Original Paper

Glucagon-Like Peptide-1 Modulates Cholesterol Homeostasis by Suppressing the miR-19b-Induced Downregulation of ABCA1

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Key Words

Glucagon-like peptide-1 • Cholesterol homeostasis • ATP-binding cassette transporter A1 • miR-19b

Abstract

Background/Aims: Abnormal regulation of cholesterol homeostasis is associated with type 2 diabetes mellitus (T2DM) and multiple other diseases. Glucagon-like peptide-1 (GLP-1) has unique effects on modulating hepatic lipid metabolism. However, the mechanism behind these is largely unknown. The aim of this study was to investigate the effects of GLP-1 on cholesterol-induced lipotoxicity in hepatocytes and examine the underlying mechanisms. **Methods:** Cell viability was determined by CCK-8. Caspase-3 detection was used to assess the effects of GLP-1 on cholesterol-induced apoptosis. TNF- α and IL-6 as the inflammatory markers were measured by ELISA. The alterations of miR-19b and ATP-binding cassette transporter A1 (ABCA1) resulting from high-fat diet/cholesterol incubation or GLP-1 were detected by real-time PCR and western blot. **Results:** GLP-1 markedly up-regulated the expression of ABCA1 protein, but didn't affect peroxisome proliferator-activated receptor α (PPAR- α) protein. The miR-19b levels were significantly down-regulated in GLP-1-treated groups. The inhibition and overexpression of miR-19b were established to explore the effects of a GLP-1-mediated alteration in miR-19b. Cholesterol transport assays revealed that treatment with GLP-1 alone or together with miR-19b inhibitor significantly enhanced ABCA1-dependent cholesterol efflux, resulting in reduced total cholesterol. Further, histological examination was used to detect lipid accumulation. Cholesterol significantly attenuated cell viability, promoted hepatic cell apoptosis, and facilitated lipid accumulation, and these effects could be reversed by GLP-1. **Conclusion:** GLP-1 may affect cholesterol homeostasis by regulating the expression of miR-19b and ABCA1.

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Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disease characterized by insulin resistance. Dysregulation of lipid metabolism in liver cells is a main pathogenic risk factor for insulin resistance [1–3]. Hepatic cholesterol metabolism is an important process in the maintenance of total body cholesterol homeostasis. Cholesterol homeostasis is critical for normal cell function, and its improper distribution or metabolism can induce serious consequences for cells and organisms [4, 5]. Because of this, cells have developed complex mechanisms to regulate the abundance and distribution of sterols cholesterol cells.

ATP-binding cassette transporter A1 (ABCA1) is an integral membrane protein, which plays an essential role in mediating cholesterol efflux to apolipoprotein A-I, as a major housekeeping mechanism for cellular cholesterol homeostasis [6, 7]. Cholesterol efflux via ABCA1 is a gatekeeper for cells to eliminate excess cholesterol and prevent cellular cholesterol accumulation [8–11]. Several epidemiological studies have revealed that inappropriate cholesterol accumulation leads to nonalcoholic fatty liver disease, obesity, T2DM, atherosclerosis, and even increases the risk of multiple types of cancer [5, 12, 13]. The activity of the ABCA1 pathway is elaborately modulated, and an increase in ABCA1 activity will prevent cholesterol accumulation in cells [6, 7].

Glucagon-like peptide 1 (GLP-1) as a novel anti-diabetic drug with multiple effects, not only has hypoglycaemic effects, but also relieves lipotoxicity at the cellular level [14, 15, 16]. GLP-1 has unique abilities to modulate lipid metabolism by affecting lipid assimilation and transport, fat formation and decomposition, hepatic lipid metabolism and reverse cholesterol transport [17, 18, 19]. The apolipoprotein A-I gene, encoding the primary anti-atherogenic factor in high-density lipoprotein (HDL) particles, is increased in both GLP-1 and exendin-4-treated HepG2 cells, and this phenomenon is accompanied by similar changes in the expression of ABCA1 [20]. Research has shown that GLP-1 and exendin-4 promote ABCA1 expression in multiple cell lines, including glomerular endothelial cells, pancreatic β cells and adipocytes. GLP-1R-mediated signalling pathways (e.g., Ca^{2+} /calmodulin-dependent protein kinase, cAMP/PKA, ERK1/2 and PI3K/AKT) are involved in cholesterol efflux by regulating ABCA1 expression [21–23]. However, the mechanism by which GLP-1 improves cholesterol homeostasis via ABCA1 in hepatic cells remains unclear.

