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Original Article

Sitagliptin downregulates retinol-binding protein 4 and upregulates glucose transporter type 4 expression in a type 2 diabetes mellitus rat model

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Abstract: The present study was designed to investigate the effects of sitagliptin on metabolic parameters as well as the expression levels of retinol-binding protein 4 (RBP4) and glucose transporter type 4 (GLUT4) in a rat model of type 2 diabetes mellitus. A rat model of type 2 diabetes mellitus was established by a combination of a high-fat diet and intraperitoneal injection of low-dose streptozotocin. Rats were divided into three groups: normal control group, diabetes group, and diabetes + sitagliptin group. Body weight, glycemic parameters, lipid profiles, fasting insulin (FINS) and serum RBP4 levels were assessed at baseline and after 6 weeks of therapy. Western blotting was used to detect the tissue RBP4 and GLUT4 expression levels. After treatment for 6 weeks, the diabetes + sitagliptin group displayed significantly improve levels of blood sugar, blood grease, and insulin sensitizing functions ($P < 0.05$) than the diabetes group. Sitagliptin markedly down regulated RBP4 expression levels and up-regulated GLUT4 expression levels in adipose tissue and skeletal muscle. The results indicate that sitagliptin can modulate the RBP4-GLUT4 system in adipose tissue and skeletal muscle. Modulation of the RBP4-GLUT4 system may be one of the mechanisms by which sitagliptin ameliorates the symptoms of type 2 diabetes mellitus.

Keywords: Sitagliptin, type 2 diabetes mellitus, RBP4, GLUT4

Introduction

Type 2 diabetes mellitus (T2DM) affects 285 million people worldwide, with the number of cases predicted to rise by 54% over the next 20 years [1]. Increased adipose tissue mass is strongly associated with the pathogenesis of T2DM [2]. Adipose tissue may be viewed as an endocrine organ that secretes many types of adipokines (such as visfatin, leptin, interleukin 6, and adiponectin) that modulate the action of insulin in other tissues [3-6]. Moreover, studies on adipose-specific glucose transporter type 4 (GLUT4) knockout mice have revealed that retinol-binding protein 4 (RBP4) provides a link between obesity and insulin resistance [7-10].

A major cause of type 2 diabetes is impaired insulin action in adipose tissue, skeletal muscle, and liver. Even in the absence of diabetes, insulin resistance is a major risk factor for early mortality in cardiovascular disease [11]. Resistance to insulin-stimulated glucose transport in adipose tissue and skeletal muscle is one of the earliest defects detected in patients with insulin-resistant status [12]. Transport of glucose by GLUT4 is the rate-limiting step in glucose use by muscle and adipose tissue [12]. With the development of insulin resistance, GLUT4 expression is down regulated in adipose tissue [12, 13]. Down-regulation of GLUT4 expression is an almost universal factor in diseases with insulin-resistant status, including obesity, T2DM, and metabolic syndrome [12].

In T2DM patients, the effect of glucose-dependent insulinotropic polypeptide (GIP) as well as the secretion of glucagon-like peptide-1 (GLP-1) are diminished or absent, contributing to insulin secretion deficiency [14]. These two incretins are secreted by the intestine [15] and stimulate insulin secretion by β-cells in a glucose-depen-

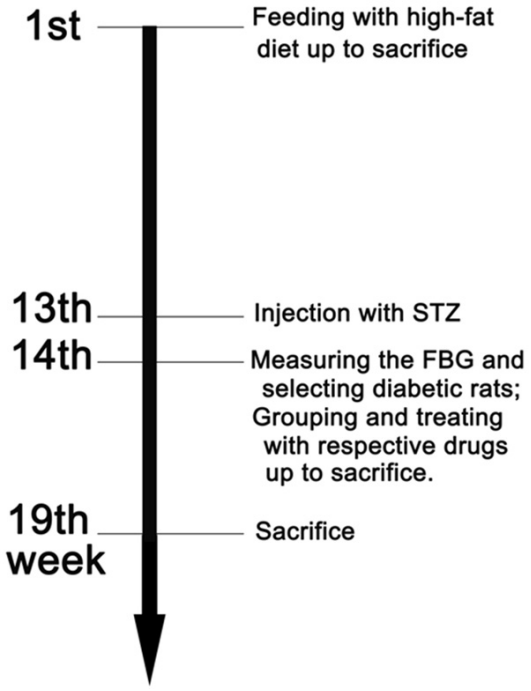

Figure 1. Schematic diagram of the induction and treatment schedule of diabetic rats.

