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ORIGINAL RESEARCH

ZG02 Improved Hepatic Glucose Metabolism and Insulin Sensitivity via Activation of AMPK/Sirt1 Signaling Pathways in a High-fat Diet/Streptozotocin-induced Type 2 Diabetes Model

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Purpose: The aim of the present study was to investigate the hypoglycemic activity and potential mechanism of tetrahydrocarbazole derivatives ZG02 in high-fat diet/streptozotocin-induced type 2 diabetes model.

Methods: C57BL/6 mice (n=30) were randomly assigned to three groups: control group (n=10) was fed with normal diet, the diabetes group (n=10) was fed with high-fat diet for eight weeks followed by intraperitoneal injection of streptozotocin (25 mg/kg) and the ZG02 group (n=10) injected intraperitoneally with ZG02 (30 mg/kg/day) for two weeks after successful modeling. The changes of weight, fasting blood glucose, oral glucose tolerance and fasting blood insulin levels in each group were evaluated. In addition, we also assessed the expression level of total AMPK, phosphorylation AMPK, *SIRT1*, PGC-1 and the activity of *G6PC* in liver.

Results: The results demonstrated that ZG02 could significantly antagonize the high-fat diet/streptozotocin-induced fasting hyperglycemia, restore fasting blood insulin levels and also improve activity of *G6PC* in liver. The results from Western blot indicated that ZG02 significantly restored the expression level of phosphorylation AMPK, Sirt1 and PGC-1.

Conclusion: ZG02 improve hepatic glucose metabolism and insulin sensitivity via activation AMPK/Sirt1 signaling pathways in type 2 diabetes mice model.

Keywords: type 2 diabetes, glucose metabolism, ZG02, AMPK, high fat

Introduction

Type 2 diabetes mellitus (T2DM)[1] is a chronic metabolic disease,[2,3] mainly characterized by relative insulin deficiency caused by pancreatic β-cell dysfunction and insulin resistance in target organs.[4] T2DM is the leading cause of morbidity and mortality worldwide and a major economic burden.[5] The prevalence of T2DM is increasing year by year due to the global rising tide of obesity, physical inactivity, and caloric excess.[6] Recently, a survey estimated that there will be more than 700 million people (aged -18–99 years) suffering from diabetes in nearly all countries by the year 2045.[7] Currently, the majority of oral hypoglycemic drugs, such as sulfonylureas[8] (SUs), meglitinides[9] (glinides), thiazolidinediones[10] (TZDs), α-glucosidase inhibitors,[11] glucagon-like peptide (GLP)-1 receptor (GLP-1R) agonists,[12] dipeptidyl peptidase (DPP)-4 inhibitors,[13] and sodium glucose transporter[14] (SGL). Although treatment of diabetes has a hypoglycemic effect, it has demonstrated side effects and adverse

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reactions. Hence, treatment of diabetes used for the major research areas is discovering and developing alternate drugs with fewer side effects.

The liver is a particularly important metabolic organ for controlling blood glucose and ~90% of endogenous glucose is produced by the hepatic system.[15] It can maintain blood glucose balance by regulating various pathways such as gluconeogenesis,[16,17] glycogenolysis,[18,19] glycogen synthesis and glycolysis.[20] Numerous studies show that gluconeogenesis contributes approximately half of the total hepatic glucose production (HGP) in humans following an overnight fast and inhibition of hepatic gluconeogenesis can significantly improve blood glucose in individuals with T2DM.[21–23] Our previous work has revealed that a tetrahydrocarbazole derivatives compound ZG02[24,25] (Figure 1) improves glucose metabolism via inhibition of gluconeogenesis in HepG2 cells.[26,27] Nevertheless, the antidiabetic effect of ZG02 in vivo and its potential underlying mechanisms is still unclear. Consequently, this study attempted to demonstrate the potential of the antidiabetic effects of ZG02 and their underlying mechanisms in high-fat diet/streptozotocin (STZ)-induced T2DM mice.

Materials and Methods

Materials

Streptozotocin (S0130, 500 mg) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Primary antibodies to AMPK (ab131512), p-AMPK (ab45174), Sirt1 (ab110304), and PGC-1α (ab191838) were purchased from Abcam (Cambridge Science Park, Cambridge, UK). Anti-rabbit IgG (no. 5151) and anti-mouse IgG (no. 5257) were purchased from CST (Cell Signaling Technology, Inc., Danvers, MA, USA). Mouse insulin (INS) ELISA kit (CSB-E05071m) was purchased from CUSABIO (Wuhan, China). Compound ZG02 (Figure 1) was synthesized in our lab.

