

Original Article

Vildagliptin reduces lipid levels possibly by mechanisms associated with HMGCR and CYP7A1

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Abstract: Objective: The present study aims to investigate the mechanism by which dipeptidyl peptidase 4 (DPP-4) inhibitors influence lipid profile. Methods: C57BL/6 mice were fed a high fat diet (HFD), and HepG2 cells were treated with vildagliptin, a DPP-4 inhibitor. Liver cholesterol/triglyceride content assay was performed to determine the content of liver cholesterol and triglyceride. Western blot was used to determine the expression of protein. To show the structure of the liver, liver samples were stained with H&E, oil red O and Filipin. Results: Vildagliptin failed to modify normal plasma glucose levels. Meanwhile, vildagliptin improved lipid profiles, reducing both low-density lipoprotein (LDL) in blood and total cholesterol (TC) in the liver. Vildagliptin increased the expression of both 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and cholesterol 7 alpha-hydroxylase (CYP7A1) in the liver from mice fed a HFD. *In vitro*, vildagliptin increased the expression of HMGCR and CYP7A1 in HepG2 cells. By contrast, there were no clear changes in the levels of TC, free cholesterol, and TG after treatment with Vildagliptin for 24 h. Conclusion: The study demonstrates that vildagliptin improves lipid profile possibly by mechanisms that are associated with functional expression of HMGCR and CYP7A1.

Keywords: Dipeptidyl peptidase 4 (DPP-4) inhibitors, vildagliptin, lipid, liver damage

Introduction

Diabetes mellitus (DM) is an independent risk factor for developing cardiovascular disease (CVD) [1]. Moreover, diabetic adults are two to four times more likely to develop heart disease or stroke than those without diabetes [1]. As we know, dipeptidyl peptidase 4 (DPP-4) inhibitors, which play important roles in reducing the breakdown of glucagon-like peptide-1 (GLP-1) and improving glycemic control by increasing insulin secretion, have been widely used as new hypoglycemic agents [2]. Their principal advantage, due to glucose-dependent insulin secretion, is low incidence of hypoglycemia, especially in elderly patients and patients with cardiac diseases [3].

Lipid profile is an important determinant of cardiovascular risk in type 2 diabetes mellitus

(T2DM) [4]. In diabetic patients, β -cell function is precipitated and worsened by toxicological effects of hyperglycemia (i.e., glucotoxicity) and elevated levels of free fatty acids (i.e., lipotoxicity) [5]. DPP-4 inhibitors can reduce visceral adipose tissue, macrophage content and inflammation by blocking monocyte activation/chemotaxis, and preventing monocyte migration and actin polymerization *in vitro*, thus exert anti-atherosclerotic effects [6]. Moreover, several studies have shown that DPP-4 inhibitors might improve lipid profile with potential favorable cardiovascular implications [7].

As one of the DPP-4 inhibitors, vildagliptin is shown to reduce both lipotoxicity that is secondary to reduced fasting lipolysis, and apo-B-48 production that induces less fat extraction from the gut and mobilizes and burns fat during meals [8]. Additionally, vildagliptin incre-

ases the secretory capacity of β -cells [9]. It is previously shown that vildagliptin significantly decreases total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL) levels [10, 11]. Moreover, vildagliptin significantly improves body weight, body mass index (BMI), TC, TG and LDL-cholesterol (LDL-C) levels in non-alcoholic, fatty liver disease patients that present dyslipidemia following vildagliptin treatment [12].

Most investigators under-appreciate whether the dyslipidemic effect of vildagliptin is related to GLP-1. Currently, little research has been carried out to determine whether vildagliptin therapy can improve the physiological lipid profile. In addition, clear evidence of the effectiveness of DPP-4 inhibitors affecting lipid metabolism is still lacking but is clearly warranted by empirically designed *in vivo* and *in vitro* studies. In the present study, we employ a mouse model and a HepG2 cell model to determine whether vildagliptin improves physiological lipid profiles, and try to explore the possible mechanisms that are involved in lipid metabolism following treatment with vildagliptin.