dent manner [16], preventing hyperglycemia. In animal models, continuous infusion of GLP-1 or injection of long-acting GLP-1 mimetics, such as exendin-4, has shown remarkable glucose-lowering efficacy, together with an ability to increase β-cell neogenesis, and reduce apoptosis and alpha-cell glucagon secretion [17-19]. Despite the beneficial actions of GLP-1 and GIP, their use as antidiabetic agents (mimetics) is impractical owing to their short half-lives as a result of their rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) [20, 21]. Thus, orally administered DPP-IV inhibitors have emerged as a new class of anti-hyperglycemic agents with the ability to extend the biological effects of incretin hormones through the inhibition of their degradation [22, 23], along with the advantage of higher stability and bioavailability when compared with mimetics.

Sitagliptin, an orally available DPP-IV inhibitor that was developed to be used as a once-daily treatment for T2DM, has shown beneficial effects on glycemic control, reduction in HbA1c levels, and prevention of hypoglycemia, as well as on islet mass and function, with no relevant adverse effects [24, 25]. Considering the vast range of physiological actions promoted by the incretins, related not only to the control of glucose levels through insulin and glucagon regulation, but also to peripheral insulin sensitization, cardiac and neuronal protection and β-cell preservation, the use of an incretin enhancer (such as sitagliptin) may present beneficial effects on diabetes pathophysiology and on prevention of its serious complications. This deserves better elucidation. However, the effects of sitagliptin on RBP4 expression remain poorly understood.

In this context, we evaluated the effects of sitagliptin on RBP4 and GLUT4 expression levels in diabetic rats. We also evaluated the effects of sitagliptin on glycemic control, insulin resistance, insulin secretion, and lipid profiles in diabetic rats.

Materials and methods

Animals and diets

Thirty-five male Sprague Dawley rats (100±20 g; 4 weeks old) were purchased from the Experimental Animal Center of Anhui Medical University (China Hefei). The animals were housed in standard cages and maintained under controlled room temperature (22±2°C) and humidity with a 12/12-hour light-dark cycle. The rats had free access to water and rodent chow, either standard rat chow (formulated by the Experimental Animal Center of Anhui Medical University) or a high-fat diet (ingredients: 55% normal diet, 12% lard, 5% sucrose, 8% milk, 5% peanut, 10% egg, 3% sesame oil, and 2% salt). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Anhui Medical University.

Induction of a diabetic model

The rats were divided into a normal diet group (NC, n = 10) and a high-fat diet group (HFD, n = 25) after one week's adaptation. The NC group was fed a normal diet until the end of the experiment; the HFD group was given high-fat diet until the end of the experiment. After being fed on a high-fat diet for 12 weeks, the HFD group was injected peritoneally with a low dose (35