Animals

Five-week-old male C57BL/6 mice (20±2 g) were supplied by the Experimental Animal Centre of Guizhou Medical University (permissions SYXK, 2019–0001). All the mice were fed with standard food and water under constant environment with 25±2°C and 12/12 h light/dark cycle (light on at 08:00 am) and adaptively raised for a week before experiments. All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China.

Diet/Streptozotocin-Induced Type 2 Diabetes Model

After acclimation for a week, C57BL/6 mice were randomly separated into two groups. Control group (n=10) was fed with normal diet containing 10% kcal fat and the model group (n=20) was fed with high-fat diet consisting of 60% kcal fat (D12492, Research diets, BioPike, China). For the establishment of a C57BL/6 mice model of T2DM, streptozotocin

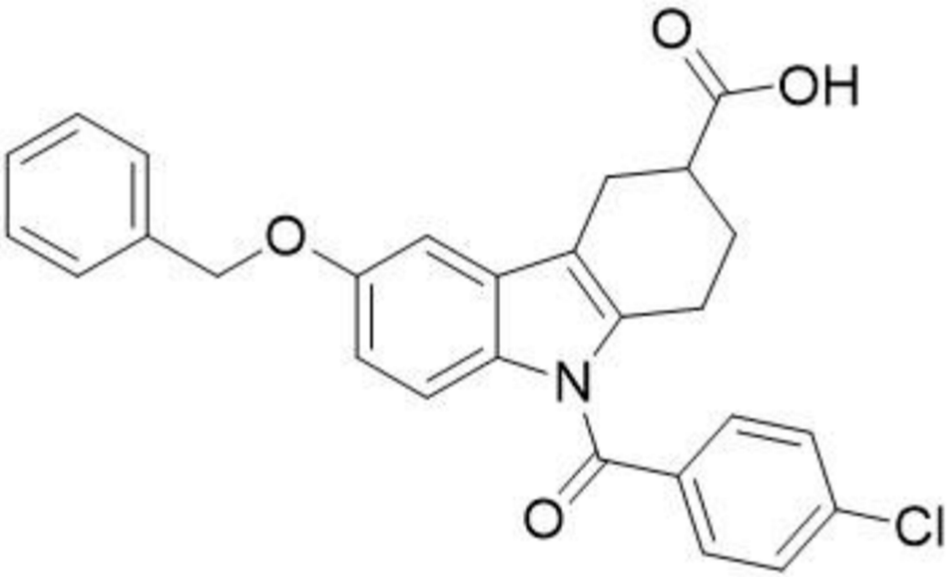

Figure 1 ZG02 chemical formula.

(STZ) (25 mg/kg^{-1} dissolved in 100 mM citrate buffer (pH 4.4), once every other day, four times) was injected intraperitoneally (ip) after eight weeks of high-fat diet. The control group were given an identical volume of vehicle citrate buffer. At four weeks after STZ injection, the successfully established animal models (fasting blood glucose ≥200 mg/dL or ≥11.1 mmol/mL) were randomly divided into the diabetic group (n=10) and ZG02 group (n=10). The ZG02 group was administered ZG02 (30 mg/kg/day) by ip injection once a day for 14 days. The other group was concurrently administered an equivalent dose of sterile water. Diabetic group and ZG02 group continued to be fed the high-fat diet. The experimental procedure is shown in Figure 2.

Blood Glucose and Oral Glucose Tolerance Tests

For measurement of fasting blood glucose (FBG) levels, mice were fasted overnight for 16 h and FBG levels were obtained from a small drop of blood from tail snip using a Glucometer (Yuwell, Jiangsu, China). The oral glucose tolerance test (OGTT) was performed by glucose gavage with 50% 2 g·kg^{-1} at 30 min after mice fasted overnight for 16 h. The blood glucose levels were determined at 0.5, 1, or 2 h after glucose loading, and OGTT was expressed by the area under curve (AUC).

