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ARTICLE

SL010110, a lead compound, inhibits gluconeogenesis via SIRT2-p300-mediated PEPCK1 degradation and improves glucose homeostasis in diabetic mice

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Suppression of excessive hepatic gluconeogenesis is an effective strategy for controlling hyperglycemia in type 2 diabetes (T2D). In the present study, we screened our compounds library to discover the active molecules inhibiting gluconeogenesis in primary mouse hepatocytes. We found that SL010110 (5-((4-allyl-2-methoxyphenoxy) methyl) furan-2-carboxylic acid) potently inhibited gluconeogenesis with 3 μM and 10 μM leading to a reduction of 45.5% and 67.5%, respectively. Moreover, SL010110 caused suppression of gluconeogenesis resulted from downregulating the protein level of phosphoenolpyruvate carboxykinase 1 (PEPCK1), but not from affecting the gene expressions of PEPCK, glucose-6-phosphatase, and fructose-1,6-bisphosphatase. Furthermore, SL010110 increased PEPCK1 acetylation, and promoted PEPCK1 ubiquitination and degradation. SL010110 activated p300 acetyltransferase activity in primary mouse hepatocytes. The enhanced PEPCK1 acetylation and suppressed gluconeogenesis caused by SL010110 were blocked by C646, a histone acetyltransferase p300 inhibitor, suggested that SL010110 inhibited gluconeogenesis by activating p300. SL010110 decreased NAD^+/NADH ratio, inhibited SIRT2 activity, and further promoted p300 acetyltransferase activation and PEPCK1 acetylation. These effects were blocked by NMN, an NAD^+ precursor, suggested that SL010110 inhibited gluconeogenesis by inhibiting SIRT2, activating p300, and subsequently promoting PEPCK1 acetylation. In type 2 diabetic *ob/ob* mice, single oral dose of SL010110 (100 mg/kg) suppressed gluconeogenesis accompanied by the suppressed hepatic SIRT2 activity, increased p300 activity, enhanced PEPCK1 acetylation and degradation. Chronic oral administration of SL010110 (15 or 50 mg/kg) significantly reduced the blood glucose levels in *ob/ob* and *db/db* mice. This study reveals that SL010110 is a lead compound with a distinct mechanism of suppressing gluconeogenesis via SIRT2-p300-mediated PEPCK1 degradation and potent anti-hyperglycemic activity for the treatment of T2D.

Keywords: SL010110; gluconeogenesis; primary mouse hepatocytes; PEPCK1; acetylation; degradation

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INTRODUCTION

Hyperglycemia is one of the most remarkable pathological features of type 2 diabetes (T2D) [1]. The liver plays a critical role in maintaining glucose homeostasis by regulating glycogenesis, glycogenolysis, and gluconeogenesis throughout the fed-fasting cycle [2]. Excessive hepatic gluconeogenesis has been recognized as one of the factors that contribute the most to the occurrence of hyperglycemia in T2D [3, 4]. Metformin, a drug that is widely used in the treatment of diabetes, improves glucose metabolism mainly by inhibiting hepatic gluconeogenesis [5]. Thus, targeting the suppression of hepatic gluconeogenesis has been regarded as an effective strategy for the discovery of active lead compounds for the treatment of T2D.

Hepatic gluconeogenesis is mainly controlled by key rate-limiting enzymes, specifically phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6pase), and fructose-1,6-bisphosphatase (FBPase) [6, 7]. Two isozymes of PEPCK are expressed in the liver: a cytoplasmic form (PEPCK1) and a mitochondrial isoform (PEPCK2). PEPCK1 is responsible for 95% of the PEPCK activity in the liver and thus is considered the main isoform involved in gluconeogenesis [8, 9]. siRNA-mediated knockdown of PEPCK1 resulted in a decrease in glucose production [10]. Targeting key gluconeogenesis enzymes directly or indirectly through the modulation of their transcription factors or coactivators, such as PPARγ coactivator 1α (PGC-1α) and cAMP-response element-binding protein (CREB), was shown to be an effective strategy for regulating hepatic gluconeogenesis and controlling hyperglycemia [11–13]. Recently, several studies revealed that posttranslational modification of PEPCK could promote its degradation and affect hepatic gluconeogenesis both in vitro and in vivo [14–18]. Zhao and colleagues found that some lysine residues of PEPCK1 could be acetylated in response to the availability of extracellular fuels, and this acetylation allowed PEPCK1 to interact with the ubiquitin protein ligase E3 component n-recognin 5 (UBR5) and then be degraded in the proteasome [15]. Subsequent studies showed that sumoylation or phosphorylation of PEPCK1 also caused the protein to become labile and led to its degradation [19, 20].

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In recent years, many compounds, such as metformin, epigallocatechin gallate, linderane, and phanginin A, have been reported to suppress gluconeogenesis by inhibiting the gene expression of PEPCK and G6pase [5, 21–23]. However, to date, few molecules have been reported to suppress gluconeogenesis by modulating PEPCK1 protein stability.

p300, which is known as a transcription factor that regulates gluconeogenesis by binding to the pck1 promoter, can also act as an acetyltransferase, catalyzing the acetylation of PEPCK1 and affecting its protein stability [15, 24]. Co-transfection of PEPCK1 with p300, but not its catalytically inactive mutant, stimulated the acetylation of PEPCK1 in HEK293T cells, indicating that the acetyltransferase activity of p300 is necessary [15]. Consistently, p300 knockdown significantly decreased PEPCK1 acetylation and increased steady-state PEPCK1 levels [15]. PEPCK1 protein stability could also be modulated by SIRT2, an NAD^+-dependent deacetylase. The overall levels of acetylated PEPCK1 were decreased by incubating the prokaryotically expressed SIRT2 protein with a peptide containing two acetylated sites (Lys70 and Lys71) of PEPCK1 [17]. Sirtinol, a SIRT2 inhibitor, abolished the effect of SIRT2 on the deacetylation of a PEPCK1 peptide [17]. Since SIRT2 could deacetylate p300 and regulate its activity [25], it would be interesting to investigate whether the regulation of PEPCK1 by SIRT2 could be mediated by p300.

Given the importance of hepatic gluconeogenesis for whole-body glucose homeostasis, we screened our compound library to identify active molecules that inhibit gluconeogenesis in primary mouse hepatocytes. SL010110 (5-((4-allyl-2-methoxyphenoxy) methyl) furan-2-carboxylic acid), a compound that has never been reported to have any biological effects, showed potent inhibition of gluconeogenesis. Moreover, SL010110 showed no effect on the gene expression of PEPCK and G6pase but significantly downregulated the protein expression of PEPCK1, suggesting a distinct mechanism of SL010110 in the suppression of hepatic gluconeogenesis. In the present study, we elucidated the underlying molecular mechanisms by which SL010110 inhibits gluconeogenesis through SIRT2-p300-mediated PEPCK1 acetylation and degradation. In addition, the acute and chronic effects of SL010110 were evaluated in *ob/ob* and *db/db* mice to investigate the anti-hyperglycemic efficacy of SL010110 and the value of promoting PEPCK1 degradation as an effective approach of glycemic control in the treatment of diabetes.

MATERIALS AND METHODS

Chemicals and materials

SL010110 (5-((4-allyl-2-methoxyphenoxy) methyl) furan-2-carboxylic acid, $M_r = 288.3$) was synthesized by J.H Shen's group, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (SIMM, CAS). Metformin, forskolin, and β-nicotinamide mononucleotide (NMN) were purchased from Sigma-Aldrich (Hesse, Germany). MG132, ketoconazole, C646, and sirtinol were purchased from Med Chem Express (New Jersey, USA). Antibodies against GAPDH (#2118), p300 (#86377), BAT3 (#8523), ubiquitin (#3933), acetylated lysine (#9441), and acetyl-p300 (K1499) (#4771) were purchased from Cell Signaling Technology (Massachusetts, USA). Antibodies against PEPCK1 (16754-1-AP) were purchased from Proteintech (Illinois, USA). HRP-conjugated goat anti-rabbit Ig (H + L) antibodies (SH-0032) were obtained from Dingguo Changsheng Biotechnology (Beijing, China).

Animals

Male C57BL/6 J mice were supplied by Ling Chang Biotechnology (Shanghai, China) and fed a standard diet. B6.V-*Lep*^{ob}/*Lep*^{ob}mice and B6.Cg-m*Lepr*^{db}/*Lepr*^{db}mice (Jackson Laboratory, Maine, USA) were bred at SIMM, CAS and fed a high-fat diet (Cat. P1400F, Puluteng, Shanghai, China). All the mice were housed in a specific pathogen-free class laboratory and maintained under a standard 12 h-light/12 h-dark cycle. All the animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Utilization Committee, SIMM, CAS.

Culture of primary mouse hepatocytes

Primary hepatocytes were isolated from C57BL/6 J mice by Seglen's two-step method with some modifications [26]. The overnight-fasted mice were anesthetized by intraperitoneal injection of 5% (w/v) chloral hydrate (SCR, Shanghai, China), and their livers were perfused with ethylene glycol tetra acetic acid buffer and collagenase digestion buffer. The mouse hepatocytes were isolated by filtration and centrifugation. Then, the hepatocytes were seeded in gelatin-coated plates with minimum essential medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10 nM insulin, and 10 nM dexamethasone (Sigma-Aldrich, Hesse, Germany), and cultured at 37 °C in 5% CO_2.