Materials and methods

Animals

Seven-week-old C57BL/6 mice were obtained from Vital River, Charles River, China, and acclimatized to the environment before being fed *ad libitum* with either chow diet (CD; 14.09 KJ/g; Beijing KeAo XieLi Feed Co., Ltd., Beijing, China) or high-fat diet (HFD; 17.05 KJ/g; Beijing KeAo XieLi Feed Co., Ltd., Beijing, China). HFD contained 82.7% CD, 2% cholesterol, 5% fat (31.29% kJ), and 0.3% sodium cholate. After being fed with HFD for 8 weeks, the mice in the HFD control group received treatment by saline for 12 weeks, and the mice in HFD+vildagliptin group received HFD (composition as described above) and vildagliptin (50 mg/Kg/day).

All animals were housed in groups of 6-8 per cage with access to food and water *ad libitum* under $21 \pm 2^\circ\text{C}$ and a 12 hour light/dark cycle. Mice were sacrificed after fasting for 8 h. Blood and hepatic tissues were collected. Blood was centrifuged for 10 min at $12,000 \times g$ and 4°C , before separating plasma, which was stored at 70°C . All experiments were carried out in accordance with the recommendations provid-

ed by the animal care and use guidelines of Shandong Provincial Hospital Affiliated to Shandong University, P.R. China.

Liver cholesterol/triglyceride content assay

Liver cholesterol/triglyceride contents were measured using a cholesterol/triglyceride assay kit (E1015, E1016, E013-15; Applygen Technologies, Beijing, China) according to the manufacturer's instructions. Briefly, liver tissues were extracted with lysis buffer, and centrifuged at $2000 \times g$ for 5 min at room temperature after being heated to 70°C for 20 min. Then, 10 μl supernatant was transferred to a vial containing 190 μl working solution for cholesterol/triglyceride assay. Absorbance was measured at a wavelength of 550 nm, after the sample was incubated at 37°C for 15-20 min. Protein concentrations were quantified using bicinchoninic acid assay (BCA assay; P1511; Applygen Technologies, Beijing, China). Cholesterol content was expressed as mmol/g protein.

Cells

HepG2 cells were obtained from Cell Bank of Type Culture Collection, Chinese Academy of Sciences (CBTCCAS, Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 $\mu\text{g}/\text{ml}$ penicillin (Thermo Fisher Scientific, Waltham, MA, USA), and 100 U/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). HepG2 cells were incubated at 37°C in an atmosphere of 5% CO_2 and full humidity. When HepG2 cells were 70-80% confluent, the cells were differentially dosed with vildagliptin (Novartis, Basel, Switzerland)/GLP-1 (Sigma-Aldrich, St. Louis, MO, USA) and stimulated for 24 h in serum-free DMEM.

Western blot

Equal amount of homogenate proteins from each sample were separated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) transfer membrane (Merck Millipore, Burlington, MA, USA). Non-

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Table 1. Characteristics of mice treated with CD, HFD or HFD+Vildagliptin

| | CD | HFD | HFD+Vildagliptin |
|--------------|----------------|----------------|------------------|
| Number | 8 | 6 | 7 |
| ALT (U/L) | 40.66 ± 7.43 | 37.22 ± 4.51 | 43.24 ± 16.79 |
| AST (U/L) | 155.56 ± 18.03 | 168.12 ± 21.76 | 180.19 ± 36.68 |
| GLU (mmol/L) | 6.42 ± 0.42 | 7.70 ± 1.34* | 7.461 ± 1.22 |
| TC (mmol/L) | 2.25 ± 0.69 | 3.99 ± 0.64** | 3.87 ± 0.97 |
| TG (mmol/L) | 0.36 ± 0.11 | 0.23 ± 0.05** | 0.25 ± 0.09 |
| HDL (mmol/L) | 1.82 ± 0.86 | 2.36 ± 0.46 | 2.73 ± 0.47 |
| LDL (mmol/L) | 0.32 ± 0.11 | 0.61 ± 0.11** | 0.47 ± 0.07** |