ELISA Determination of Insulin

For measurement of fasting blood insulin levels, mice were fasted overnight for 16 h and blood was collected from the caudal vein. Plasma insulin levels were detected by insulin ELISA kit (CUSABIO, Wuhan, China) according to kit instructions. The Homeostasis Model Assessment of IR (HOMA-IR) has proved to be a robust tool for the surrogate assessment of insulin resistance.[28] HOMA-IR can be calculated with the following formula: HOMA-IR index=fasting glucose (mmol/L)×fasting insulin (mU/L)/22.5.[29,30]

Western Blotting

The liver tissues were homogenized in RIPA lysis buffer and the sample maintained on ice for 60 min. The homogenate was collected and centrifuged at 12,000×g for 30 min at 4°C. The final supernatant was stored at −80°C until further use. The protein concentrations of the supernatants were analyzed by the BCA protein assay kit (Beyotime).

The supernatants were diluted to 40 μg/lane with sample buffer and heated at 95°C for five minutes. The protein mixtures were loaded on a SDS-PAGE gel. The separated proteins were transferred to the PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked by 5% nonfat milk in TBST for two hours, followed by probing with primary antibodies at 4°C overnight in 2% nonfat milk-TBST: anti-AMPK (1:2000), anti-p-AMPK (1:3000), anti-Sirt1 (1:1000), anti-PGC-1α (1:1000). Subsequently, the blots were incubated with anti-rabbit IgG or anti-mouse IgG for one hour at room temperature. The immunoreactivity was detected with infrared fluorescence and images captured by Odyssey Imagers (LI-COR Biosciences, USA). The relative quantity of protein expression was analyzed by the software Image J and rectified by the reference protein β-actin.

Statistical Analysis

All the results are expressed as the mean ±SD. Inter-group statistical significance was determined by one-way ANOVA using STATA 14.0 (StataCorp, College Station, TX, USA) with a statistical significance set at $p<0.05$.

Results

Effects of ZG02 on Body Weight

The results showed that the weight was remarkably increased in diabetes group ($p<0.01$) compared to the control group. ZG02 treatment significantly restored the weight level compared to the diabetes group ($p<0.05$). The weight of each group is shown in Figure 3A.

Effects of ZG02 on FBG

The FBG levels of each group are shown in Figure 3B. Compared with control group, the levels of FBG were significantly higher in the diabetes group ($p<0.05$). ZG02 treatment could decrease the FBG levels compared to the diabetes group.

Effects of ZG02 on OGTT

The OGTT was performed after an overnight fast (16 h) at week 15 of the experimentation. Following oral administration of glucose, blood glucose levels reached a maximum

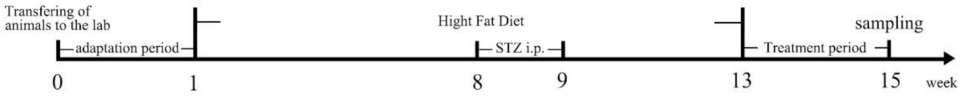

Figure 2 The experimental procedure.

