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Original Article

Cucurbitacin-D inhibits hepatic gluconeogenesis through activation of Stat3 signaling

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Abstract: Background: Cucurbitacins belong to a class of highly oxidized tetracyclic triterpenoids, which are widely distributed in the plant. Objective: In this study, we addressed the roles of Cucurbitacin-D (CuD) in diabetic db/db mice and streptozotocin (STZ)-treated C57BL/6 mice. Methods: C57BL/6 mice were injected with streptozotocin for induction of diabetes followed by determination of gluconeogenesis. Western blot and real time PCR were performed to measure the gene expression involved in signaling transduction. Results: We found CuD improved serum glucose concentrations, even without improving insulin sensitivity. Consistently, hepatic phosphoenolpyruvate carboxykinase (PEPCK) and Glucose-6-phosphatase (G6Pase), two key enzymes in the gluconeogenesis, were down-regulated by CuD. Besides, hepatic triglyceride contents were also reduced, accompanied by decreased expression of lipogenic genes. At the molecular level, CuD was shown to activate signal transducer and activator of transcription 3 (Stat3) signaling *in vivo* and *in vitro*. Conclusions: Our data suggest that CuD improves fasting blood glucose by direct inhibition of gluconeogenesis in liver, suggesting a new therapeutic approach for the treatment of type 2 diabetes.

Keywords: Cucurbitacin-D, gluconeogenesis, Stat3, PEPCK, G6Pase

Introduction

Fasting blood glucose is determined by de novo glucose production, which takes place mainly in the liver. It is essential for maintain blood glucose levels within the physiologic range and providing the sole fuel source for other organs [1, 2]. On the other hand, enhanced gluconeogenesis and glucose output usually contributes to hyperglycemia in the presence of obesity and insulin resistance [3]. Indeed, expression or activity of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two key enzymes in the hepatic gluconeogenesis, is dramatically up-regulated in obese humans and mice [4, 5]. Therefore, inhibition of hepatic glucose overproduction has become an important therapeutic approach for the treatment of hyperglycemia. For instance, metformin is currently the drug of first choice for the treatment of type 2 diabetes, and increasing evidence suggests that its primary function is to reduce hepatic glucose production, mainly by inhibiting gluconeogenesis [6, 7].

Cucurbitacins belong to a class of highly oxidized tetracyclic triterpenoids, which are widely distributed in the plant [8]. Recent studies reveal their promising anticancer activities including anti-proliferation, induction of apoptosis and cell-cycle arrest [9]. Cucurbitacin-D (CuD) is shown to induce growth inhibition, cell cycle arrest, and apoptosis in human endometrial and ovarian cancer cells [10]. In the present study, we examined the effects and molecular mechanism of hepatic gluconeogenesis in response to CuD in diabetic *db/db* mice and streptozotocin (STZ)-treated C57BL/6 mice. We found: (1) CuD decreased fasting blood glucose and expression of key gluconeogenic genes, including PEPCK and G6Pase; (2) CuD activated signal transducer and activator of transcription 3 (Stat3) signaling *in vivo* and *in vitro*.

Materials and methods

Mice and cell culture

Male C57BL/6 and db/db mice, 8-10 weeks of age, were obtained from Shanghai Laboratory

Animal Company (Shanghai, China). All mice were housed and bred according to international standard conditions with a 12-hr dark, 12-hr light cycle. For induction of diabetes mellitus, C57BL/6 mice were fasted overnight and intraperitoneal injected (i.p injection) with streptozotocin (Sigma-aldrich, 0.25 g/kg) in 0.05 M citrate. 2 hr after the injection, mice were fed ad libitum. Induction of diabetes was determined by measuring insulin levels. The animal protocol was reviewed and approved by the Shanghai Jiaotong University Council on Animal Care Committee, China. The hepatocellular carcinoma cell line HuH7 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA).

Cucurbitacin-D treatment

The Cucurbitacin-D (CuD, Sigma-aldrich) solution was prepared in phosphate-buffered saline (PBS) and delivered by oral gavage at dosage of 200 mg/kg/day. The control group was given vehicle control (PBS).

Blood and liver collection

After three weeks' CuD treatment, mice were fasted overnight. Liver and blood were collected from each group of mice under anesthetized. Blood was collected from abdominal aorta using syringe puncture. Liver was removed and divided into several pieces, which were subjected to RNA, protein and lipids extraction.

