. Control Loxp/Loxp and *βLamtor1*-KO male mice were obtained by mating homozygous *Lamtor1*-floxed mice (*Lamtor1*$^{F/F}$) with RIP-Cre mice, on a C57BL/6N background. All animal experiments were performed in accordance with the ARRIVE guidelines.

Glucose tolerance test. Mice received a 20% glucose solution (Sangon Biotech Co., Ltd.; 1.5 g/kg) by intraperitoneal (i.p.) injection. Blood glucose levels were measured at 0, 30, 60, 90 and 120 min after injection.

Hyperglycemic clamp. The hyperglycemic clamp was conducted strictly according to standardized procedures. Chronic cannulation of the right jugular vein was performed 4-5 days prior to intravenous glucose infusion. The mice were fasted for 6 h in a restrainer before the experiment. The experimental mice were subsequently infused with 20% glucose until plasma glucose levels reached approximately 16-18 mM.

Dynamic assessment of insulin secretion. To assess insulin secretion, a perfusion system equipped with a peristaltic pump was used to deliver Krebs-Ringer bicarbonate (KRB) buffer (Sigma-Aldrich; Merck KGaA) to isolated pancreatic islets. Fifty size-matched islets were placed in columns and perfused at a flow rate of 100 µl/min with KRB buffer at 37˚C. Perfusion with 2.8 mM glucose was used to balance and measure basal secretion before the islets were exposed to different treatments. The medium was transferred to 96-well plates, and insulin levels were measured by the high sensitive mouse Insulin ELISA kit (cat. no. 32270; ImmunoDiagnostics), normalized to total islet DNA or protein as indicated.

Mitochondrial analysis. The real-time oxygen consumption of mitochondrial preparations was measured using a Seahorse XF analyzer (Agilent Technologies, Inc.). Islets (50/well) were cultured in 24-well plates in a medium consisting of unbuffered DMEM, 1% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 2.8 mM glucose at 37˚C without CO_2. The islets were then incubated in a high level of glucose (16.7 mM) and continuously treated with 1M FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone] and 5 M rotenone plus 5 M antimycin. The oxygen consumption rate (OCR) was calculated using an XF analyzer AKOS algorithm (Agilent Technologies, Inc.) and normalized to basal levels or to total protein content. Protein was extracted with a radioimmune precipitation lysis buffer, and the total protein content was determined using a Pierce BCA Protein Assay kit (Beyotime Institute of Biotechnology).

Western blotting. Protein was extracted from fresh islets using the radioimmune precipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with protease and phosphatase inhibitors (leupeptin, aprotinin and vanadate). Total protein was determined using a Pierce BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 µg) were separated by SDS-PAGE on 8 and 10% gels. Electrophoresis was performed under identical running and transferring conditions for all samples. Proteins were subsequently transferred to immun-Blot PVDF membrane and blocked using 5% w/v non-fat dry milk in TBST (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Membranes were incubated with the primary antibodies for 16-20 h at 4˚C. Antibody information is presented in Table I. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated antibodies, specifically anti-rabbit (1:2,000; cat. no. 7074) and anti-mouse IgG (1:2,000; cat. no. 7076; both from Cell Signaling Technology, Inc.), for 1 h at room temperature. Immunoreactive signals were detected using enhanced chemiluminescence reagents (EMD Millipore; Merck KGaA). Finally, densitometric analysis of the protein strips was performed using ImageJ version 1.46 (NIH).

Quantitative PCR. Total RNA from fresh islets was extracted and purified with Trizol (Sigma-Aldrich; Merck KGaA) and collected by centrifugation at 10,000 g for 10 min at 4˚C. Following quality analysis of the RNA samples, cDNA was synthesized by reverse transcription using 200 ng RNA and a High-capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Quantitative PCR was performed using 1X SYBR-Green Universal PCR Mastermix (Takara Bio, Inc.). The following primer sequences were used: PDX1, forward 5'-GGTATAGCCGGA GAGATGC-3', reverse 5'-CTGGTCCGTATTGGAACG-3';

Table I. Antibody information.

Antibody	Manufacturer	Catalogue number	Type	Species	Dilution factor
Anti-LAMTOR1	Abcam	ab229760	Polyclonal	Rabbit	1:1,000
Anti-Tubulin	Abcam	ab6046	Polyclonal	Rabbit	1:500
Anti-Hsp90	Santa Cruz	sc-13119	Monoclonal	Mouse	1:1,000
Anti-p-AMPK	Cell Signaling Technology	4186	Polyclonal	Rabbit	1:1,000
Anti-AMPK	Cell Signaling Technology	5831	Monoclonal	Rabbit	1:1,000
Anti-p-ACC1	Cell Signaling Technology	3661	Polyclonal	Rabbit	1:1,000
Anti-ACC1	Cell Signaling Technology	4190	Polyclonal	Rabbit	1:1,000
Anti-β-actin	Cell Signaling Technology	4967	Polyclonal	Rabbit	1:1,000

ACC1, acetyl-CoA carboxylase 1; p-ACC1, phosphorylated ACC1 Lamtor1, late endosomal/lysosomal adaptor MAPK and mTOR activator 1; AMPK, adenosine 5'-monophosphate-activated protein kinase; p-AMPK, phosphorylated AMPK.

INS2, forward 5'-TGGAGGCTCTCTACCTGGTG-3', reverse 5'-TCTACAATGCCACGCTTCTG-3'; GLUT2, forward 5'-CTTGGTTCATGGTTGCTGAAT-3', reverse 5'-GCAATG TACTGGAAGCAGAGG-3'; GCK, forward 5'-ATCTTCTGT TCCACGGAGAGG-3', reverse 5'-GATGTTAAGGATCTG CCTTCG-3'. The thermocycling conditions were as follows: 95˚C for 10 min; 40 cycles at 95˚C for 7 sec, 57˚C for 30 sec and extension at 72˚C for 30 sec. Reactions were performed in triplicate in 96-well plates, using the CFX96 real-time System (Bio-Rad Laboratories, Inc.). Transcript levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to the expression of internal reference gene GAPDH (13).

