ORIGINAL

Berberine ameliorates hyperglycemia in alloxan-induced diabetic C57BL/6 mice through activation of Akt signaling pathway

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Abstract. Recently, it is implicated that the abnormality of Akt signaling pathway is involved in the diabetic pathology. Previous studies have demonstrated that berberine could decrease blood glucose by elevating liver glycogen synthesis. However, the underlying mechanism is still unclear. In the present study, we investigated the effects of berberine on fasting blood glucose, liver glycogen, Akt, Glycogen synthase kinase-3, glucokinase and insulin receptor substrate (IRS) in alloxan-induced diabetic mice, exploring its possible hypoglycemic mechanism. We found that in alloxan-induced diabetic mice, the high blood glucose was significantly lowered by berberine treatment. Liver glycogen content, the expression and activity of glucokinase and the phosphorylated Akt and IRS were all significantly reduced in diabetic mice whereas berberine blocked these changes. Berberine also depressed the increasing of phosphorylated GSK-3β in diabetic mice. Collectively, Berberine upregulates the activity of Akt possibly via insulin signaling pathway, eventually lowering high blood glucose in alloxan-induced diabetic mice.

Key words: Diabetic mouse, Berberine, Akt signaling pathway, GSK-3β, Glucokinase

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DIABETES Mellitus (DM) is the worldwide-recognized metabolism syndrome with increasing morbidity and mortality. About 171 million people (2.8% of the population) suffer from diabetes [1]. Impairment of β-cell or insulin resistance is the major cause or character of all kinds of diabetes in which insulin-induced glucose disposal is defective, then, glucose output enhancement and hyperglycemia are found. Continuing hyperglycemia leads to severe diabetic complications and further impairs glucose metabolism such as dysfunction of insulin-stimulated glycogen synthesis and increased glucose output. Researches show that inducing glycogen synthesis is an important way to attenuate hyperglycemia [2, 3].

Recent studies have confirmed the implication of

Akt signaling pathway in glucose metabolism [4-6]. Serine/threonine kinase B (Akt), involved in various signal cascades, is a vital mediator to insulin-induced glucose and lipid metabolism. Under physiological condition, Akt can be activated by insulin and some growth factors. It is believed that insulin-stimulated Akt activation promotes glycogen synthesis *via* inactivation of glycogen synthase kinase-3β (GSK-3β). In addition, glucokinase, another downstream factor of Akt found recently is a rate-limiting enzyme to catalyze glycogen synthesis [7]. In hepatocytes, the activation of Akt increases glucokinase gene expression and glycogen synthesis to maintain the euglycemia [8]. Krook A et al revealed the possible role of Akt in DM [9]. The defect of Akt activity has been found in muscle biopsies from type 2 diabetes mellitus patients[10]. In addition, over-expression of Akt in endotheliocyte ameliorates vascular impairment caused by hyperglycaemia [11]. All those findings indicate that the depression of Akt pathway contributes to pathological progression of DM and its complications. It has been highlighted that the activation of Akt signal pathway is a potential strategy

in treatment of diabetes and its complications [12].

Berberine (BBR, $[C_{20}H_{18}NO_4]^+$, Fig. 1), one of the main constituents of *Coptidis rhizoma* and *Cortex phellodendri*, is an isoquinoline alkaloid with multiple pharmacological activities. BBR has exhibited hypoglycemic, hypolipidemic, anti-oxidation, antitumor and anti-aldose reductase effect, which presents wide clinical values and perspectives in treating diabetes and its complications [13-18]. Our previous studies demonstrated that BBR could efficiently ameliorate hyperglycaemia in streptozotocin-induced diabetic rats [19, 20]. However, the underlying hypoglycemic mechanism of BBR is not fully elucidated yet. In the present study, we aim to investigate the novel hypoglycemic mechanism of BBR by examining fasting plasma glucose, liver glycogen, Akt and its upstream factor IRS and downstream factors GSK-3β and glucokinase in alloxan-induced diabetic mice.