Gluconeogenesis

The primary mouse hepatocytes were seeded in 48-well plates 4 h before the experiment. Then, the hepatocytes were incubated in 0.1% bovine serum albumin in glucose-free Dulbecco's modified Eagle's medium supplemented with 0.1% dimethyl sulfoxide (DMSO) or different doses of SL010110 or 500 μM metformin for 1.5 h. Then, the hepatocytes were incubated for 4 h with or without gluconeogenic substrates (2 mM sodium pyruvate and 20 mM sodium lactate) (Sigma-Aldrich, Hesse, Germany) in the presence or absence of 20 μM forskolin. For the experiments with inhibitors, the hepatocytes were pretreated with different inhibitors for 0.5 h, and then, the procedure described above was followed. The medium was collected at the end of the incubation. The glucose content was determined with a colorimetric glucose assay kit (RSbio, Shanghai, China) and normalized to the cellular protein concentration. Gluconeogenesis was calculated based on the difference between the groups cultured with and without gluconeogenic substrates.

PEPCK, G6pase, and FBPase activity assays

The PEPCK, G6pase, or FBPase activity in primary mouse hepatocytes and livers from *ob/ob* mice was measured with a PEPCK Activity Kit (BC3310), a G6pase Activity Kit (BC3320), or a FBPase Activity Kit (BC0920), respectively, from Solarbio Life Sciences (Beijing, China) according to the manufacturer's instructions.

p300 acetyltransferase activity assay

The p300 activity was determined by a HAT (p300) Assay Kit (AS-72172, AnaSpec, California, USA) with some modifications [27]. After treatment with the compounds, the p300 protein was immunoprecipitated from the primary mouse hepatocytes with anti-p300 antibody and then incubated with acetyl-CoA and substrate peptide at 37 °C for 15 min. The reaction was terminated by the Stop Solution, followed by further incubation with the Developer Solution at room temperature for 30 min. The p300 acetyltransferase activity was determined by measuring the fluorescence intensity at Ex/Em = 389/513 nm.

Sirt activity assay

The cytoplasm and nucleus were separated from the primary mouse hepatocytes and livers from *ob/ob* mice by extraction reagents (78835, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. The Sirt activities in the nucleus (mainly SIRT1) and cytoplasm (mainly SIRT2) [28] were detected by a SIRT Activity Assay Kit (P-4036-48, Epigentek Group, New York, USA). Briefly, after adding the Assay Buffer, Sirt substrate, NAD^+, TSA, and extracted samples into the strip wells, the test plate was covered with an adhesive film and incubated at 37 °C for

90 min. Then, the reaction solution was removed, and the capture antibody was added and incubated for 60 min. After washing, the detection antibody was added and incubated for 30 min, followed by one additional wash. Subsequently, a developing reaction was conducted with the Developer Solution for 10 min and then stopped with the Stop Solution. The Sirt activity was assessed by detecting the absorbance at 450 and 655 nm.

NAD^+/NADH measurement

The NAD_{total} (NAD^+ and NADH) and NADH contents in the hepatocytes and liver tissues from the *ob/ob* mice were measured with a commercial NAD^+/NADH assay kit (S1075, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The NAD^+ content was based on the difference between the NAD_{total} and NADH contents. Then, the NAD^+/NADH ratio was calculated based on the ratio of the NAD^+ content to the NADH content.

Real-time PCR

Total RNA was extracted from the primary mouse hepatocytes or livers from *ob/ob* mice by TRIzol reagent (15596018, Thermo Fisher Scientific, Massachusetts, USA), according to the manufacturer's instructions, and the RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit supplemented with gDNA eraser (RR037, Takara Bio, Kyoto, Japan). PCRs were performed using SYBR® Premix Ex Taq (RR420, Takara Bio, Kyoto, Japan). The gene expression levels were normalized against the β-actin expression levels, and the relative expression compared to the experimental controls was determined using the $2^{-\Delta\Delta CT}$ method. The primer sequences are shown in Table 1.

Immunoprecipitation and immunoblotting

For immunoprecipitation, primary hepatocytes or *ob/ob* mouse livers were lysed and mixed with the indicated primary antibodies overnight at 4 °C. Then, these solutions were mixed with protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology, California, USA) for 3 h. The immunoprecipitants were washed five times with lysis buffer and subjected to immunoblotting or activity assays.

For immunoblotting, the proteins from the lysed hepatocytes or homogenized livers or the immunoprecipitants were denatured, separated by SDS-PAGE, and then transferred to polyvinylidene fluoride membranes. After blocking with 7.5% (w/v) defatted milk (Sangon, Shanghai, China), the membranes were stained with the indicated primary and secondary antibodies. With ECL development and detection, the specific bands of the indicated proteins were quantified using Quantity One Software (Bio-Rad Laboratories, California, USA).

Acute treatment of *ob/ob* mice with SL010110

Male *ob/ob* mice (7–8 weeks old) were fasted for 4 h and divided into two groups based on body weight ($n = 8$). A single dose of SL010110 (100 mg/kg) or vehicle control (0.25% sodium carboxymethylcellulose, CMC-Na) (SCR, Shanghai, China) in a volume of 10 mL/kg was administered to these mice via gavage. After 1.5 h, the mice were anesthetized by intraperitoneal injection of chloral hydrate, and the livers were dissected for NAD^+/NADH ratio determination, PEPCK and Sirt activity assays, PCR and immunoblotting analysis.

Pyruvate tolerance test in *ob/ob* mice

The pyruvate tolerance test (PTT) was performed in mice to evaluate gluconeogenesis in vivo [29]. After 6 h of fasting, male *ob/ob* mice (8–9 weeks old) were divided into three groups according to blood glucose and body weight ($n = 8$). The mice were subjected to treatment with a volume of 10 mL/kg vehicle control (0.25% CMC-Na), SL010110 (100 mg/kg), or metformin (100 mg/kg) via gavage 30 min before intraperitoneal injection of pyruvate sodium (1.5 g/kg). Blood was obtained by tail clipping, and the glucose levels were measured at 0, 15, 30, 60, and 120 min post-injection with an ACCU-CHEK Performa Glucose Monitor (Roche, Basel, Switzerland).

Table 1. The sequences of primers for real-time PCR.

Genes	Forward (5′→3′)	Reverse (5′→3′)
pck1	CATATGCTGATCCTGGGCATAAC	CAAACTTCATCCAGGCAATGTC
g6pc	ACACCGACTACAGCAACAG	CCTCGAAAGATAGCAAGAGTAG
fbp	GTGTCAACTGCTTCATGCTG	GAGATACTCATTGATGGCAGGG
β-actin	TGACAGGATGCAGAAGGAGA	GCTGGAAGGTGGACAGTGAG

Pharmacokinetic study of SL010110 in *ob/ob* mice

Female and male *ob/ob* mice (11–12 weeks old) were fasted overnight and fed 2 h after dosing with free access to water. SL010110 was dissolved in 0.25% CMC-Na and administered to the female mice and male mice at a dose of 15 mg/kg. Blood samples were collected from the female *ob/ob* mice at 0.25, 0.5,1, 2, 4, 8, and 24 h and liver samples were collected at 0.5, 2, 4, 8, and 24 h after dosing. Blood samples were collected from the male *ob/ob* mice at 0.083, 0.25, 0.75, 2, 4, 8, and 24 h and liver samples were collected at 0.25, 2, 4, 8, and 24 h after dosing. The concentrations of SL010110 in the serum and liver were determined by LC-MS/MS.

Chronic treatment of *ob/ob* mice with SL010110

The chronic effect of SL010110 on abnormalities in glucose metabolism was evaluated in male *ob/ob* mice (7–8 weeks old). Briefly, the mice were assigned to three groups based on body weight, blood glucose, HbAc1, and serum insulin levels ($n = 8$). Vehicle control (0.25% CMC-Na, 10 mL/kg) and SL010110 (15 or 50 mg/kg, 10 mL/kg) were administered to the mice via gavage twice daily for 25 days. The random and fasting blood glucose levels were measured at days 0, 4, 8, 12, 16, 20, and 25 with an ACCU-CHEK Performa Glucose Monitor using samples collected by tail clipping. The body weight and food intake were measured once daily throughout the treatment. On the last day of the chronic treatment, the mice were fasted for 6 h and anesthetized by intraperitoneal injection of chloral hydrate. Blood samples were collected, and HbA1c was determined by a commercial assay kit (Roche Diagnostics, Basel, Switzerland). The livers were dissected and stored at −80 °C for further analysis.

Chronic treatment of *db/db* mice with SL010110

The chronic effect of SL010110 on abnormalities in glucose metabolism was also assessed in *db/db* mice (7–8 weeks old, half male and half female). The mice were divided into four groups based on body weight, blood glucose, HbAc1, and serum insulin levels ($n = 8$) and subjected to treatment with vehicle control (0.25% CMC-Na, 10 mL/kg), SL010110 (15 or 50 mg/kg, 10 mL/kg), or metformin (250 mg/kg, 10 mL/kg) via gavage once daily for 32 days. The random and fasting blood glucose levels were determined at days 0, 8, 12, 16, 20, 24, and 32 with an ACCU-CHEK Performa Glucose Monitor using samples collected by tail clipping. The body weight and food intake were measured once daily throughout the treatment. On the last day of the chronic treatment, the mice were fasted for 6 h and anesthetized, and blood samples were collected for HbA1c measurement.

Statistical analysis

All the statistical data are presented as the mean ± SEM and were analyzed with GraphPad Prism software (GraphPad, California, USA). Student's *t* test was used in the analysis after confirming the normal distribution of data. A $P < 0.05$ was considered statistically significant.