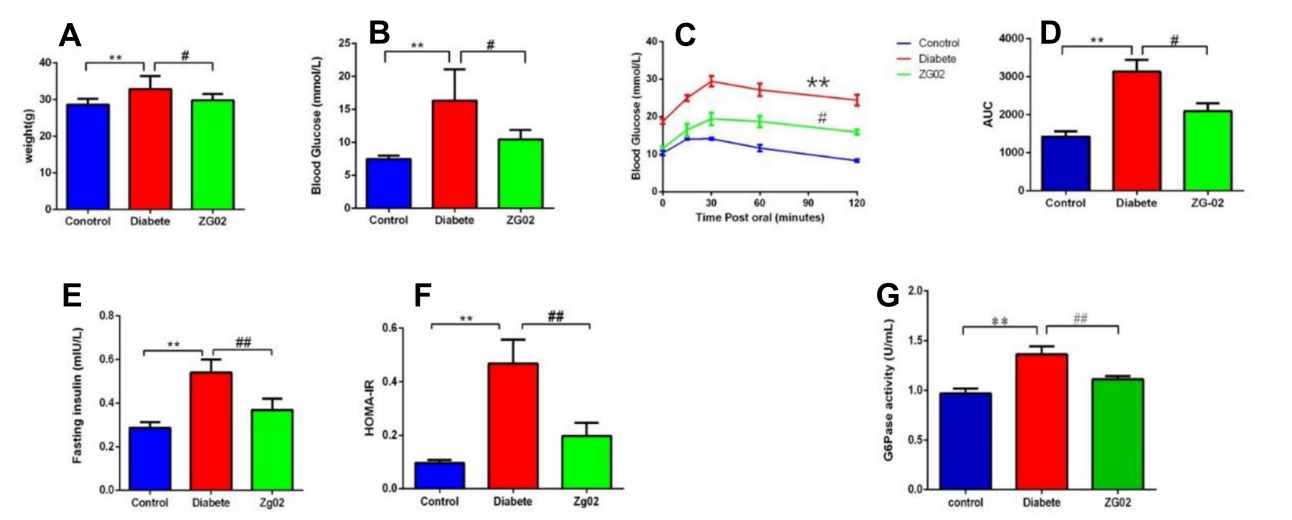

Figure 3 (**A**) The levels of weight in each group. (**B**) The levels of FBG in each group. (**C**) The levels of OGTT in each group. (**D**) The AUC values of each group. (**E**) The levels of blood insulin in each group. (**F**) The levels of HOMA-IR in each group. *p<0.01 vs control group; **p<0.05 vs diabetes group. (**G**) The activity of *G6PC* in the liver in each group. *p<0.01 vs control group; ***p<0.01 vs diabetes group.

at 15–30 min postglucose gavage in the control group, and then gradually decreased. Blood glucose levels in diabetic mice were significantly higher than in the control group at 0, 15, 30, 60, and 120 min. After ZG02 treatment, the blood glucose levels in the ZG02 group were significantly lower than in the diabetes group at each time point (Figure 3C). The corresponding AUC profile in (Figure 3D) is shown in (Figure 3F). The AUC values of the diabetes group were obviously increase 220% compared with the control group. The AUC values of the ZG02 group were obviously reduced 67% compared with the diabetes group (Figure 3D).

Effects of ZG02 on Serum Insulin Levels and HOMA-IR

Compared with the control group, serum insulin levels were significantly increased in the diabetes groups (p<0.001). Serum insulin levels of the ZG02 group were reduce compared with the diabetes group (Figure 3E). Similarly, the HOMA-IR in diabetes group were significantly increased compared with the control group (p<0.001). After ZG02 treatment, the HOMA-IR in the ZG02 group was significantly lower (p<0.001) compared with the diabetes group (Figure 3F). These results indicated that ZG02 can ameliorate insulin resistance in diabetic mice.

Effects of ZG02 on the Activity of G6PC

Compared with the control group (Figure 3G), the activity of *G6PC* in the liver was significantly increased in the diabetes groups (p<0.001). After ZG02 treatment, the activity of *G6PC* in the ZG02 group was significantly lower than in the diabetes group (p<0.001).

Effects of ZG02 on the Levels of Protein of AMPK-*SIRT1*-PGC-1 Pathway

Compared with the control group (Figure 4A and B), the expression levels of phosphorylation AMPK, *SIRT1* and PGC-1 were significantly decreased in the diabetes groups (p<0.01, p<0.01, p<0.01). After ZG02 treatment, the expression levels of phosphorylation AMPK, *SIRT1*, FoxO1 and PGC-1 were significantly increased (p<0.01, p<0.01, p<0.01). The expression levels of total AMPK were not changed in each group.

Discussion

The aim of the present study was to assess the importance of tetrahydrocarbazole derivatives ZG02 in improving hepatic glucose metabolism and insulin sensitivity via activation AMPK/Sirt1/FoxO1/PGC-1α signaling pathways in mice on a high-fat diet with streptozotocin-induced type 2 diabetes.

AMPK (adenosine monophosphate-activated protein kinase) belongs to the RD (Arg-Asp) kinases.[31] It is known as a serine/threonine kinase that functions as an intracellular energy sensor and has been implicated in the modulation of glucose and fatty acid metabolism. AMPK plays an important role in maintaining energy homeostasis and adaptive response to energy stress. It