Blood biochemistry

Serum levels of insulin were determined using commercial kits from Millipore, USA. Pyruvate tolerance tests (PTTs) were performed in a state of fasting for 12 hr, with injection of i.p. sodium pyruvate in saline (1.0 g/kg). Blood glucose was determined in the tail vein blood using portable glucometer (Roche, Basel, Switzerland). Hepatic and serum triglyceride, free fatty acid, alanine aminotransferase (ALT) and aspartate transaminase (AST) were determined using automatic biochemistry analyzer.

Western blot

Liver tissues and cells were lysed in radioimmunoprecipitation buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 2 mmol/L EDTA, 1 mmol/L NaF, 1% NP40, and 0.1% SDS. Protein was resolved in 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane. The antibodies to PEPCK, G6Pase, Stat3, CREB, FoxO1, FoxO6, HNF4, PGC-1α, GR and SHP were purchased from the Santa Cruz Biotechnology (CA, USA). Expression of GAPDH was employed as a loading control using a rabbit GAPDH antibody (Abcam, USA)

RNA isolation and real-time PCR

Total RNA was extracted from liver tissues or cells using the TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA). Reverse transcription of 2 μg RNA was carried out according to the instructions of Prime Script™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The real-time PCR reaction was conducted in 20 μl (SYBR® Premix Ex TaqTM, TaKaRa Japan). The result was normalized against GAPDH mRNA signal.

Statistical analysis

The results were expressed as the means ± SE. The significant difference in two groups was statistically analyzed using the Student's t test. The significant difference in more than two groups was statistically analyzed using ANOVA. Statistical significance was set at $P<0.05$.

Results

CuD treatment decreased fasting blood glucose in db/db mice

To explore the roles of CuD in hepatic glucose metabolism, we used db/db mice, a standard model for hyperglycemia and type 2 diabetes [11]. Mice were treated with CuD or vehicle control (PBS) through oral gavage for 3 weeks, and then subjected to variety of tests. While body weight, food intake, serum triglycerides, cholesterol, ALT and AST contents were not altered (**Figure 1A-F**), fasting blood glucose levels were significantly reduced in mice with CuD treatment (**Figure 1G**). PTT results showed that glucose levels were decreased in these mice after