Methods

Chemicals and reagents

Alloxan was supplied by BoLi Biology Co. Ltd (Guangzhou, China). Berberine was purchased from Shanxi Scidoor Hi-tech Biology Co. Ltd (Batch number 20050220; Xi'An, China) and the purity of the compound was 95.13% by high-performance liquid chromatography. All primers involved in this study were synthesized by Sangon Biotech co. Ltd (Shanghai, China). Rabbit monoclonal anti-phosphorylated, total GSK-3β, phosphorylated Akt antibodies and GAPDH mouse monoclonal antibody were all from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat polyclonal anti-total Akt and rabbit polyclonal anti-glucokinase antibodies were both supplied by Santa Cruz Biotechnology Co. (CA, USA). Kits for glycogen and glucokinase measurement were supplied by Jiancheng Bioengineering Research Institute (Nanjing, China).

Healthy SPF male C57BL/6 mice, weighing 23±2g, were purchased from Center of Experimental Animals, Sun Yat-Sen University, Guangzhou, China. The animals were housed in barrier system and given free access to water and standard laboratory chow during study period. The animal experiments were carried out in accordance with the China Animal Welfare Act and ethics approval was obtained from the Sun Yat-Sen University Committee on Ethics in the Care and Use of Laboratory Animals. After fed for 3 days, 30 Fasted mice were injected intraperitoneally once in a
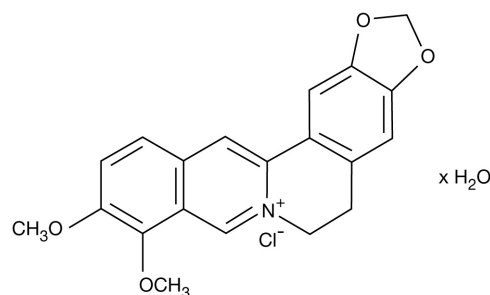

Fig. 1 Chemical structure of berberine (BBR)

single dose of 200 mg/kg alloxan (Sigma,USA) dissolved in Sodium Chloride buffer to induce diabetes. Blood glucose levels were measured 72 h after alloxan injection using a One-Touch glucose meter (Lifescan Co., USA). The mice with fasting blood glucose level of above 11.1 mM were considered diabetic and 16 of them were randomly divided into berberine treatment group and diabetic model group, 8 mice per group. Mice in berberine treatment group were orally administrated with 300 mg/kg berberine dissolved in CMC-Na daily between 8:30 and 9:30 am for 12 weeks, and diabetic model group were given the solvent by lavage. Healthy mice with normal blood glucose were put into normal control group and given the same volume of distilled water. The fasting blood glucose was determined by glucose meter (Lifescan, USA) at the end of 0, 4th, 8th, 12th week after model construction. All animals were sacrificed at the end of the 12th week. Mice liver samples were rapidly excised and frozen in liquid nitrogen, then stored in -80°C until analysis.

Blood glucose, liver glycogen and glucokinase activity

Fasting blood glucose was tested by ONETOUCH glucose meter (USA.), and the unit was represented as mM. Glycogen content was measured by anthrone assay, then colorimetry involved to evaluated the concentration of sample solutions compared to standard colour of glucose solution. One little piece of mouse liver was dissected and put into boiled concentrated alkaline solution leading to its degradation, then the mixed solution with color reagent diluted by distill water was boiled for 5 minutes. Absorbance was detected spectrophotometrically at the wavelength of 620 nm. The unit was presented by mg/g.

Glucokinase activity was detected by enzyme substrate method according to the manufacture's instruction. The unit was represented with U/g protein.

Real-time PCR assay

Real-time PCR was used to determine the gene expression of Akt, GSK-3β and glucokinase in mice livers. Total RNA was purified from livers using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed into single stranded DNA with AMV RNase Reverse Transcriptase XL and Random Primer (9mer) programmed as follows: 30°C, for 10 min, 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min for 1 cycle. The real-time PCR quantitative amplification with reagents SYBR Premix Ex Taq (TAKARA, Japan) enzyme and primers (As shown in Table 1) was performed on a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, US).

