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Effect of siglidine on glucose lipid metabolism and the expression of iNOS and GLP-1 receptors in diabetic rats

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) has become the major complication of diabetes. The progression of the disease impedes the efficacy of DN treatment. Therefore, the strategies to inhibit or reverse kidney damage in DN patients are of critical importance. We aim to investigate the effect of sitagliptin on DN within rat model and analyze the associated metabolism and expression of iNOS/GLP-1 receptor.

MATERIALS AND METHODS: Diabetic model was generated by using Sprague-Dawley (SD) rats, which received an intra-peritoneal injection of 30 mg/kg streptozotocin. Rats were then treated with saline or 15 mg/(kg.d) sitagliptin by gavage. After 12 weeks, fasting blood glucose and insulin resistance were measured. Rat glucose and lipid metabolism were evaluated by high triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). Western blot was used to measure expression of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) related with glucose-lipid metabolism, and expression of iNOS and GLP-1 expression in kidney tissues.

RESULTS: After 12 weeks of feeding, the levels of blood glucose and lipid in sitagliptin group were significantly decreased compared to those in control group ($p<0.05$), whilst insulin sensitivity was enhanced ($p<0.05$). Western blot showed that sitagliptin downregulated the expressions of glucose-lipid metabolism proteins such as G6Pase, PEPCK, ACC and FAS in rat livers, inhibited iNOS expression in kidneys and elevated GLP-1 receptor activity ($p<0.05$).

CONCLUSIONS: Sitagliptin effectively stabilizes blood glucose and lipid levels in DN rats, significantly improves glucose-lipid metabolism and protects kidney and vascular endothelial cells during DN pathogenesis through inhibiting iNOS expression and elevating GLP-1 receptor activity.

Key Words:

Sitagliptin, Type II diabetes, Rat diabetic model, Glucose-lipid metabolism, iNOS, GLP-1 receptor.

Introduction

Diabetes mellitus belongs to a type of metabolic disorder caused by insulin secretion deficits or other biological effects leading to persistently high blood glucose level[1]. The rise of blood glucose level can result in abnormal function of major body organs. Diabetic nephropathy (DN), as a major complication for diabetes, remains a challenging issue once progressing into terminal stage compared to other renal diseases[2]. Researchers[3,4] showed the correlation between DN pathogenesis and abnormal glucose-lipid metabolism in diabetic patients, and DN was also related with growing expression of inducible nitric oxide synthase (iNOS) at early stage of DN. Sitagliptin represents a novel dipeptidyl peptidase-4 (DDP-4) inhibitor for the treatment of diabetes. It significantly improves the damage of renal tissue from diabetic patients at early stage[5,6]. Currently, the protective mechanism of sitagliptin on renal cells in DN is still unclear. Green et al[7] suggested that sitagliptin mainly mediated blood glucose level via protecting endogenous incretin and enhancing its function. Such process is achieved predominantly via the function of glucose dependent insulinotropic peptide (GIP) and glucagon like peptide-1 (GLP-1)[8]. GLP-1 is a brain-gut peptide produced by ileum secretory cells, and can stimulate islet B cells to secrete insulin through binding onto GLP-1 receptor, a target for drugs against type 2 diabetes. iNOS expression is induced by tissue injury, and its level can be used to evaluate inflammatory condition of body. Researches showed that at early stage DN, renal cells maintain at minimal inflammatory status, while the increase of iNOS concentration is involved in injury of renal cells. Therefore, we aimed to investigate the effect of sitagliptin on glucose/lipid metabolism and expression of iNOS and GL-1 receptor.

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Materials and Methods

Animal Grouping and Diabetic Model Generation

A total of 30 male Sprague Dawley (SD) rats (12 weeks old, average body weight = 203.4±5.3 g) were purchased from Laboratory Animal Center, Kunming Medical University, and were randomly divided into control (N=10) and model (N=20) groups. Control group received normal diet whilst model group was fed by high-fat diet. After 4-week feeding, all rats were fasted for 12 h, followed by intra-peritoneal injection of 30 mg/kg Streptozotocin in model group, or equal volume of citric acid buffer in control group. After 4-week feeding, fasted blood glucose level was measured every week after 12 h fasting. Those rats with averaged fasted blood glucose higher than 7.8 mmol/L were identified as successful model generation. A total of 16 rats (16/20) showed diabetic syndrome after 4 weeks. Those diabetic rats were randomly divided into model control group and sitagliptin treatment group (N=8 each). Treatment group received 15 mg/kg sitagliptin by gavage daily, whilst model control group received equal volume of saline. All rats were fed for 12 consecutive weeks[9]. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of First People's Hospital of Yunnan Province (Kunming, Yunnan, China).