Statistical analysis. The data were analyzed using an unpaired two-tailed Student's t-test for experiments with only two groups and presented as mean ± standard deviation (SD). For multiple group comparisons, one-way ANOVA followed by Tukey's post hoc test was used. SPSS version 20.0.0 (IBM Corp.) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Production of βcell-specific Lamtor1-KO mice. To determine the physiological role of LAMTOR1 in pancreatic β-cells, β cell-specific *Lamtor1*-KO mice (*βLamtor1*-KO) were bred by crossing *Lamtor1*-floxed mice (*Lamtor11*$^{F/F}$) with RIP-Cre mice (Fig. 1). Immunoreactivity analysis confirmed the deletion of a floxed sequence from the primary islet cell genomic DNA of *βLamtor1*-KO mice. LAMTOR1 protein expression in the pancreatic islet cells of *βLamtor1*-KO mice was almost undetectable by western blotting compared with that in their *Lamtor1*$^{F/F}$ littermates (Fig. 2A).

Comparison of glucose tolerance between the two experimental animal groups. Glucose tolerance levels of 10-week-old mice in the *βLamtor1*-KO and *Lamtor1*$^{F/F}$ groups were determined by an *in vivo* i.p. glucose tolerance test (IPGTT). Comparative analysis revealed that the glucose tolerance of *βLamtor1*-KO mice was significantly lower than that of *Lamtor1*$^{F/F}$ mice at 30 and 60 min after glucose injection (Fig. 2B). As previously described, pancreatic β-cells play an important role in the development of diabetes, since glucose-induced insulin secretion is the key physiological function of these cells (14). Therefore, the potential involvement of LAMTOR1 in the regulation of β-cell function was evaluated. Quantitative PCR was performed to determine the mRNA expression levels of several related genes including *Pdx1*, *Ins2*, *Glut2* and *Gck*. *Pdx1* and *Ins2* are key regulators in insulin biosynthesis, while *Glut2* and *Gck* are associated with glucose uptake and glucose metabolism, respectively (15). The results suggest that their mRNA expression levels were similar between the two groups (Fig. 2C). In addition, there were no significant differences in the total pancreatic insulin content between the *βLamtor1*-KO and *Lamtor1*$^{F/F}$ groups of mice, whether they were fasted or fed (Fig. 2D). These results suggest that enhanced glucose-stimulated insulin secretion may explain the improvement in β-cell function observed in the *β Lamtor1*-KO group.

Lamtor1 knockout improves glucose-stimulated insulin secretion. To determine whether insulin secretion is enhanced in the absence of LAMTOR1, hyperglycemic clamping experiments were performed. The hyperglycemic clamp is the gold standard test for detecting pancreatic β-cells and can be used to identify both the first and second phases of insulin secretion (16). The hyperglycemic clamp experiments were conducted 4-5 days after the external jugular vein of 10-week-old mice had been catheterized. The two groups of mice were then injected with a 20% glucose solution to maintain their baseline blood glucose level at around 16-18 mM for 90 min (Fig. 3A). The mean glucose infusion levels were 21±1.1 mg/kg/min in the *βLamtor1*-KO mice, and 13±1.2 mg/kg/min in the *Lamtor1*$^{F/F}$ mice, at an overall duration of 90 min (Fig. 3B). The resulting curves showed that the temporal pattern of insulin secretion was normal in the two groups of mice, however the *βLamtor1*-KO mice released higher insulin levels compared with the *Lamtor1*$^{F/F}$ mice during both the first and second phases of the experiment (Fig. 3C). These data suggest that the loss of *Lamtor1* enhances insulin secretion in pancreatic β-cells.

To further confirm this conclusion, islet perfusion experiments were performed. The GSIS response curves revealed that insulin secretion in perfused *βLamtor1*-KO islets was

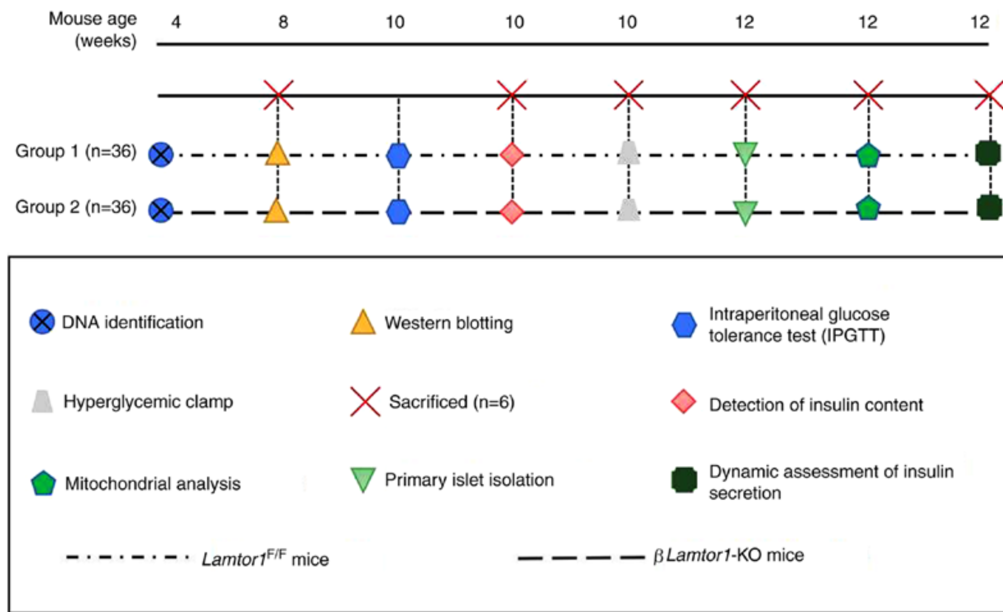

Figure 1. Experimental design. Mice were obtained by mating homozygous *Lamtor1*-floxed mice (*Lamtor1$^{F/F}$*) with RIP-Cre mice on a C57BL/6N background, and divided into two groups (n=36): Group1, Lamtor1-floxed mice; Group2, *βLamtor1*-KO mice. Lamtor1, late endosomal/lysosomal adaptor MAPK and mTOR activator 1; KO, knockout.