Note: CD, chow diet; HFD, high fat diet; Vildagliptin dose, 50 mg/Kg. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLU, glucose; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein. * $p < 0.05$ and ** $p < 0.01$ compared with CD group; && $p < 0.01$ compared with HFD.

specific binding sites were blocked in 5% (w/v) non-fat dried milk in Tween (0.1%)/phosphate-buffered saline (PBS-T, pH 7.4) for 2 h. Next, the blots were incubated overnight at 4°C with a targeted commercial antibody, including anti-HMGCR (1:1000; ab174830; Abcam, Cambridge, UK), anti-LDLR (1:1000; ab30532; Abcam, Cambridge, UK), anti-CYP7A1 (1:2000; ab79847; Abcam, Cambridge, UK), anti-ACAT2 (1:1000; ab37467; Abcam, Cambridge, UK), anti-SR-B1 (1:1000; sc-67099; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-SREBP2 (Ser-79) (1:1000; 10007663; Cayman Chemical, Ann Arbor, MI, USA). After three washes in 5% (w/v) non-fat dried milk in PBS-T (pH 7.4) for 10 min, and incubation with corresponding secondary antibodies (Zsbio, Ltd, China), immune complexes were detected using enhanced chemiluminescence plus (ECL-plus) detection system (Amersham Life Sciences; GE Healthcare, Pittsburgh, PA, USA). The same membrane was reincubated with GAPDH (i.e., a mouse monoclonal antibody, 1:7500; Anbo Biotechnology Co., Ltd., San Francisco, CA, USA). Immunoreactive bands were quantified using Alpha Imager 2200 (Alpha Innotech, San Leandro, CA, USA).

Oil red O staining

Oil red O staining indicates the presence of neutral lipids in the deposit region, with hematoxylin counterstaining performed according to previously reported literature [13]. In brief, 0.5 g oil red O was dissolved by 100 ml isopropanol to make a saturated solution and 30 ml of the saturated solution was dissolved into 50 ml by isopropanol before use. Pretreated specimen

slices (8 µm) were immersed in oil red stain for 8 min, polarized by being extracted 10 times in 60% ethanol, stained by hematoxylin for nuclear visualization and observed under an inverted microscope (CKX31-A11RC; Olympus, Tokyo, Japan) following glycerol mounting.

Filipin staining

Filipin staining was carried out according to the procedures described in the Filipin fluorescence staining kit instructions (GMS800-59.03; Genmed Co. Ltd., Shanghai, China). In brief, cells/frozen sections were washed three times in

PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, stained with filipin according to the manufacturer's protocol, and viewed by fluorescence microscopy (Axio Imager A2; Zeiss, Jena, Germany) using a UV filter set.

Statistical analysis

Measurement data were expressed as means ± standard deviation, and SPSS software (version 20.0; IBM, Armonk, NY, USA) was used for statistical analysis. Unpaired Student's t-test was applied to test statistically significant differences between two experimental groups, and one-way ANOVA was used for comparisons among multiple groups. An alpha value of $p < 0.05$ was considered statistically significant.

Results

Vildagliptin reduces LDL level but does not alter GLU level in mice treated with HFD

Blood specimens from the mice were tested by automatic biochemical assay after different dietary treatment for 20 weeks. No difference was found for serum ALT and AST levels among the mice treated with CD, HFD and HFD+Vildagliptin ($p > 0.05$), suggesting that liver injury was not induced by food or therapeutic treatment. Compared with mice in the CD group, the mice fed with HFD for 8 weeks had higher levels of GLU ($p < 0.05$), LDL ($p < 0.01$) and TC ($p < 0.01$), and a lower level of TG ($p < 0.01$). After treatment with vildagliptin for 12 weeks, mice had decreased level of LDL as