RESULTS

SL010110 inhibits gluconeogenesis and decreases PEPCK1 protein levels in primary mouse hepatocytes

The structure of SL010110 is shown in Fig. 1a. SL010110 inhibited gluconeogenesis in primary mouse hepatocytes under basal condition in a dose-dependent manner (Fig. 1b), and 3 and 10 μM SL010110 led to reductions of 45.5% and 67.5%, respectively. Under stimulation with the adenylate cyclase activator forskolin, SL010110 also led to strong inhibition of gluconeogenesis, and 3 and 10 μM SL010110 resulted in decreases of 62.8% and 82.5%, respectively (Fig. 1b). Metformin (500 μM), used as a positive control here, caused reductions of 55.2% and 64.9% under basal and forskolin-stimulated conditions, respectively. The mRNA expression levels of the gluconeogenic genes pck1, g6pc, and fbp were not affected by SL010110 treatment in either the basal or forskolin-treated states, while metformin

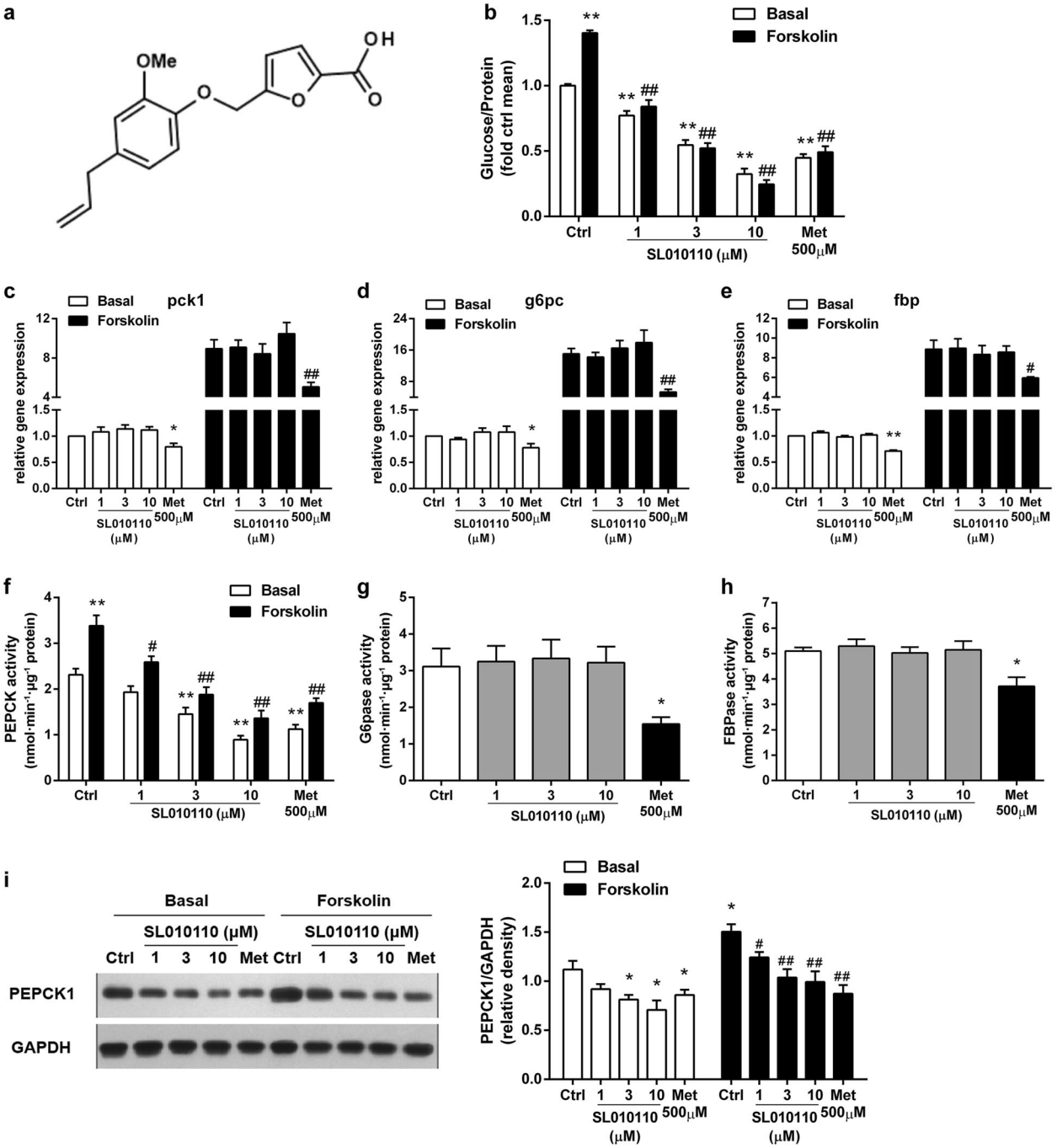

Fig. 1 SL010110 inhibits gluconeogenesis and decreases PEPCK1 protein levels in primary mouse hepatocytes. a Chemical structure of SL010110. Gluconeogenesis (**b**), mRNA expression of pck1(**c**), g6pc (**d**), fbp (**e**), and PEPCK activity (**f**) were measured in primary mouse hepatocytes treated with SL010110 or metformin (Met) at the indicated concentrations in both basal and forskolin (20 μM)-treated conditions. G6pase activity (**g**) and FBPase activity (**h**) in primary hepatocytes were also tested under basal condition. **i** PEPCK1 protein levels were analyzed by Western blotting analysis of primary mouse hepatocytes treated with SL010110 or metformin under basal and forskolin-treated conditions. The data are shown as the mean ± SEM from three or five independent experiments; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs the corresponding controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the forskolin-treated controls.

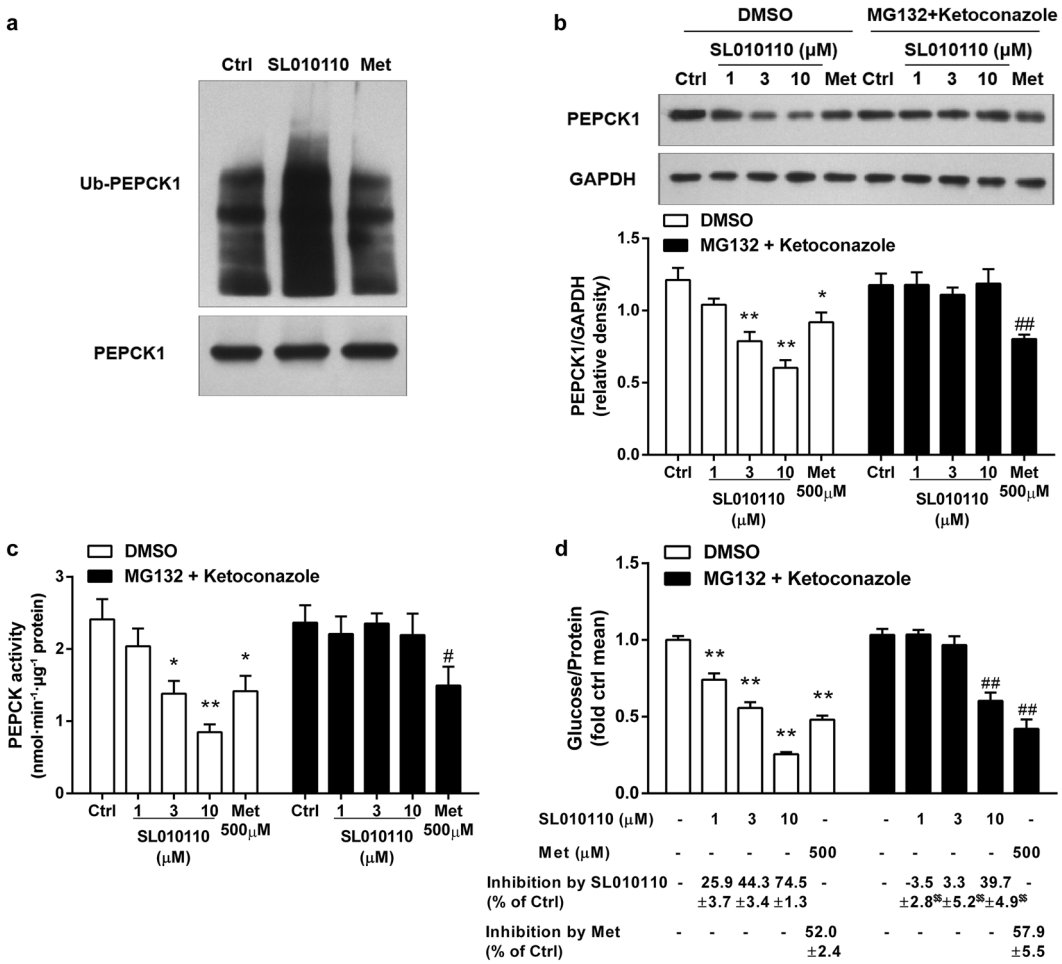

Fig. 2 SL010110 suppresses gluconeogenesis by promoting PEPCK1 ubiquitination and degradation. a PEPCK1 ubiquitination in primary mouse hepatocytes was detected by immunoblotting after treatment with 3 μM SL010110 or 500 μM metformin. The primary mouse hepatocytes were pretreated with MG132 (10 μM) and ketoconazole (5 μM) for 30 min, followed by cotreatment with SL010110 or metformin for 4 h. Then, the PEPCK1 protein levels (**b**), PEPCK activity (**c**), and gluconeogenesis (**d**) were determined. The values are shown as the mean ± SEM from three or five independent experiments; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs the DMSO-treated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the MG132- and ketoconazole-treated controls; $^{\$\$}P < 0.01$, vs reduction caused by SL010110 under DMSO conditions (shown as a percentage of the control).

significantly inhibited the expression of these genes (Fig. 1c, d, e). The activities of PEPCK, G6pase, and FBPase in primary mouse hepatocytes were measured. SL010110 decreased the PEPCK activity under both basal and forskolin-stimulated conditions in a dose-dependent manner but did not affect the G6pase activity or FBPase activity; however, metformin decreased the activities of all three enzymes (Fig. 1f, g, h). We further evaluated the protein level of PEPCK1, which is a main functional form of PEPCK. As shown in Fig. 1i, SL010110 decreased the PEPCK1 protein level under both basal and forskolin-stimulated conditions in a dose-dependent manner.

