Sitagliptin reduces insulin resistance and improves rat liver steatosis via the SIRT1/AMPKα pathway

TIAN SHEN, BILIN XU, TAO LEI, LIN CHEN, CUIPING ZHANG and ZHENHUA NI

Department of Endocrinology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062, P.R. China

Received February 7, 2017; Accepted November 10, 2017

DOI: 10.3892/etm.2018.6554

Abstract. Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease and is frequently identified among individuals with metabolic diseases, including obesity and diabetes. NAFLD is primarily characterized by the accumulation of triacylglycerol in the liver. Since insulin resistance and fat metabolism dysregulation are major causes of type 2 diabetes and NAFLD, anti-diabetes agents are widely considered as potential therapy strategies for NAFLD. Sitagliptin, an inhibitor of dipeptidyl peptidase-4, has been developed as an oral anti-hyperglycemic agent. In the present study, the effect of sitagliptin on the progression of NAFLD was evaluated in a rat model fed with a high fat diet (HFD). It was identified that sitagliptin significantly suppressed lipid accumulation in rat blood and liver and improved insulin resistance. Furthermore, it was revealed that sitagliptin reactivated the HFD-suppressed SIRT1/AMPK axis pathway and upregulated its downstream target genes, modulating fatty acid metabolism. These findings demonstrate a preventive effect of sitagliptin on hepatic lipid dysregulation and suggest that sitagliptin has potential as a clinical therapeutic strategy for NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease, and encompasses a spectrum of liver conditions, including simple steatosis, steatohepatitis and end-stage liver disease (1,2). NAFLD is now the liver disease associated with the highest mortality rate, as a consequence of increased risk of cardiovascular disease and hepatocellular carcinoma (3). NAFLD is also associated with metabolic diseases, including diabetes mellitus, obesity and hypertension (4,5). In a 5-year retrospective review, participants with NAFLD had higher risks of impaired fasting glucose and type 2 diabetes mellitus (T2DM) compared with NAFLD-free controls (2).

NAFLD is generally asymptomatic at presentation and frequently identified among individuals with conditions including obesity, T2DM, metabolic syndrome and pathological alterations of liver tissues (1). NAFLD is primarily characterized by accumulation of triacylglycerol in the liver (2). Ingestion of high-fat foods is a key inducer of excessive fat accumulation in the liver, resulting in insulin resistance (IR), dyslipidemia and NAFLD (3,6,7). Current treatments for NAFLD include weight reduction by lifestyle change, insulin sensitizer agents, lipid-lowering drugs and antioxidants (5,6,8,9). Antidiabetic drugs, which improve IR, have a notable effect on NAFLD and slow down the progression of symptoms (4,5).

Sirtuin 1 (SIRT1), a mammalian sirtuin, is an NAD+-dependent protein deacetylase, which functions as an important regulator of energy hemostasis in response to nutrient availability (10). Adenosine monophosphate-activated protein kinase (AMPK) acts as a cellular metabolic switch in regulating fatty acid synthesis and oxidation, maintaining the balance of metabolism in cells and the body (11). AMPK relies on SIRT1 activity to regulate the gene expression of fatty acid metabolism, and AMPK and SIRT1 are important in maintaining energy hemostasis and regulating fatty acid metabolism (12,13).

Dipeptidyl peptidase-4 (DPP-4) is a serine protease that contributes to inactivation of incretin hormones, including glucagon-like peptide-1 (GLP-1) (14,15). DPP-4 inhibitors have been developed as oral anti-hyperglycemic agents (16). DPP-4 inhibitors increase GLP-1 levels and inhibit glucagon release, which in turn enhances insulin secretion, and ameliorates liver enzymes and hepatocyte ballooning in non-alcoholic steatohepatitis patients with T2DM (15-18). Sitagliptin, a recently developed DPP-4 inhibitor, has been widely used to treat T2DM and has also been evaluated in diabetic patients with NAFLD symptoms (6,18,19). However, the effect of sitagliptin on reducing fatty liver in NAFLD patients requires further investigation and its mechanism remains unknown.
In the present study, a rat model of NAFLD was established by administration of a high-fat diet (HFD), and the effect of sitagliptin on the progression of NAFLD was evaluated. With this model, the preventive and therapeutic efficacy of sitagliptin on lipid accumulation in blood and liver was evaluated. Furthermore, the underlying mechanisms involving the SIRT1/AMPK signaling pathway were investigated.