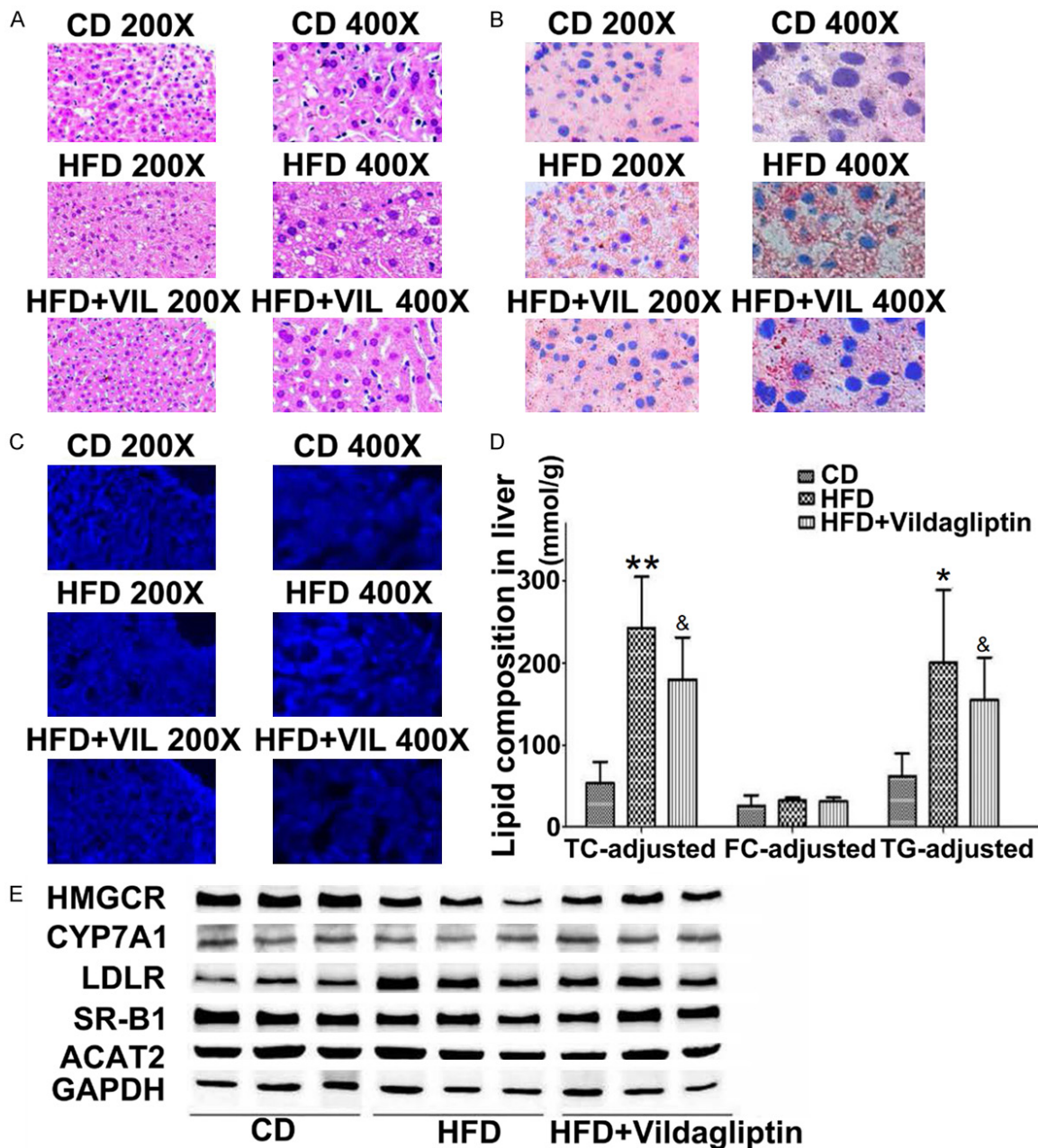


Figure 1. Vildagliptin reduced cholesterol and triglyceride in the liver of mice fed with HFD. A. Representative H&E staining of liver sections from indicated groups. B. Oil red O staining of neutral lipids in liver tissues from indicated groups. Red, triglyceride; Blue, nucleus. C. Filipin staining of free cholesterol in liver tissues from indicated groups. Blue, free cholesterol. D. Lipid contents in the liver from indicated groups. Liver cholesterol/triglyceride contents were measured using cholesterol/triglyceride assay kit. E. Expression of cholesterol metabolism-related proteins, including HMGCR, CYP7A1, LDLR, SR-B1 and ACAT2. CD, chow diet; HFD, high fat diet; vildagliptin dose, 50 mg/Kg. * $p < 0.05$ and ** $p < 0.01$ compared with CD group; & $p < 0.05$ compared with HFD group.

