SIRT1 Mediates the Effect of GLP-1 Receptor Agonist Exenatide on Ameliorating Hepatic Steatosis

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Running title: Exenatide improves hepatic steatosis via SIRT1

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Abstract
GLP-1 and incretin mimetics, such as exenatide, have been shown to attenuate hepatocyte steatosis both in vivo and in vitro, while the specific mechanism underlying is unclear. SIRT1, a NAD⁺-dependent protein deacetylase, has been considered as a crucial regulator in hepatic lipid homeostasis by accumulated studies. Here, we speculate that SIRT1 might mediate the effect of GLP-1 receptor agonist exenatide (exendin-4) on ameliorating hepatic steatosis. After 8-week exenatide treatment in HFD induced male SIRT1+/− mice and their wild-type (WT) littermates, we found that lipid deposition and inflammation in liver, which were improved dramatically in WT group, diminished in SIRT1+/− mice. In addition, the protein expression of SIRT1 and p-AMPK were up-regulated, while lipogenic related protein including SREBP-1c and PNPLA3 were down-regulated in WT group after exenatide treatment. However, none of these changes were observed in SIRT1+/− mice. In HepG2 cells, exendin-4-reversed lipid deposition induced by palmitate was hampered when SIRT1 was silenced by SIRT1 RNAi. Our data demonstrate that SIRT1 mediates the effect of exenatide on ameliorating hepatic steatosis, suggesting GLP-1 receptor agonist could serve as a potential drug for NAFLD, especially in T2DM combined with NAFLD, and SIRT1 could be a therapeutic target of NAFLD.

Key Words: GLP-1; Sirtuin1; liver steatosis; inflammation; NAFLD; T2DM.
Abbreviations:

GLP-1, glucagon-like peptide 1; HFD, high-fat diet; AMPK, AMP-activated protein kinase; SREBP-1c, sterol regulatory element binding protein 1c; PNPLA3, patatin-like phospholipase domain containing 3; T2DM, type 2 diabetes; MCP-1, monocyte chemotactic protein-1; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; FGF21, fiberblast growth factor 21; NF-κB, nuclear factor kappa B
Introduction

Nonalcoholic fatty liver disease (NAFLD) is a burgeoning health problem that begins with the aberrant accumulation of triglyceride in the liver. It includes isolated fatty liver and nonalcoholic steatohepatitis (NASH), the latter of which can progress to cirrhosis and liver cancer in some individuals (1). Additionally, NAFLD is mostly common in obesity and metabolic syndrome, both of which are strongly associated with insulin resistance. What challenging us most is that there have been no drug therapy approved for NAFLD so far (2).

Glucagon-like peptide-1 (GLP-1), an incretin hormone, is a gut-derived peptide secreted by intestinal L-cells after meal. It has pleiotropic functions in mammals as to promote insulin secretion of pancreatic β-cells, suppress inappropriate glucagon secretion, slow gastric emptying and induce insulin mediated glucose uptake (3). As a new kind of anti-diabetic drugs, incretin mimetics, such as exenatide (exendin-4), have been proved by increasing amount of evidences that they could improve lipid deposition in liver effectively (4-6). However, the specific mechanism underlying is little known. It was reported that GLP-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro (5). A recent study found that exendin-4 could reduce inflammation in liver by inhibiting macrophage recruitment and activation (7). Nevertheless, the exact mechanism as to signaling pathway of GLP-1 and its mimetics on improving hepatic steatosis is not fully understood.

SIRT1, mammalian sirtuin 1, is a kind of NAD$^+$-dependent protein deacetylase. It is an important regulator of energy homeostasis in response to nutrient availability (8; 9). We have previously found that loss of SIRT1 leads to more serious liver steatosis in $SIRT1^{+/-}$ mice compared with wild-type (WT) mice after HFD induction
(10). Other study also showed that hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation (11). On the other hand, hepatic over-expression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver (12). Activating SIRT1 signaling program by resveratrol or SRT1720 relieves fatty liver with reduced lipid synthesis and increased rate of fatty acid oxidation (13; 14). A recent published study reported that SIRT1 mediated the activation of FGF21 which could prevent liver steatosis caused by fasting (15). Another one demonstrated that hepatic deletion of SIRT1 promoted steatosis and inflammation in response to ethanol challenge via lipin-1, a transcriptional regulator of lipid metabolism (16). All above indicate that SIRT1 is vital in the lipid homeostasis of liver.