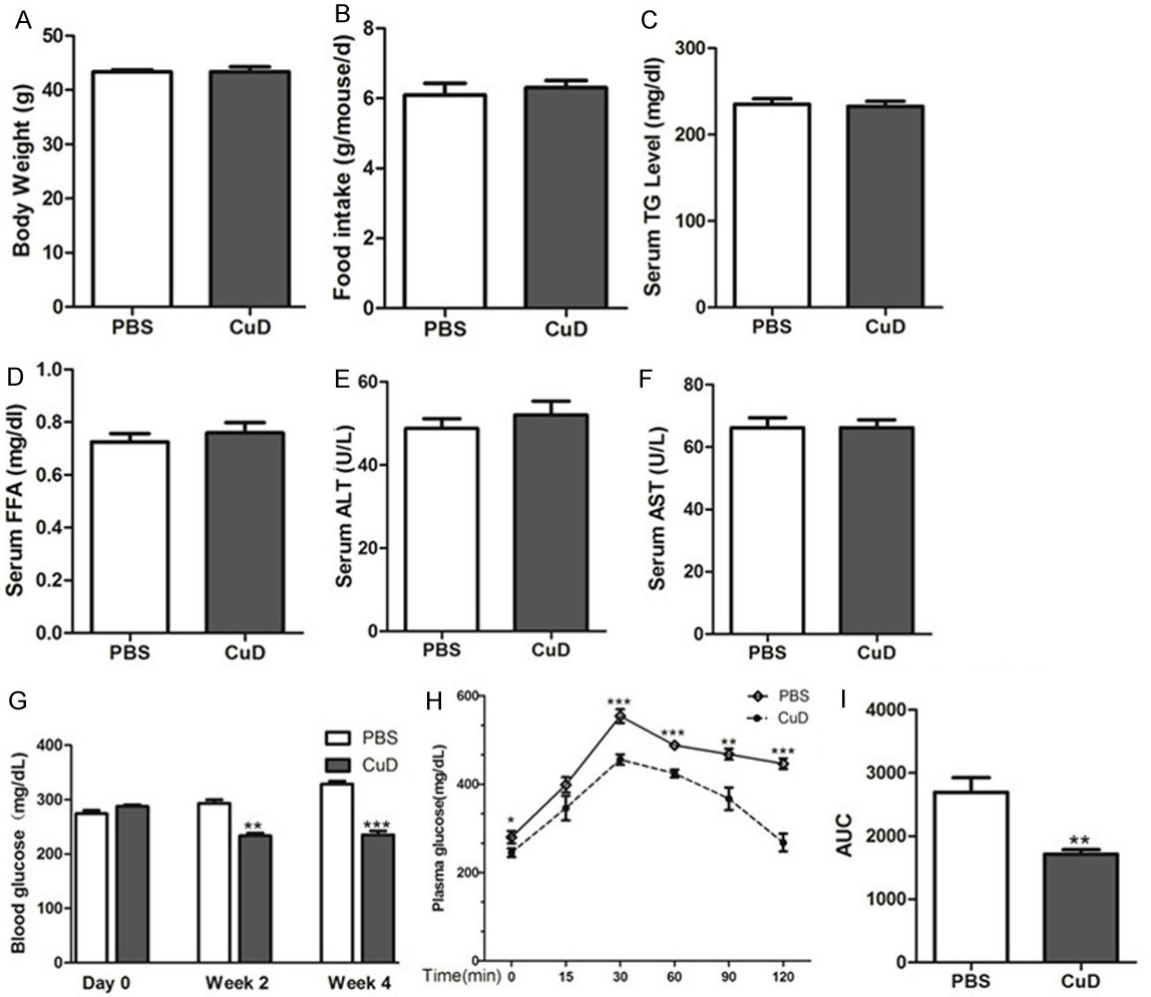

Figure 1. CuD treatment decreased fasting blood glucose in *db/db* mice. (A-F) Body weight (A), food intake (B), serum triglyceride (C), free fatty acid (C), ALT and AST levels in db/db mice treated with CuD (200 mg/kg/d) or vehicle control (PBS) for three weeks. N=8. (G) Fasting blood glucose levels in two groups of mice. Mice were fasted overnight and blood glucose levels were measured at the indicated time. N=8. (H-I) Blood glucose profiles (H) and area under curve (AUC) (I) of PTT analysis. Blood glucose was determined after a 12-hr fast at week 4. N=8. *P<0.05, **P<0.01, ***P<0.001.

pyruvate administration (**Figure 1H**, **1I**), Therefore, our data suggest that CuD potentially reduced fasting blood glucose in db/db mice.

CuD inhibits the expression of gluconeogenic genes in liver

To investigate hepatic gluconeogenesis, we examined expression of PEPCK and G6Pase, two rate-limiting enzymes in the hepatic gluconeogenic process. As shown in **Figure 2A**, both enzymes were reduced significantly in the livers from mice treated with CuD. Besides, the changes were also confirmed by western blot analysis (**Figure 2B**). Moreover, hepatic triglyceride contents were also reduced, accompanied by decreased expression of lipogenic genes including SREBP-1c, FASN and SCD-1 (Supplementary Figure 1A, 1B).

CuD activates Stat3 signaling in the liver

We sought to investigate the molecular basis for the observed phenotypic changes in mice with CuD treatment. Hepatic gluconeogenesis and gluconeogenic enzymes are tightly controlled by a variety of transcription factors, including cAMP-responsive element-binding protein (CREB), forkhead factor O1 (FoxO1), FoxO6 and signal transducer and activator of transcription 3 (Stat3), glucocorticoid receptor (GR), hepatocyte nuclear factor 4 α (HNF4α) and transcriptional cofactors such as small het-