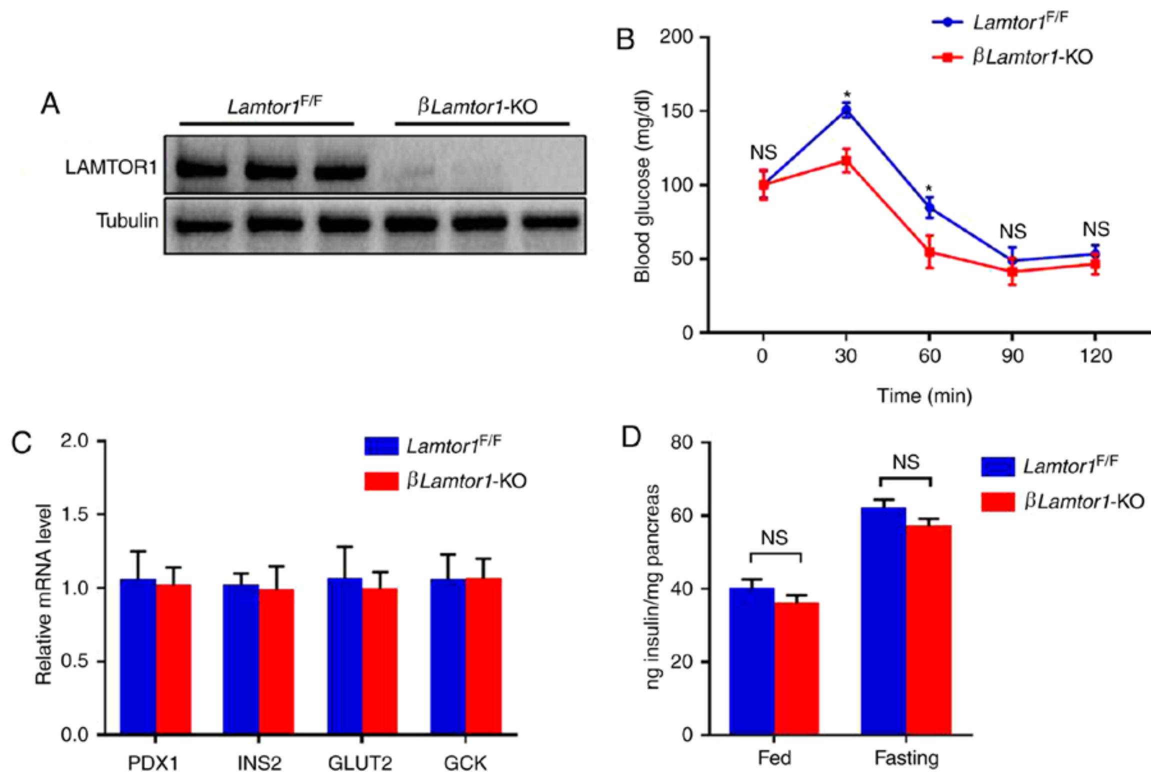

Figure 2. Effect of *Lamtor1* deficiency on insulin production. (A) Demonstration of successful *Lamtor1* knockout in *βLamtor1*-KO mice. Western blot showing the depletion of *Lamtor1* in islet lysates. (B) IPGTT was conducted on 8-week-old mice after 12 h of fasting, and blood glucose levels were measured. Data are shown as mean ± SD. *P<0.05, n=6. (C) Expression of Pdx1, Ins2, Glut2, and Gck was detected by real-time fluorescence quantitative-PCR, n=6. (D) Insulin content from the islets of fed and fasted *βLamtor1*-KO mice and *Lamtor1$^{F/F}$* littermate controls. Insulin content is presented relative to that of the pancreas. n=6. IPGTT, intraperitoneal glucose tolerance test; Lamtor1, late endosomal/lysosomal adaptor MAPK and mTOR activator 1; KO, knockout; NS, no significance.

similar to that in the control animals at low glucose concentrations (2.8 mM). However, increased glucose concentrations (16.7 mM) significantly enhanced insulin secretion in the islets of *βLamtor1*-KO mice (Fig. 3D). In addition, this experiment produced similar results to the hyperglycemic clamping experiment in that the mice in the *βLamtor1*-KO group secreted higher levels of insulin during both phases of the experiment.