Since SIRT1 plays such an essential role in the lipid metabolism of liver, whether GLP-1 receptor agonist exenatide ameliorating hepatic steatosis is mediated by SIRT1 remains to be investigated. Here, we presume that exenatide improves liver steatosis via SIRT1 pathway. In our study, SIRT1 +/- mice and their WT littermates were challenged with HFD following by exenatide treatment. We found that GLP-1 receptor agonist treatment could reverse liver steatosis in WT mice but not in SIRT1 +/- mice, which indicates that loss of SIRT1 significantly impairs the effect of GLP-1 receptor agonist. It is, for the first time to our knowledge, to point out that SIRT1 plays an indispensable role in mediating the effect of GLP-1 receptor agonist exenatide on ameliorating hepatic steatosis.
Materials and Methods

Animals and diets. SIRT1<sup>+/−</sup> mice in C57BL/6J gene background were offered as a gift by Prof. Jianping Ye from Pennington Biomedical Research Center, Louisiana State University (10). Seven-eight weeks old C57BL/6J breeders were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Male SIRT1<sup>+/−</sup> mice and their WT littermates were used in the study. The mice were maintained at 22 ± 2°C and 50% ± 5% relative humidity with a 12-h light, 12-h dark cycle. All the mice had ad libitum access to rodent chow diet (5% fat wt/wt, Guangdong Medical Laboratory Animal Center) and water. High-fat diet (HFD, 36% fat wt/wt, D12492, Research Diets) were used to induce obesity and fatty liver. After 12 weeks chow diet feeding or HFD challenge, mice were divided randomly into the following 5 groups: WT+chow diet, WT+HFD+saline, WT+HFD+exenatide, SIRT1<sup>+/−</sup>+HFD+saline and SIRT1<sup>+/−</sup>+HFD+exenatide. Mice were treated with intraperitoneal injection of exenatide (24 nmol/kg, Eli Lilly and Company, Indianapolis, USA) or normal saline control daily for 8 weeks. Food intake and body weight were monitored once per two weeks during this period. By the end of the 20<sup>th</sup> week, all the animals were fasted for 8h and anesthetized with ether first and then sacrificed for blood and tissue collection. All experiments were approved by the Animal Ethics Committees of the Sun Yat-Sen University.

Cell culture and treatments. HepG2 human hepatoma cells obtained from American Type Culture Collection (ATCC) were cultured in MEM medium containing 10% (vol/vol) fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 100 U/mL penicillin and 100mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were grown to 70% confluence and incubated in serum free
medium for 4 h before treatments. To knock down SIRT1 in HepG2 cells, cells were transfected with lentivirus vector expressing SIRT1 shRNA sequence and the control vector as scramble (Genechem, Shanghai, China). HepG2 cells were treated with palmitate (#P9767, Sigma-Aldrich), and exendin-4 (#E7144, Sigma-Aldrich) or resveratrol (#R5010, Sigma-Aldrich) if indicated. Cell lysates were collected for the western blot analysis.

**Intraperitoneal glucose tolerance test (ip GTT) and insulin tolerance test (ip ITT).** For GTT, mice were fasted overnight and administered with glucose (2.5g/kg wt) intraperitoneally at the next morning. ITT was conducted by intraperitoneal injection of insulin (0.75U/kg wt, Novolin R, Novo Nordisk INC.) in mice after 4 hours fasting as previously described. Tail vein blood glucose was measured at 0 min, 30 mins, 60 mins and 120 mins with Optium Xceed glucometer (Abbott Diabetes Care INC., USA) both in GTT and ITT.

**Quantitative real-time PCR.** Tissues were collected and kept in liquid nitrogen, then stored at -80°C. Total RNA was extracted from liver using TRIzol reagent (Invitrogen, Shanghai, China). RNA was reverse transcribed to cDNA using the Prime ScriptTM RT reagent kit (Takara, Japan). The primers were from the Applied Biosystems (Foster City, CA). These include: F4/80 (Mm00802530_m1), TNF-α (Mm00443258_m1), MCP-1 (Mm00441242_m1). The quantitative RT-PCR (qRT-PCR) was conducted with Real-Time PCR System (Lightcycler 480 II, Roche, Germany).

**Western blot.** Livers were rinsed with ice cold PBS, and then stored at -80°C until western blot analysis. Liver tissues were homogenized in the whole cell lysis buffer. Antibodies include these against: SIRT1 (#2496, Cell Signaling, USA), p-AMPK
Liver lipids test. Liver tissues were homogenized in PBS (1g: 20ml). The lipids were extracted from the liver tissue lysates using a chloroform/methanol (2:1) mixture (17). TG and glycerol were determined using Serum Triglyceride Determination Kit (TR0100, Sigma). Cholesterol was determined with Cholesterol Reagent (80015, Biovison) according to instructions by the manufacturer.

Hematoxylin and eosin (H&E) staining. Fresh liver tissues were collected and fixed in 4% neutral buffered formalin solution (HT50-1-2, Sigma). The tissue slides were obtained through serial cross-section cutting at 6-8μm thickness and processed with a standard procedure of hematoxylin and eosin staining.

Oil red-O staining. Accumulation of triglyceride content both in liver and the treated HepG2 cells was visualized by oil red-O (Sigma-Aldrich) staining as previously described (18). The lipids accumulation was photographed with a microscope (BX51WI, Olympus, Japan).