SL010110 suppresses gluconeogenesis by promoting PEPCK1 ubiquitination and degradation

The PEPCK1 ubiquitination level was measured in primary mouse hepatocytes after incubation with 3 μM SL010110 or 500 μM metformin. As shown in Fig. 2a, SL010110 obviously increased PEPCK1 ubiquitination, whereas metformin treatment caused no change. Thus, we speculated that the SL010110-induced decrease in the PEPCK1 protein levels might be mediated by protein degradation via the ubiquitin-proteasome system (UPS). To test this hypothesis, the proteasome inhibitor MG132 was used. As reported, MG132 is easily metabolized by the metabolic enzyme cytochrome P450 3A4 (CYP3A4) in primary hepatocytes [30]. Ketoconazole, an inhibitor of CYP3A4, was simultaneously added to enhance the stability of MG132. After pretreatment with MG132 and ketoconazole, the SL010110-induced reduction in the PEPCK1 protein levels was inhibited, while metformin still exerted its inhibitory effect on the PEPCK1 protein content (Fig. 2b). Similarly, the SL010110-mediated suppression of PEPCK activity was also abolished (Fig. 2c). With inhibitors treatment, the suppression of gluconeogenesis by 1 or 3 μM SL010110 was completely abrogated, and the suppression of gluconeogenesis by 10 μM SL010110 was significantly diminished (74.5% vs 39.7%, without or with MG132 and ketoconazole, $P < 0.01$, Fig. 2d); however, the metformin-mediated suppression of gluconeogenesis was unaffected (Fig. 2d). These data suggested that SL010110 suppressed hepatic gluconeogenesis mainly by promoting PEPCK1 ubiquitination and degradation.

SL010110 promotes PEPCK1 acetylation and indirectly increases p300 acetyltransferase activity

Treatment with 3 μM SL010110 clearly increased PEPCK1 acetylation in primary mouse hepatocytes, while metformin showed no effect (Fig. 3a). By immunoprecipitation with a PEPCK1 antibody,

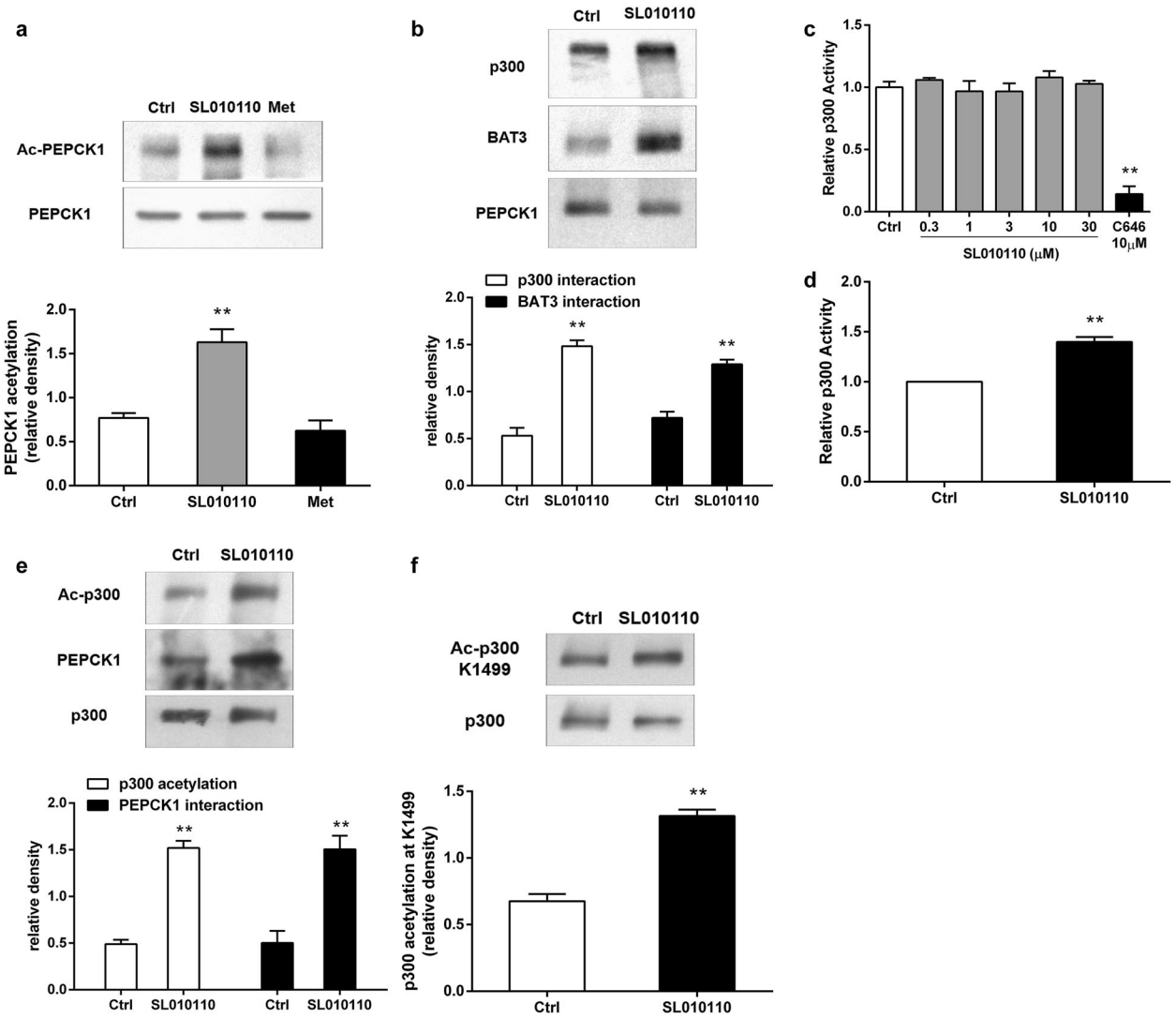

Fig. 3 SL010110 promotes PEPCK1 acetylation and indirectly increases p300 acetyltransferase activity. a PEPCK1 acetylation in primary mouse hepatocytes pretreated with 3 μM SL010110 or 500 μM metformin was detected by immunoprecipitation. **b** p300 and BAT3 coimmunoprecipitated with PEPCK1 from hepatocytes pretreated with 3 μM SL010110 were quantified. **c** SL010110 did not directly increase p300 acetyltransferase activity. Primary mouse hepatocytes were treated with 3 μM SL010110, and the p300 acetyltransferase activity (**d**), p300 acetylation level, PEPCK1 protein interaction with p300 (**e**), and p300 acetylation at K1499 (**f**) were detected. The values are presented as the mean ± SEM from three or five independent experiments; $^{**}P < 0.01$ vs the controls.

we found that higher amounts of p300 and its chaperone protein, HLA-B-associated transcript 3 (BAT3), bound to PEPCK1 after treatment with 3 μM SL010110 (Fig. 3b); these results indicated that p300 might be involved in PEPCK1 acetylation. Then, the effect of SL010110 on the acetyltransferase activity of p300 was further detected. SL010110 did not affect the acetyltransferase activity of p300 when directly incubated with the p300 protein (Fig. 3c) but significantly increased the acetyltransferase activity of p300 in primary mouse hepatocytes (Fig. 3d), suggesting that SL010110 might indirectly activate p300. By immunoprecipitation with a p300 antibody, we found that compared with the control treatment, 3 μM SL010110 induced a significant 3-fold increase in p300 acetylation in primary mouse hepatocytes (Fig. 3e). A comparable increase in PEPCK1 coprecipitates with p300 was also observed after SL010110 treatment (Fig. 3e). The acetylation of lysine at position 1499 (K1499) in the HAT pocket of p300 was also stimulated by treatment with SL010110 (Fig. 3f). These results suggested that SL010110 might increase the acetylation of PEPCK1 by indirectly activating the acetyltransferase activity of p300.

SL010110 inhibits gluconeogenesis through the activation of p300 acetyltransferase and the acetylation of PEPCK1

C646 is a selective and competitive inhibitor of the histone acetyltransferase p300. Pretreatment with C646 abolished SL010110-stimulated p300 activity (Fig. 4a). C646 blocked the SL010110-induced binding of p300 to PEPCK1 and thereby abolished the augmentation of PEPCK1 acetylation (Fig. 4b). The SL010110-induced reduction in the PEPCK1 protein levels and inhibition of the PEPCK1 activity were blocked by C646, while the effects of metformin did not change (Fig. 4c, d). Furthermore, the suppression of gluconeogenesis by 1 or 3 μM SL010110 was completely abrogated by treatment with C646, the suppression of gluconeogenesis by 10 μM SL010110 was significantly diminished (75.1% vs 37.0%, without or with C646, $P < 0.01$, Fig. 4e), and the suppression of gluconeogenesis by metformin was unaffected (Fig. 4e).