Materials and methods

Animals. The following animal studies were approved by the Animal Care and Ethics Committee of Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (20). Six-week old male Sprague-Dawley rats (~200 g) were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and bred under 25°C and 60% relative humidity with a 12-h light/dark cycle and unlimited food and water supplied. Rats were randomly divided into 2 groups: Normal control (NC) group (n=16, fed with normal diet: 10 kcal% fat, 20 kcal% protein and 70 kcal% carbohydrate; cat. no. DI2450B) and high fat (HF) group (n=26, fed with HFD: 45 kcal% fat, 20 kcal% protein and 35 kcal% carbohydrate; cat. no. DI2451) (both Research Diets, Inc., New Brunswick, NJ, USA) (21). After 12 weeks of feeding, 6 rats from each group were randomly selected and analyzed in order to confirm the establishment of the NAFLD model in the HF group compared with the NC group (22). Other rats in the HF group were then divided into 2 subgroups: Sitagliptin-treated group (HF + XI) (n=10, HFD-fed and 100 mg/kg/day sitagliptin) and HF only group (n=10, HFD-fed and an equal volume of saline) (23-25). Rats were treated through gavage every day for the next 8 weeks. During the experiments, body weight and food intake were monitored twice per week. At week 20, rats were fasted overnight and sacrificed. Blood samples were collected from the abdominal aorta. Half of the liver tissues were excised, flash frozen in liquid nitrogen, and stored at -80°C until further processing. The other halves of the liver tissues were fixed in 10% formalin solution for 24 h at room temperature.

Serum analysis. Sera were separated from blood samples by centrifugation at 1,500 x g at 20°C for 15 min after coagulation. Alanine transferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC), fasting blood glucose (FBG) and free fatty acid (FFA) in the serum were analyzed using an automatic biochemical analyzer (Dimension® RxL Max®; Siemens Healthineers, Erlangen, Germany). The fasting serum insulin level was measured using a rat insulin ELISA kit (cat. no. RAB0904-1KT; Sigma-Aldrich; Merck, KGaA, Darmstadt, Germany). Homeostatic model assessment of IR (HOMA-IR) was calculated as previously described (26).

Liver lipid test and histological analysis. Liver tissues were weighed and homogenized in PBS (20 ml/g of tissue). Lipids were then extracted from the liver tissue lysates using a chloroform/methanol (2:1) mixture (21). TG was determined using the Serum Triglyceride Determination kit (TR0100, Sigma-Aldrich; Merck KGaA).

Livers tissues fixed in formalin were embedded in paraffin and serial sections (5 µm thickness) were cut from each block. Sections were stained with hematoxylin and eosin and images were captured using an Olympus BX51WI microscope (magnification, x100; Olympus Corporation, Tokyo, Japan) and then used for histological feature analysis in a blind manner by two pathologists (21). Features examined included steatosis, inflammation and hepatocellular ballooning and were evaluated with the previously described scoring systems (27,28). Steatosis and lobular inflammation was scored from 0 to 3, respectively. Hepatocyte ballooning was scored from 0 to 2. NAFLD activity score (NAS) was the sum of steatosis, inflammation and ballooning scores and ranged from 0 to 8 as previously described (29). Oil Red O staining was performed as previously described and images were captured with an Olympus BX51WI light microscope at magnification, x100 (21,29).

Reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was isolated from liver tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was reverse transcribed using a Prime Script RT Reagent kit (TaKaRa Bio, Inc., Otsu, Japan). qPCR was conducted using LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) with the LightCycler 480II Real-Time PCR system (Roche Diagnostics) under the following conditions: Denature at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Relative expression levels of tested genes were calculated and normalized to GAPDH using the 2^ΔΔCq method (21). Primers used for qPCR were as follows: SIRT1, forward, 5'-GATGATGTCGACACCGGA-3' and reverse, 5'-AGT TCCCAATGCTGTTGGAGG-3'; AMPKα1, forward, 5'-GAG CCCCTGAATTGCTTTC-3' and reverse, 5'-TGTCCG TTCTATGGCCTGG-3'; acetyl CoA carboxylase 1 (ACC1), forward, 5'-GGCGGCTCTGGAGGTGTATGGTT-3' and reverse, 5'-CTATGGCTAGTGGTGGAGG-3'; carnitine palmitoyltransferase 1 (CPT1), forward, 5'-GTCTGAGCCATGAGGTTG-3' and reverse, 5'-CACAGCTTCTCCTCCTGTG-3'; GPDH, forward, 5'-TGATGGGTTGTGACACCAG-3' and reverse, 5'-ATACGCGCACAGTTCCAG-3'.