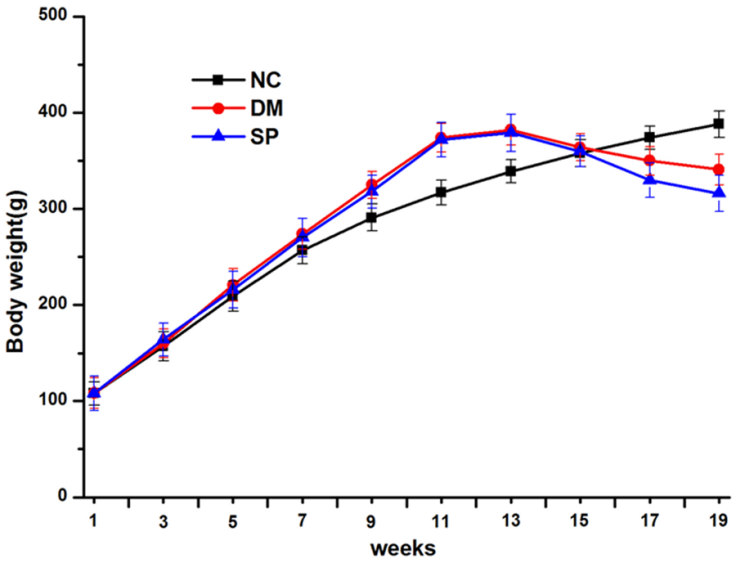

Figure 2. Effects of sitagliptin on body weight in diabetic rats.

mg/kg) of streptozotocin (STZ, Sigma-Aldrich (Shanghai) Trading Co, Ltd, Shanghai, China). One week later, fasting blood glucose levels were tested and rats with fasting blood glucose ≥ 11.1 mmol/L were chosen as T2DM models (n = 20) [26].

Treatment protocol

The 20 diabetic rats were randomly divided into two groups: a diabetes group (DM, n = 10) and a sitagliptin intervention group (SP, n = 10). The SP group was treated with sitagliptin (10 mg/kg/day); the sitagliptin was dissolved in 2 ml of sodium carboxymethylcellulose and administered intragastrically once daily for 6 weeks. The NC group and the DM group were administered an equivalent volume of sodium carboxymethylcellulose solution. A schematic diagram of the induction and treatment schedule is shown in **Figure 1**. The rats' body weight was measured every 2 weeks, and fasting blood glucose (FBG), triglyceride (TG), total cholesterol (TCH), low-density lipoprotein cholesterol (LDL-C), fasting insulin (FINS), and serum RBP4 levels were measured before and after 6 weeks of treatment.

Specimen collection and processing

Blood was collected from the tail vein at the baseline and from the inferior vena cava at the end of treatment. The blood samples were kept at room temperature for 5 minutes to coagulate. Subsequently, the serum was obtained from the coagulated blood by centrifugation at 1600 × g at 4°C for 15 min. The liver, epididymal adipose, and skeletal muscle were collected and stored in a liquid nitrogen container for later analysis.

Blood biochemical examination

Serum concentrations of FBG, TG, TCH, and LDL-C were measured using an automatic blood chemistry analyzer (Roche Modular DPP, Basel, Switzerland). FINS was determined using a radioimmunoassay kit (Beijing Atomic Tech Company, Beijing, China) and serum RBP4 was determined using an enzyme-linked-immunosorbent assay kit (Bio-Technology Co., Ltd. Shanghai Yuanye, Shanghai, China). Insulin resistance and β-cell function were evaluated by the homeostasis model assessment (HOMA) method; in particular, we used the homeostasis model assessment insulin resistance index (HOMA-IR) and the homeostasis model assessment β-cell function index (HOMA-β).

Western blot analysis

The tissue samples were added to the tissue lysate solution (Tris-HCl, pH 7.14, 150 mmol /L NaCl, 1 mmol/L EDTA, 1 % Triton, 0.1% SDS, 5 mg/ml Leupeptin, 1 mmol/L PMSF) at a ratio of 1:20. The lysates were centrifuged at 14000 × g at 4°C for 30 min. The protein concentrations of the samples were measured by Micro-BCA Protein Assay Reagent Kit (Pierce, USA). Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the separated proteins were transferred to a polyvinylidene fluoride (PVDF, Beyotime Biotechnology, Jiangsu, China) membrane and treated with 5% non-fat milk in TPBS (phosphate-buffered saline containing 0.05% Tween 20) for 2 h to block any non-specific binding sites. The PVDF membranes were incubated with primary antibodies overnight at 4°C. After washing three times (for 10 min each time) with TPBS, the PVDF membranes were then incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary anti-