Researches shows that ABCA1 is comprehensively regulated by intracellular microRNAs (miRNAs) [24, 25]. These non-coding RNAs have been identified as important post-transcriptional regulators of cholesterol homeostasis. Several miRNAs, such as miR-33, miR-122, miR-370, miR-378, miR-146b, miR-27 and miR-19b, show effects on ameliorating cholesterol homeostasis and fatty acid metabolism [5, 26, 27]. Circulating miR-19b is correlated with biomarkers of lipid metabolism [28]. It has been reported that miR-19b binds to the 3'-UTR of ABCA1 transporters, and this novel interaction has multiple potential roles in cholesterol metabolism [29]. In this context, this study investigates the efficacy of GLP-1 on improving hepatic cells cholesterol homeostasis through the miR-19b/ABCA1 interaction *in vivo* and *in vitro*.

Materials and Methods

Animal experiments

60 male SD rats 3 to 4 weeks old were purchased from the Experimental Animal Center of Second Affiliated Hospital of Harbin Medical University. After a 7-day adaptation period, rats were assigned to two dietary treatments in a completely randomized design, namely, a control diet and high-fat diet (D12108) (Research Diets Inc., New Brunswick, NJ). A total of 60 rats were fed the control diet (20 rats) and the high-fat diet (40 rats) for 16 weeks. At 4 weeks after raising, the rats in control diet group were randomly subdivided into two groups (10 rats per group): with or without daily subcutaneous injection of GLP-1 (20 $\mu\text{g}/\text{kg}$, Sigma, USA). Meanwhile, the rats in high-fat diet group were randomly subdivided into four groups (10 rats per group): high-fat diet alone (HFD), HFD+GLP-1 (HG), HFD+GLP-1+agomiR-19b (HG-Ago), and

HFD+GLP-1+antagomiR-19b groups (HG-Ant). The rats from the HG-Ago group and HG-Ant group received tail vein injections of 80 mg/kg agomiR-19b/antagomiR-19b, on days 1-3 of every other week after starting the high-fat diet [29, 30]. Rats were sacrificed by exsanguination after isoflurane anaesthesia. Serum and livers were frozen in liquid nitrogen instantly and stored at -80°C for biochemical analysis, and aliquots of the livers were fixed for histological examination. The experiments were performed with the approval of the Animal Care and Use Committee of the Harbin Medical University of China according to the institutional guidelines for the use and care of laboratory animals [31].

Cell culture and treatments

Human HepG2 cells (a kind gift from the Harbin Medical University Cancer Institute) were cultured as previously described [32], in RPMI 1640 medium (HyClone, USA) supplemented with 10% foetal bovine serum (GIBCO, USA), 100 IU/ml penicillin and 100 g/ml streptomycin. The cells were cultured at 37°C in a 5% CO_2 environment. The cells were cultured to 80% confluence, incubated with or without 5 mmol/L soluble cholesterol (Sigma, USA) medium (CHO) for 24h, and then maintained with or without 10 nmol/L GLP-1 (Sigma, USA) for the next 24h.

Cytokine assays and measurement of serum biochemical parameters

The serum concentrations of TNF- α (Huatan Institute of Biotechnology, Beijing, China) and IL-6 (Eiaab Science Co., Ltd, Wuhan, China) were measured by ELISA according to the manufacturer's instructions. Cholesterol was quantitated in HepG2 cells using a cholesterol quantitation kit (BioVision, CA, USA). In addition, aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), and total cholesterol (TC) concentrations were determined by an automatic biochemistry analyser (COBAS80000, c702, Swit).

Measurement with the Cell Counting Kit-8 (CCK-8)

This assay was to detect the viability of HepG2 cells. The cells were exposed to various concentrations of cholesterol (2.5, 5 and 10 mmol/L), with or without 10 nmol/L GLP-1 for 24 h. After replacing the RPMI 1640 medium, 10 μl of CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the 96-well plate was incubated in the dark at 37°C for 1.5 h. The absorbance was measured at 450 nm in a microplate reader (Tecan). All the experiments were repeated three times.