Immunohistochemistry staining. Fresh liver tissues were fixed in neutral buffered formalin, dehydrated, and embedded in paraffin. Thin tissue slides (3-5 um) were
deparaffinized, blocked, and incubated overnight at 4°C with antibody of mouse anti-rat F4/80 (sc-71087; Santa Cruz Biotechnology, Inc.). The immunoreactions were revealed using OneStep Polymer HRP anti-mouse/rat/rabbit Detection System (GTX83398, GeneTex Inc.) and using DAB as chromogen. Microphotographs were taken under microscope (DM 2500B, Leica, Germany) with X20 and X40 object lens, respectively.

**Statistical analysis.** The data were presented as the mean ± SEM from multiple samples (n = 5-12 for each group in animal study). All of the in vitro experiments were replicated three times at least. Two-tailed, unpaired Student’s t test was used in the statistical analysis with significance P ≤ 0.05.
Results

The effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in SIRT1+/− mice

First, SIRT1+/− mice and their littermate WT mice were induced with HFD for 12 weeks, and then treated with exenatide or saline as normal control for another 8 weeks. Body weight of WT mice on HFD challenge was significantly higher than that of on chow diet since 8 weeks till 12 weeks. From 10 weeks to 12 weeks, SIRT1+/− mice became statistically heavier than WT mice on HFD (Fig. 1A). No significant difference was observed on food intake (kcal/kg/hour) between wild type and SIRT1+/− mice on HFD (Fig. 1B). After 4 weeks exenatide administration, WT mice began to show a significant reduction of body weight compared with the saline control, which last till the end of the intervention period. However, this body weight reducing effect diminished in SIRT1+/− mice reflected by no significant difference observed between exenitide treated mice and control ones (Fig. 1C). Food intake declined slightly after exenatide treatment both in WT and SIRT1+/− group, yet still remained statistically comparable among all groups (Fig. 1D).

Both ip GTT and ip ITT were carried out after 8 weeks of exenatide administration. The fasting blood glucose (FBG) level of HFD challenged WT mice was higher than that on chow diet, and showed a significant reduction after exenatide treatment. SIRT1+/− mice had higher FBG compared with WT mice on HFD and this difference was sustained after exenatide treatment in two groups. But FBG level of SIRT1+/− mice didn’t show any statistical difference either with exenatide treatment or not (Fig. 1E). After administration of glucose, all trends above were maintained or even stronger at 30 mins and 60 mins (Fig. 1E). Area under the curve (AUC) also revealed
a significant improved glucose tolerance in WT mice after exenatide treatment. However, the impaired glucose tolerance of SIRT1\(^{+/−}\) mice on HFD was not improved as much as that in WT mice after exenatide treatment (Fig. 1E). During ip ITT test, administration of insulin led to a significant decrease of blood glucose levels in WT mice after exenatide treatment but not in SIRT1\(^{+/−}\) mice (Fig. 1F). AUC showed statistical improved insulin sensitivity in WT mice after exenatide treatment. However, no statistical improvement of insulin sensitivity was observed in SIRT1\(^{+/−}\) mice on HFD after exenatide treatment (Fig. 1F).

All above indicate that the effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in SIRT1\(^{+/−}\) mice.

**Exenatide-improved liver weight, FBG and lipid profile are weakened in SIRT1\(^{+/−}\) mice**

After all of the treatments, we collected and weighed livers, detected FBG and fasting insulin level, and tested lipids profile in these mice. As expected, the liver weight in WT mice on HFD group was dramatically decreased after exenatide treatment compared with the saline control. However, this effect was weakened in SIRT1\(^{+/−}\) mice. The same trend was observed in FBG change. Fasting insulin level didn’t show significant difference among these groups due to big variation within groups (Table 1).

Next, the lipids profile including triglyceride, glycerol, and total cholesterol in serum were tested. Exenatide treatment decreased the triglyceride, glycerol, and total cholesterol level by 37%, 29%, and 9% in HFD induced WT mice compared with the saline control, respectively (Table 1). SIRT1\(^{+/−}\) mice had significantly higher lipids level in serum compared with WT mice on HFD, and this difference was sustained
after exenatide treatment in two groups (Table 1). However, no statistical changes were observed in $SIRT1$+/− mice regardless of exenatide treatment (Table 1).

**Exenatide-ameliorated liver steatosis disappears in $SIRT1$+/− mice**

Although recent study showed GLP-1 receptor agonist could relieve hepatic steatosis by up-regulation of SIRT1 in C57BL/6J mice (19). Yet, whether exenatide induced hepatic steatosis attenuation is directly mediated by SIRT1 is still unclear. To test this possibility, we examined morphology, histology and lipids content of liver both in $SIRT1$+/− mice and their littermate WT mice either with exenatide treatment or not. The general pictures of liver exhibited bigger size and white coloring in $SIRT1$+/− mice irrespectively of exenatide treatment (Fig. 2A). Both H&E staining and Oil red-O staining showed a significant increase of lipid droplets in hepatocytes of $SIRT1$+/− mice compared with WT mice on HFD (Fig. 2B, X40). The white coloring and lipid droplets in hepatocytes were dramatically improved in HFD induced WT mice after exenatide treatment compared with the saline control; however, all these effects disappeared in $SIRT1$+/− mice (Fig. 2A-B). Consistently, triglyceride, glycerol and total cholesterol contents in liver were decreased by 38%, 29%, and 13% in HFD induced WT mice with exenatide compared with the saline control, respectively. But no change was observed in lipid contents in HFD induced $SIRT1$+/− mice after exenatide treatment (Fig. 2C-E).