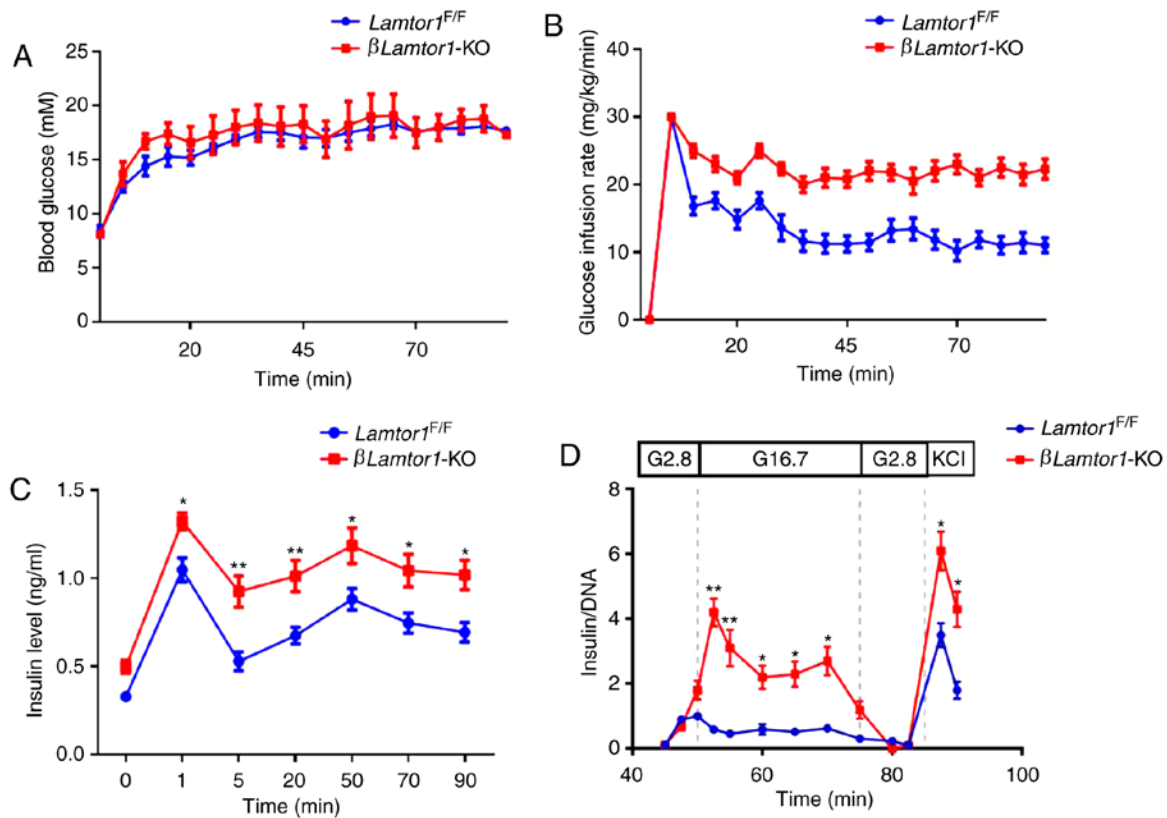

Figure 3. Loss of *Lamtor1* enhances glucose-stimulated insulin secretion. Result of hyperglycemic clamp in *Lamtor1*$^{F/F}$ and *βLamtor1*-KO mice. (A) Blood glucose levels. A total of 20% glucose was injected through the jugular vein catheter to maintain blood glucose levels at the 16.0-18.0 mM for 90 min. (B) Glucose infusion rate. Glucose infusion rates calculated by calculating the average amount of glucose (mg/kg/min) in 90 min. (C) Detection of insulin secretion levels. 20-30 µl blood samples were collected from the tail vein at 0, 1, 5, 20, 50, 70 and 90 min after glucose infusion to detect insulin levels. Data are shown as mean ± SD. *P<0.05, **P<0.01, n=6. (D) Dynamic insulin secretion assay. The levels of insulin secretion were measured after adding 2.8 mM glucose and 16.7 mM glucose respectively during perfusion of isolated islets. The addition of 30 mM KCl led to membrane depolarization, which increased insulin secretion. The insulin measured for each sample was normalized to total DNA. n=6.

Effect of the triggering pathway on insulin secretion in the islets of βLamtor1-KO mice. Triggering and amplification are two action pathways that regulate insulin secretion in response to glucose (17,18), in which cytosolic Ca^{2+} is a triggering signal. The potential enhancement of insulin secretion via the triggering pathway in *Lamtor1*-deficient β-cells was evaluated using islet perfusion experiments (19). The *βLamtor1*-KO perfused islets were found to secrete more insulin than the control islets during stimulation with 16.7 mM glucose (Fig. 4A). Treating mice with diazoxide, a reagent that effectively opens K_{ATP} channels (20), completely inhibited glucose-induced insulin secretion in both experimental groups. On this basis, the secretion of insulin was also monitored in the two groups following the addition of potassium chloride (KCl). In these experiments, higher insulin levels were secreted in the *βLamtor1*-KO group than in the control group, which is consistent with previous results. Subsequently, to further analyze the problem, the K_{ATP} channels were closed by glyburide treatment. Higher insulin secretion was observed in the *βLamtor1*-KO group than in the control group during stimulation with either basal (2.8 mM) or high glucose concentrations (16.7 mM) (Fig. 4B). Thus, higher insulin levels were produced in the *βLamtor1*-KO group with a similar degree of membrane depolarization, suggesting that K_{ATP} channels are not responsible for enhancing insulin secretion in the *Lamtor1*-deficient β-cells.

Perfused islets were also treated with nifedipine, an inhibitor of voltage-gated calcium channels. Results showed that the addition of nifedipine led to a significant reduction in insulin secretion in both groups (Fig. 4C). The results suggest that VDCCs are necessary for insulin secretion in the islets of *βLamtor1*-KO mice. Combined with our conclusions about K_{ATP} channels, the triggering pathway does not sufficiently explain the effect of glucose on insulin secretion in *βLamtor1*-KO islets.

Deletion of Lamtor1 leads to mitochondrial dysfunction. It was hypothesized that the deletion of *Lamtor1* would enhance mitochondrial function, and thus experiments were designed to verify this possibility. However, the results of the present study could not confirm our initial hypothesis. While the ATP content increased in both the *βLamtor1*-KO and control groups, after stimulation with high glucose concentrations, it was markedly lower in the *βLamtor1*-KO group than in the control group, indicating that insulin secretion did not enhance mitochondrial function in the β-cells of *βLamtor1*-KO mice (Fig. 4D).

In addition, the oxygen consumption rate (OCR), an indicator of the extent of aerobic glucose metabolism was assessed. OCR at the basal glucose level was slightly increased in the *βLamtor1*-KO islets but did not reach a level of statistical significance (Fig. 4E). However, *Lamtor1* deletion eliminated