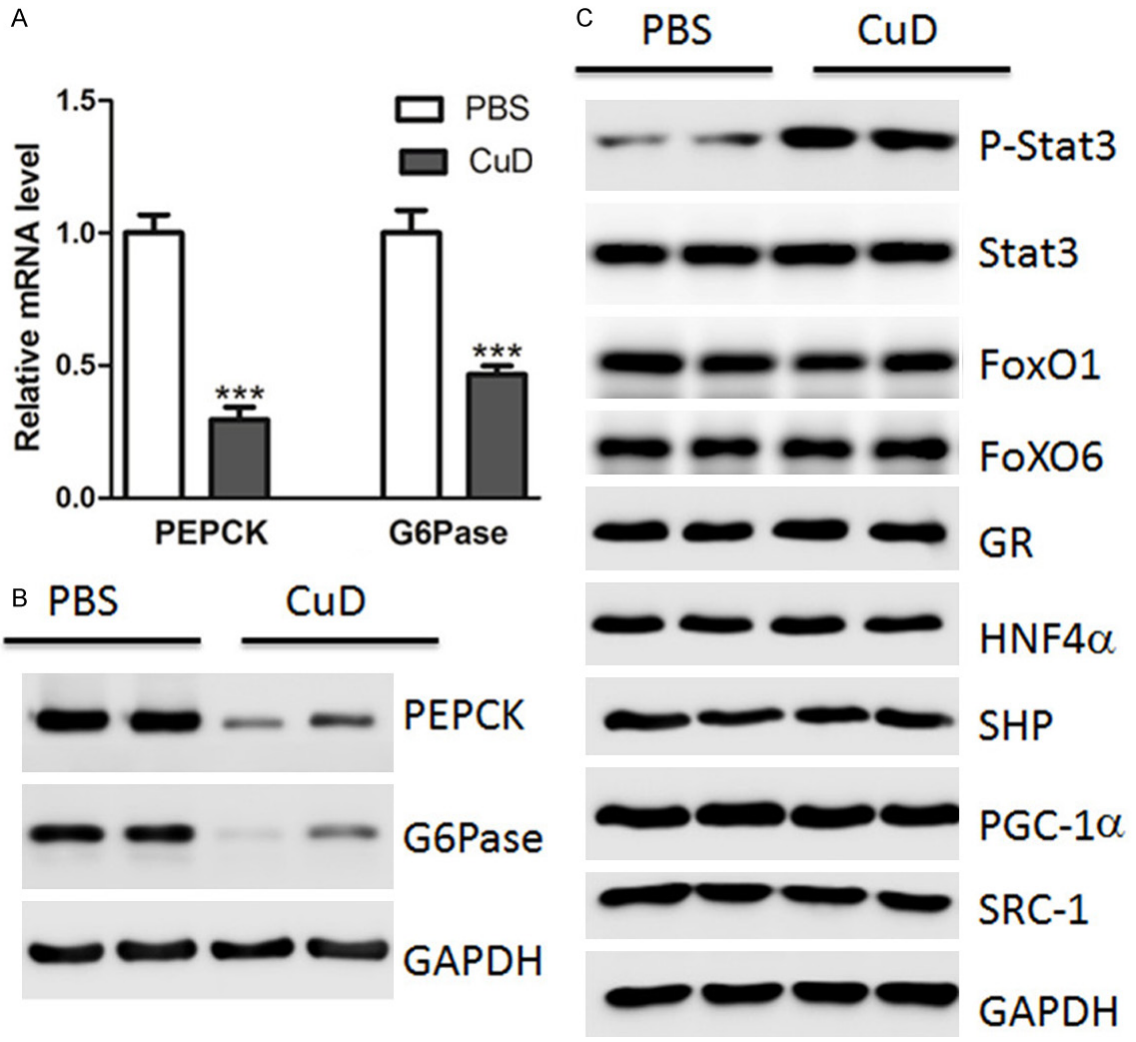

Figure 2. CuD inhibits the expression of gluconeogenic genes in liver. A, B. mRNA and representative protein levels of PEPCK and G6Pase were determined in db/db mice with CuD or vehicle control (PBS) treatment. N=8. C. Representative protein levels of phosphorylated Stat3, FoxO1, FoxO6, GR, HNF4, SHP, PGC-1 and SRC-1 were shown. Protein contents of Stat3 and GAPDH were used as loading controls. ***P<0.001.

erodimer partner (SHP), peroxisome proliferator activated receptor coactivator 1 (PGC-1α) and steroid receptor coactivator-1 (SRC-1) [12-14]. To understand the mechanism of PEPCK and G6Pase inhibition by CuD, we examined protein levels of these molecules in the liver. Stat3 signaling was highly activated by CuD as shown by its phosphorylation status, while other molecules remained unaffected (**Figure 2C**).

To further confirm this result in an independent setting, HuH7 cells (a cell line cells derived from hepatocellular carcinoma) and mouse primary hepatocytes (MPH) were administrated with CuD or vehicle control (PBS). As shown in **Figure 3A**, CuD treatment led to a dramatic increase of Stat3 phosphorylation, as well as inhibition of PEPCK and G6Pase expression (**Figure 3B**, **3C**). Similar results were also observed in MPH (**Figure 3D**, **3F**). Together, our data implicate that CuD regulates gluconeogenesis through activation of Stat3 signaling.