**SIRT1 is required by exenatide to alleviate inflammation in liver**

As we know, activation of inflammatory processes was considered to be a consequence of fatty acids accumulation in liver (20). Our previously research showed that inflammatory genes expression were enhanced in liver of mice on HFD indicated by F4/80 and TNF-α gene expression (10). Here, the results demonstrated
that exenatide could reduce inflammatory gene expression including TNF-α, F4/80 and MCP-1 in liver of HFD induced WT mice. However, no obvious change was observed in all those genes expression in SIRT1\(^{+/−}\) mice either with exenatide treatment or not (Fig. 3A-C). Macrophage infiltration was also determined using F4/80 protein expression in liver. Immunohistochemical staining of F4/80 suggested that F4/80 protein was remarkably reduced in liver of WT mice after exenatide treatment compared with saline control, but not in SIRT1\(^{+/−}\) mice (Fig. 3D). The data suggest that SIRT1 is required by exenatide to relieve inflammation in liver.

**Exenatide-reduced hepatic steatosis depends on SIRT1/AMPK pathway**

AMPK is a metabolic fuel gauge that regulates lipid metabolism through phosphorylation by sensing changes in the intracellular AMP/ATP ratio, especially in liver (21). To test whether the actions of exenatide are mediated by SIRT1 through AMPK, protein expression of SIRT1 and AMPK were examined. In accordance with the up-regulation of SIRT1 by exenatide in HFD induced WT mice, p-AMPK expression was also remarkably increased compared with saline control. But no change was observed in liver of SIRT1\(^{+/−}\) mice irrespective of exenatide injection (Fig. 4A-C).

We then verified the above hypothesis in HepG2 cell line *in vitro*. Since exenatide is a synthetic version of exendin-4, we used exendin-4 for the treatment in HepG2 cells to exclude the influence of auxiliary material. Intracellular lipid detection by Oil red-O staining showed that exendin-4 could reverse palmitate induced lipid accumulation in HepG2 cells (Fig. 4D). And exendin-4 (20 nmol/L and 200 nmol/L, respectively) increased both SIRT1 and p-AMPK protein levels significantly in palmitate induced HepG2 cells (Fig. 4E). The phosphorylation of ACC, a substrate enzyme of AMPK,
was up regulated in parallel with p-AMPK (Fig. 4E). After silencing SIRT1 using SIRT1 RNAi, the effect of exendin-4 (20 nmol/L) was attenuated sharply (Fig. 4F). Resveratrol (50 µmol/L), a SIRT1 activator, was provided to compare with the effect of exendin-4 on palmitate induced HepG2 cells. As shown, the effect of exendin-4 on activating SIRT1 and p-AMPK was comparable with resveratrol (Fig. 4G).

Since SIRT1 is NAD$^+$ dependent, whether exendin-4 acts by altering the level of NAD$^+$ or ratio of NAD$^+$/NADH were further investigated in HepG2 cells in vitro. The results showed that exendin-4 did reverse the palmitate-reduced NAD$^+$ level and NAD$^+$/NADH ratio in HepG2 cells in a dose dependent way (Fig. 4H), which suggest exendin-4 could induce up-regulation of not only SIRT1 protein expression but also its activation through increasing the NAD$^+$/NADH ratio.

These data support our speculation that exenatide-reduced hepatic steatosis depends on SIRT1/AMPK pathway.

**SIRT1 is required by exenatide to ameliorate lipogenesis through inhibiting SREBP-1 in liver**

SREBP-1c is one of the master transcription factors of de novo lipogenesis in liver (22). PNPLA3, a target gene of SREBP-1c, also plays a role in lipogenesis in mouse liver (23). Both of these two factors were examined in our study. The results showed a significant decrease of SREBP-1 and PNPLA3 protein expression in HFD challenged WT mice with exenatide treatment compared with saline control. Not surprisingly, again neither SREBP-1 nor PNPLA3 protein expression changed in SIRT1$^{+/-}$ mice whether with exenatide treatment or not (Fig. 5A-C). In vitro, exendin-4 (20 nmol/L and 200 nmol/L) significantly decreased SREBP-1 protein expression in palmitate induced HepG2 cells (Fig. 5D). After knocking down SIRT1 in HepG2 cells, the
effect of exendin-4 (20 nmol/L) on inhibiting SREBP-1 expression was weakened dramatically (Fig. 5E). Besides, Resveratrol (50 µmol/L) was provided to treat with palmitate challenged HepG2 cells. As shown, the effect of exendin-4 on inhibiting SREBP-1 was comparable with resveratrol (Fig. 5F).