Table 1. Comparison of metabolic parameters and RBP4 levels among the three groups before intervention (mean ± SD)

Group	NC (n = 10)	DM (n = 10)	SP (n = 10)
TG (mmol/l)	0.43±0.07	1.43±0.44*	1.43±0.50*
TCH (mmol/l)	1.78±0.59	2.71±0.48*	2.82±0.62*
LDL-C (mmol/l)	0.92±0.30	2.21±0.27*	2.28±0.73*
FBG (mmol/l)	5.39±0.84	12.74±0.98*	12.44±2.28*
FINS (mI U/L)	7.10±0.84	14.88±2.66*	14.65±1.60*
HOAM-IR	1.70±0.34	8.43±1.63*	8.09±1.67*
HOAM-β	88.40±36.22	32.52±6.60*	34.57±7.39*
RBP4 (umol/l)	30.97±5.59	49.27±8.17*	48.72±6.95*

*P < 0.05 vs. NC group. NC: Normal control; DM: diabetes group; SP: sitagliptin intervention group.

Table 2. Comparison of metabolic parameters and RBP4 levels among the three groups after intervention (mean ± SD)

Group	NC (n = 10)	DM (n = 10)	SP (n = 10)
TG (mmol/l)	0.50±0.15	1.51±0.30*	0.55±0.03#
TCH (mmol/l)	1.70±0.66	2.76±0.39*	2.07±0.58#,*
LDL-C (mmol/l)	0.90±0.25	2.22±0.26*	1.37±0.46#,*
FBG (mmol/l)	5.00±0.84	13.36±2.07*	8.09±1.12#,*
FINS (mI U/L)	6.45±0.74	14.87±2.92*	10.02±0.37#,*
HOAM-IR	1.44±0.24	8.88±2.55*	3.61±0.54#,*
HOAM-β	90.21±18.75	31.08±8.11*	46.54±13.71#,*
RBP4 (umol/l)	31.31±4.14	52.84±8.70*	40.85±5.33#,*

*P < 0.05 vs. NC group, #P < 0.05 vs. DM group. NC: Normal control; DM: diabetes group; SP: sitagliptin intervention group.

bodies at a 1:4000 dilution and then stained with enhanced chemiluminescence reagents (Pierce, USA).

Statistical analysis

SPSS 17.0 software was used to analyze the data. Data were described as mean ± standard deviation. All comparisons were conducted by ANOVA (one-way analysis of variance). P < 0.05 was considered statistically significant.

Results

Body weight

As shown in **Figure 2**, the body weight of the NC group increased during the study. The body weight of the rats fed the high-fat diet was higher than that of the normal control rats before the injection of STZ. However, the body weight of the DM group was significantly lower than that of the normal control group after injection of STZ. Sitagliptin did not significantly alter the body weight in diabetic rats during the 6 weeks of treatment compared with the DM group.

Biochemical analysis

In order to evaluate the possible side effects of sitagliptin, we measured the basic biochemical parameters of rats before sitagliptin treatment. As shown in **Table 1**, the FBG, TG, TCH, LDL-C, FINS, and RBP4 levels, and HOMA-IR of each group in the diabetic model were significantly higher than those in the NC group, while the HOMA-β in each group in the diabetic model was significantly lower than that in the NC group (P < 0.05). There was no statistically significant difference between the DM group and the SP group. After 6 weeks of treatment, FBG, TG, TCH, LDL-C, FINS, and BP4 levels, and HOMA-IR in the SP group were significantly lower compared with those in the DM group, while HOMA-β was significantly higher compared with that in the DM group (**Table 2**).

Effects of sitagliptin treatment on RBP4 expression levels in different tissues

As shown in **Figure 3B**, the level of RBP4 expression in the adipose tissue of the DM group was higher than that in the NC group, which was also observed in the skeletal muscle (**Figure 3C**). After the diabetic rats had been treated for 6 weeks, the levels of RBP4 expression in the adipose tissue and skeletal muscle of the SP group were lower compared with those of the DM group, especially in the adipose tissue (**Figure 3B** and **3C**). However, there were no significant differences in the levels of RBP4 expression in the liver between the three groups (**Figure 3A**). **Figure 3D** shows the statistical analysis of RBP4 levels in the three tissues.