Caspase 3 analysis

The expression of caspase-3 is enhanced when cell apoptosis occurs and represents the degree of apoptosis to some extent. The activation level of caspase-3 was assayed to explore cholesterol-induced apoptosis using the caspase-3 Activity Kit (Solarbio, China). In short, after extraction of total cell proteins of each group, 10 μl protein was incubated with 90 μl of provided reaction buffer and 10 μl Ac-DEVD-pNA (2 mM) in 96-well plates at 37°C for 2 h. Afterwards, the reaction mixtures were measured at a wavelength of 405 nm in a microplate reader.

miRNA transfection

Oligonucleotide analogue and inhibitor which were chemically modified and synthesized were used to increase and decrease the expression of miR-19b in HepG2 cells. The microRNA-19b inhibitor, microRNA-19b mimics, microRNA NC-FAM (GenePharm Co. Ltd, China) and lipofectamine™ 3000 (Invitrogen, USA) were diluted in serum-free RPMI 1640 without antibiotics prior to being incubated at room temperature for 5 minutes. Next, diluted microRNAs were added to each tube of diluted Lipofectamine™ 3000 Reagent (1:1 ratio) separately. The oligonucleotide-lipo complexes were added to the cells for 24h. Then cholesterol and GLP-1 were taken every other 24 h before further analysis. The sequences of mimics and inhibitors used are shown below: hsa-miR-19b mimics, sense 5'-UGUGCAAUCCAUGCAAAACUGA-3'; antisense 5'-AGUUUUGCAUGGAUUUGCACA-3'; hsa-miR-19b inhibitor, 5'-UCAGUUUUGCAUGGAUUUGCACA-3'; microRNA NC-FAM, sense 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense 5'-ACGUGACACGUUCGGAGAATT-3'.

Table 1. Primers used for this study

Parameter	Sprague-Dawley rats	HepG2 cells
ABCA1	Forward: 5'-AATGGTCAATGGGAGGTTCA-3'; Reverse: 5'-TGGACAGGCTTTAGGTCAGG-3'	Forward: 5'-GACTAAAGCCATTGTCTGTG-3'; Reverse: 5'-CCAACAGAACTGTCACAGC-3'
GAPDH	Forward: 5'-GCCAGCCGAGCCACAT-3'; Reverse: 5'-GGATCTCGCTCCTGGAAGAT-3'	Forward: 5'-AACTTTGGCATTGTGGAAGG-3'; Reverse: 5'-ACACATTGGGGGTAGGAACA-3'
miR-19b	RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGAT Forward: 5'-CGAGTTTTCGAGGTTTGCATCCAG-3' Reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3'	RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGTT Forward: 5'-CGCTGGTGTGCAATCCATGCAA-3' Reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3'
U6	RT primer: CGCTTCACGAATTTGCGTGTGTCAT Forward: 5'-GCTTCGGCAGCATATACTAAAT-3' Reverse: 5'-CGCTTCACGAATTTGCGTGTGTCAT-3'	RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA Forward: 5'-AGAGAAAGATTAGCATGGCCCTG-3' Reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3'

RNA extraction and quantitative PCR

Total RNA was extracted from rat livers and HepG2 cells using the Trizol reagent (Invitrogen, Waltham, USA). Reverse transcription was carried out using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). qRT-PCR reactions were detected using the Fast Start Universal SYBR Green Master (Roche, Germany) with CFX96 real time PCR detection system (Bio-Rad, USA). U6 small RNA and GAPDH were used as the reference gene. Each reaction was performed in triplicate according to the manufacturer's protocol, and the qRT-PCR results were calculated using the $2^{-\Delta\Delta Ct}$ method [33]. All primer sequences are shown in Table 1.

Protein extraction and western blotting

For protein extraction, rat livers and HepG2 cells were lysed with 200 μ l of modified RIPA Lysis Buffer (Beyotime institute of Biotechnology, China) containing 1% PMSF (Beyotime institute of Biotechnology, China) on ice. Splitted decomposition with ultrasonic wave for 30 second, then centrifugalized at 14,000 g at 4°C for 15min, the proteins were transferred to the fresh tube. The proteins were determined using the BCA method. Subsequently, proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, USA). The membrane was incubated with primary antibodies to ABCA1 (1:500, ab18180), PPAR- α (1:500, ab8934) and GAPDH (1:10000, ab181602) (Abcam, USA). After the secondary antibody (Cell Signaling Technology, Danvers, USA) was probed for 2 h, the blots were developed using an enhanced chemiluminescence kit (Beyotime institute of Biotechnology, China) and then visualized and photographed with the ChemiDoc imaging system (Tanon, Beijing, China).