SL010110 inhibits cytoplasmic Sirt activity to promote p300 acetyltransferase activation and suppress gluconeogenesis

Since p300 activity is regulated by the deacetylase Sirts [25], we examined the effect of SL010110 on the Sirt activity in primary

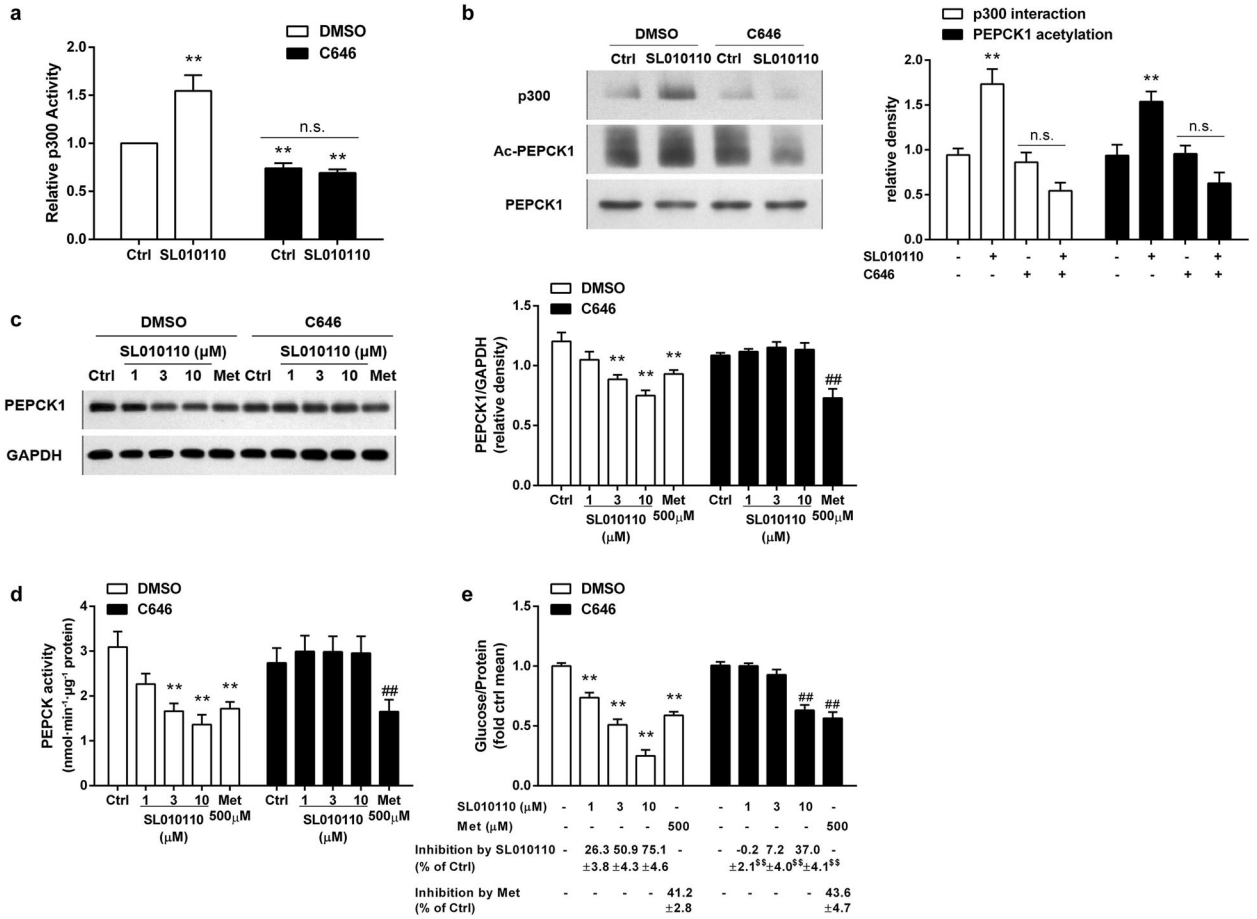

Fig. 4 SL010110 inhibits gluconeogenesis through the activation of p300 acetyltransferase and the acetylation of PEPCK1. Primary mouse hepatocytes were pretreated with the histone acetyltransferase p300 inhibitor C646 (10 μM) and cotreated with SL010110. Then, the p300 acetyltransferase activity (**a**), the p300 interaction with PEPCK1, PEPCK1 acetylation (**b**), PEPCK1 protein level (**c**), PEPCK activity (**d**), and gluconeogenesis (**e**) were analyzed. The results are presented as the mean ± SEM from three or five independent experiments; $^{**}P < 0.01$ vs the DMSO-treated controls; $^{\#\#}P < 0.01$ vs the C646-treated controls; $^{\$\$}P < 0.01$ vs the reduction caused by SL010110 under DMSO conditions (shown as a percentage of the control).

mouse hepatocytes. Mammalian Sirts display distinct subcellular localizations, and SIRT1 is mainly localized in the nucleus while SIRT2 is mainly localized in the cytoplasm [28]. As shown in Fig. 5a, cytoplasmic Sirt activity (mainly SIRT2 activity) was significantly inhibited by the positive controls, namely, the SIRT2 inhibitor sirtinol and the pan Sirt inhibitor NAM. SL010110 at concentrations of 1, 3, and 10 μM also significantly reduced cytoplasmic Sirt activity, with reductions of 19.8%, 32.1%, and 38.2%, respectively (Fig. 5a). SL010110 and sirtinol did not change the nuclear Sirt activity (mainly SIRT1 activity) in primary hepatocytes, while NAM exerted an inhibitory effect (Fig. 5b). SL010110 decreased the NAD^+/NADH ratio in primary mouse hepatocytes in a dose-dependent manner (Fig. 5c). NMN is an NAD^+ synthesis precursor that has been reported to upregulate the cellular NAD^+/NADH ratio [31–33]. After cotreatment with NMN, the SL010110-mediated suppression of SIRT2 activity was completely abolished (Fig. 5d). Furthermore, NMN fully blocked the SL010110-stimulated p300 acetyltransferase activity and PEPCK1 acetylation (Fig. 5e, f). In addition, NMN completely abolished the suppression of gluconeogenesis by 1 or 3 μM SL010110, and the reduction in gluconeogenesis due to 10 μM SL010110 was also significantly diminished (69.9% vs 28.8%, without or with NMN, $P < 0.01$, Fig. 5g); however, the inhibition of gluconeogenesis by metformin was unaffected (Fig. 5g). These results suggested that SL010110 inhibited gluconeogenesis through the inhibition of SIRT2 and the subsequent activation of p300 in primary mouse hepatocytes.

Sirtinol suppresses gluconeogenesis and increases PEPCK1 acetylation by activating p300 acetyltransferase

To further confirm the involvement of p300 in the SIRT2-induced acetylation of PEPCK1, additional studies were performed with sirtinol, a SIRT2 inhibitor, in primary mouse hepatocytes. As shown in Fig. 6a, treatment with 25 and 50 μM sirtinol significantly inhibited gluconeogenesis by 25.1% and 49.6% under basal condition and by 49.8% and 73.4% under forskolin-stimulated condition, respectively. Sirtinol at a concentration of 50 μM significantly promoted p300 acetyltransferase activity and increased p300 acetylation in primary hepatocytes (Fig. 6b, c). Moreover, sirtinol markedly stimulated PEPCK1 acetylation, and this effect was abolished in the presence of the p300 inhibitor C646 (Fig. 6d). In addition, the inhibition of gluconeogenesis by sirtinol could also be blocked by C646 (Fig. 6e). These results suggested that the SIRT2 inhibitor suppressed gluconeogenesis by activating p300 acetyltransferase activity and subsequently increasing PEPCK1 acetylation.

Acute treatment with SL010110 inhibits hepatic SIRT2 activity, increases p300 and PEPCK1 acetylation and suppresses gluconeogenesis in *ob/ob* mice

The effects of a single oral dose of SL010110 (100 mg/kg) on the hepatic NAD^+/NADH ratio, SIRT2 activity, p300 acetylation, and PEPCK1 acetylation and degradation were investigated in *ob/ob* mice with T2D, and a PTT was conducted to evaluate the effect of