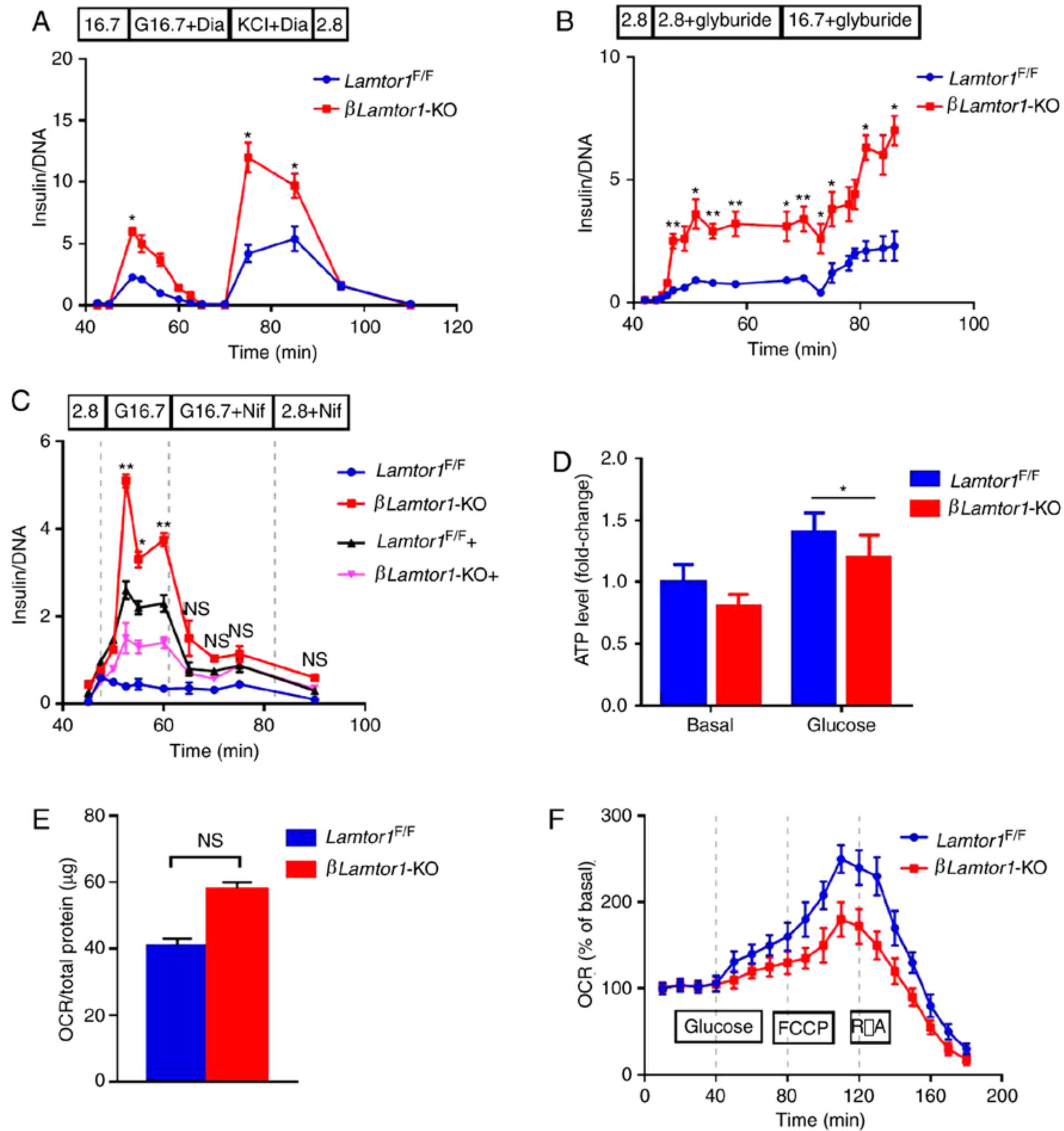

Figure 4. Lamtor1 deficiency increases insulin secretion but impairs mitochondrial function. (A) Insulin levels measured during the perifusion of isolated islets. *Lamtor1*-deficient islets secrete higher levels of insulin than the control under 16.7 mM glucose stimulation, and administration of 100 μM diazoxide eliminates this difference; however, the addition of 30 mM KCl causes a second peak in insulin secretion. (B) Insulin secretion followed by glyburide treatment. 1 μM glyburide induces higher levels of insulin secretion from *Lamtor1*-deficient islets, under both low and high glucose conditions. (C) Glucose-stimulated insulin secretion from perifused islets treated with nifedipine. Black and pink lines; nifedipine was added before high glucose. Blue and red lines; nifedipine was added 15 min after the addition of high glucose. Data are shown as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, n=6. (D) Adenosine triphosphate levels of islets responsive to basal and high glucose (16.7 mM). $^{*}P<0.05$. (E) Basal OCR measured by the Seahorse XF24 analyzer in the presence of 2.8 mM glucose. n=6. (F) OCR measured over a time period of 180 min. Glucose (20 mM), FCCP (1 μM) and rotenone plus antimycin A (5 μM each) were added at the indicated times. Día, diazoxide; Nif, nifedipine; ATP, adenosine triphosphate; OCR, oxygen consumption rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; R/A, rotenone plus antimycin A; NS, no significance.

the high glucose-induced OCR (Fig. 4F). Moreover, the OCR was still lower after the addition of the mitochondrial un-coupler FCCP than in the control islets. Taken together, these results indicate that LAMTOR1 deficiency leads to mitochondrial dysfunction in pancreatic β-cells, further supporting the conclusion that the enhanced insulin secretion from *βLamtor1*-KO islets is not caused by the triggering pathway of insulin secretion.

Deletion of Lamtor1 amplifies insulin secretion by increasing glutamate. Previous results have demonstrated that enhanced insulin secretion in the absence of LAMTOR1 is not associated with the triggering pathway. Thus, the next step in the present study was to examine the role of the amplifying pathway. Glutamate is derived from the malate-aspartate shuttle upon glucose stimulation and has an enhanced effect on calcium function (21-23). To determine whether glucose-stimulated insulin secretion in *βLamtor1*-KO mice was enhanced by increasing glutamate production, the ^{13}C enrichment of glutamate was measured in whole cells and the cytosol of islet cells with or without aminooxyacetate [AOA, an inhibitor of the malate-aspartate shuttle (24)] treatment,