Serum Biochemical Assays

All rats were fed for 12 h, and were sacrificed by cervical dislocation after 12 h fasting. Blood samples were collected from all groups, and were centrifuged at 3000 g for 10 min. The upper phase of plasma was saved for 8000 g centrifugation for 10 min, for further detection of fasted blood glucose, high triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels using test kits (Senbeijia Biotech, Nanjing, Jiangsu, China) following manual instruction. Measurement was performed on AU680 fully automatic biochemical analyzer (Beckman, Brea, CA, USA).

Expression Assay of Rat Liver Lipid Contents and Glucose-Lipid Metabolism

Liver tissues were collected from rat liver tissues after they were sacrificed, and were homogenized in pre-cold saline, followed by 2000 g cold centrifugation for 10 min. The supernatant was collected for measuring TG, TC and free fatty acids (FFA) contents. Protein quantification was performed using coomassie brilliant blue.

mRNA Expression Assay for iNOS and GLP-1 Receptor (qRT-PCR assay)

Rat kidney tissues were collected and total RNA was extracted by using TRIzol method. qRT-PCR was used to measure mRNA expression of iNOS and GLP-1 receptor in rat renal tissues. PCR primers for iNOS and GLP-1 receptor genes were firstly designed as Table I. Reverse transcription was performed to obtain cDNA in a system containing 10 μL RNA, 5 μL RT-Buffer, 1 μL RNase Mix, and 9 μL ddH_2O. Reverse transcription was performed under 37°C for 60 min, followed by PCR in a system containing 2 μL cDNA, 10 μL qPCR Mix, 2 μL forward/reverse primers, and 4 μL ddH_2O. The reaction was performed under 95°C for 3 min, and 40 cycles each containing 95°C for 15 s and 60°C for 30 s. Using β-actin as the internal reference gene, relative expression level was presented as $2^{-\Delta\Delta Ct}$ values.

Western Blot

Tissues were mixed with protein lysis buffer. After 12000 g centrifugation for 10 min, the supernatant was collected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Total proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Western blot was used to measure expression levels of liver glucose or lipid metabolism related factors, including glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS), acetyl co-A carboxylase (ACC). The membrane was blocked using 5% defatted milk powder for 1 h at 37°C, followed by 4°C overnight incubation in primary antibody against G6Pase, PEPCK, FAS, ACC, iNOS, GLP-1 and β-actin (Cell Signaling Technology, Danvers, MA, USA, 1:200 dilutions). Tris-buffered saline and Tween-20 (TBST) was used to wash out extra primary antibody, and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (1:1000) was added for 1 h room temperature incubation. The

Table I. qRT-PCR primers

Name	Sequence
iNOS-F	5' GGGGCATACAGCCATAC 3'
iNOS -R	5' CGCCAAGGAACGAAA 3'
GLP-1R-F	5' TGCCCTCCTCCAACCACTAT 3'
GLP-1R -R	5' GAATCCATTGAAAGCCACCC 3'
Actin-F	5'GTACCACTG GCATCGTGAT 3'
Actin-R	5' TGTTGGCGTACAGGTCTTTG 3'

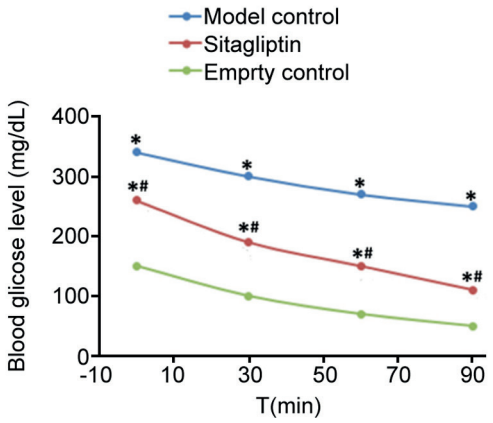

Figure 1. Effects of sitagliptin on fasted blood glucose and insulin resistance. *, $p<0.05$ compared to empty control group; #, $p<0.05$ compared to model control group.

membrane was rinsed in TBST, and was incubated in 3,3'-diaminobenzidine (DAB) chromogenic buffer for 10 min. Images were processed by a gel imaging analysis system (Bio-Rad, Hercules, CA, USA) to reveal band density value of grey[10].