BODIPY-Cholesterol efflux assay (Cholesterol transport assay)

A BODIPY-Cholesterol efflux assay was performed as previously described [23, 34]. In brief, HepG2 cells were incubated with 2.5 mM of BODIPY-cholesterol in culture medium at 37°C for 2 h. Cells were rinsed twice with Krebs-Ringer buffer and incubated at 37°C for 1 h. The cell supernatant was centrifuged for 5 min at 6800 g, and the BODIPY fluorescence intensity in the supernatants was measured using a TECAN GENios Pro Microplate Reader (Tecan US, Inc., Morrisville, NC, USA) at excitation 490 ± 10 nm and emission 535 ± 20 nm.

Oil red O staining

For lipid droplets observation, the HepG2 cells were plated in 6-well plates. After stimulation with cholesterol and GLP-1 for 24 h, each group was rinsed three times in PBS, fixed in 4% paraformaldehyde for 30 min, stained in freshly diluted oil red O for 15 min, decolorized in 70% ethanol solution for 15 sec, re-dyed in haematoxylin staining solution for 30 sec and rinsed in PBS twice. Finally, the intracellular lipid droplets were observed and captured with an inverted microscope (Leica, Wetzlar, Germany).

Haematoxylin and eosin (HE) staining

The liver specimens were embedded in 10% formaldehyde-buffered solution, mounted in paraffin blocks, cut in 5 μ m slices, and stained with HE. The pathological changes of samples were observed and photographed under an optical microscope and scored according to the NAFLD activity score (NAS) [35] for the degrees of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2).

Immunohistochemistry (IHC)

Rat livers were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4- μ m sections. After deparaffinization, the sections were boiled in citrate buffer (pH 6.0) for 5 min, treated with 3% hydrogen peroxide for 5 min to endogenous peroxidase activity, then incubated for 5 min in Tris buffer (pH 7.6). They were incubated with a monoclonal antibody for ABCA1 (1:200, ab18180) (Abcam, USA) followed by anti-rabbit IgG antibody (1:2000, ab16891) (Abcam, USA) according to the manufacturer's protocol.

Statistics

The data were obtained from three independent experiments, presented as the mean $\pm$ standard deviation (SD), and analyzed using SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). To compare the significance of two groups, Student's t test was performed. A $p < 0.05$ value was considered statistically significant.

Results

GLP-1 ameliorates metabolic characteristics and inflammatory markers in Sprague-Dawley (SD) rats on a high-fat diet

Sixteen weeks after starting the diet treatments, the body weight, liver wet weight, and liver index were increased in the high-fat diet group (HFD) compared to the control diet group (Control). Rats treated with GLP-1 showed a decrease in body weight, liver wet weight, and liver index, both in the normal diet combined with GLP-1 injection group (GLP-1) and the high-fat diet combined with GLP-1 injection group (HG) (Fig. 1A, B and C). After 16 weeks of the high-fat diet, ALT and AST as indicators of liver function were increased in HFD group compared to Control group. After 12 weeks of GLP-1 injection, ALT and AST were improved in the HG group compared to the HFD group. However, no difference in ALT and AST were observed between the Control group and GLP-1 group (Fig. 1D). The HFD group demonstrated significant increases in TC and TG compared to the Control group, and the treatment with GLP-1 decreased TC in both the GLP-1 group and the HG group. Meanwhile, GLP-1 injection decreased of TG in the HG group compared to the HFD group, but there was no difference between the Control group and GLP-1 group (Fig. 1E). Serum TNF- α and IL-6 were increased significantly in the HFD group compared to the Control, and GLP-1 treatment decreased them in the HG group compared to HFD group. While, no significant differences between the Control group and the GLP-1 group (Fig. 1F and G). Hepatic histological evaluation showed that the HFD group exhibited steatosis, lobular inflammation, and hepatocellular ballooning. After 12 weeks of GLP-1 injection, NAS was improved in the HG group compared to HFD group. No evidence of hepatic steatosis was observed in the rats fed a regular diet without (Control group) or with GLP-1 (GLP-1 group, Fig. 1H and I). These results reveal that the high-fat diet caused metabolic and inflammatory disorders in SD rats, and GLP-1 significantly improved these imbalances.