Effects of sitagliptin on GLUT4 expression levels in different tissues

The levels of expression of GLUT4 in the adipose tissue and skeletal muscle were lower in

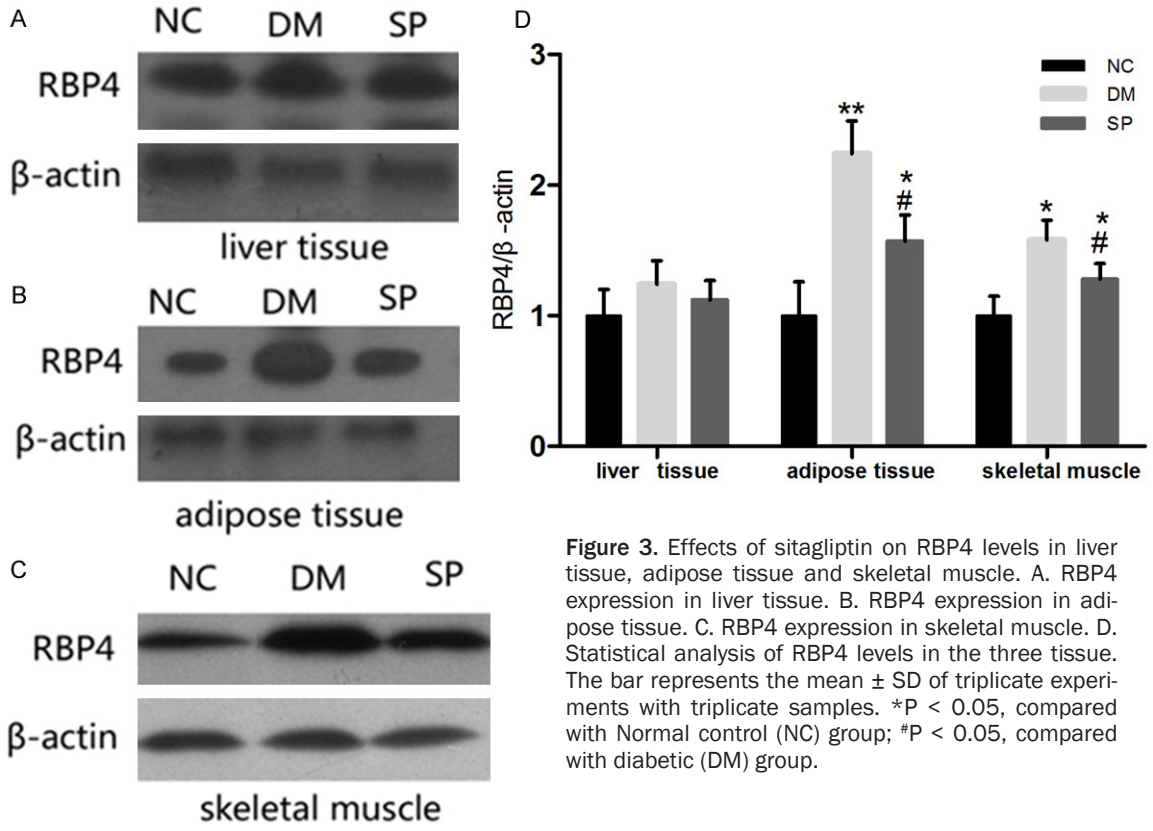

Figure 3. Effects of sitagliptin on RBP4 levels in liver tissue, adipose tissue and skeletal muscle. A. RBP4 expression in liver tissue. B. RBP4 expression in adipose tissue. C. RBP4 expression in skeletal muscle. D. Statistical analysis of RBP4 levels in the three tissue. The bar represents the mean ± SD of triplicate experiments with triplicate samples. *P < 0.05, compared with Normal control (NC) group; #P < 0.05, compared with diabetic (DM) group.