These results indicate that SIRT1 is required by exenatide to ameliorate lipogenesis via inhibiting SREBP-1 in liver.
Discussion

NAFLD has become a worldwide health concern since global incidence of obesity has increased. Epidemiological studies showed that NAFLD is strongly associated with T2DM—each is highly predictive of the other (24; 25). The coincident occurrence of hepatic steatosis and insulin resistance also leads to the hypothesis that excess triglyceride in liver causes insulin resistance which contributes to T2DM (2). Since ideal treatment for NAFLD has not been established, novel approaches aimed at reducing lipotoxicity and inhibiting proinflammatory are urgently needed. Incretin hormone GLP-1 and its mimetics, a new kind of antidiabetic drugs, show pleiotropic functions in both pancreatic β-cells and extra-pancreatic organs in mammals (26; 27). Accumulated evidences demonstrate that it could improve lipid deposition and inflammation in liver effectively (6; 7; 28), which indicates GLP-1 and its mimetics could be a potential drug for the treatment of NAFLD, especially in NAFLD combined with T2DM (29; 30). Here, we report a new mechanism of GLP-1 receptor agonist on improving liver steatosis, which demonstrates that SIRT1 mediates the effect of exenatide (exendin-4) on ameliorating hepatic steatosis.

SIRT1 plays a vital role in hepatic lipid metabolism by deacetylation of acetylated lysine residues on histones and various transcriptional regulators (8). Complete deletion of the SIRT1 gene leads to developmental defects and postnatal lethality (31; 32), which implies that SIRT1−/− mice are not appropriate for the study for medication intervention. SIRT1+/− mice are normal in development and postnatal growth (31; 32). As our animal experiments were proceeding, a study was reported that GLP-1 receptor agonist exendin-4 attenuated fatty liver through activation of
SIRT1 in HFD induced C57BL/6J mice (19). However, whether the effect of GLP-1 receptor agonist on improving fatty liver is mediated by SIRT1 has never been proved on genetically SIRT1 knockout model, and the exact mechanisms underlying remains elusive. Our data showed that hepatic steatosis, which was improved dramatically in WT group, diminished in \textit{SIRT1}^{+/−} mice after exenatide treatment (Fig. 2B-D). It indicates that exenatide-improved lipid deposition in liver is indeed mediated by SIRT1. With regard to why steatosis is not improved at all in response to exendin-4 in \textit{SIRT1}^{+/−} mice, we inferred that the effect of exenatide on improving liver steatosis may not only require the existence of SIRT1, but also a certain amount of SIRT1 expression. Over 50% loss of SIRT1 protein level was not able to exert the effect of exenatide on hepatocytes. After knocking down SIRT1 by RNAi in HepG2 cells (Fig.4F), there was still very low SIRT1 expression though much less compared with scramble control, but exendin-4 was not able to up regulate SIRT1 and its downstream factors. It indicates that low expression of SIRT1 is not enough to mediate the effect of exendin-4.

AMPK is a metabolic fuel gauge that regulates lipid metabolism through phosphorylation by sensing changes in the intracellular AMP/ATP ratio, especially in liver (21). One recent study demonstrates that SIRT1 plays an essential role in the ability of moderate doses of resveratrol to stimulate AMPK and improve mitochondrial function (33). SIRT1 also mediates the effect of alpha-lipoic acid on regulating lipid metabolism through activation of AMPK (34). In the current study, we tested whether the actions of exenatide were mediated by SIRT1 through activation of AMPK. Our data showed that exenatide up regulated SIRT1 and p-AMPK in liver of HFD induced WT mice, but not in \textit{SIRT1}^{+/−} mice (Fig. 4A-C). After silencing SIRT1 by lentivirus expressing SIRT1 RNAi in palmitate induced HepG2
cells, the effect of exendon-4 on increasing both SIRT1 and p-AMPK protein level was attenuated sharply as well (Fig. 4E-F). The results support our speculation that exenatide’s role in hepatic steatosis alleviation is mediated by SIRT1 through AMPK.