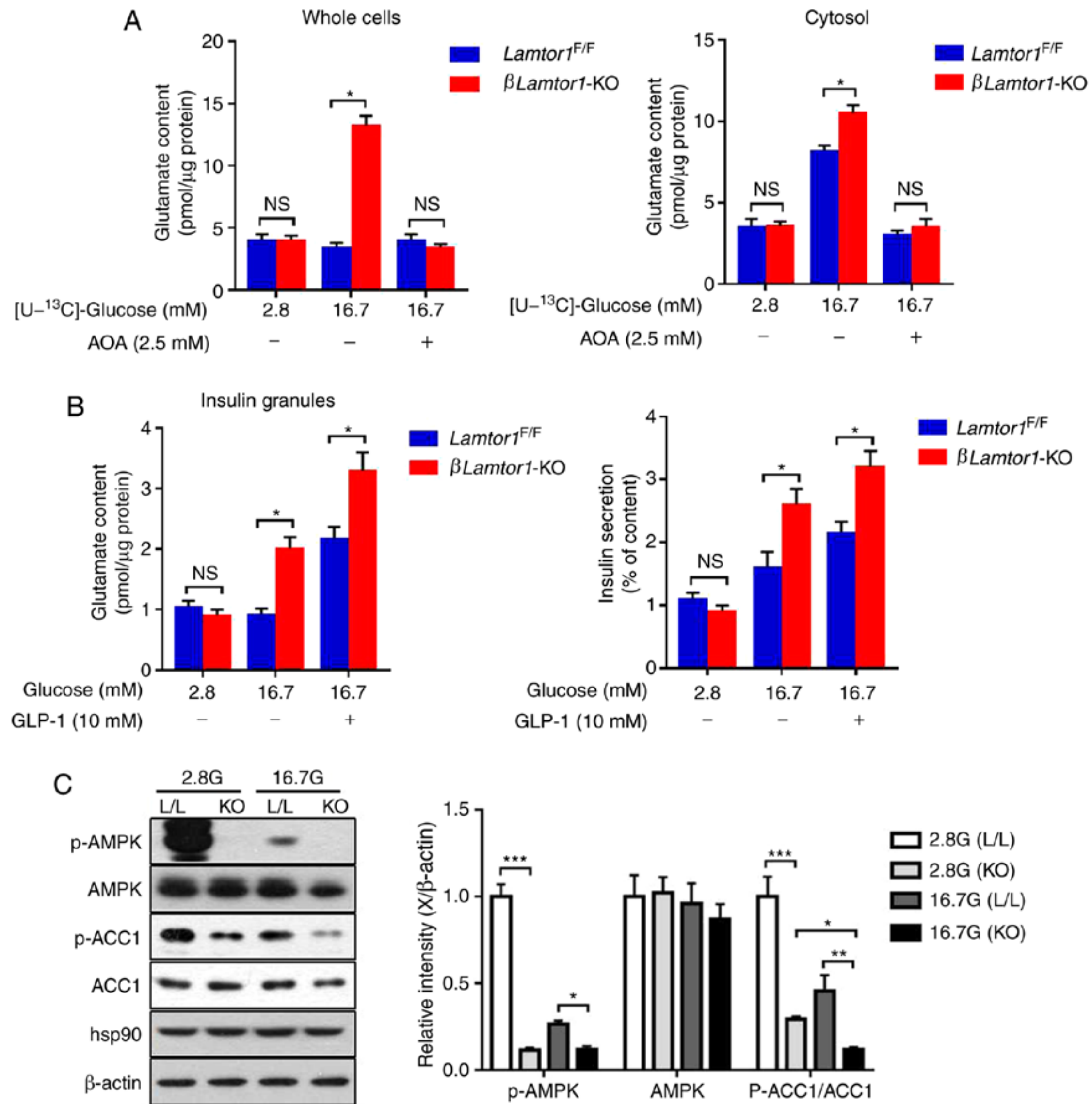

Figure 5. *Lamtor1* deficiency in pancreatic β-cells amplifies insulin secretion by increasing glutamate production and ACC1 activity. (A) Effects of aminooxyacetate (2.5 mM) on the content of glutamate isotopomers in whole cells and the cytosol in isolated *Lamtor1*-deficient and control pancreatic β-cells. n=6. (B) Changes in glutamate content in the insulin granules and insulin secretion under glucose stimulation in the absence or presence of glucagon-like peptide 1 (10 nM). (C) Western blots showing phosphorylated adenosine 5'-monophosphate-activated protein kinase, total adenosine 5'-monophosphate-activated protein kinase, phosphorylated ACC1, total ACC1, heat-shock protein 90 and β-actin protein levels in $Lamtor1^{F/F}$ and *βLamtor1*-KO mouse islets under low- and high-glucose stimulation. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, n=6. ACC1, acetyl-CoA carboxylase 1; p-ACC1, phosphorylated ACC1; AOA, aminooxyacetate; GLP-1, glucagon-like peptide 1; AMPK, adenosine 5'-monophosphate-activated protein kinase; p-AMPK, phosphorylated AMPK; hsp90, heat-shock protein 90; NS, no significance.

while stimulating with [U-^{13}C]-glucose (Fig. 5A). The glutamate levels in the whole cells and cytosol were compared between the *βLamtor1*-KO and control groups after treatment with high glucose, AOA and glucagon-like peptide 1 (GLP-1). We found that the glutamate content in both the whole cells and the islet cytosol of *βLamtor1*-KO mice was significantly higher than that of the controls, when exposed to high glucose (16.7 mM). In addition, treatment with AOA significantly inhibited glutamate production in both the whole cells and the cytosol. The results indicated that *Lamtor1* deletion led to increased glutamate production. Moreover, the glutamate content of the insulin granules and the insulin secretion in the *βLamtor1*-KO group were further increased by the addition of GLP-1 (Fig. 5B). This indicated that *Lamtor1* deletion increased GLP-1-stimulated insulin secretion.

Lamtor1 knockout affects ACC1 activity in pancreatic β-cells. AMPK phosphorylation negatively regulates ACC1 and ACC2 enzyme activity *in vivo*, inhibiting the activity of ACC1 and ACC2 (25). It has been reported that *Lkb1* deletion inhibits the phosphorylation of AMPK, which in turn inhibits ACC1 phosphorylation, leading to the increase of ACC1 activity and promotion of insulin secretion (10). Therefore, we speculated that LAMTOR1 affects the activity of ACC1. Western blotting analysis showed that the level of AMPK phosphorylation in the *βLamtor1*-KO group was lower than that in the control group under both low and high glucose conditions, resulting in a decrease in the ratio of p-ACC1/ACC1 in the *βLa*mtor1-KO group, thereby increasing the ACC1 activity. The level of phosphorylation of ACC1 decreased with increasing glucose concentration, leading to an increase in