compared to their non-treated counterparts ($p < 0.01$). By contrast, vildagliptin did not improve the levels of GLU and other sub-fractions of lipid-protein (Table 1). The results suggest that vildagliptin reduces LDL level but does not alter GLU level in mice treated with HFD.

Vildagliptin can reduce lipid content of the liver and may improve lipid profile by a mechanism that is dependent on the regulation of CYP7A1 and HMGCR expression

To show liver structure, liver samples in CD, HFD, and HFD+Vildagliptin groups were stain-

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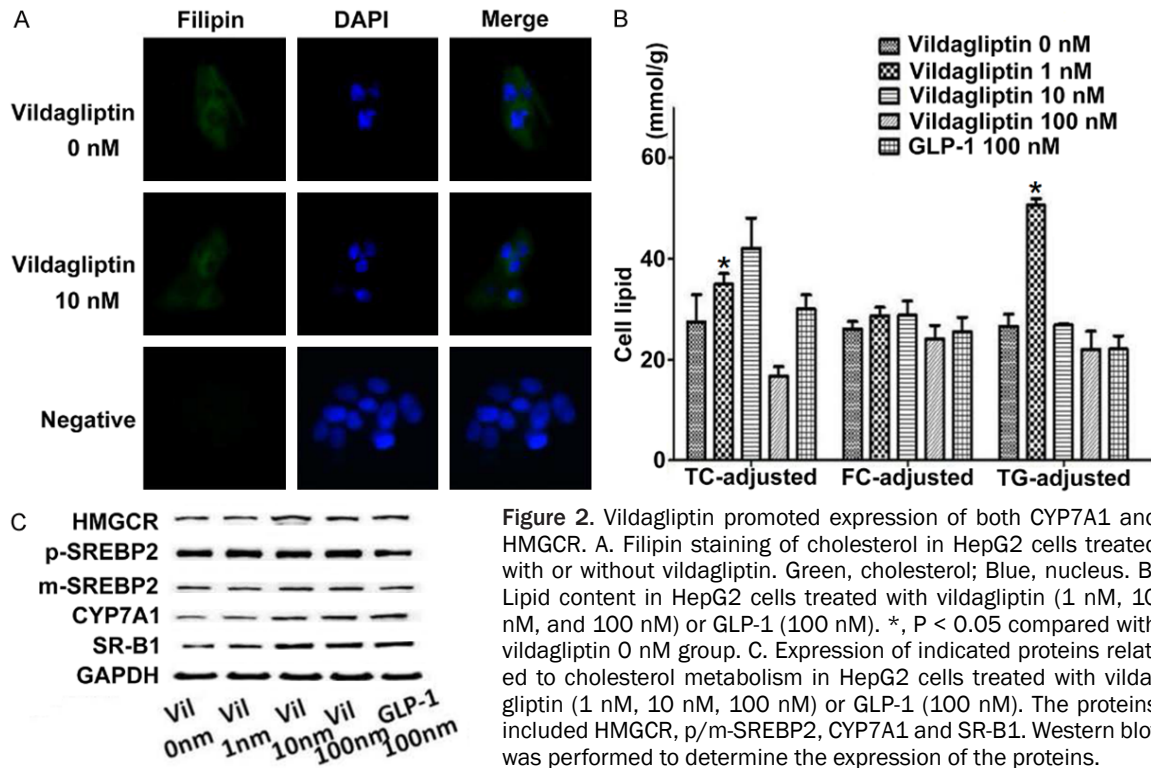