Stat3 is essential for the inhibition of gluconeogenesis by CuD

Next, we explored whether ablation of Stat3 reversed the roles of CuD. Adenoviral shRNA against a Stat3 coding region (Ad-shStat3) was adopted to silence hepatic Stat3 expression in *db/db* mice. Ad-shStat3 or Ad-shGFP as a negative control was delivered to mice via tail-vein injection. Hepatic Stat3 was almost completely

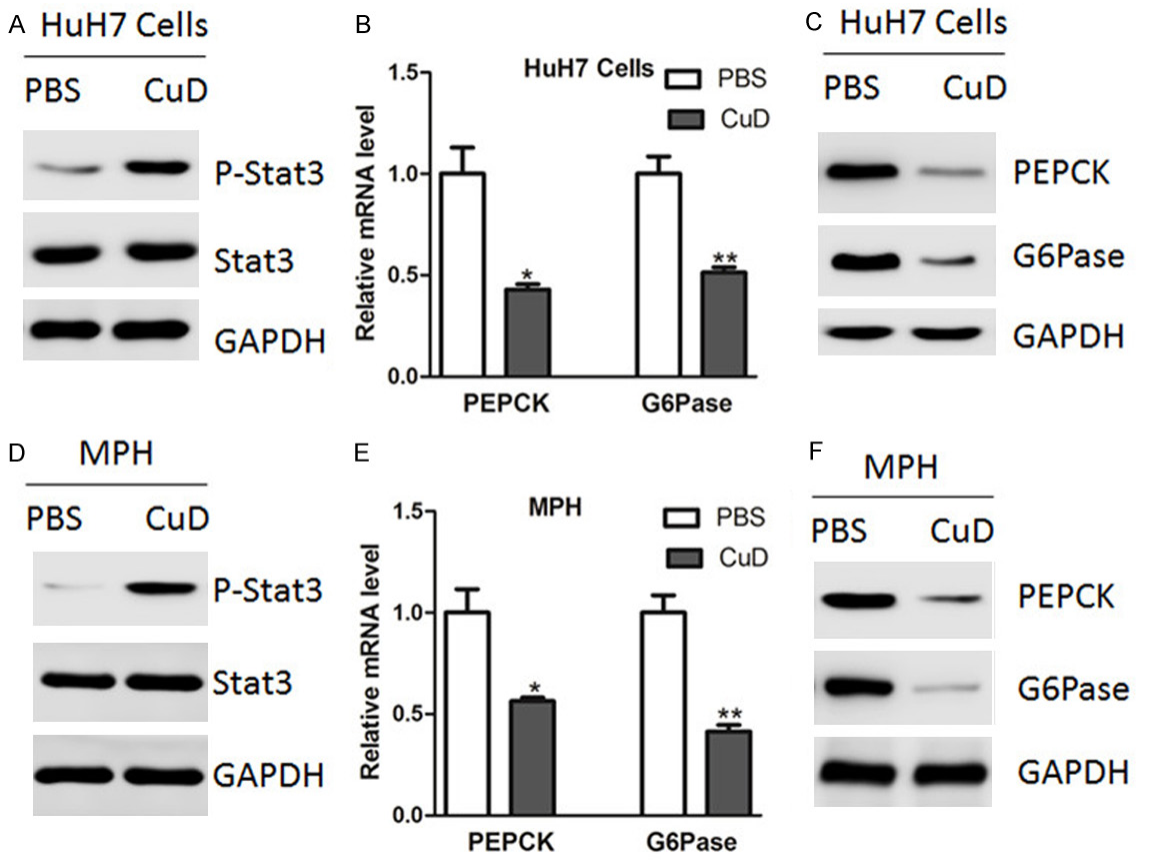

Figure 3. CuD activates Stat3 signaling *in vitro*. (A) Representative protein levels of phosphorylated Stat3 in HuH7 cells treated with CuD (500 nM) or vehicle control (PBS) for 2 hr. (B, C) mRNA (B) and representative protein (C) levels of PEPCK and G6Pase were determined in HuH7 cells treated with CuD (500 nM) or vehicle control (PBS) for 12 or 24 hr, respectively. (D) Representative protein levels of phosphorylated Stat3 in mouse primary hepatocytes (MPH) treated with CuD (500 nM) or vehicle control (PBS) for 2 hr. (E, F) mRNA (E) and representative protein (F) levels of PEPCK and G6Pase were determined in mouse primary hepatocytes (MPH) treated with CuD (500 nM) or vehicle control (PBS) for 12 or 24 hr, respectively. *P<0.05, **P<0.01.

abolished in Ad-shStat3 mice as compared with Ad-shGFP mice (**Figure 4A**, **4B**). As a result, Ad-shStat3 largely attenuated the inhibition roles of CuD on the blood glucose levels (**Figure 4C**). Moreover, reduced expression contents of PEPCK and G6Pase were also reversed by Stat3 deficiency (**Figure 4D**, **4E**), suggesting that the roles of CuD in the gluconeogenesis is dependent on Stat3 activation.