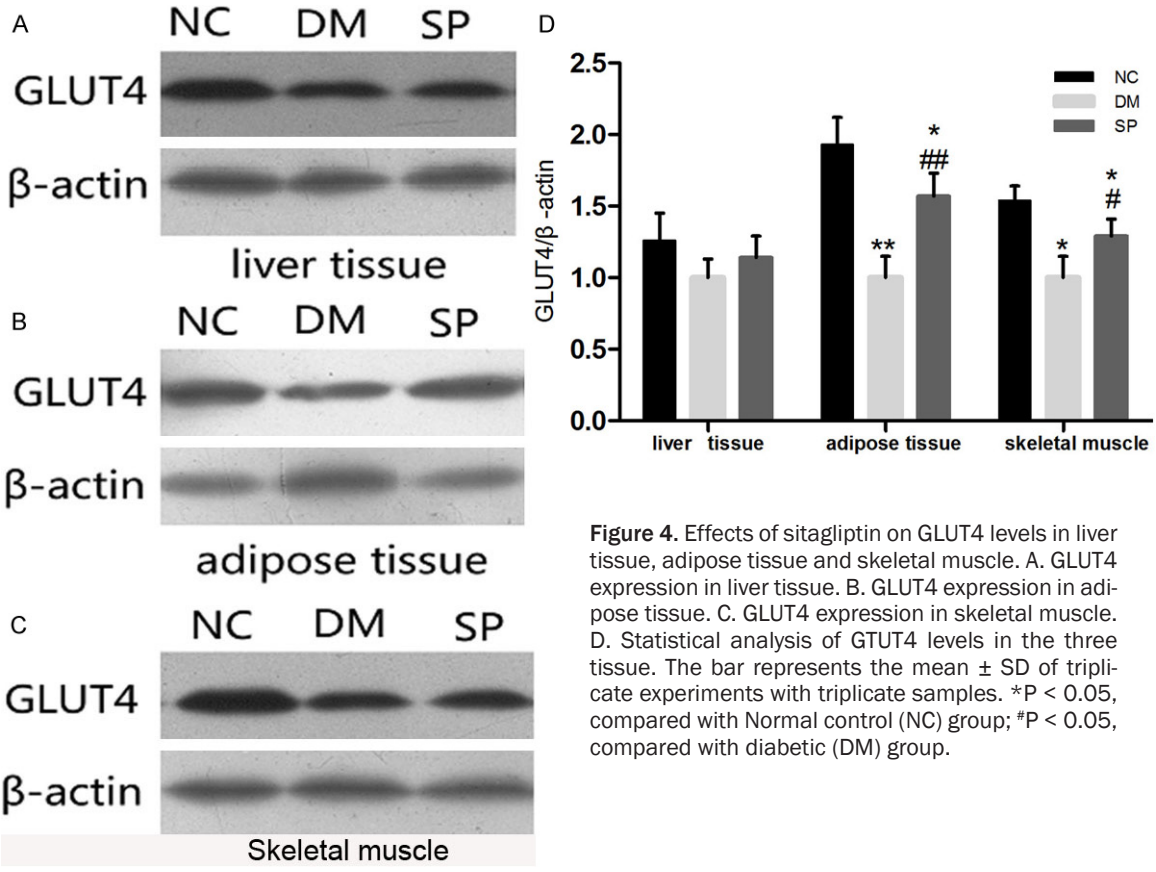

Figure 4. Effects of sitagliptin on GLUT4 levels in liver tissue, adipose tissue and skeletal muscle. A. GLUT4 expression in liver tissue. B. GLUT4 expression in adipose tissue. C. GLUT4 expression in skeletal muscle. D. Statistical analysis of GTUT4 levels in the three tissue. The bar represents the mean ± SD of triplicate experiments with triplicate samples. *P < 0.05, compared with Normal control (NC) group; #P < 0.05, compared with diabetic (DM) group.

the DM group than in the NC group (**Figure 4B** and **4C**). After the rats has been treated for 6 weeks, the GLUT4 expression levels in the adipose tissue and skeletal muscle of the SP group were higher compared with those in the DM group (**Figure 4B** and **4C**). There were no significant differences in the GLTU4 expression levels in the liver between the three groups, similar to what was observed for the RBP4 expression levels in the liver (**Figure 4A**). **Figure 4D** shows the statistical analysis of the GLUT4 levels in the three tissues.