Statistical Analysis

All data were presented as mean±standard deviation (SD), and were analyzed by SPSS 20.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Between-group comparison was performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. A statistical significance was defined when $p<0.05$.

Results

Effects of Sitagliptin on Rat Fasted Blood Glucose Levels

All rats were fed for 12 weeks and blood samples from tail veins were collected. Faster blood glucose level was shown in Figure 1. Compared to model control group, sitagliptin treatment significantly elevated the blood glucose level ($p<0.05$). Insulin resistance assay also showed the blood glucose level in sitagliptin group was significantly decreased after insulin injection compared to model control group ($p<0.05$).

Effects of Sitagliptin on Rat Blood Lipid Levels

To evaluate the effect of sitagliptin on blood lipid level of rat, we collected serum samples from all rats and measured key indexes for evaluation of blood lipid levels, including TG, TC, LDL-C and HDL-C. As shown in Table II, we found that after 12-week feeding, serum TG, TC, LDL-C and HDL-C levels were all significantly elevated in model control group, whilst serum indexes in sitagliptin treatment group were remarkably decreased, indicating that sitagliptin treatment effectively suppressed rat serum lipid level and improved blood lipid metabolism ($p<0.05$).

Effects of Sitagliptin on Glucose/Lipid Metabolic Related Gene Expression

To better evaluate the effect of sitagliptin on glucose/lipid metabolic related gene expression, we extracted proteins from liver tissues of experiment rats. Using β-actin as the reference, protein factor level was calculated (Figure 2A and 2B). After 12-week drug delivery, sitagliptin significantly suppressed expressions of REPCK and G6P, compared to model control group ($p<0.05$). Moreover, we found FAS protein was markedly decreased, which also participates in lipid biosynthesis in rat liver tissues ($p<0.05$), whilst no statistical change of ACC expression was observed ($p>0.05$).

Effects of Sitagliptin on iNOS and GLP-1 Receptor Expression in Renal Tissues

To analyze the effect of sitagliptin on iNOS and GLP-1 receptor expression in renal tissues

Table II. Blood lipid level in rats.

Index	Empty control	Model control	Sitagliptin
N	10	8	8
Sitagliptin dosage (mg/kg•d)	0	0	15
TG (mg/dL)	231.1±65.3	584.1±158.3*	342.3±128.3*#
TC (mg/dL)	102.3±18.3	162.3±20.3*	122.3±28.3*#
LDL-C (mg/dL)	62.8±10.2	102.8±12.2*	72.6±8.6*#
HDL-C (mg/dL)	36.6±6.2	73.6±7.2*	53.6±5.2*#

Note: *, $p<0.05$ compared to empty control group; #, $p<0.05$ compared to model control group.

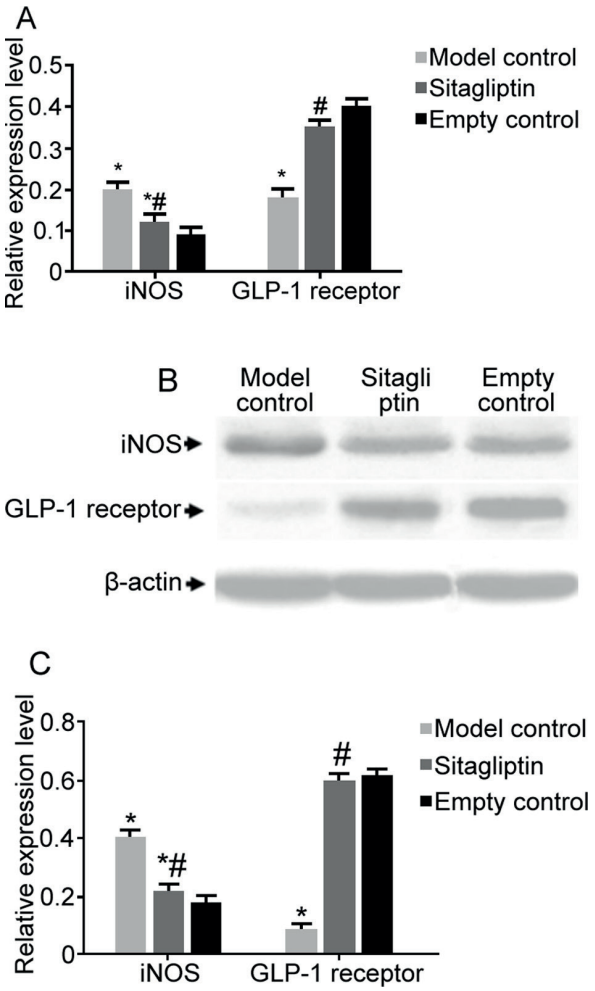

Figure 3. Expression of iNOS and GLP-1 receptor protein in diabetic renal tissues. ***(A)*** qRT-PCR for iNOS and GLP-1 receptor expression level; ***(B)*** Western blot for iNOS and GLP-1 expression level; ***(C)*** Differential expression of iNOS and GLP-1 receptor in diabetic rats at protein levels. *, $p<0.05$ compared to empty control group; #, $p<0.05$ compared to model control group.