CuD decreased fasting blood glucose in streptozotocin (STZ)-treated C57BL/6 mice

Finally, to further evaluate the contribution of CuD to diabetic hyperglycemia, we studied its effect in the liver in an acute type I diabetes mouse model. We injected 10-week-old C57BL/6 mice with streptozotocin (STZ, 0.25 g/kg) to ablate insulin-producing beta cells. Six days after STZ injection, blood glucose concentrations were over 350 mg/dl and insulin was not detectable (**Figure 5A**, **5B**). Then these mice were treated with CuD or vehicle control (PBS) for another three weeks. We found that CuD treatment significantly reduced fasting blood glucose levels in these diabetic mice (**Figure 5C**). Furthermore, mRNA and protein levels of PEPCK and G6Pase were significantly lower in the CuD-treated, STZ-injected group (**Figure 5D**, **5E**). At the molecular level, phosphorylated Stat3 was higher in mice with CuD treatment (**Figure 5F**). Therefore, these results demonstrate that CuD could also alleviate hyperglycemia in STZ-induced diabetes.

Discussion

In the present study, we have shown that Cucurbitacin-D treatment resulted in reduced

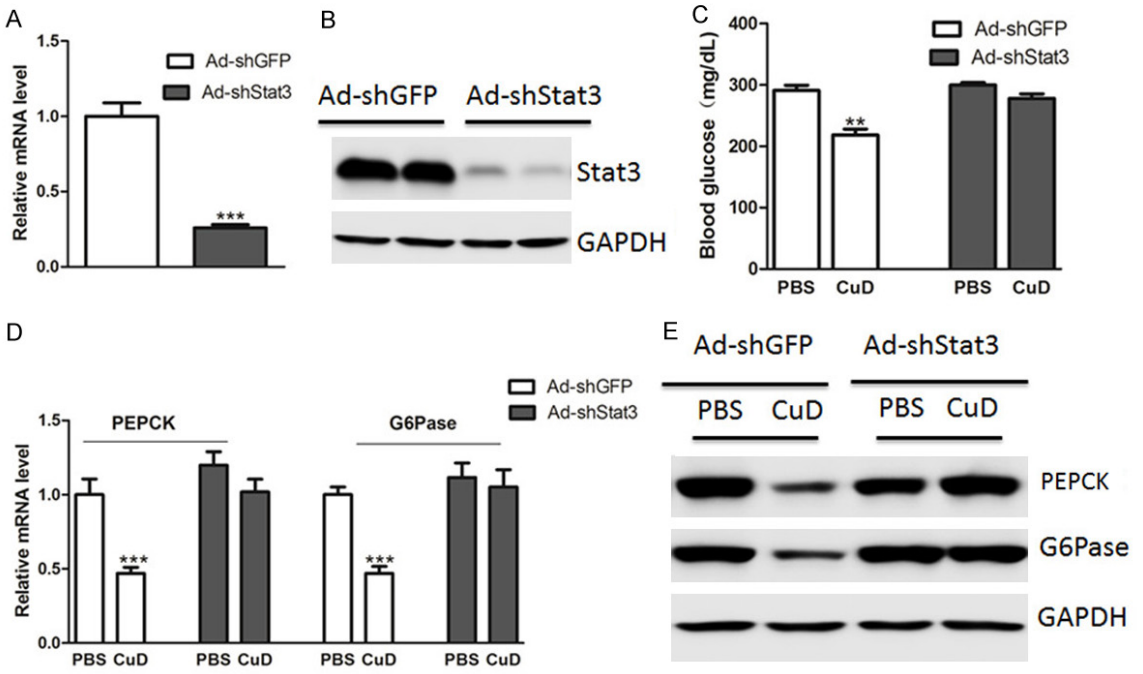

Figure 4. Stat3 knockdown reversed the roles of CuD. (A, B) mRNA (A) and representative protein (B) levels of Stat3 in livers of *db/db* mice administrated with adenoviral shRNA targeting Stat3 (Ad-shStat3) or GFP (Ad-shGFP). N=6. (C) Fasting blood glucose levels. Mice were fasted overnight and blood glucose levels were measured. N=6. (D, E) mRNA (D) and representative protein (E) levels of PEPCK and G6Pase were determined in *db/db* mice. N=6. **P<0.01, ***P<0.001.