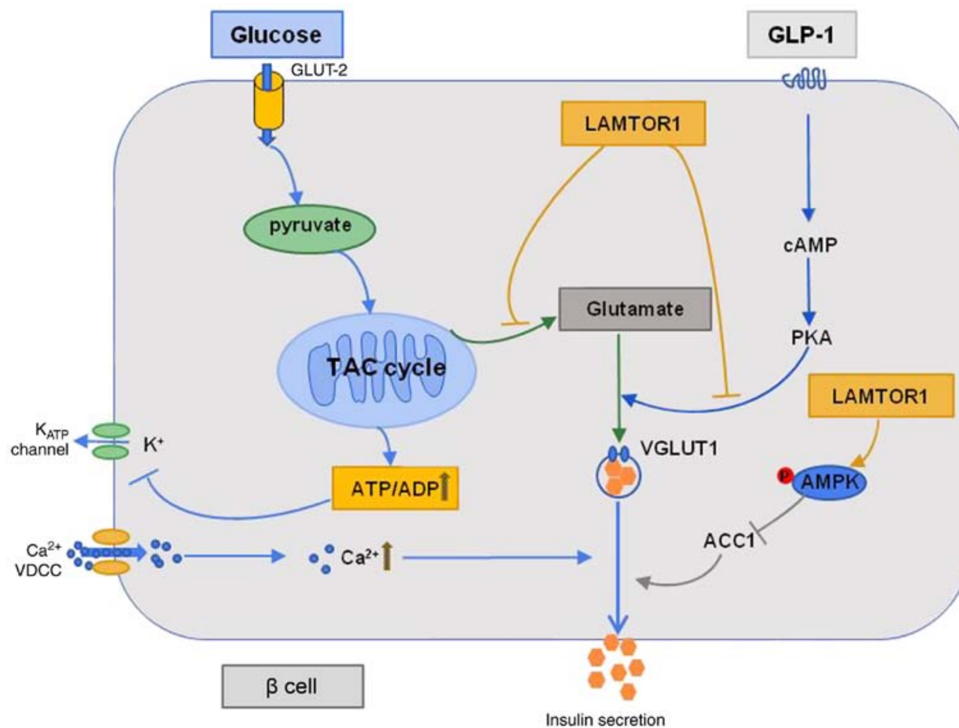

Figure 6. Lamtor1 attenuates insulin secretion by inhibiting the amplification pathways associated with glutamate and ACC1 metabolism. As demonstrated, a number of processes are required for Lamtor1 to produce this result, including: i) Inhibition of insulin secretion by reducing glutamate levels in whole cells and cytosol; ii) inhibition of incretin/cAMP signaling and prevention of cytosolic glutamate transport into insulin granules, thereby inhibiting insulin secretion (23); iii) promotion of AMPK and ACC1 phosphorylation, which leads to the inhibition of ACC1 activity and consequent decrease in insulin secretion (10). Lamtor1, late endosomal/lysosomal adaptor MAPK and mTOR activator 1; ACC1, acetyl-CoA carboxylase 1.

ACC1 activity (Fig. 5C). In agreement with this hypothesis, as demonstrated in Fig. 5C, knocking out *Lamtor1* in β-cells inhibited phosphorylation of AMPK, and resulted in increased ACC1 activity.

Discussion

Diabetes is a systemic disease characterized by abnormal glucose metabolism, which is mainly caused by insufficient insulin secretion and insulin resistance (26). In the present study, the effect of LAMTOR1 on insulin secretion, in response to stimulation by glucose and glucose metabolic pathways, was analyzed. Results suggest that *Lamtor1* deletion increases glucose tolerance and insulin secretion, which is associated with certain side effects, leading to mitochondrial dysfunction. In addition, this study explored the possible mechanisms of action employed by LAMTOR1 in the context of glucose-stimulated insulin secretion. It was found that LAMTOR1 deficiency stimulated an alternative pathway that amplified insulin secretion, thus compensating for the effects of the classical triggering pathway.

Mitochondria are the processing plants for cellular energy metabolism. Their main function is to remove hydrogen from glucose, fat and protein molecules that are being metabolized as foodstuffs by oxidation-phosphorylation to form ATP, thus fueling the body. Mitochondria combine glucose metabolism with extracellular insulin secretion through the tricarboxylic acid (TCA) cycle (27). Mitochondrial dysfunction decreases ATP production and reduces the ATP/ADP ratio in pancreatic β-cells, opening the ATP-sensitive K^+ channels in the cell membrane. This enhances the efflux of intracellular K^+ and the hyperpolarization of the cell membrane, inhibiting insulin secretion. In the present study, opposite results were identified. The deletion of *Lamtor1* in pancreatic β-cells caused a decrease in mitochondrial function but an increase in insulin secretion. Moreover, the glucose oxidation-dependent triggering pathway was found to be defective in *βLamtor1*-KO mice, implying that other more effective insulin secretion mechanisms may be involved, such as the amplifying pathway in *Lamtor1*-deficient β-cells.

In order to further characterize the pathways by which glucose stimulates insulin secretion, an amplifying pathway was analyzed by increasing glutamate production, which could in turn promote the action of calcium. It was found that the glutamate content in the whole cells and the islet cytosol of *βLamtor1*-KO mice was higher than that in the control groups, indicating that the amplifying pathway may play an important role in enhanced glucose-stimulated insulin secretion in the *Lamtor1*-deficient β-cells. Therefore, it appears that the amplifying effect is not mediated by increased ATP production but instead by glutamate. It is well known that incretin/cAMP signaling stimulates insulin secretion in a glucose-dependent manner. Cytosolic glutamate is transported into the insulin granules via incretin/cAMP signaling and amplifies insulin release (23). Our results indicate that *Lamtor1* deletion enhanced insulin release by increasing glutamate content in insulin granules via incretin-induced cAMP/PKA signaling.

Increasing ACC1 activity represents an additional pathway for the enhancement of insulin secretion in the absence of

LAMTOR1. ACC1 catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which inhibits mitochondrial fatty acid oxidation and results in insulin secretion (28). Inhibition of ACC1 activity leads to a decrease in pyruvate cycle activity, resulting in a significant drop in glucose-stimulated insulin secretion, suggesting that ACC1 may play a role in GSIS through pyruvate metabolism (29).