Western blotting assay

The protein levels of p-Akt, t-Akt, p-IRS, t-IRS, p-GSK-3β, t-GSK-3β and glucokinase were detected by western-blot analysis. Liver supernatant was loaded at 80μg (glucokinase at 40μg) protein/well and separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were electroblotted onto a PVDF membrane, and then washed in TBS-T buffer (0.1% Tween 20 in TBS) and incubated for 1 h under room temperature in 20 mL of blocking buffer containing 5% skimmed milk in TBS-T (150 mmol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.6) and incubated with antiphospho-Akt(1:1000, Cell Signaling, USA), antiphospho-IRS(1:700, Santa Cruz, USA), antiphospho-GSK-3β (1:1000, Cell Signaling, USA), or anti-glucokinase(1:800, Santa Cruz, USA) antibody in TBS-T containing 5% BSA buffer overnight at 4°C. After further washing, blots were incubated for 1~1.5h at room temperature with peroxidase conjugated goat anti-mouse/goat anti-rabbit IgG (1:10000, in TBS-T containing 5% BSA, Promega, USA). Membranes were washed again and membrane-bound antibody was detected by enhanced chemiluminescence (ECL) method with Supersignal West Pico hemiluminescent substrate (Pierce, USA) and captured on X-ray film. Following the phosphorylated proteins and glucokinase detection, blots were analyzed for equivalence of protein loading. Blots were incubated with 20 mL of blocking buffer for 1 h and then with anti-total Akt/GSK-3β/GAPDH antibody (1:1000, Cell Signaling, USA) or IRS (1:700, Santa Cruz, USA) in TBS-T containing 5% BSA overnight, and the following steps were taken as above. The densitometry assay was performed using UVP's Gel Documentation System GDS8000 and analyzed using Gel works LABWORK 4.0 Analysis Software.

Table 1 Primer sequences for Glucokinase, Akt and GSK-3β.

Gene	Primer Sequence 5' → 3'
Glucokinase	Forward: ACCGGCACTGCTGAGAT Reverse: CCCTTGGTCCAGTTGAGA
Akt	Forward: GAGCATCATCCCTGGGTTAC Reverse: CTCCTTCACAATGGCTACG
GSK-3β	Forward: TTATTTGACCGCATAGTTC Reverse: AAGCACCTGACTTTCCTC

Statistical analysis

All experiments were performed in at least triplicate with similar result. The data were expressed as means ± SD and were assessed by SPSS 13.0. The One-Way ANOVA (Post-Hoc Multiple Comparisons, LSD) and t-test were used for statistical comparison between groups and within group. $P<0.05$ was considered to be statistically significant.

Results

Berberine reduced fasting blood glucose (FBG) in alloxan-induced diabetic mice

In diabetic mice, FBG level was nearly 3-fold higher than that of control mice ($P<0.05$). Although a little decline was observed in diabetic mice in 2nd and 3rd month, FBG level was still significantly higher than control mice and beyond the threshold (11.1mM) thought as diabetic. Berberine treatment for 1, 2 and 3 months markedly reduced FBG level (Fig. 2, $P<0.05$).

Berberine increased liver glycogen in diabetic mice

As shown in Fig. 3, liver glycogen content in diabetic mice was decreased compared to control by almost 3-fold ($P<0.05$), whereas the remarkable increase by nearly 2-fold in diabetic mice was observed after berberine treatment ($P<0.05$).

Effect of berberine on mRNA and protein expression of Akt in diabetic liver

To explore hypoglycemic mechanism of berberine, we measured the mRNA and protein levels of Akt in the liver by real-time PCR and Western-blot assay, respectively. The mRNA levels of Akt were not different between control and diabetic mice (Fig. 4A). Phosphorylated Akt was significantly decreased in dia-
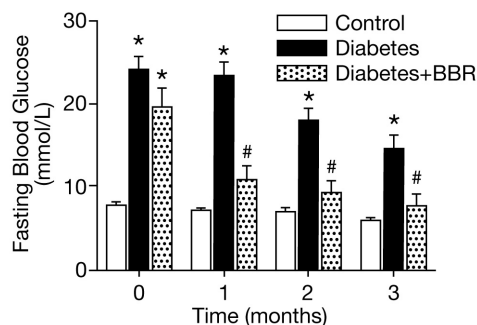
Fig. 2 Berberine reduced fasting blood glucose (FBG) in alloxan-induced diabetic mice. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks, and the FBG were tested at the end of 4th, 8th and 12th week. Date are means ± SD, n=8. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.
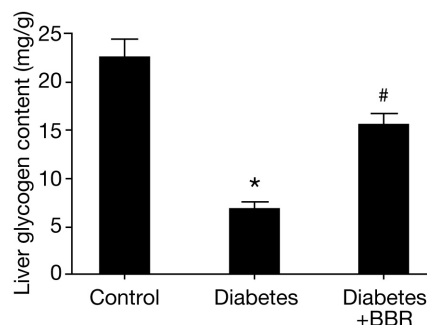