fasting blood glucose in diabetic mice. Our data indicate that CuD inhibits expression levels of gluconeogenic enzymes including PEPCK and G6Pase in coordination with Stat3 activation. Although the mechanisms how CuD could activate Stat3 signaling remain unexplored now, the present results suggest that CuD might be a glucose-lowering compound, which might provide additional insight into the development of new anti-diabetes drugs.

Numerous studies have demonstrated that the process of gluconeogenesis is controlled by a variety of transcription factors [12-16]. Among them, Stat3 is known to suppress expression of gluconeogenic genes [17, 18]. Consistently, interleukin-6 (IL-6) treatment promotes Stat3 to bind with the promoters of the PEPCK and G6Pase, accompanied by a decrease in polymerase II recruitment and histone H4 acetylation [17]. Besides, ectopic expression of Stat3 in *db/db* mice reverses hyperglycemia and diabetes [19]. In agreement, mice with liver-specific Stat3 deficiency exhibit hyperglycemia and insulin resistance when fed a high-fat-diet [19]. Moreover, expression of Stat3 in the livers of *db/db* mice also reduces liver triglycerides contents and expression of lipogenic genes [19], which is consistent with our observations in CuD-treated *db/db* mice.

Family of Cucurbitacin is shown to regulate various critical cellular processes through several signaling pathways such as MAPK, AKT and Wnt/-Catenin [20-22]. Here, using mice liver specific Stat3 knockdown system, our results reveal that Stat3 signaling is required for the anti-diabetes of CuD. Stat3 activation has been viewed as crucial for multiple tumor growth and progression while CuD could repress tumor cell proliferation [23, 24]. Therefore, the molecular mechanisms of CuD might be cell or tissue-specific.

Overall, our results strongly implicate CuD as a potential glucose-lowing compound to treat hyperglycemia and diabetes. Identification of the specific role for CuD in hepatic gluconeogenesis would help to develop a potential treatment for metabolic disorders.

Disclosure of conflict of interest

None.