The toxicity of cholesterol accumulation and the effect of GLP-1 treatment on cell apoptosis and cholesterol homeostasis

To evaluate the toxic effect of cholesterol accumulation on HepG2 cells, the cells were incubated with different concentrations of cholesterol (2.5, 5.0, or 10.0 mmol/L) for 24 h. With the increase of cholesterol concentration, the cell viability gradually decreased (Fig. 2A). To detect the therapeutic and protective effects of GLP-1 on cells suffering from lipotoxicity, the HepG2 cells were incubated with or without GLP-1, or exposed to 5mmol/L cholesterol with or without GLP-1. We found that cell viability was substantially increased after GLP-1 treatment, suggesting that GLP-1 was effective to overcome the toxicity of cholesterol accumulation (Fig. 2B). We further explored the potential impact of cholesterol accumulation and GLP-1 treatment on the apoptosis of HepG2 cells by analysis of caspase-3 activity. The relative activity of caspase-3 was significantly increased in the cholesterol group compared to the negative control, but this activity was markedly reduced by co-incubation with 5 mmol/L cholesterol and 10 nmol/L GLP-1 for 24 h. There was no statistically significant difference

Fig. 1. GLP-1 ameliorates metabolic characteristics and inflammatory markers of SD rats. (A). The body weight of rats for 16 weeks. (B). The liver wet weight of rats for 16 weeks. (C). The liver index of rats for 16 weeks. (D). Comparison of serum ALT and AST levels for 16 weeks. (E). Comparison of serum TC and TG levels for 16 weeks. (F). Comparison of serum TNF-α levels. (G). Comparison of serum IL-6 levels. (H). HE staining of liver sections. Representative photomicrographs (×200) are shown for each group. Arrows points out the hepatocellular ballooning in HE staining. (I). The pathological changes of livers were scored according to the NAS. The data are shown as the mean ± SD (n = 10). *p<0.05, **p<0.01 compared to control group, #p<0.05 compared to HFD group.

between the control group and GLP-1 group, although the activity of caspase-3 was slightly lower after treatment with GLP-1 (Fig. 2C). HepG2 cells treated with GLP-1 decreased the relative expression of intracellular cholesterol than the control group. In addition, treatment with cholesterol led to a marked increase in cellular cholesterol concentration. Compared with these in cholesterol-treated cells, intracellular cholesterol was decreased when HepG2 cells were treated with GLP-1 and cholesterol (Fig. 2D). There was a negative association between cellular cholesterol efflux and lipid accumulation. BODIPY-Cholesterol provides an

efficient and sensitive probe for ABCA1-mediated efflux. In our study, the BODIPY-Cholesterol efflux assay indicated that treatment with both cholesterol and GLP-1 significantly increased cholesterol efflux compared to cholesterol only. In the control diet group, GLP-1 showed the same effect (Fig. 2E). Moreover, we observed a large quantity of red lipid droplets in the HepG2 cells in the high-cholesterol group, whereas after the high cholesterol group was treated with GLP-1, the cells displayed a significant reduction in the number of visible droplets. However, almost no lipid droplets were detected in the HepG2 cells in the negative control and GLP-1 groups (Fig. 2F). These results demonstrate that GLP-1 improves apoptosis and cholesterol homeostasis in HepG2 cells.

The effect of GLP-1 treatment on ABCA1 and miR-19b expression

The effect of GLP-1 on ABCA1 expression in SD rat livers and HepG2 cells was determined by quantitative PCR and western blotting. The mRNA expression of ABCA1 was significantly enhanced in GLP-1-treated tissue and cells compared to the untreated control group (Fig. 3A and B). This increase in mRNA expression was also associated with a concomitant increase

Fig. 2. The toxicity of cholesterol accumulation and the effect of GLP-1 treatment on cell apoptosis and cholesterol homeostasis. (A). HepG2 cells were treated with 2.5, 5.0 or 10.0 mmol/L cholesterol for 24 h, and cell viability was detected by CCK-8 assay. (B). The cells were incubated with or without GLP-1, and exposed to 5mmol/L cholesterol with or without GLP-1. Then, cell viability was detected by CCK-8 assay. (C). The cells were incubated with or without GLP-1, and exposed to 5mmol/L cholesterol with or without GLP-1 before determine the relative activation of caspase 3. (D). The cells were incubated with or without GLP-1, and exposed to 5mmol/L cholesterol with or without GLP-1 before examine the relative expression of intracellular cholesterol. (E). The cells were incubated with or without GLP-1, and exposed to 5mmol/L cholesterol with or without GLP-1 before measured BODIPY-Cholesterol efflux. (F). The cells were incubated with or without GLP-1, and exposed to 5mmol/L cholesterol with or without GLP-1 before stained with oil red O. Representative photomicrographs (×200) are shown for each group. The data are shown as mean ± SD (n = 3) *p<0.05, **p<0.01, ***p<0.001 compared to control group, #p<0.05 compared to CHO group.