Fig. 3 Berberine enhanced liver glycogen in alloxan-induced diabetic C57BL/6 mice. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks, and the liver glycogen content were evaluated by anthrone assay. Date are means ± SD, n=8. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.
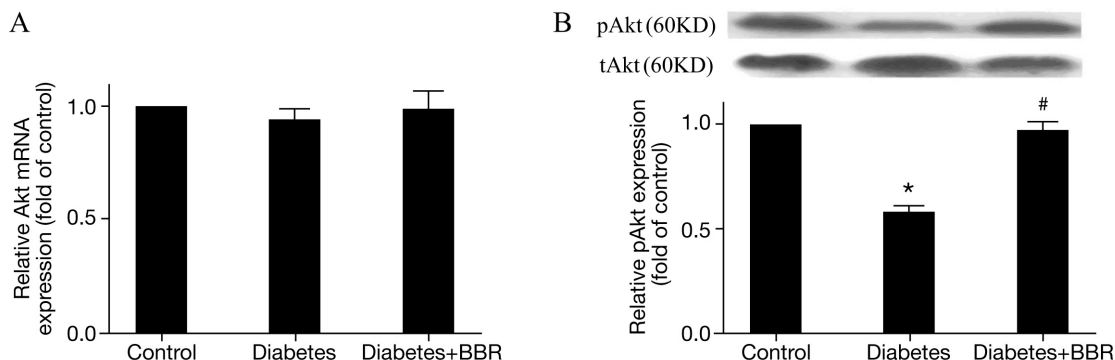

Fig. 4 Effect of berberine on mRNA (A) and protein (B) levels of Akt in diabetic liver. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks. The mRNA and protein level of Akt in the liver were evaluated by real-time PCR assay and western blotting experiment, respectively. Data are means ± SD for five independent experiments. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.

betic liver, and berberine markedly enhanced the phosphorylated Akt level (Fig. 4B, $P<0.05$) by 1.7 fold.

Effect of berberine on mRNA and protein expression of GSK-3β in the liver of diabetic mice

GSK-3β, one of downstream factors regulated by Akt, plays an important role in glucose metabolism decreasing synthesis of glycogen. There were no differences in the mRNA level of GSK-3β among three groups (Fig. 5A). The active protein form-phosphorylated GSK-3β was significantly increased in diabetic liver compared to control, and berberine markedly reduced the phosphorylated GSK-3β level nearly 50% in the liver from diabetic mice (Fig. 5B, $P<0.05$).

Berberine enhanced the mRNA and protein expression of glucokinase(GCK) as well as its activity in diabetic liver

GCK is an important rate-limiting enzyme of glycogen synthesis regulated by Akt. We measured mRNA, protein level and activity of GCK in liver, respectively. The mRNA, protein expression and activity of GCK were obviously decreased in diabetic mice, and berberine markedly reversed these inhibitions (Fig. 6, $P<0.05$).

Effect of berberine on protein expression of IRS in diabetic liver

To further examine whether berberine ameliorates Akt signaling pathway involving IRS, we measured the protein levels of IRS in the liver by Western-blot
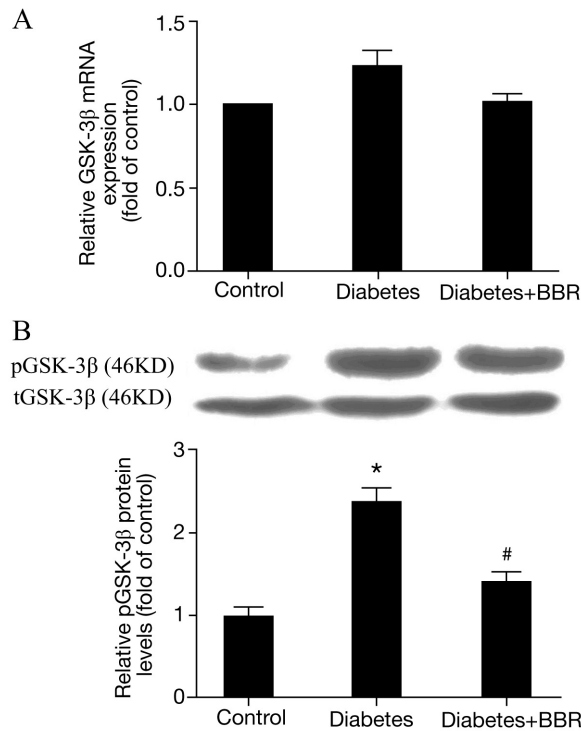
A