Discussion

The present study demonstrated that sitagliptin could decrease RBP4 expression levels in a rat model of type 2 diabetes. The key findings of this study include: (A) Sitagliptin can downregulate RBP4 levels in serum, adipose tissue and skeletal muscle; (B) Sitagliptin can up-regulate GLUT4 levels in adipose tissue and skeletal muscle; and (C) Sitagliptin can significantly improve insulin sensitivity and glucose and lipid metabolism.

Many studies have reported that long-term high-fat diets lead to insulin resistance and hyperinsulinemia, and under the strain of compensatory hyperinsulinemia, the β-cells are easily damaged by low doses of STZ [27, 28]. In other words, the high-fat diet combined with low doses of STZ induced the onset of the characteristics of later-stage T2DM in diabetic rats, including hyperglycemia, insulin resistance, moderate impairment of insulin secretion, and abnormalities in lipid metabolism [29]. In the present study, we induced diabetes in rats by feeding them with a high-fat diet for 12 weeks and injecting them with low doses of STZ to mimic the diabetic model, and then investigated the antidiabetic effects and potential mechanisms of sitagliptin in this model.

RBP4 is a serum protein that is mainly synthesized in the liver, adipose tissue, and skeletal muscle [30, 31]. The physiological role of RBP4 is to transport retinol (vitamin A) from the liver to extrahepatic tissues to support many crucial biological functions [32]. RBP4 gained much clinical attention after the early notion that it played an important role in mediating adipose tissue communication with other insulin target tissues in rodent models of insulin resistance and diabetes. Clinical investigations have revealed a positive correlation between circulating RPB4 levels and the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, and type 2 diabetes [8, 10, 33]. Furthermore, the severity of glucose intolerance in women with previous gestational diabetes is also associated with high RBP4 concentrations [34]. Injection of RBP4 or transgenic overexpression in mice impairs insulin signaling in skeletal muscle and induces the expression of gluconeogenic enzymes in the liver [7]. Thus, RBP4 has been recognized as an adipokine that is positively correlated with metabolic syndrome, and a risk factor for type 2 diabetes [35].

In our study, we noted significantly higher RBP4 levels in diabetic rats compared with the controls. We found that the levels of expression of RBP4 in the adipose tissue and skeletal muscle of the DM group were higher than those of the NC group, especially in the adipose tissue. This indicates that the increased serum RBP4 level mainly comes from the adipose tissue in diabetic rats. These results present the suggestion that RBP4 might be an effective predictive biomarker for insulin resistance and T2DM, and lowering RBP4 levels may be an effective strategy for the prevention and treatment of T2DM.

It is well known that uptake of glucose into cells depends on the assistance of membrane GLUT4, and decreased expression of muscle and adipose GLUT4 in diabetic individuals is one of the most important causes of insulin resistance [12]. In our study, we also observed that the expression of GLUT4 levels in the adipose tissue and skeletal muscle of the DM group were lower than those of the NC group. We can therefore deduce that drugs probably up-regulate the expression of GLUT4 in peripheral tissues, which can eventually improve the insulin activity in peripheral tissues and enhance the uptake and utilization of glucose. However, the effect of hypoglycemic treatment on the RBP4-GLUT4 system has not been thoroughly studied until now. Thus, the effect of sitagliptin treatment on the expression of RBP4 and GLUT4 was observed in our study.