in the corresponding protein levels. A significant increase in ABCA1 protein was observed following GLP-1 treatment compared to the untreated control levels. However, the expression of peroxisome proliferator-activated receptor α (PPAR- α), the main transcriptional regulator of ABCA1 gene [36], was not altered in GLP-1-treated groups (Fig. 3C and D). These results suggested a potent regulatory role for GLP-1 in ABCA1 expression *in vivo* and *in vitro*. To investigate the relationship between GLP-1 and miR-19b, the changes in the expression of miR-19b were assessed by quantitative PCR. It has been reported that miR-19b dramatically represses ABCA1 expression through partial complementary binding to the 3'-UTR of ABCA1 mRNA [27]. To investigate whether miR-19b was involved in GLP-1-induced ABCA1 expression, we examined miR-19b levels in rat livers and HepG2 cells. As shown in Fig. 3E–H, GLP-1 significantly decreased miR-19b *in vivo* and *in vitro*. To detect the functional role of miR-19b in the effects of GLP-1 on ABCA1 expression, we further carried out the study under the condition of the gain or loss of functions of miR-19b. The data of this study proved that GLP-1 regulates the expression of genes and miRNAs related to cholesterol homeostasis.

The regulation of ABCA1 expression and cholesterol homeostasis by miR-19b

To further investigate the interplay between GLP-1, miR-19b and ABCA1, miR-19b inhibition and overexpression experiments were done to explore the effects of GLP-1-mediated reduction in miR-19b on the mediator of cholesterol transport: ABCA1. As shown in Fig. 4A and B, the HG-Ant group had a significant increase compared with the HG group in both ABCA1 mRNA and protein. Similarly, the HFD and HG-Ago groups showed a significant reduction in ABCA1 expression. The same alterations were also observed in immunohistochemical staining of ABCA1. ABCA1 expression was enhanced in rats treated with GLP-1 injection, namely the GLP-1 group and HG group. Expression of ABCA1 trended to further increase in rats treated with GLP-1 together with antagomiR-19b, and significantly decreased in the GLP-1+agomiR-19b-treated rats when compared with the HG group (Fig. 4C). To manipulate the expression of miR-19b in HepG2 cells, a miR-19b mimics (40nM), a miR-19b inhibitor (100nM) and a negative control (miRNA-NC-FAM) were used for transfection. Flow cytometry indicated that the transfection efficiency reached about 80% in the cells (Fig. 4D and E). After transfected with miR-19b mimics, HepG2 cell viability was decreased and cell apoptosis was increased compared to the CHO+mimics-NC group. On the contrary, transfecting miR-19b inhibitor facilitated cell viability and inhibited cell apoptosis compared to the CHO+inhibitor-NC group. (Figure 4F and G). As shown in Figure 5A and B, exposure of HepG2 cells to miR-19b-inhibitor resulted in a significant increase in both the expression of ABCA1 mRNA and protein. In contrast, HepG2 cells exposed to miR-19b-mimics showed a significant reduction in ABCA1 expression. Taken together, these findings reveal that GLP-1 regulates ABCA1 expression by inhibiting the level of miR-19b. As shown in Figure 5C, exposing the HepG2 cells to the miR-19b-inhibitor resulted in a significant reduction in intracellular cholesterol measurement. In contrast, the HepG2 cells that were exposed to miR-19b-mimics showed a significant increase. The Oil red O staining and cholesterol measurement showed similar results (Figure 5D). Additionally, the miR-19b-inhibitor group significantly increased cholesterol efflux compared to the miR-19b-inhibitor negative control (Scrambled) group, and the miR-19b-mimics group showed the opposite results (Figure 5E). These results demonstrate that miR-19b regulates ABCA1 expression and cholesterol homeostasis *in vivo* and *in vitro*.