Western blot analysis. Lysates were extracted from liver tissues using a radioimmunoprecipitation buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with a Complete® mini protease inhibitor (Roche Diagnostics). Protein concentration was determined using a BCA kit (Thermo Fisher Scientific, Inc.) and 40 µg of total protein per lane was fractionated on SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were then blocked with 5% bovine serum albumin (cat. no. A9647) in Tris-buffered saline (pH 8.0) with 0.1% Tween-20 (all Sigma-Aldrich; Merck KGaA) for 1 h at 20°C. Primary antibodies targeting pAMPKα (Thr-172; cat. no. 2531, 1:1,000), AMPKα (cat. no. 2532, 1:1,000), ACC1 (cat. no. 4190, 1:1,000), pACC1 (Ser-79) (cat. no. 3661, 1:1,000), FAS (cat. no. 3189, 1:1,000) (all Cell Signaling Technology, Inc., Danvers, MA, USA), CPT1A (cat. no. ab83862, 1:1,000, Abcam, Cambridge, MA, USA).
Sitagliptin improves HFD-induced abnormal lipid accumulation in blood and liver. To generate a rat model of NAFLD, rats were fed with HFD for 20 weeks, which mimicked the long-term ingestion of HFD content in obese individuals. Compared with the NC group, HFD-fed rats had significantly increased wet liver weight and higher liver/body weight ratio, although there was no significant difference in their body weights (Table I). Serological analysis demonstrated that HFD induced significantly higher levels of glucose, insulin, TG and FFA compared with a normal diet. HOMA-IR analysis suggested that HFD significantly induced IR in rats. Significantly higher levels of serum ALT and AST activity in the HF group compared with the NC group indicated abnormal liver function was induced by HFD. These results suggest that HFD successfully induced NAFLD-like symptoms, including abnormal lipid accumulation in the serum and liver dysfunction.

Furthermore, it was investigated whether sitagliptin could affect these physiological and biochemical parameters and improve IR induced by HFD in the rat NAFLD model (Table I). Notably, sitagliptin significantly suppressed the increase of insulin induced by HFD without affecting the FBG level. Sitagliptin also significantly suppressed serum TG and FFA induced by HFD, resulting in improved IR, as demonstrated by significantly decreased HOMA-IR in the HF + XI group compared with the HF group. Sitagliptin treatment also exhibited mild effects on other abnormal alterations induced by HFD, but these were not observed to be significant. These findings indicate that sitagliptin improves NAFLD-like symptoms induced by HFD in a rat model.

**Results**

**Sitagliptin suppresses HFD-induced pathological changes in the rat liver.** To determine whether sitagliptin could directly affect fat accumulation at liver, TG level was determined in rat liver. Pathological development of fatty liver was evaluated (Fig. 1A). Fatty infiltration was observed in<5% of rat liver tissues, and ballooning and inflammation were not observed in rat liver tissues from the NC group. Sitagliptin treatment greatly suppressed the accumulation of TG in the rat liver, significantly reduced ballooning (Fig. 1D), and improved IR induced by HFD without affecting the FBG level. These results suggest that Sitagliptin treatment (HF + XI group) significantly reduced ballooning (Fig. 1D),

**Table I. Effect of sitagliptin on characteristics of the high fat diet-induced non-alcoholic fatty liver disease model.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NC (n=10)</th>
<th>HF (n=10)</th>
<th>HF + XI (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>597.4±56.47</td>
<td>608.9±40.32</td>
<td>593.2±48.94</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>16.33±3.79a</td>
<td>22.77±4.39</td>
<td>20.67±3.34</td>
</tr>
<tr>
<td>Liver/body weight ratio</td>
<td>2.74±0.67a</td>
<td>3.56±1.87</td>
<td>3.27±1.39</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>6.03±0.41a</td>
<td>7.12±0.63</td>
<td>6.92±0.55</td>
</tr>
<tr>
<td>Insulin (µU/l)</td>
<td>26.13±8.48a</td>
<td>33.35±9.41</td>
<td>28.61±7.56a</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>6.98±1.92a</td>
<td>7.69±3.27</td>
<td>7.26±2.45a</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.38±0.13a</td>
<td>0.72±0.24</td>
<td>0.42±0.54a</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>2.34±0.94</td>
<td>2.67±0.40</td>
<td>2.58±0.78</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>36.93±8.62a</td>
<td>49.24±10.04</td>
<td>45.63±9.34</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>45.23±9.34</td>
<td>56.65±12.31</td>
<td>52.22±13.42</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>9.76±2.21a</td>
<td>11.88±3.36</td>
<td>8.54±2.76a</td>
</tr>
<tr>
<td>Liver TG (mmol/l)</td>
<td>11.86±4.61a</td>
<td>15.32±2.24</td>
<td>9.67±2.66a,b</td>
</tr>
</tbody>
</table>