There is limited data about the effect of sitagliptin on the RBP4-GLUT4 system. Derosa et al. [36] observed a decrease in serum RBP4 levels with a combination of sitagliptin + metformin compared to placebo + metformin. A

prior study [37] found that another DPP-4 inhibitor, vildagliptin, also reduced serum RBP4 levels in patients with type 2 diabetes. In type 2 diabetic rats, GLP-1 treatment significantly increased adipose tissue and skeletal muscle levels of GLUT4 expression in diabetic rats [38].

In the present study, we observed for the first time that sitagliptin significantly modulates the RBP4-GLUT4 system in serum, adipose tissue, and skeletal muscle. There is one question about how sitagliptin affects the RBP4-GLU4 system in the adipose tissue and skeletal muscle. It was reported that the administration of MCP-1 strongly reduced the expression of GLUT4 and the ability for insulin-stimulated glucose uptake in 3T3-L1 adipocytes [39]. Another study has shown that sitagliptin treatment significantly decreased MCP-1 expression in diabetic rats [40]. Thus, a decrease in the expression of MCP-1 by sitagliptin can contribute to the inhibition of the down-regulation of GLUT4 expression. There is another possibility for the regulation of GLUT4 expression. AMPK activation stimulates glucose transport through increased GLUT4 translocation in adipocytes [41]. A prior study demonstrated that sitagliptin activates AMPK in adipocytes [42]. Based on these reports, it is speculated that downregulation of MCP-1 by sitagliptin blocks the down-regulation of GLUT4 gene expression and activation of AMPK by sitagliptin may contribute to the increase in the protein expression of GLUT4. These significant changes of GLUT4 may modulate the expression of RBP4, but we did not see significant changes in the levels of RBP4 expression or GLUT4 expression in the three groups. More experiments are required to investigate the effect of sitagliptin on the RBP4-GLUT4 system and the mechanism through which sitagliptin affects the RBP4-GLUT4 system.

In our study, we found the body weight of rats fed with the high-fat diet was higher than that of the NC rats; however, their body weight was reduced to significantly less than the NC rats after injection with STZ. Treatment with sitagliptin did not alter the body weight significantly compared to the DM group. These data are in line with a previous report that DPP-4 inhibitors have a neutral effect on body weight [43].

In the present study, the blood glucose level of diabetic rats was significantly decreased when treated with sitagliptin. Furthermore, we also observed a larger improvement of fasting measures of FINS, HOMA-β, and HOMA-IR after treatment with sitagliptin. Regarding the lipid profiles, we observed that sitagliptin appears to have a beneficial effect on TG, TC, and LDL-C levels. Our results are quite similar to those reported by Sakamoto et al, who reported sitagliptin to be a suitable agent for use in the comprehensive treatment of patients with T2DM because it improves not only glycemic control, but also blood pressure and lipid profiles [44]. However, some reports have shown sitagliptin to have no effect on TG, TC, LDL-C, or high-density lipoprotein cholesterol levels in obese mice [45, 46]. Therefore, the definitive effect of the DPP-4 inhibitor sitagliptin on the blood lipid profiles of mice remains contentious.

The limitations of our study included: (1) Our sample size was limited; (2) We did not record if the improvement of metabolic parameters and the modulation of the RBP4-GLUT4 system were maintained after the sitagliptin treatment was over; and (3) We did not observe the effects of treatment on diabetic rats with different doses of sitagliptin. However, to the best of our knowledge, this is the first study evaluating the effects of sitagliptin treatment on RBP4 and GLUT4 expression levels in different tissues.

Conclusion

In conclusion, this investigation has demonstrated that sitagliptin leads to the down-regulation of RBP4 expression in serum, adipose tissue, and skeletal muscle, and the up-regulation of GLUT4 expression in adipose tissue and skeletal muscle, as well as the improvement of glycemic and lipid profiles. Modulation of the RBP4-GLUT4 system may be one of the mechanisms by which sitagliptin ameliorates the symptoms of T2DM.

Acknowledgements

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Disclosure of conflict of interest

None.

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