Fig. 5 Effect of berberine on mRNA (A) and protein (B) levels of GSK-3β in the liver of diabetic mice. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks. The mRNA and protein levels of GSK-3β in the liver were evaluated by real-time PCR assay and Western blotting assay, respectively. Data are means ± SD for three independent experiments. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.
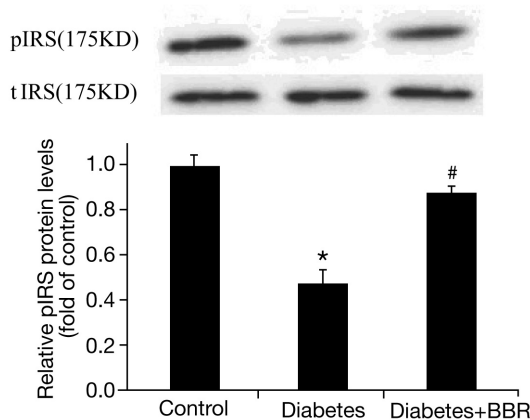

Fig. 7 Effect of berberine on protein levels of IRS in diabetic liver. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks. The protein levels of IRS in the liver were evaluated by western blotting analysis. Data are means ± SD for five independent experiments. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.
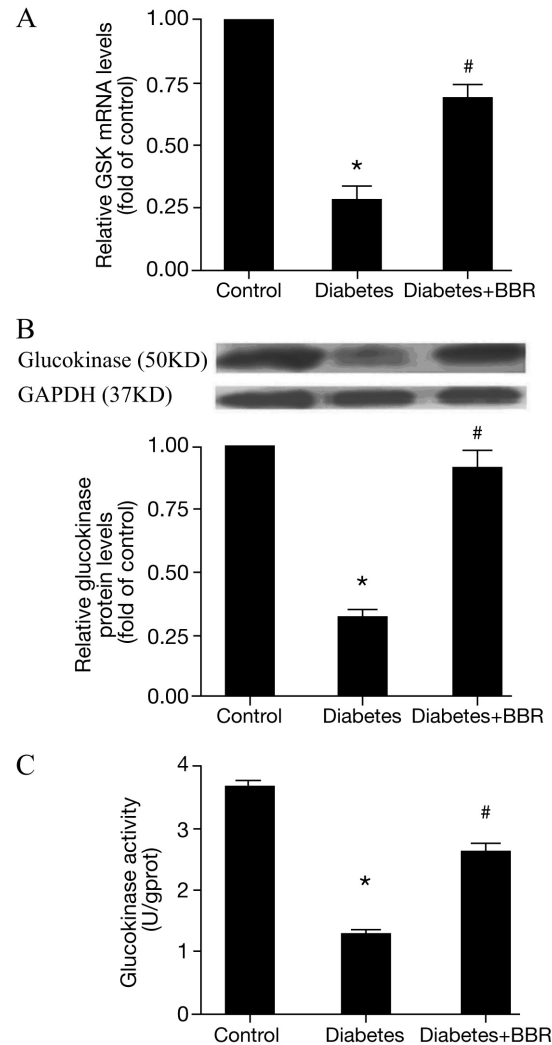
A

Fig. 6 Effect of berberine on mRNA and protein levels of glucokinase (GCK) as well as GCK activity in diabetic liver. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks. The mRNA levels of GCK in the liver were evaluated by real-time PCR assay (A); the protein levels of GCK in the liver were evaluated by Western blotting assay (B); and the GCK activity in the liver measured by enzyme assay (C). Data are means ± SD for three independent experiments. *$P<0.05$ *vs.* control group, #$P<0.05$ *vs.* diabetic group by ANOVA.

assay. Phosphorylated IRS was significantly decreased in diabetic liver whereas berberine markedly enhanced the phosphorylated IRS level in diabetic liver (Fig. 7, $P<0.05$).