Hepatic steatosis could result from an increase of de novo lipogenesis. SREBP-1c is one of the master lipogenic transcription factors of de novo lipogenesis in liver (22). PNPLA3, a target gene of SREBP-1c, also plays a role in lipogenesis in mouse liver (23). In our study, both SREBP-1 and PNPLA3 protein expression was decreased remarkably in HFD challenged WT mice after exenatide treatment, while no changes were observed in SIRT1+/− mice with exenatide treatment (Fig. 5A-C). In vitro, the effect of exendon-4 on inhibiting SREBP-1 was mediated by SIRT1 using RNAi method to knock down SIRT1 expression in palmitate induced HepG2 cells (Fig. 5E). We also found that exendin-4 reduced the precursor of SREBP-1 (−P) (Suppl. 1A) and this effect was mediated by SIRT1 (Suppl. 1B). Meanwhile, exendin-4 inhibited the nuclear translocation of SREBP-1 (Suppl. 2). Besides, SREBP-1 Ser 372 phosphorylation didn’t change under the concentration of 20nM exendin-4 and was up-regulated with the concentration of 200nM (Suppl. 1A). Our data indicated that exendin-4 inhibited both the synthesis of the precursor SREBP-1 (−P) and the nuclear translocation of SREBP-1 instead of affecting its proteolytic processing. Previous study verified SREBP-1c is an in vivo target of SIRT1 and SIRT1 deacetylates and inhibits SREBP-1c activity in regulation of hepatic lipid metabolism (35). Our results indicate that SIRT1 was required by exenatide to ameliorate lipogenesis via inhibiting the synthesis of the precursor of SREBP-1c in liver. Whether exenatide affects the acetylation/deacetylation of SREBP-1c through SIRT1 remains to be investigated.
NAFLD includes isolated fatty liver and nonalcoholic steatohepatitis (NASH). Hepatic steatosis is usually accompanied with chronic inflammation indicated by inflammatory cell infiltration during NAFLD progression according to liver histology findings (2). A recent study finds that exendin-4 reduces inflammation in liver by reducing macrophage recruitment and activation (7). In T2DM patients, administration of liraglutide, a GLP-1 analogue, improves liver inflammation and altered liver fibrosis (28). Here in our study, inflammation was reduced by exenatide in liver of WT mice, and this effect disappeared in liver of SIRT1+/− mice (Fig. 3). Recent studies have demonstrated SIRT1’s properties in anti-inflammation (36-38). SIRT1 knockdown led to enhanced IκB kinase phosphorylation and NF-κB activation in adipocytes stimulated by lipopolysaccharide (LPS). It also resulted in increased expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (36). In line with SIRT1’s role of anti-inflammation, it’s not hard to understand that SIRT1 is required by exenatide to alleviate inflammation in liver in our study, though the exact mechanism remains to be investigated further.

In conclusion, the present study demonstrates that SIRT1 mediates the effect of GLP-1 receptor agonist exenatide on relieving liver steatosis. And the actions of exenatide on ameliorating lipogenesis mediated by SIRT1 are through activating AMPK pathway and inhibiting SREBP-1c simultaneously. Moreover, SIRT1 is required by exenatide to alleviate inflammation in liver as well. Our study indicates, for the first time to our knowledge, SIRT1 is essential for GLP-1 receptor agonist exenatide on reducing hepatic steatosis in mice, which suggests GLP-1 receptor agonist could serve as a potential drug for NAFLD, especially in T2DM combined with NAFLD, and SIRT1 might be a therapeutic target of NAFLD.
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Contribution statement F.X. contributed to the study design, acquisition of data, data interpretation and manuscript writing. Z.L. researched data, performed animal studies and analyzed data. X.Z. researched data, performed cell culture and lentivirus transfection, and contributed to the data analysis. H. Liu contributed to the performance of animal studies. H. Liang contributed the study design and data analysis. H.X. contributed to the data interpretation and manuscript writing. Z.C. contributed to the performance of animal studies. K.Z. contributed to the acquisition of data. J.W. contributed to the study design, acquisition of data, revision of the manuscript, and approval of the version to be submitted.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.
Reference

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Table 1. Exenatide-improved liver weight, FBG and lipid profile is weakened in

\textit{SIRTI}^{+/−} \textit{mice}. After 12 wks HFD induction and 8 wks exenatide treatment, mice
were fasted overnight and tail vein blood glucose concentrations were measured at the
next morning. Then mice were euthanized before sacrificed. Blood was collected first,
and then livers were collected and weighed. Insulin, triglyceride, glycerol, and
cholesterol in serum were determined. N=5-12, values are mean±SEM. “∗” means
WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD;
P<0.05. “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means
SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. SHK: SIRT1 heterozygous knockout
mice; WT: wild-type mice; HFD: high fat diet.

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<th>Assay</th>
<th>WT+chow</th>
<th>WT+HFD</th>
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<th>WT+HFD+Exe</th>
<th>SHK+HFD+Exe</th>
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<td>Liver weight (g)</td>
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<td>1.26 ± 0.06∗</td>
<td>1.36 ± 0.07#</td>
<td>0.98 ± 0.05§</td>
<td>1.21 ± 0.06†</td>
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<td>FBG (mmol/L)</td>
<td>7.60 ± 0.38</td>
<td>11.55 ± 0.58∗</td>
<td>12.19 ± 0.61#</td>
<td>10.10 ± 0.51§</td>
<td>11.51 ± 0.58†</td>
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<td>Insulin (pg/ml)</td>
<td>2747.43 ± 393.71</td>
<td>2379.88 ± 763.22</td>
<td>2663.23 ± 859.33</td>
<td>2173.86 ± 976.46</td>
<td>1351.72 ± 602.80</td>
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<td>Serum TG (mg/dl)</td>
<td>12.94 ± 0.65</td>
<td>20.82 ± 1.04∗</td>
<td>28.86 ± 1.45#</td>
<td>13.10 ± 0.67§</td>
<td>28.40 ± 1.53†</td>
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<td>Serum glycerol (mg/dl)</td>
<td>8.58 ± 0.43</td>
<td>14.79 ± 0.80∗</td>
<td>21.96 ± 1.09#</td>
<td>10.52 ± 0.53§</td>
<td>21.10 ± 1.10†</td>
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<td>Serum cholesterol (mg/dl)</td>
<td>157.23 ± 7.82</td>
<td>199.39 ± 9.97∗</td>
<td>218.30 ± 10.92#</td>
<td>181.11 ± 9.06§</td>
<td>218.78 ± 10.88†</td>
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Figure Legends