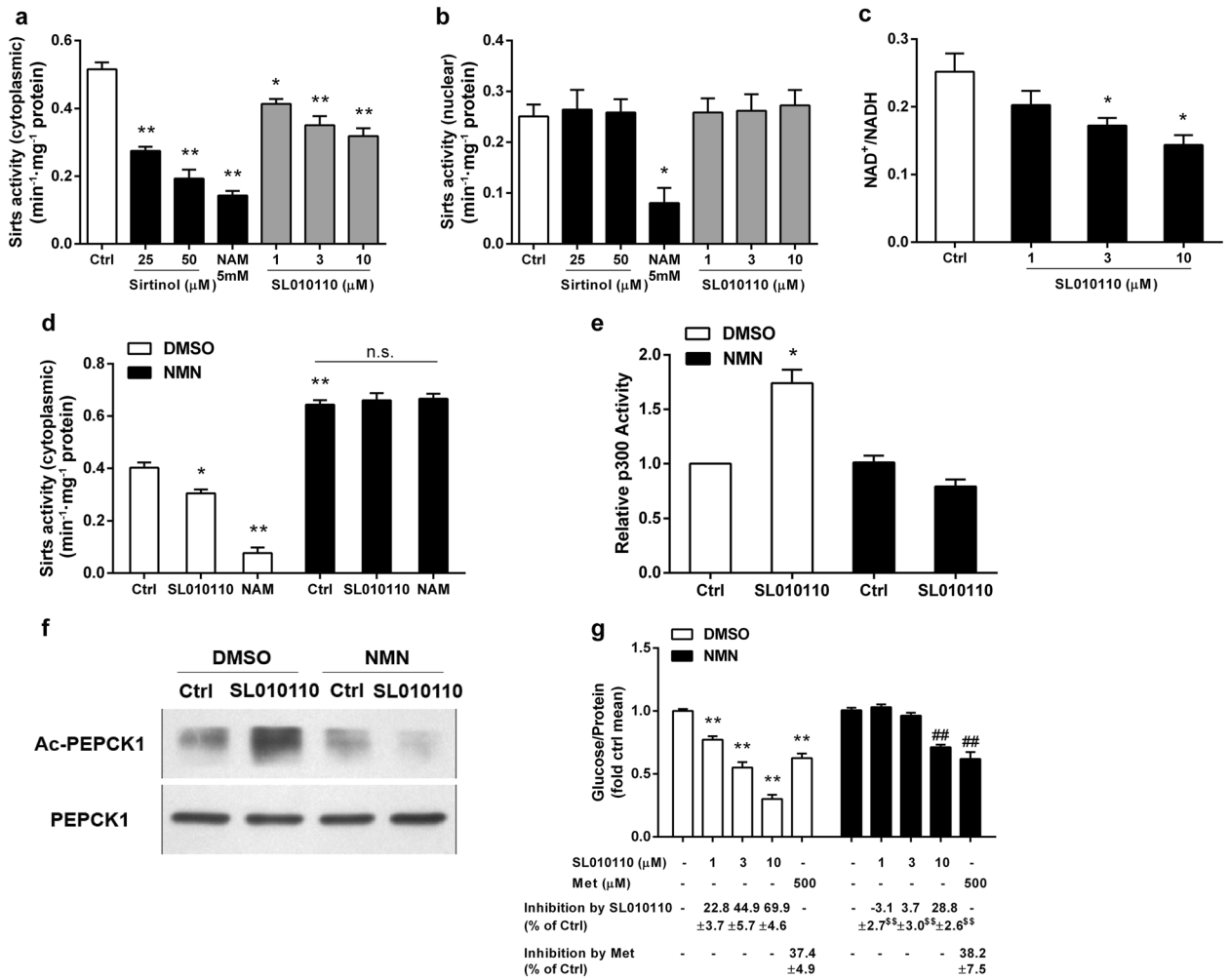

Fig. 5 SL010110 inhibits cytoplasmic Sirt activity to promote p300 acetyltransferase activation and suppress gluconeogenesis. The activities of cytoplasmic Sirts (mainly SIRT2) (**a**) and nuclear Sirts (mainly SIRT1) (**b**) were measured in primary mouse hepatocytes treated with the indicated compounds. **c** The NAD^+/NADH ratio was measured in hepatocytes treated with SL010110 at the indicated concentrations. Primary mouse hepatocytes were pretreated with the NAD^+ synthesis precursor NMN (500 μM) and cotreated with 3 μM SL010110, and the cytoplasmic Sirt activity (**d**), p300 acetyltransferase activity (**e**), PEPCK1 acetylation (**f**), and gluconeogenesis (**g**) were detected. The values are shown as the mean ± SEM from three to five independent experiments; NAM, niacinamide, as a positive control of Sirt activity; $^*P < 0.05$ and $^{**}P < 0.01$ vs the DMSO-treated controls; $^{\#\#}P < 0.01$ vs the NMN-treated controls; $^{\$\$}P < 0.01$ vs the reduction caused by SL010110 under DMSO conditions (shown as a percentage of the control).

SL010110 on gluconeogenesis in vivo. As shown in Fig. 7a and b, the NAD^+/NADH ratio in the mouse livers was reduced by 37.2%, and the hepatic SIRT2 activity was decreased by 25.7%, after a single dose of SL010110. Correspondingly, SL010110 treatment caused a 1.8-fold increase in the acetylation of p300 and a 2.5-fold increase in the acetylation of PEPCK1 in the liver (Fig. 7c, d). The hepatic PEPCK1 protein level and PEPCK activity were both reduced by SL010110 in *ob/ob* mice, but the mRNA levels of PEPCK and G6Pase were unchanged (Fig. 7e-g). Furthermore, treatment with SL010110 significantly reduced the blood glucose levels at 15, 30, and 60 min after the intraperitoneal administration of sodium pyruvate, suggesting the in vivo inhibition of whole-body gluconeogenesis (Fig. 7h).

Pharmacokinetic properties of SL010110 in the serum and liver of *ob/ob* mice

A pharmacokinetic study of SL010110 was conducted in both female and male *ob/ob* mice with a dose of 15 mg/kg. The serum and hepatic concentration-time curves of SL010110 in the female mice are shown in Fig. 8a, b, and the curves of SL010110 in the male mice are shown in Fig. 8c, d. The pharmacokinetic parameters of SL010110 were calculated (Table 2). In the female *ob/ob* mice, the serum concentration of SL010110 was 58642 ± 4313 ng/mL 15 min after administration, and the AUC_{0-24} was 63529 ± 8728 h*ng/mL. In the male *ob/ob* mice, the serum concentration of SL010110 was 82353 ± 5173 ng/mL 5 min after administration, and the AUC_{0-24} was 73335 ± 11080 h*ng/mL. In the female *ob/ob* mice, the liver concentration of SL010110 was 34980 ± 3579 ng/g 30 min after administration. In the male *ob/ob* mice, the liver concentration of SL010110 was 125733 ± 3579 ng/g 15 min after administration. The results indicated that SL010110 was quickly absorbed after oral administration, and the AUC_{0-24} values in the two sexes were quite similar; however, some differences could be observed in the serum or liver concentrations of SL010110, which were due to the different test time points.

Chronic administration of SL010110 improves glucose homeostasis in diabetic *ob/ob* and *db/db* mice

The chronic glucose-lowering potential of SL010110 (15 or 50 mg/kg, orally, bid) was evaluated in *ob/ob* mice. Both doses of SL010110 significantly lowered the random blood glucose level beginning on day 8, with reductions of 33.3% and 34.8% at day 25,

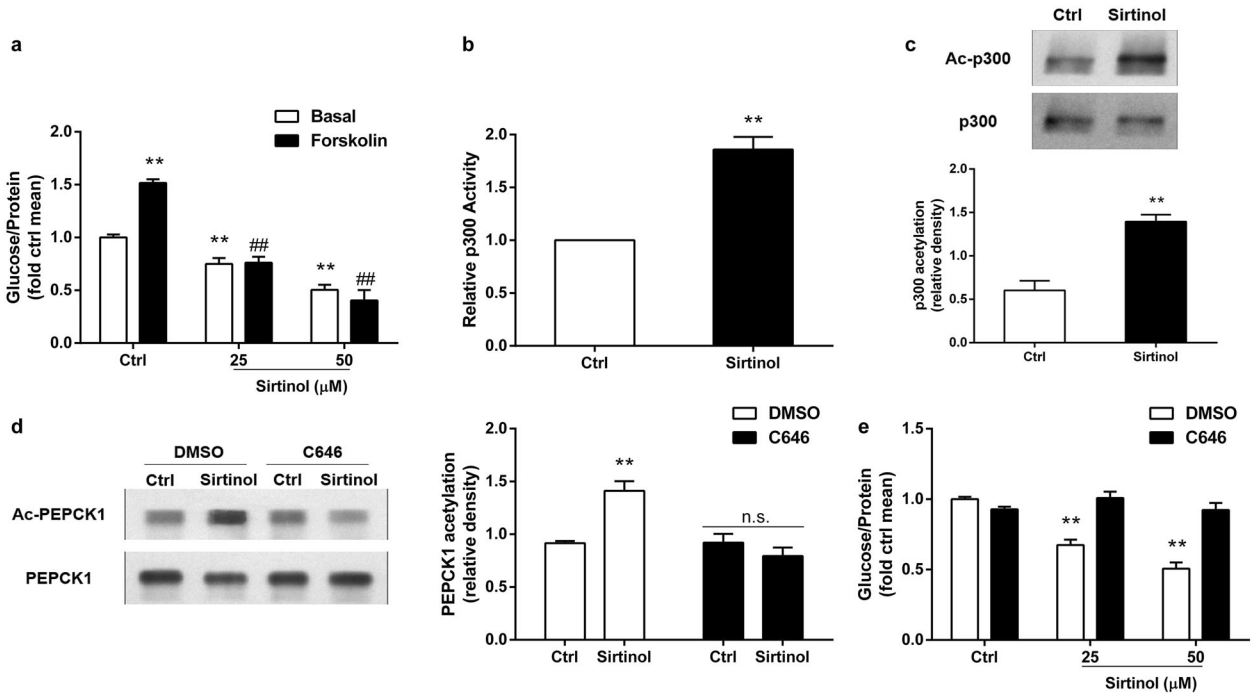

Fig. 6 Sirtinol suppresses gluconeogenesis and increases PEPCK1 acetylation by activating p300 acetyltransferase. a Sirtinol inhibited gluconeogenesis in primary mouse hepatocytes under basal and forskolin-induced conditions. p300 acetyltransferase activity (**b**) and acetylation (**c**) were measured in primary mouse hepatocytes treated with 50 μM sirtinol. Primary mouse hepatocytes were pretreated with C646 (10 μM) and cotreated with 50 μM sirtinol, and PEPCK1 acetylation (**d**) and gluconeogenesis (**e**) were detected. The results are shown as the mean ± SEM from three to five independent experiments; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs the corresponding controls; $^{\#\#}P < 0.01$ vs the forskolin-treated control.

respectively (Fig. 9a). The fasting blood glucose levels were also significantly decreased by SL010110 treatment, with decreases of 37.2% and 41.9%, respectively, at the end of treatment (Fig. 9b). The chronic glycemic index HbA1c was clearly lowered by 50 mg/kg SL010110 treatment (Fig. 9c). Body weight and food intake were unaffected by both doses of SL010110 (Fig. 9d, e). Chronic SL010110 administration did not alter hepatic pck1 or g6p gene transcription in the *ob/ob* mice (Fig. 9f). However, long-term treatment with 50 mg/kg SL010110 markedly reduced the PEPCK activity by 41.8%, and chronic administration of 15 or 50 mg/kg SL010110 clearly decreased the PEPCK1 protein levels by 52.3% and 49.1%, respectively (Fig. 9g, h). The chronic effect of SL010110 (15 or 50 mg/kg, orally, qd) on abnormalities in glucose metabolism was also assessed in *db/db* mice, and metformin (250 mg/kg, orally, qd) was used as a positive control. In the course of treatment, 15 and 50 mg/kg SL010110 markedly reduced the random blood glucose levels, with reductions of 31.6% and 46.1% at the end of treatment, respectively (Fig. 9i). The fasting blood glucose levels were also lowered by SL010110 treatment, with decreases of 17.5% and 38.9%, respectively, at the end of the treatment (Fig. 9j). The random and fasting blood glucose levels of the metformin-treated mice were also significantly decreased by 41.8% and 53.6%, respectively (Fig. 9i, j). The HbA1c levels of the *db/db* mice also showed a decreasing trend after treatment with 50 mg/kg SL010110 (Fig. 9k). Body weight and food intake were unaffected by both doses of SL010110 (Fig. 9l, m).