Taken together, LAMTOR1 is important for the maintenance of mitochondrial function in pancreatic β-cells. Although *Lamtor1* deletion impairs insulin secretion via the triggering pathway, it improves insulin secretion via the amplifying pathways associated with glutamate and ACC1 metabolism. These amplifying pathways compensate for the defective triggering pathway, and ultimately lead to an increase in glucose-stimulated insulin secretion (Fig. 6). These findings emphasize the importance of LAMTOR1 in modulating insulin secretion and propose the deletion of *Lamtor1* as a viable therapeutic strategy for diabetes and the improvement of pancreatic β-cell function.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QH, QG and TW performed the experiments and wrote the manuscript. SF, JX and JL assisted in the conduct of the experiments. XH, QL and JH performed the literature search and designed the study. LZ contributed to project design, data acquisition and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Maechler P, Li N, Casimir M, Vetterli L, Frigerio F and Brun T: Role of mitochondria in beta-cell function and dysfunction. Adv Exp Med Biol 654: 193-216, 2010.
2. Thorens B: GLUT2, glucose sensing and glucose homeostasis. Diabetologia 58: 221-232, 2015.
3. MacDonald PE, Joseph JW and Rorsman P: Glucose-sensing mechanisms in pancreatic beta-cells. Philos Trans R SocLond B Biol Sci 360: 2211-2225, 2005.
4. Tarasov A, Dusonchet J and Ashcroft F: Metabolic regulation of the pancreatic beta-cell ATP-sensitive K+ channel: A pas de deux. Diabetes 53 (Suppl 3): S113-S122, 2004.
5. Fridlyand LE and Phillipson LH: Mechanisms of glucose sensing in the pancreatic β-cell: A computational systems-based analysis. Islets 3: 224-230, 2011.
6. Lei Y, Gong L, Tan F, Liu Y, Li S, Shen H, Zhu M, Cai W, Xu F, Hou B, *et al*: Vaccarin ameliorates insulin resistance and steatosis by activating the AMPK signaling pathway. Eur J Pharmacol 851: 13-24, 2019.
7. Hardie DG, Ross FA and Hawley SA: AMPK: A nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13: 251-262, 2012.
8. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR and Hardie DG: Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2: 28, 2003.
9. Granot Z, Swisa A, Magenheim J, Stolovich-Rain M, Fujimoto W, Manduchi E, Miki T, Lennerz JK, Stoeckert CJ Jr, Meyuhas O, *et al*: LKB1 regulates pancreatic beta cell size, polarity, and function. Cell Metab 10: 296-308, 2009.
10. Fu A, Robitaille K, Faubert B, Reeks C, Dai XQ, Hardy AB, Sankar KS, Ogrel S, Al-Dirbashi OY, Rocheleau JV, *et al*: LKB1 couples glucose metabolism to insulin secretion in mice. Diabetologia 58: 1513-1522, 2015.
11. Bar-Peled L, Schweitzer LD, Zoncu R and Sabatini DM: Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell 150: 1196-1208, 2012.
12. Zhang CS, Jiang B, Li M, Zhu M, Peng Y, Zhang YL, Wu YQ, Li TY, Liang Y, Lu Z, *et al*: The lysosomal v-ATPase-Ragulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism. Cell Metab 20: 526-540, 2014.
13. Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008.
14. Fu Z, Gilbert ER and Liu D: Regulation of insulin synthesis and secretion and pancreatic beta-cell dysfunction in diabetes. Curr Diabetes Rev 9: 25-53, 2013.
15. Li X, Cheng KKY, Liu Z, Yang JK, Wang B, Jiang X, Zhou Y, Hallenborg P, Hoo RL, Lam KSL, *et al*: The MDM2-p53-pyruvate carboxylase signalling axis couples mitochondrial metabolism to glucose-stimulated insulin secretion in pancreatic β-cells. Nat Commun 7: 11740, 2016.
16. Henquin JC, Nenquin M, Stiernet P and Ahren B: In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: Pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells. Diabetes 55: 441-451, 2006.
17. Henquin JC, Ravier MA, Nenquin M, Jonas JC and Gilon P: Hierarchy of the beta-cell signals controlling insulin secretion. Eur J Clin Invest 33: 742-750, 2003.
18. Henquin JC, Dufrane D and Nenquin M: Nutrient control of insulin secretion in isolated normal human islets. Diabetes 55: 3470-3477, 2006.
19. Swisa A, Granot Z, Tamarina N, Sayers S, Bardeesy N, Philipson L, Hodson DJ, Wikstrom JD, Rutter GA, Leibowitz G, *et al*: Loss of liver kinase B1 (LKB1) in beta cells enhances glucose-stimulated insulin secretion despite profound mitochondrial defects. J BiolChem 290: 20934-20946, 2015.
20. Misler S, Gee WM, Gillis KD, Scharp DW and Falke LC: Metabolite-regulated ATP-sensitive K+ channel in human pancreatic islet cells. Diabetes 38: 422-427, 1989.
21. Maechler P: Glutamate pathways of the beta-cell and the control of insulin secretion. Diabetes Res Clin Pract 131: 149-153, 2017.
22. Murao N, Yokoi N, Honda K, Han G, Hayami T, Gheni G, Takahashi H, Minami K and Seino S: Essential roles of aspartate aminotransferase 1 and vesicular glutamate transporters in β-cell glutamate signaling for incretin-induced insulin secretion. PLoS One 12: e0187213, 2017.

23. Gheni G, Ogura M, Iwasaki M, Yokoi N, Minami K, Nakayama Y, Harada K, Hastoy B, Wu X, Takahashi H, *et al*: Glutamate acts as a key signal linking glucose metabolism to incretin/cAMP action to amplify insulin secretion. Cell Rep 9: 661-673, 2014.
24. Eto K, Tsubamoto Y, Terauchi Y, Sugiyama T, Kishimoto T, Takahashi N, Yamauchi N, Kubota N, Murayama S, Aizawa T, *et al*: Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. Science 283: 981-985, 1999.
25. Fullerton MD, Galic S, Marcinko K, Sikkema S, Pulinilkunnil T, Chen ZP, O'Neill HM, Ford RJ, Palanivel R, O'Brien M, *et al*: Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nat Med 19: 1649-1654, 2013.
26. Tripathy D and Chavez AO: Defects in insulin secretion and action in the pathogenesis of type 2 diabetes mellitus. Curr Diab Rep 10: 184-191, 2010.
27. Supale S, Li N, Brun T and Maechler P: Mitochondrial dysfunction in pancreatic β cells. Trends Endocrinol Metab 23: 477-487, 2012.
28. Zhang S and Kim KH: Essential role of acetyl-CoA carboxylase in the glucose-induced insulin secretion in a pancreatic beta-cell line. Cell Signal 10: 35-42, 1998.
29. Ronnebaum SM, Joseph JW, Ilkayeva O, Burgess SC, Lu D, Becker TC, Sherry AD and Newgard CB: Chronic suppression of acetyl-CoA carboxylase 1 in beta-cells impairs insulin secretion via inhibition of glucose rather than lipid metabolism. J Biol Chem 283: 14248-14256, 2008.

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