Figure 2. Vildagliptin promoted expression of both CYP7A1 and HMGCR. A. Filipin staining of cholesterol in HepG2 cells treated with or without vildagliptin. Green, cholesterol; Blue, nucleus. B. Lipid content in HepG2 cells treated with vildagliptin (1 nM, 10 nM, and 100 nM) or GLP-1 (100 nM). *, $P < 0.05$ compared with vildagliptin 0 nM group. C. Expression of indicated proteins related to cholesterol metabolism in HepG2 cells treated with vildagliptin (1 nM, 10 nM, 100 nM) or GLP-1 (100 nM). The proteins included HMGCR, p/m-SREBP2, CYP7A1 and SR-B1. Western blot was performed to determine the expression of the proteins.

ed with H&E, oil red O and Filipin. To detect cholesterol pathway, Western blotting was performed. H&E staining showed that the structural organization of the liver in different groups was normal (Figure 1A). It is clear that vildagliptin can decrease triglyceride and free cholesterol levels, which were elevated in the HFD group when compared with the CD group (Figure 1B and 1C). Lipid content in the liver showed that vildagliptin significantly reduced total cholesterol in the liver as compared to mice fed with only HFD ($p < 0.05$). However, total cholesterol in the HFD+Vildagliptin group was increased as compared to that in the CD group ($p < 0.05$). The same statistically significant tendency was found in terms of triglyceride levels after vildagliptin treatment (Figure 1D). These results showed that vildagliptin could reduce the lipid content of the liver. Western blot showed that the expression of both CYP7A1 and HMGCR was decreased in the HFD group compared with the CD group, and expression of HMGCR and CYP7A1 was increased in HFD+Vildagliptin group as compared with the HFD group (Figure 1E). These results demonstrated that vildagliptin can reduce lipid content of the liver and may improve lipid profile by a mechanism that is dependent on the regulation of CYP7A1 and HMGCR expression.

Vildagliptin promotes expression of CYP7A1 and HMGCR

With the different outcomes of lipid content in the liver and expression of the cholesterol pathway, we dose-dependently treated HepG2 cells with vildagliptin (0 nM, 1 nM, 10 nM and 100 nM) and GLP-1 (100 nM), and cell lipid content was assayed after 24 h. The data showed no significant difference in contents of free or total cholesterol or triglyceride between cells treated with vildagliptin and those not treated with vildagliptin ($p > 0.05$) (Figure 2A). Short of GLP in the media, it was perplexing to find significantly increased levels of both triglyceride and total-cholesterol in cells treated with 1 nM vildagliptin. This means that vildagliptin affected liver cells directly (Figure 2B). Western blot showed that vildagliptin increased protein expression of both CYP7A1 and HMGCR (Figure 2C), which was consistent with the results found *in vivo*. The results suggest that vildagliptin promotes the expression of CYP7A1 and HMGCR.

Discussion

The mechanism of action whereby DPP-4 inhibitors modulate the cellular lipid profile is poorly understood. In this current study, our

objective was to explore the likely mechanisms involved in lipid metabolism after treatment with DPP-4 inhibitor vildagliptin. We did not find any change in the activities of ALT and AST between mice treated with and without vildagliptin, and this indicates that liver damage is not induced by treatment with vildagliptin. We also discovered that vildagliptin improves cellular lipid profiles by reducing the levels of LDL in the blood and TC in the liver. Moreover, vildagliptin enhances HMGCR and CYP7A1 expression in the liver in animals fed with HFD. In addition, vildagliptin increases the expression of HMGCR and CYP7A1 by HepG2 cells *in vitro*. By contrast, we have not observed any changes in levels of TC, free cholesterol, and TG after treatment with vildagliptin for 24 h.