Fig. 3. The effect of GLP-1 treatment on ABCA1 and miR-19b expression. (A). The relative expression of ABCA1 mRNA after 20 μ g/kg GLP-1 injection for 12 weeks in control diet-fed rats. (B). The relative expression of ABCA1 mRNA after treatment with or without 10 nmol/L GLP-1 for 24 h. (C). The expression of ABCA1 and PPAR- α protein after 20 μ g/kg GLP-1 injection for 12 weeks in control diet-fed rats. (D). The expression of ABCA1 and PPAR- α protein after treatment with or without 10 nmol/L GLP-1 for 24 h. (E). The relative expression of miR-19b mRNA after 20 μ g/kg GLP-1 injection for 12 weeks in control diet-fed rats. (F). The relative expression of miR-19b mRNA after 20 μ g/kg GLP-1 injection for 12 weeks in high fat diet-fed rats. (G). The relative expression of miR-19b mRNA after treatment with or without 10 nmol/L GLP-1 for 24 h. (H). The relative expression of miR-19b mRNA after treatment with or without 10 nmol/L GLP-1 in the presence of 5 mmol/L cholesterol for 24 h. The data are shown as mean $\pm$ SD (A, C, E and F, n = 10, B, D, G and H, n = 3). *p<0.05 compared to control group.

Fig. 4. The regulation of ABCA1 expression and cholesterol homeostasis by miR-19b. (A). The relative expression of ABCA1 mRNA in high-fat diet alone (HFD), HFD+GLP-1 (HG), HFD+GLP-1+agomiR-19b (HG-Ago), and HFD+GLP-1+antagomiR-19b group (HG-Ant). (B). The expression of ABCA1 and PPAR- α protein in high-fat diet alone (HFD), HFD+GLP-1 (HG), HFD+GLP-1+agomiR-19b (HG-Ago), and HFD+GLP-1+antagomiR-19b group (HG-Ant). (C). The immunohistochemical staining of ABCA1 of rat liver slices. (D). The transfection efficiency was detected by flow cytometry. (E). The relative expression of miR-19b was measured after transfection by real-time quantitative PCR. (F). The cells transfected with mimics-NC, miR-19b mimics, inhibitor-NC or miR-19b inhibitor were incubated with 5.0 mmol/L cholesterol. The relative cell viability was detected by CCK-8. (G). The cells transfected with mimics-NC, miR-19b mimics, inhibitor-NC or miR-19b inhibitor were incubated with 5.0 mmol/L cholesterol. The relative activation of caspase 3 was detected. The data are shown as mean $\pm$ SD. (A-C, $n = 10$, D-G, $n = 3$) E: * $p < 0.05$ compared to mimics-NC group, # $p < 0.05$ compared to inhibitor-NC group; F and G: * $p < 0.05$ compared to CHO+mimics-NC group, # $p < 0.05$ compared to CHO+inhibitor-NC group.

Fig. 5. (A). The relative expression of ABCA1 mRNA was analyzed after overexpression and inhibition of miR-19b by transfection. (B). The expression of ABCA1 and PPAR- α protein were analyzed after overexpression and inhibition of miR-19b by transfection. (C). The relative expression of intracellular cholesterol in HepG2 cells. (D). The HepG2 cells were stained with oil red O. (E). Relative expression of BODIPY-cholesterol in HepG2 cells. The data are shown as mean $\pm$ SD. (A-E, n = 3) C and E: * p < 0.05 compared to mimics-NC group, # p < 0.05 compared to inhibitor-NC group.

Discussion

T2DM as a metabolic disease, is usually accompanied by abnormal lipid metabolism. The toxicity of cholesterol enriches the connotation of lipotoxicity, which contributes to the development of T2DM [37, 38]. GLP-1 as a promising anti-diabetic drug, could improve lipid accumulation and inflammation in hepatocytes [19, 20, 39]. However, the mechanism of this effect is poorly understood. In this study, we investigated the protective effect and the molecular mechanisms underlying GLP-1's effects on cholesterol homeostasis in HepG2 cells and high-fat diet rats. As illustrated in Fig. 4 and 5, GLP-1 up-regulated ABCA1 by suppressing miR-19b and induced ABCA1-dependent cholesterol efflux from HepG2 cells, resulting in the reduction in cholesterol deposition.