Fig.1 The effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in SIRT1\textsuperscript{+/−} mice. A) Body weight during HFD feeding detected per two weeks. “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05. B) Average food intake during HFD feeding before exenatide treatment. C) Body weight during exenatide treatment detected per two weeks. D) Average food intake during exenatide treatment. E) Intraperitoneal glucose tolerance test (ip GTT) after exenatide treatment and area under the curve. F) Intraperitoneal insulin tolerance test (ip ITT) after exenatide treatment and area under the curve. For C), E) and F), “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05; “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. N=5-12, values are mean±SEM. SHK: SIRT1 heterozygous knockout mice; WT: wild-type mice; HFD: high fat diet.

Fig.2 Exenatide-ameliorated liver steatosis disappears in SIRT1\textsuperscript{+/−} mice. A) General pictures and liver size. B) Liver sections with H&E staining (top) and Oil red-O staining (bottom). Pictures were taken using a microscopy with 40X object lense. C)-E) hepatic triglyceride, glycerol and cholesterol contents were determined. N=5-12, values are mean±SEM. “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05; “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. SHK: SIRT1 heterozygous knockout mice; WT: wild-type mice; HFD: high fat diet.
Fig. 3 SIRT1 is required by exenatide to relieve inflammation in liver. A-C) Relative mRNA expression of TNF-α, F4/80 and MCP-1 in liver. D) Immunohistochemical staining with macrophage marker F4/80 in liver. N=5-12, values are mean±SEM. “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05; “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. SHK: SIRT1 hetrozygous knockout mice; WT: wild-type mice; HFD: high fat diet.

Fig. 4 Exenatide reduced hepatic steatosis depends on SIRT1/AMPK pathway. A) Total protein extracted from liver lysates was used in western blot. SIRT1, p-AMPK and total AMPK were detected with specific antibodies. B) and C) Ratios of SIRT1 to β-actin and pAMPK to AMPK, respectively, were quantified in three independent experiments per condition. Data are expressed as the mean±SEM (n=3). D) Oil red-O staining of HepG2 cells treated with PA (0.3mmol/L) and Ex-4 (20nmol/L) as indicated for 24 h. E) HepG2 cells were treated with PA (0.3mmol/L) and Ex-4 (20nmol/L and 200nmol/L, respectively) as indicated for 24 h. F) HepG2 cells were transfected with lentiviral vectors expressing SIRT1 RNAi for 12 h and the medium was changed, and cells were cultured for another 48 h. Then transfected cells were treated with PA (0.3mmol/L) and Ex-4 (20nmol/L) as indicated for 24 h. G) HepG2 cells were treated with PA (0.3mmol/L) and resveratrol (50μM) or Ex-4 (20nmol/L) as indicated for 24 h. H) Intracellular levels of the NAD+/NADH ratio, NAD+ and NADH in HepG2 cells treated with PA (0.3mmol/L) and Ex-4 (20nmol/L and 200nmol/L, respectively) as indicated were quantified in three independent experiments per condition. Data are expressed as the mean±SEM (n=3). For H), “*”
means compared with control, P<0.05; “#” means compared with PA, P<0.05; “§” means compared with Ex-4 (20 nmol/L), P<0.05. For B) and C), “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05; “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. SHK:SIRT1 hetrozygous knockout; WT:wild type; HFD: high-fat diet; PA:palmitate; Ex-4: exendin-4.

**Fig.5 SIRT1 is required by exenatide to ameliorate lipogenesis through inhibiting SREBP-1 in liver.** A) Total protein extracted from liver lysates was used in western blot. SREBP-1 and PNPLA3 were detected with specific antibodies. B) and C) Ratios of SREBP-1 to β-actin and PNPLA3 to β-actin, respectively, were quantified in three independent experiments per condition. Data are expressed as the mean±SEM (n=3). D) HepG2 cells were treated with PA (0.3mmol/L) and Ex-4 (20nmol/L and 200nmol/L, respectively) as indicated for 24 h. E) HepG2 cells were transfected with lentiviral vectors expressing SIRT1 RNAi for 12 h and the medium was changed, and cells were cultured for another 48 h. Then transfected cells were treated with PA (0.3mmol/L) and Ex-4 (20nmol/L) as indicated for 24 h. F) HepG2 cells were treated with PA (0.3mmol/L) and resveratrol (50uM) or Ex-4 (20nmol/L) as indicated for 24 h. “*” means WT+HFD versus WT+chow, P<0.05; “#” means SHK+HFD versus WT+HFD, P<0.05; “§” means WT+HFD+Exe versus WT+HFD, P<0.05; “†” means SHK+HFD+Exe versus WT+HFD+Exe, P<0.05. SHK:SIRT1 hetrozygous knockout; WT:wild type; HFD: high-fat diet; PA:palmitate; Ex-4: exendin-4.
The effect of exenatide on reducing body weight and maintaining glucose homeostasis is attenuated in SIRT1+/− mice.
Fig. 2: Exenatide-ameliorated liver steatosis disappears in SIRT1+/− mice.