*P<0.05 vs. HF group; *P<0.05 vs. NC group. NC, negative control; HF, high fat; XI, sitagliptin treatment; FBG, fasting blood glucose; HOMA-IR, homeostatic model assessment of insulin resistance; TG, triglycerides; TC, total cholesterol; ALT, alanine transferase; ALT, aspartate aminotransferase; FFA, free fatty acid.
Sitagliptin rescues the HFD-suppressed SIRT1/AMPK pathway. To explore the underlying mechanism of the effect of sitagliptin, the expression levels of proteins in the SIRT1/AMPK pathway were evaluated in rat liver lysates (Fig. 2). HFD significantly suppressed the protein level of SIRT1 and AMPKα1, and significantly reduced the phosphorylation of AMPKα1 at Thr-172 and of ACC at Ser-79. Sitagliptin treatment rescued the expression of SIRT1 and total AMPKα1, and also enhanced the phosphorylation of AMPKα1 (Fig. 2A and B).

The expression levels of FAS, ACC1 and CPT1 proteins, downstream targets of the SIRT1/AMPK pathway, were also evaluated. HFD induced significantly increased levels of FAS protein and significantly decreased expression of CPT1 and ACC1 phosphorylation (p-ACC1Ser79) in rat liver. Sitagliptin treatment significantly reduced the expression of FAS protein to untreated control level and rescued the expression of CPT1 and ACC1 phosphorylation (p-ACC1Ser79) in rat liver (Fig. 2C and D). These results demonstrated that sitagliptin
reactivates AMPK pathway downstream targets and restores fatty acid oxidation in the rat liver.

**Sitagliptin restores AMPK pathway activity suppressed by HFD.** To further explore activity of the AMPK pathway, mRNA expression of AMPK pathway genes in rat livers was evaluated (Fig. 3). HFD significantly suppressed the mRNA level of AMPKα1 and ACC1 and significantly upregulated the mRNA level of FAS. Sitagliptin treatment restored the mRNA levels of AMPKα1, ACC1, CPT1 and FAS. These results were consistent with the alterations observed in the expression level of ACC1, CPT1 and FAS proteins. These findings indicate that sitagliptin restores the AMPK/CPT pathway to enhance fatty acid oxidation, suppress fatty acid synthesis and block TG accumulation in the rat liver.

**Discussion**

NAFLD is the most common cause of liver disease in western countries, presenting in >30% of the general population (30,31). The prevalence in Asian populations ranges from 6 to 25% (3,22). The prevalence rate of NAFLD has doubled in Chinese cities in the past two decades (22). Previous results have indicated that IR serves a pathogenic function in T2MD and NAFLD (32). The pathogenesis of NAFLD was originally described by the ‘two-hit hypothesis’, and subsequently modified as the ‘multi-hit hypothesis’, which considers multiple injuries acting together to induce NAFLD and provides a more accurate explanation of NAFLD pathogenesis (33,34). IR serves a central function in hepatic injury as a result of dysregulation of fatty acid metabolism, leading to steatosis (26). High levels of fat content in the diet also contribute to lipid deposition in the liver (5,17,18,21,35). Approximately 80% of FFA, originating from the diet, TG decomposition of adipose tissue or liver fatty acid de novo synthesis, is primarily transported to the liver and causes intracellular accumulation of lipid metabolites and hepatic TG deposition (3,5,32,36,37).
Therefore, improving IR and decreasing the level of FFA are essential for arresting the development of NAFLD.