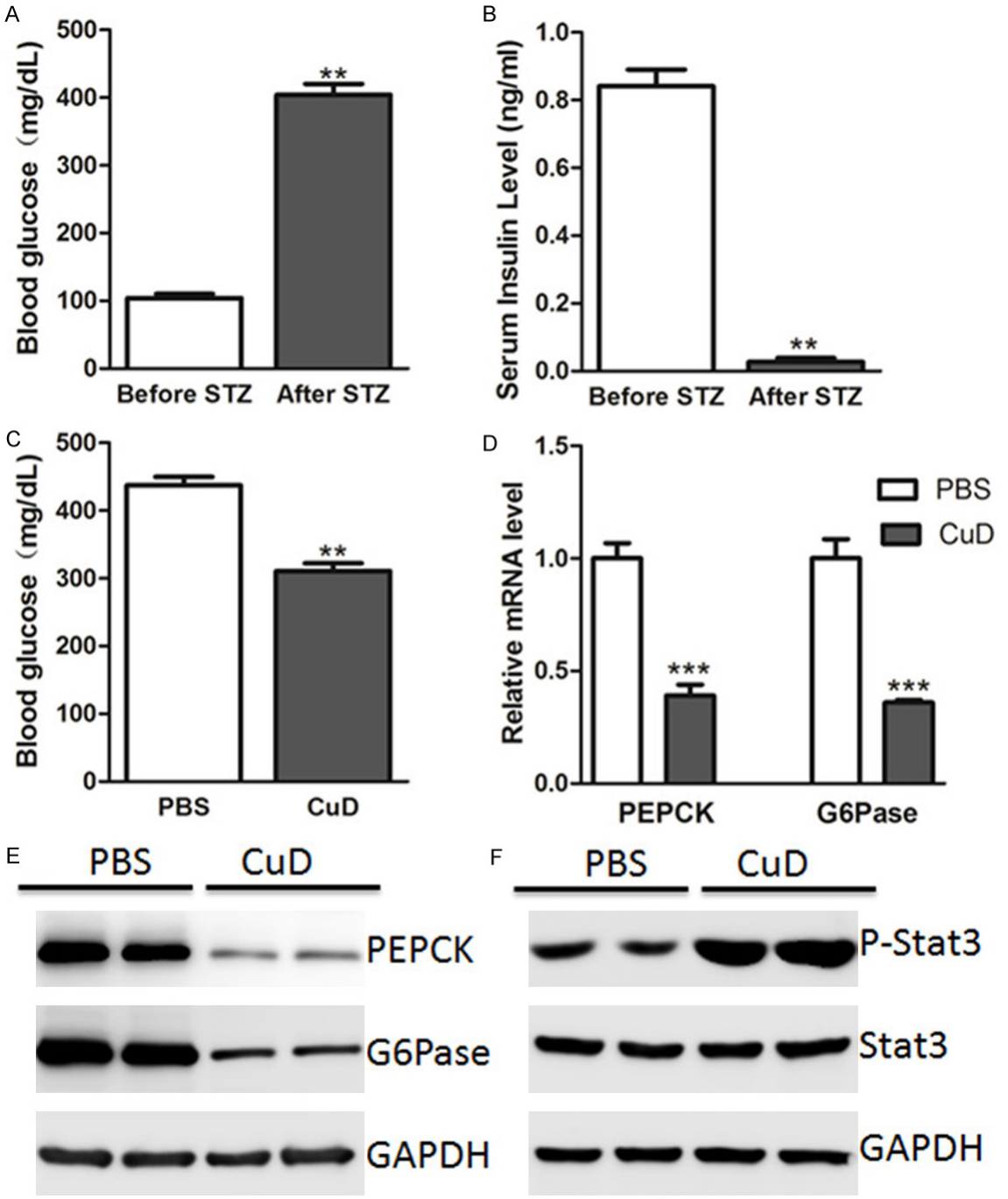

Figure 5. CuD treatment reduced fasting blood glucose in STZ-treated mice. (A, B) Fasting blood glucose and serum insulin levels in C57BL/6 mice before or six days after STZ treatment. N=8. (C) Fasting blood glucose levels. Mice were fasted overnight and blood glucose levels were measured. N=8. (D) mRNA (D) and representative protein (E) levels of PEPCK and G6Pase were determined. N=8. (F) Representative protein levels of phosphorylated Stat3 were shown. Protein contents of Stat3 and GAPDH were used as loading controls. **P<0.01, ***P<0.001.

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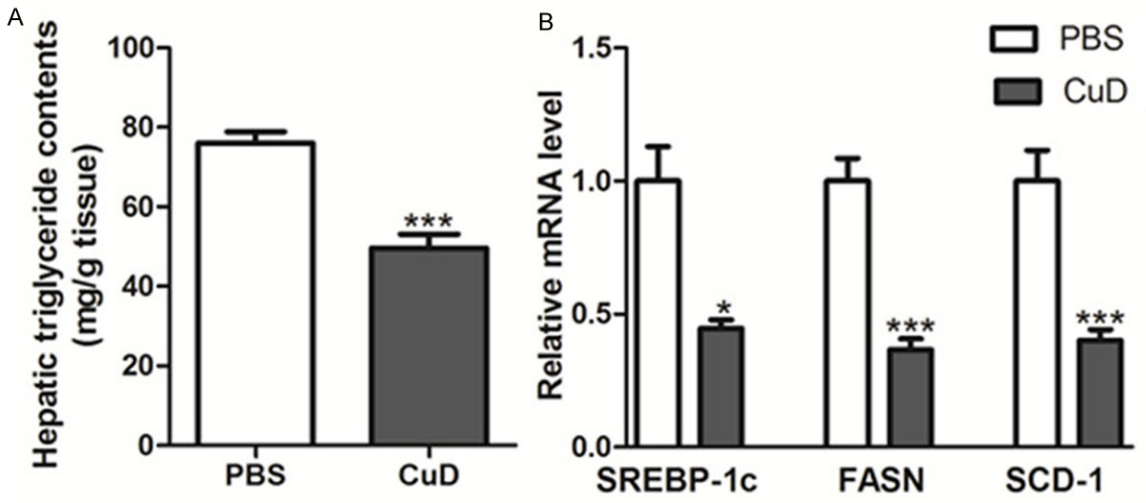

Supplementary Figure 1. A. Hepatic triglyceride contents were determined in *db/db* mice treated with CuD (200 mg/kg/d) or vehicle control (PBS). N=8. B. mRNA levels of SREBP-1c, FASN and SCD-1 were determined in two groups of *db/db* mice. N=8.