DISCUSSION

Given that the liver is a critical organ in glucose homeostasis, dysregulated hepatic gluconeogenesis contributes to abnormal glucose metabolism in T2D [34]. Hence, the development of active molecules to suppress gluconeogenesis is an attractive method of glycemic control in the treatment of diabetic patients [35]. In the discovery of new compounds with glucose-lowering effects, many molecules, including the widely used drug metformin, were found to inhibit gluconeogenesis by downregulating the gene expression of PEPCK and G6pase. However, compounds targeting the protein stability of PEPCK to regulate gluconeogenesis have rarely been reported. In the present study, we found that a lead compound, SL010110, could potently inhibit hepatic gluconeogenesis by promoting PEPCK1 acetylation and degradation. More importantly, we demonstrated that SL010110 suppressed SIRT2 activity and reduced p300 deacetylation, thus promoting p300 acetyltransferase activity and PEPCK1 acetylation. In addition, SL010110 showed potent glucose-lowering effects in *ob/ob* and *db/db* mice with T2D.

PEPCK1 is the main functional isoform of the rate-limiting enzyme PEPCK in gluconeogenesis [9], and it can be regulated either by regulating its gene expression or protein stability. SL010110 clearly reduced the PEPCK1 protein levels, and no alterations occurred in the PEPCK1 gene expression levels. The UPS is one of the major pathways responsible for protein degradation in the maintenance of cellular homeostasis [36]. Since PEPCK1 was reported to be degraded through the UPS [15, 19, 20], we assessed the effect of SL010110 on the ubiquitination of PEPCK1. As expected, PEPCK1 ubiquitination was markedly increased by SL010110 treatment. The proteasome inhibitor MG132 abolished the inhibitory effect of SL010110 on gluconeogenesis. Thus, the inhibition of gluconeogenesis by SL010110 in primary mouse hepatocytes was dependent on the increase in PEPCK1 proteasomal degradation.

The ubiquitination and proteasomal degradation of PEPCK1 can be modulated by various factors, including acetylation by p300 [15], sumoylation by ubiquitin-conjugating enzyme 9 (Ubc9) [19], and phosphorylation by glycogen synthesis kinase 3β (GSK3β) [20]. Here, SL010110 significantly increased the acetylation of PEPCK1 and the interaction between PEPCK1 and

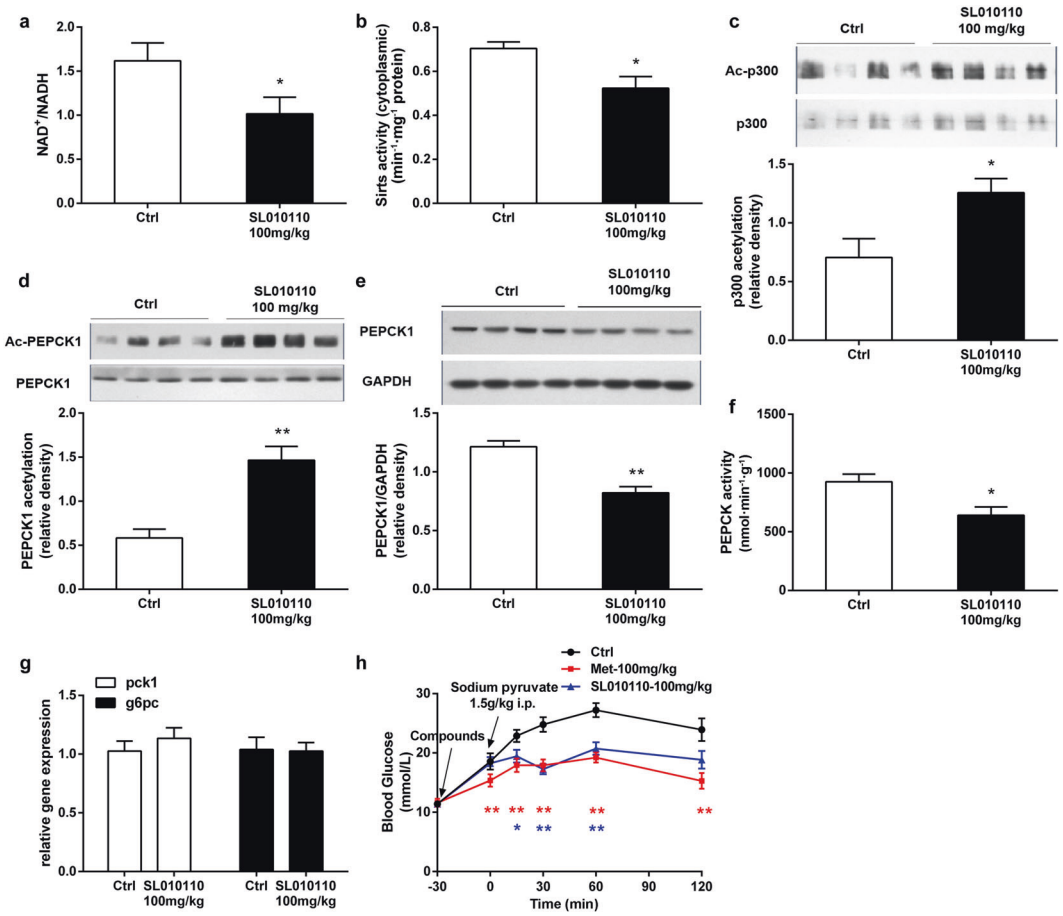

Fig. 7 Acute treatment with SL010110 inhibits hepatic SIRT2 activity, increases p300 and PEPCK1 acetylation and suppresses gluconeogenesis in *ob/ob* mice. After a single oral dose of SL010110 (100 mg/kg), the NAD^+/NADH ratio (**a**), cytoplasmic Sirt activity (**b**), p300 acetylation (**c**), PEPCK1 acetylation (**d**), PEPCK1 protein levels (**e**), PEPCK activity (**f**), and gluconeogenic gene expression (**g**) in the livers of *ob/ob* mice were detected and quantified. **h** Blood glucose levels were measured in *ob/ob* mice subjected to a pyruvate tolerance test. The data are presented as the mean ± SEM for $n = 8$ mice; $^*P < 0.05$ and $^{**}P < 0.01$ vs the vehicle control mice.

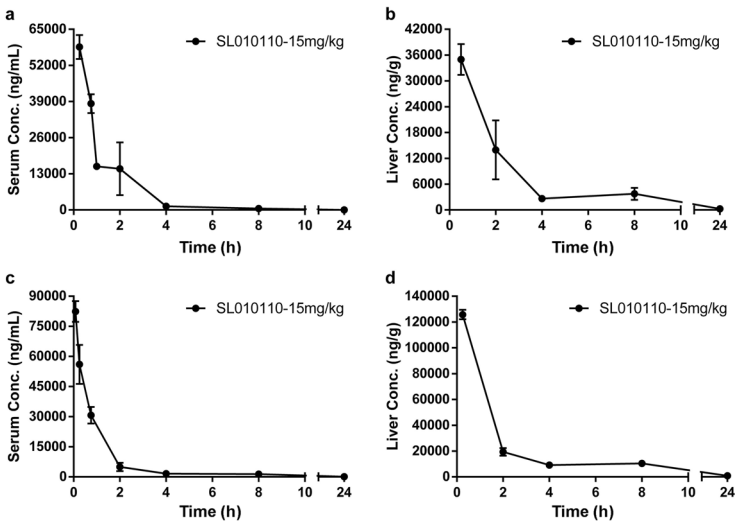

Fig. 8 Pharmacokinetic changes of SL010110 in the serum and liver after administration to *ob/ob* mice by gavage. Pharmacokinetic changes of SL010110 in the serum and liver after administration to *ob/ob* mice by gavage. Serum concentration-time curve (**a**) and liver concentration-time curve (**b**) of SL010110 in female *ob/ob* mice and serum concentration-time curve (**c**) and liver concentration-time curve (**d**) of SL010110 in male *ob/ob* mice after gavage with 15 mg/kg SL010110. The data are shown as the mean ± SEM of three mice.

p300. As an enhancer of p300, BAT3 always binds to p300, forming a complex to strengthen the activity of p300 [37, 38]. Here, SL010110 treatment increased the BAT3–PEPCK1 interaction, further suggesting that p300 was involved in the induction of PEPCK1 acetylation by SL010110. p300 can act either as a transcription coactivator to regulate downstream gene expression or as an acetyltransferase to acetylate certain substrates. Here, SL010110 stimulated p300 activity but did not affect gluconeogenic gene expression in primary mouse hepatocytes. We speculated that p300 acts as an acetyltransferase to regulate the SL010110-mediated induction of PEPCK1 acetylation. As expected, the competitive p300 inhibitor C646 abolished the PEPCK1 acetylation and degradation induced by SL010110 and attenuated the inhibition of gluconeogenesis by SL010110 in primary mouse hepatocytes, suggesting that p300 is required for SL010110 to acetylate PEPCK1 and inhibit gluconeogenesis.