Because of the key role played by GLP-1 in mediating lipid metabolism, much researches has aimed to understand the cellular and molecular mechanisms involved [18, 19, 40]. Fat accumulation in the liver is regarded as a key pathogenic factor of metabolic diseases. High-fat diet resulting in weight gain is associated with increased liver weight and liver index. It was reported that administration of GLP-1 reversed hepatic steatosis in an obese mouse model [41, 42]. In this study, the rats gained weight and showed dyslipidaemia on the high-fat diet, while after GLP-1 treatment for 12 weeks, their weight and liver index were improved. Our data from this study also indicate that, persistent improvement in ALT, AST, TC and TG were observed in the HG group compared to the HFD group. Our histopathological findings demonstrate the damage of the high-fat diet to the liver structure, with many lipid droplets in hepatocytes, while GLP-1 injection reversed this phenomenon. TNF- α and IL-6, two cytokines are involved in the pathology of diseases, were involved in the toxicity of cholesterol accumulation and in the improvement caused by GLP-1 treatment.

Hao et al. established a cholesterol overload model by incubating pancreatic β -cells with 10 mmol/L soluble cholesterol [43]. HepG2 cells were treated with 5 mmol/L soluble cholesterol, which was higher than normal culture medium, and this did not lead to more rapid cell death or damage than 10 mmol/L cholesterol. Our results suggest that redundant intracellular cholesterol inhibited hepatic cell viability, and disturbed the internal and external balance of cholesterol *in vitro*. Caspase-3, a marker of cell apoptosis, was involved in the toxicity of cholesterol. GLP-1 treatment significantly improved the cell viability and moderated cholesterol metabolism in numerous ways. The results are consistent with previous studies on SD rats. Thus, our findings may help explain the improved lipid profile seen in patients receiving GLP-1 treatment.

However, it is not clear that how ABCA1 expression is responsive to GLP-1 in hepatic cells. MiRNAs regulate gene expression post-transcriptionally by binding to 3'-UTRs and repress protein production by destabilizing the mRNA or by translational silencing [24, 25]. MiR-19b potentially inhibits ABCA1 expression by binding to the 3'-UTR of ABCA1 mRNA [29]. ABCA1 is important in HDL particle assembly at the surface of the hepatocyte [44]. Based on this, we measured the role of miR-19b in GLP-1-induced ABCA1 expression. Our observation that GLP-1 decreased miR-19b and enhanced ABCA1 mRNA and protein levels suggests that the peptide regulates cholesterol homeostasis in multiple ways. PPAR- α , an important transcriptional regulator of genes involved in lipid metabolism [45, 46], was not affected by GLP-1, suggesting that PPAR- α expression cannot account for the alteration of ABCA1 with GLP-1 treatment.

Subsequently, we explored the impact of miR-19b knockdown and overexpression on ABCA1 expression in hepatocytes. GLP-1 increased the expression of ABCA1 by suppressing miR-19b *in vivo* and *in vitro*, then accelerated cholesterol efflux and improved lipid metabolism. Consistent with these results, hepatic lipid deposition dramatically decreased in rats treated with GLP-1 or GLP-1 together with antagomiR-19b. Our findings further demonstrate the regulatory effect of GLP-1 is intimately associated with its role in modulating ABCA1 expression and cholesterol homeostasis in hepatocytes. All these mechanisms contribute to the action of GLP-1.

Although cholesterol is an important component of most cells, its inappropriate accumulation leads to diabetes, obesity, atherosclerosis and cancers [47–50]. GLP-1 has particularly strong effects in regulating lipid metabolism [51, 52]. However, the molecular mechanism linking the two is largely missing. Our earlier results in rats' experiments led us to further anticipate the mechanism underlying the molecular link between GLP-1 and cholesterol homeostasis. In this study, we demonstrated that GLP-1 affects cholesterol homeostasis by regulating the expression of miR-19b and ABCA1. Our data point to miR-19b as an upstream regulator of the transcriptional network involved in lipid mechanism and suggest antagomiR-19b could treat dyslipidaemia by the view of increasing the expression of ABCA1. In summary, our findings provide mechanistic evidence that therapeutic intervention with GLP-1 decreases hypercholesterolaemia-associated impairment, possibly by regulating miR-19b level, which in turn enhances ABCA1 expression, thus shedding more light on potential novel therapeutic approaches to reverse lipid mechanism disorders. However, our understanding of the relationship between miR-19b and GLP-1 is still at an early stage. A better understanding of the mechanisms whereby miR-19b regulates lipid metabolism by targeting different signalling pathways may enable the development of miR-19b-mediated therapy.

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Disclosure Statement

The authors declare that they have no competing interests.

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