A. WT + chow    WT + HFD    SHK + HFD    WT + HFD + Exe    SHK + HFD + Exe

B. WT + chow    WT + HFD    SHK + HFD    WT + HFD + Exe    SHK + HFD + Exe

C-D. E. Liver triglyceride (mg/g)  Liver glycerol (mg/g)  Liver cholesterol (mg/g)

WT+chow  WT+HFD  SHK+HFD  WT+HFD+Exe  SHK+HFD+Exe

*  #  †  §  †

Diabetes
Fig. 3 SIRT1 is required by exenatide to alleviate inflammation in liver.

A. TNF-α

B. F4/80

C. MCP-1

D. Immunohistochemistry images at 20X and 40X magnification for each condition.
Fig. 4 Exenatide-reduced hepatic steatosis depends on SIRT1/AMPK pathway

A. SIRT1, β-actin, p-AMPK, T-AMPK, β-actin

B. Relative SIRT1/β-actin protein expression

C. Relative p-AMPK/T-AMPK protein expression

D. Control, PA, PA+Exendin-4

E. PA (0.3 mM), Ex-4 (20 nM), Ex-4 (200 nM)

F. SIRT1, p-AMPK, AMPK, p-ACC, ACC, GAPDH

G. PA (0.3 mM), Res (50 μM), Ex-4 (20 nM)

H. NAD⁺/10⁶ cells, NAD/NADH, NADH/10⁶ cells
Fig. 5. SIRT1 is required by exenatide to ameliorate lipogenesis through inhibiting SREBP-1 in liver.

A. Relative SREBP1 / β-actin protein expression

B. Relative PNPLA3 / β-actin protein expression

C. Relative SREBP1 / β-actin protein expression

D. SREBP-1

E. SREBP-1

F. SREBP-1
Suppl. 1 Exendin-4 reduces the precursor of SREBP-1 via SIRT1
Suppl. 2 Exendin-4 inhibits the nuclear translocation of SREBP-1

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<tr>
<td>Ex-4 (nM)</td>
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Diabetes
Supplemental methods and figure legends

Supplementary Methods

NAD⁺/NADH ratio assay. The NAD⁺/NADH ratio was measured from whole-cell extracts of HepG2 cells by using the Biovision NAD⁺/NADH Quantification Colorimetric Kit (Biovision, San Francisco, CA, USA). Total NADt (NAD⁺ and NADH) was detected by transferring 50 µl extracted samples into labeled 96-well plate. To detect NADH, NAD⁺ needs to be decomposed before the reaction by heating to 60 °C for 30 min in a heating block. After the NADH standard and all the samples were transferred, 100 µl NAD Cycling Mix was added at room temperature for 5 min to convert NAD⁺ to NADH. Then 10 µl NADH developers were added into each well at room temperature for 1 to 4 hrs and the plate was read at OD 450 nm. The readings were applied to the NADH standard curve and then the amounts of NADt and NADH can be calculated. These readings were normalised to the cell number and the NAD⁺/NADH ratio was calculated as (NADt−NADH)/NADH.
Supplementary Figure legends

Figure S1. Exendin-4 reduces the precursor of SREBP-1 via SIRT1. A) HepG2 cells were treated with palmitate (0.3mmol/L) and exendin-4 (20nmol/L and 200nmol/L, respectively) as indicated for 24 h. B) HepG2 cells were transfected with lentiviral vectors expressing SIRT1 RNAi for 12 h and the medium was changed, and cells were cultured for another 48 h. Then transfected cells were treated with palmitate (0.3mmol/L) and exendin-4 (20nmol/L) as indicated for 24 h. For A) and B), phosphorylation of SREBP-1c (pSREBP-1c) was analysed by immunoblotting with an SREBP1c-phosphorylated antibody at Ser372. P and N denote the precursor (~125 kDa) and cleaved nuclear (~68 kDa) forms of SREBP-1, respectively. PA:palmitate; Ex54:exendin-4.

Figure S2. Exendin-4 inhibits the nuclear translocation of SREBP-1. HepG2 cells were treated with PA (0.3mmol/L) and exendin-4 (Ex54, 20nmol/L and 200nmol/L, respectively) or resveratrol (Res,50uM), an activator of SIRT1 as a positive control as indicated for 24 h. Cytoplasmic and nuclear protein were extracted with a NucBuster™ Protein Extraction Kit (Merck Millipore). P and N denote the precursor (~125 kDa) and cleaved nuclear (~68 kDa) forms of SREBP-1. GAPDH and SP3 were used as control for cytoplasmic and nuclear protein, respectively. PA:palmitate; Ex-4:exendin-4.