IR has been recognized as the primary pathogenic mechanism of NAFLD and diabetes (32). Antidiabetic drugs, particularly insulin sensitizer agents, have been indicated to improve NAFLD and/or slow down its progression (6,8,19,32,37,38). Metformin treatment improves the sensitivity of insulin response in NAFLD patients, but it has no certain effects on liver histology (6). Pioglitazone, a thiazolidinedione derivative has been tested for the treatment of NAFLD in clinical trials, however it has safety issues for long-term treatment and a disadvantage of increasing body weight (39). Sitagliptin, as a DPP-4 inhibitor, has been widely used in the treatment of T2DM (39,40). It also indirectly inhibits hepatic fat accumulation and hepatic steatosis in mice and humans (39,40). A single-arm, open-label study revealed that long-term treatment with sitagliptin at 100 mg/day could reduce the body mass index and ALT levels of patients (40). Another DPP-4 inhibitor, des-fluoro-sitagliptin, reduced the accumulation of TG in the liver and blocked hepatic steatosis in mice fed with linoleic acid and sucrose diet (41). These findings reveal that sitagliptin has potential activity to improve IR and hepatic lipid dysregulation in NAFLD.

In the present study, the effect of sitagliptin on lipid dysregulation induced by HFD was evaluated in a rat model. HFD treatment induced an increase of liver weight, blood/liver TG level, FBG and insulin level, resulting in higher HOMA-IR compared with the control group. A previous study reported that HFD also causes peripheral IR and dysregulation of glucose and lipid metabolism in a mouse model (41). Sitagliptin functions as a DPP-4 inhibitor to block the degradation of GLP-1 and gastric inhibitory polypeptide (GIP) secreted by intestinal L cells, which promote the release of insulin and suppress the secretion of glucagon in order to reduce the blood glucose level (42). This blockade relies on the intestinal glucose level. In the present study, HF diet caused a slight increase of FBG, while sitagliptin reduced, instead of enhanced, the production of insulin. These results indicate that dysfunction of glucose metabolism in the tested model was not sufficient to enhance GLP-1 and GIP secretion and stimulate the generation of endogenous insulin. Furthermore, sitagliptin significantly reduced hepatic TG level in the HF + XI group and reversed lipid accumulation in the liver. Based on the combination of reduced insulin level and HOMA-IR, sitagliptin induced a notable improvement in HFD-induced hyperinsulinemia and IR.

In the present study, it was identified that sitagliptin reactivated the SIRT1/AMPK pathway, which was suppressed by HFD treatment. SIRT1, a mammalian sirtuin, is an NAD+−dependent protein deacetylase that serves a key function in regulating energy homeostasis in response to nutrient availability (10). SIRT1 can enhance insulin sensitivity, regulate liver fat metabolism, suppress oxidative stress and reduce inflammatory response in NAFLD (21,35,43). AMPK serves a central function in controlling lipid metabolism through modulating the phosphorylation of ACC and regulating the activity of CPT1 (11,18,44,45). In the present study, the presence of high levels of fatty acid suppressed SIRT1 and AMPK activity, resulting in reduced fatty acid utilization and abnormal lipid deposition in the liver. Sitagliptin reactivated the expression of SIRT1 and AMPK proteins in the rat liver.

Aside from its role in direct phosphorylation of target proteins, AMPK functions as an important transcription factor in regulating the response to stress and alterations in metabolism (18,44,46). AMPK can directly control the status of histone H2B phosphorylation, which recruits transcription factors to bind with DNA, and further regulates the transcription of AMPK pathway target genes such as Acc1 and Cpt1c (46). In the present study, it was identified that the reactivated SIRT1/AMPK pathway upregulated the transcription level of downstream target genes ACC1 and CPT1, while FAS mRNA level was downregulated. These findings are consistent with the function of sitagliptin in suppressing fatty acid synthesis and enhancing fatty acid β-oxidation. Therefore, reactivation of SIRT1/AMPK pathway is indicated to be one of the primary mechanisms of sitagliptin in the prevention of NAFLD. The present study demonstrated that sitagliptin is an effective agent in preventing the development of hepatic lipid dysregulation and has potential as a clinical therapeutic strategy for NAFLD.

Acknowledgements

Not applicable.

Funding

This study was supported by the Shanghai Municipal Commission of Health and Family Planning Project (grant no. 2013Y079), the Backup Medical Expert Cultivation Plan of Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (grant no. B-X-79) and Shanghai Putuo District Key Clinical Specialty Project, Endocrinology (grant no. 2016PTZK05).

Availability of data and materials

The datasets generated during the current study are not publicly available as they are currently being used for further research, however they are available from the corresponding author on reasonable request following the completion of this research.

Authors' contributions

TS and TL conceived and designed the research. TS, BLX, ZHN and CPZ performed the experiments and statistical analysis. TS, CPZ and LC acquired and interpreted the data. TS and LC drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Care and Ethics Committee of Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Patient consent for publication

Not applicable.
The authors declare that they have no competing interests.

References