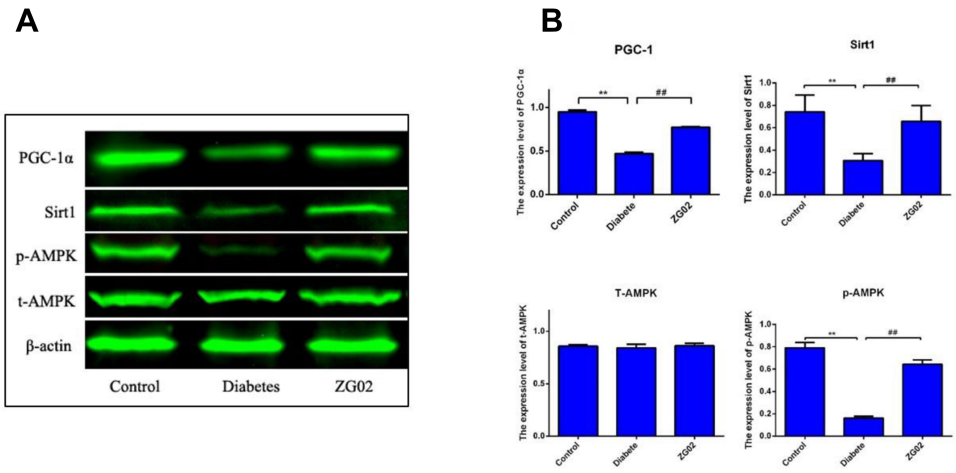

Figure 4 (**A**) Representative Western blot images of T-AMPK, p-AMPK, *SIRT1* and PGC-1 in each group; (**B**) Quantification of T-AMPK, p-AMPK, *SIRT1* and PGC-1 expression in each group. **p*<0.01 vs Control group; ***p*<0.01 vs diabetes group.

is activated in response to energy stress by sensing increases in AMP/ATP and ADP/ATP ratios and restores energy balance by inhibiting anabolic processes that consume ATP, while promoting catabolic processes that generate ATP.[32–35] Under conditions of low energy, AMPK phosphorylates specific enzymes and growth control nodes to increase ATP generation and decrease ATP consumption.The hydrolysis of ATP to ADP provides the energy for driving virtually all of the processes associated with living cells. Maintaining an adequate supply of energy is an essential requirement for survival.AMPK plays an important role in maintaining energy homeostasis in eukaryotic cells.[33,36] By activation of the AMPK, the pathway can improve the symptoms of type 2 diabetes, and currently metformin is accepted treatment for the syndrome caused by diabetes,[37–41] and enhances insulin sensitivity by a decrease in the activity of AMPK-mediated signaling.[42] The antidiabetic effect of the first-line antidiabetic medicine metformin is based on the activation of the AMPK system,[43] *SIRT1* is a NAD^+-dependent protein deacetylases/deacylases that is frequently overexpressed in a wide variety of mechanisms of biological metabolism processes,[44,45] phospho-AMPK can also lead to an increase in nicotinamide phosphoribosyltransferase (NAMPT) enzyme activity and thereby increasing NAD+/NADH ratio which in turn induces *SIRT1*.[46,47] *SIRT1* is a master repressor of inflammation in multiple organs including the liver. It was investigated whether liver *SIRT1* deficiency in C57BL/6 mice had any impact on the high-fat diet/streptozotocin-induced type 2 diabetes model responses.[48] *SIRT1* will act on the AMPK pathway, and then regulate the glycogen synthesis and gluconeogenesis,[49] PGC-1α (proliferator-activated receptor-γ coactivator 1α) is a direct substrate of *SIRT1*, maintained blood glucose concentration and insulin sensitivity by regulating the transcription of genes involved in glucose metabolism. Recent insight from different in vivo transgenic models clearly suggests that AMPK, *SIRT1* and PGC-1a might act as an orchestrated network to improve metabolic fitness, dysregulation of these pathways will lead to metabolic diseases such as type 2 diabetes and obesity.

The current study found suggest that ZG02 could reduce the level of FBG, increase insulin sensitivity, and inhibit the enzyme activity of *G6PC* in high-fat diet/streptozotocin-induced type 2 diabetes C57BL/6 mice in vivo. These significant effects of ZG02 were mediated by activation of the AMPK/*SIRT1*/PGC-1α signal pathway and the result was consistent with our previous study in HepG2 cells. In addition, a large number of studies also have confirmed the therapeutic role of AMPK/*SIRT1*/PGC-1 pathway in diabetes or its complications.

Taken together, our data demonstrate that ZG02 has a therapeutic potential for the treatment of type 2 diabetes can be attributed to its regulation of the activation of the AMPK/*SIRT1*/PGC-1α signaling pathway. These results will lay the foundation for future ZG02 into medicinal development.

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Disclosure

The authors report no conflicts of interest in this work.

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