of diabetic rats, we employed qRT-PCR and Western-Blot to measure relative gene at both mRNA and protein levels. As shown in Figure 3, the level of iNOS in sitagliptin-treated rat was significantly suppressed ($p<0.05$), whilst GLP-1 receptor expression was significantly up regulated ($p<0.05$).

Discussion

DN, as a frequent complication of diabetes, is the second common reason leading to terminal nephropathy. Currently, accumulative studies[11-13] have confirmed that sitagliptin could stabilize blood glucose level in DN patients, and improved glucose/lipid metabolic process. However, few reports have been performed regarding the role of sitagliptin in DN pathogenesis and progression. This study thus aimed to investigate the effect of sitagliptin on glucose/lipid metabolism in diabetic patients and on expressions of iNOS and GLP-1 receptor via a rat diabetic model. We firstly generated a SD rat diabetic model via streptozotocin, and compared fasted blood glucose and insulin resistance level after the treatment for 12 weeks. Results showed that sitagliptin could suppress fasted blood glucose level in experimental rats, and enhanced its sensitivity to insulin, indicating its benefit for insulin treatment. Sitagliptin is a DPP-4 inhibitor treating type 2 diabetes. Hermansen et al[14] showed that sitagliptin significantly improved blood glucose management and islet B cell function in diabetic patients. Further studies showed multiple biological activities of sitagliptin, as it can suppress apoptosis of islet beta cells and facilitate their proliferation. Thus, it decreases blood glucose level of type 2 diabetic patients[10,15]. In diabetic patients, tissue/organ dysfunctions may occur due to persistently higher blood glucose level, leading to metabolic disorder such as high blood lipid level[16]. Abnormal increase of blood lipid level in diabetic patients activates blood coagulation and damages renal glomerular cells. It further impedes micro-circulation of renal vessels, eventually leading to kidney dysfunction due to hypoxia of renal tissues[17,18]. This study found that sitagliptin effectively improved blood glucose and lipid level in diabetic rats, and regulated abnormal expression of proteins that are closely related with glucose/lipid metabolism such as G6Pase, PEPCK, FAS and ACC; thus, it contributed to the maintenance of relatively low levels of both glucose and lipid to relieve DN progression. In early study of pathological process of DN, it was found that iNOS expression in renal cells was significantly elevated, whilst GLP-1 receptor activity was decreased, suggesting that both iNOS and GLP-1 receptors are closely correlated with DN pathogenesis[19,20]. The elevation of iNOS induces the rise of NO concentration in renal tissues, which further aggravates glomerular injury of DN patients. Vaghasiya et al[21] showed that sitagliptin treatment decreased renal iNOS and NO levels in diabetic patients, and demonstrated that sitagliptin could protect renal tissues via suppressing iNOS expression. For diabetic patients, the escalation of endogenous concentration of GLP-1 or GLP-1 receptor could facilitate insulin secretion and de-

crease blood glucose, and exert certain protective effects on vascular endothelial cells[19]. Such protective effects against vascular endothelial dysfunction are mainly due to the decreasing secretion of inflammatory factor TNF-α, PAI-1 and VAM from vascular endothelial cells via the increase of GLP-1 receptor[22]. This study showed that sitagliptin could reduce iNOS level, and up regulate GLP-1 receptor level, indicating that sitagliptin could protect renal and vascular endothelial tissues of diabetic patients to certain extents, thus alleviating disease progression of DN. Further study thus should focus on the effect of sitagliptin in clinic and potential of combined use of compounds such as sodium-glucose cotransporter 2 inhibitor, resveratrol on the therapy against DN[23,24].

Conclusions

We showed that sitagliptin could effectively stabilize blood glucose and lipid level in DN rats, suppress iNOS expression, induce GLP-1 receptor activity, and play a protective role on renal and vascular endothelial cells during DN pathogenesis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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