Many other clinical studies have shown that vildagliptin dampens the levels of lipids such as triglycerides and cholesterol in the blood, and our animal modeling experiments have confirmed this. It is reported that abnormal metabolism of LDL cholesterol represents a risk factor for the development of atherosclerosis [14]. Moreover, statins are in therapeutic use to lower the levels of LDL by inhibiting the function of HMGCR and reducing coronary morbidity and mortality rates [15]. DPP-4 inhibitors are previously found to play an important role in dampening the levels of triglycerides and cholesterol [16], and the differential effect of each DPP-4 inhibitor is suggested by a meta-analysis of previously published randomized clinical trials [17].

Vildagliptin is launched onto the anti-diabetic agents market as a monotherapy for this condition [18] and is indicated in combinatorial therapies with other anti-diabetic agents [19-21]. Treatment with vildagliptin reduces the rate of lipolysis when in fasting state [22] and decreases total cholesterol levels [23]. Indeed, treatment with DPP-4 inhibitors is used as another incretin-based therapy, with the exception of GLP-1 analogues and GLP-1R agonists, which are effective in regulating blood glucose [24]. In addition to their principal advantages, DPP-4 inhibitors are effective and safe in T2DM patients with moderate to severe renal impairment [25]. This treatment also exhibits local endothelial modulating properties in wound healing of diabetic foot ulcers [26] and in reduced

incidence of macrovascular events in the primary care of diabetic patients [27]. In addition, treatment with DPP-4 inhibitors provokes a significant increase in plasma adiponectin levels [28].

Regarding the effect of vildagliptin on fasting blood glucose [29], our study demonstrates that vildagliptin decreases blood glucose levels, being consistent with other research [30-32]. This observation is explained by the knowledge that vildagliptin increases glucose-dependent insulinotropic polypeptide (GIP) [33-35] and glucagon secretion during low blood glucose levels [36, 37]. This means that vildagliptin reduces glucagon levels during hyperglycemia and simultaneously avoids inhibition of counter-regulatory glucagon responses during episodes of hypoglycemia. We have attempted to find the mechanism by which vildagliptin reduces blood lipid levels and found that the expression of HMGCR is not decreased as expected but unexpectedly increased. We also find a similar tendency in the context of the expression of CYP7A1 and SR-B1.

It is also interesting to identify differential changes in lipid levels in HepG2 cells after vildagliptin treatment. After 24 h of dose-dependent treatment with vildagliptin, we find that the levels of several cellular lipids have not changed (i.e., unaltered levels of TC, FC, and TG), and the expression of both HMGCR and CYP7A1 has displayed similar changes after treatment with vildagliptin. We speculate that vildagliptin improves lipid profile possibly through up-regulating CYP7A1 expression which translates cholesterol into bile acid. Meanwhile, HMGCR is regulated by the negative feedback of cholesterol, which means that HMGCR expression is increased when cholesterol level is decreased by vildagliptin. However, above hypothesis needs to be confirmed by further studies.

Our study also has some short-comings. First, we have not determined bile acid levels in this study, and we cannot show increased levels of circulating bile acids. Second, more comprehensive and detailed studies of the effect of vildagliptin on cells needs to be performed in the future. Moreover, the number of laboratory animals should be increased to improve the

clinical significance of any observed differences from experimental treatments and the interpretation of these data.

In conclusion, vildagliptin is able to reduce blood and liver lipid levels, possibly by mechanisms that are associated with the functional expression of HMGCR and CYP7A1.

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Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Disclosure of conflict of interest

None.

Abbreviations

CVD, cardiovascular disease; CYP7A1, cholesterol 7 alpha-hydroxylase; DM, diabetes mellitus; DPP-4, Dipeptidyl peptidase 4; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-like peptide-1; HbA1c, hemoglobin A1c; HFD, high fat diet; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides.

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