The histone deacetylase SIRT2 has been reported to deacetylate and thus regulate the activities or stabilities of certain protein substrates, including PEPCK1 and p300 [15, 25]. Here, SL010110 decreased the NAD^+/NADH ratio and inhibited the cytoplasmic Sirt activity (mainly SIRT2 activity) in primary mouse hepatocytes. The NAD^+/NADH ratio can be affected by multiple factors, such as aging and nutrient availability. It has also been documented that NADH levels are closely related to mitochondrial respiration, especially mitochondrial complex 1 activity [39]. However, whether mitochondrial complex 1 is involved in the SL010110-induced decrease in the NAD^+/NADH ratio requires further exploration. NMN is a precursor of NAD^+ synthesis and can distinctly augment cytoplasmic Sirt activity. With NMN supplementation, SL010110 failed to suppress SIRT2 activity, and the SL010110-induced PEPCK1 acetylation was abolished, indicating that the inhibition of SIRT2 activity was crucial in regulating the

Table 2. Pharmacokinetic properties of SL010110 (15 mg/kg) after oral administration to female and male *ob/ob* mice (mean ± SEM, $n = 3$).

p.o. (15 mg/kg)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-24} (h*ng/mL)
Female	2.9 ± 0.1	0.3 ± 0.06	58642 ± 4313	63529 ± 8728
Male	4.77 ± 0.22	0.083 ± 0.011	82353 ± 5173	73335 ± 11080

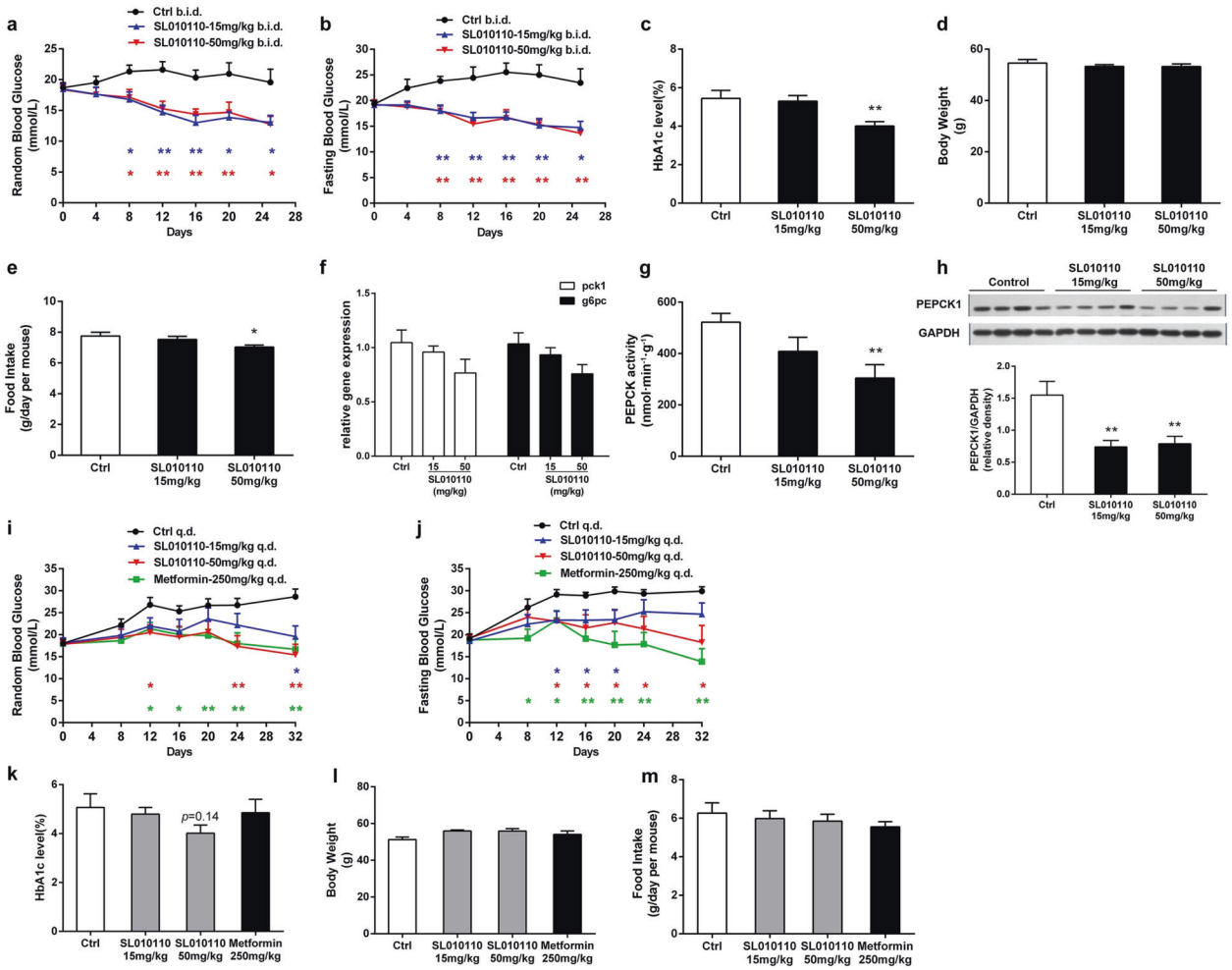

Fig. 9 Chronic administration of SL010110 improves glucose homeostasis in diabetic *ob/ob* and *db/db* mice. The *ob/ob* mice were orally gavaged with SL010110 at doses of 15 and 50 mg/kg or with vehicle twice daily for 25 days. Random blood glucose levels (**a**), fasting blood glucose levels (**b**), HbA1c levels (**c**), body weight (**d**), and average daily food intake (**e**) were determined. Hepatic pck1 and g6pc mRNA expression (**f**), PEPCK activity (**g**), and PEPCK1 protein expression (**h**) were also detected. The *db/db* mice were orally gavaged with SL010110 (15 and 50 mg/kg), metformin (250 mg/kg), or vehicle once daily for 32 days. Random blood glucose levels (**i**), fasting blood glucose levels (**j**), HbA1c level (**k**), body weight (**l**), and average daily food intake (**m**) were measured. The results are presented as the mean ± SEM for $n = 7$–8 mice; $^*P < 0.05$ and $^{**}P < 0.01$ vs the vehicle control mice.

level of PEPCK1 acetylation. Since p300 also played an essential role in the SL010110-induced acetylation of PEPCK1 and inhibition of gluconeogenesis, we assessed whether p300 might be a key mediator in the SIRT2-PEPCK1 axis. As expected, NMN supplementation completely abolished the SL010110-stimulated acetyltransferase activity of p300, suggesting that SL010110 inhibited SIRT2 in order to activate p300 to acetylate PEPCK1 for degradation. The SIRT2 inhibitor sirtinol was reported to promote PEPCK1 acetylation and degradation, thus decreasing cell gluconeogenesis [17]. However, whether p300 is involved in sirtinol-induced PEPCK1 acetylation is unknown. In the present study, sirtinol increased the acetylation and acetyltransferase activity of p300, and the p300 inhibitor C646 completely blocked the sirtinol-induced PEPCK1 acetylation and gluconeogenesis inhibition. These data demonstrated that sirtinol-induced PEPCK1 acetylation was also dependent on p300 acetyltransferase activity. These findings revealed a novel mechanism by which p300 might play a crucial role in SIRT2-mediated PEPCK1 degradation and gluconeogenesis regulation.

Further in vivo studies were performed to investigate whether SL010110 affected the SIRT2-p300-PEPCK1 axis in the livers of mice with T2D. Consistent with the results in primary mouse hepatocytes, a single oral dose of SL010110 decreased the activity of SIRT2, increased the levels of acetylated p300 and PEPCK1, and reduced the protein level and activity of PEPCK1 but exerted no effects on the expression of gluconeogenic genes in the livers of *ob/ob* mice. Aberrantly enhanced gluconeogenesis is a major cause of fasting hyperglycemia in T2D, and gluconeogenesis is significantly increased in diabetic *ob/ob* mice [40, 41]. Here, a single dose of SL010110 by gavage also markedly reduced the pyruvate-challenged blood glucose levels in diabetic *ob/ob* mice, indicating the suppression of whole-body gluconeogenesis in vivo. These findings supported the conclusion that SL010110 could repress gluconeogenesis in *ob/ob* mice through SIRT2-p300-mediated PEPCK1 degradation.

The most important goal in the treatment of T2D is to lower hyperglycemia. Chronic SL010110 treatment (15 or 50 mg/kg, orally, bid) clearly reduced the random and fasting blood glucose levels and the HbAc1 levels in *ob/ob* mice. SL010110 administration also decreased the hepatic PEPCK1 activity and protein levels but did not affect the gluconeogenic gene expression, suggesting that the promotion of PEPCK1 protein degradation could accomplish the goal of lowering blood glucose levels. We further evaluated the anti-hyperglycemic effect of SL010110 in another type 2 diabetic mouse model, namely, *db/db* mice, and SL010110 also exerted significant blood glucose-lowering effects under both random and fasting states. Taken together, we demonstrated that SL010110 could relieve glucose metabolic disorders in type 2 diabetic mice by promoting PEPCK1 degradation.

CONCLUSION

In conclusion, our study identified SL010110 as a valuable compound with a distinct mechanism of suppressing hepatic gluconeogenesis by promoting PEPCK1 degradation. SL010110 inhibited SIRT2 in order to activate p300 acetyltransferase activity, further acetylate PEPCK1, and promote PEPCK1 degradation. Chronic administration of SL010110 significantly reduced the blood glucose levels in *ob/ob* and *db/db* mice. These findings revealed a novel mechanism by which p300 might play an important role in SIRT2-mediated PEPCK1 degradation and highlighted the potential value of SL010110 as a lead compound with effective anti-hyperglycemic activity for the treatment of T2D.

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AUTHOR CONTRIBUTIONS

YL, JHS, and SLH designed the research. YRR, YLY, YF, TFX, YS, and JL performed the research. YRR, SLH, and YL analyzed and interpreted the data. YRR, SLH, and YL wrote the paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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