Discussion

Alloxan selectively impairs pancreatic β cells and causes deficiency of insulin secretion, which is respon-

sible for defect of glucose disposal and enhanced glucose output. Long-term hyperglycemia decreases insulin sensitivity *via* impairing the activity of insulin receptor kinase [21]. Meanwhile, the defect of insulin sensitivity contributes to constant hyperglycemia which breaks the balance of ATP/ADP in β-cell resulting in further dysfunction of insulin secretion [22]. The malignant feedback loop exaggerates the pathological state of hyperglycemia. Our study showed that the blood glucose level was increased up to nearly 22mM after injecting 200mg/kg alloxan, indicating induction of overt diabetes. Although there was a little decline in diabetic group at the end of 8th and 12th week as a result of self-repairment of pancreatic β cells, the glucose level was still higher than the diabetic threshold (11.1mM). Berberine treatment markedly decreased blood glucose and enhanced liver glycogen content in diabetic mice, suggesting that the amelioration of hyperglycaemia by berberine might partly attribute to the increased glycogen synthesis in liver.

Akt is an important kinase mediating insulin-stimulated glucose metabolism. It induces GLUT translocation to plasma membrane [23] and regulates downstream factors including GSK-3β and glucokinase involved in glycogen synthesis [7, 24, 25]. The impaired Akt phosphorylation occurs in liver of diabetic individual [10]. Defect of Akt phosphorylation brings many changes on enzymes and kinases leading to disturbance of glucose metabolism. Therefore, it is believed that the reduced Akt activity is one of the major pathological mechanisms on diabetes. In the current study, we found that there was no change in the mRNA level of Akt. However the active form-phoryphoslated Akt (ser^{473}) was significantly decreased in diabetic liver, indicating that the impaired activity of Akt rather than the alteration of its mRNA expression contributed to the pathological progress of DM. Berberine significantly enhanced the phosphorylated Akt level by 1.7-fold, suggesting hypoglycemic effect of berberine might benefit from recovering the activity of Akt in diabetic mice liver.

GSK-3 is a Serine/threonine kinase with two isoforms in mammals, termed GSK-3α and GSK-3β exhibiting similar biochemical properties [26]. Elevated level of GSK-3β has been observed in diabetic and obese mice [27]. The inhibition of GSK-3β activity promotes the conversion of glucose to glycogen suggesting GSK-3β plays an important role in the pathogenesis of diabetes [28]. The phosphorylation of GSK-3β on the site of Serine 9 is repressed following the activation of Akt signal pathway. In the present study, we also observed that the phosphorylated GSK-3β was strikingly increased with inhibition of Akt activity in liver of diabetic mice. While berberine markedly reduced the phosphorylated GSK-3β (ser^9) level without change in its mRNA expression, implying that berberine lowered blood glucose possibly through inhibiting GSK-3β activity to induce glycogen synthesis in diabetic mice.

Glucokinase, also termed as hexokinase D, is a critical enzyme involved in glucose metabolism, which catalyzes glucose to convert into glucose 6-phosphate. Impaired glucokinase activity was found in diabetic individuals [29, 30]. Inactivation or the activation mutation of glucokinase gene have been linked to the development of maturity-onset diabetes of the young (MODY), Type 2 (MODY2) and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [31-33]. Patrick B et al. have demonstrated that the activation of Akt signal pathway increases glucokinase gene expression [7]. In our study, we found that berberine markedly increased glucokinase expression and its activity as well as the activation of Akt signal pathway in diabetic liver, which contributed to glycogen synthesis resulting in lower of blood glucose.

Previous study showed that the expression of IRS-1, IRS-2 significantly decreased in livers of alloxan-induced diabetic rats [34]. As a downstream factor of insulin signaling pathway, the activity of Akt is regulated by insulin and its substrate. To determine whether berberine exerting its hypoglycemic effects by upregulating Akt activity directly or by insulin receptor signaling pathway, we next measured the phosphorylated IRS level in diabetic liver. The result showed that phosphorylated IRS was significantly decreased in diabetic liver, and berberine markedly enhanced the phosphorylated IRS level, indicating that berberine activates Akt signaling pathway possibly through improvement of insulin pathway.

Besides exploring that berberine acted directly to liver by Akt signaling, we investigated if berberine improved the damage of pancreas, which contributed to lowered high blood glucose. The photomicrographs of HE staining for pancreas showed that the gland alveolus and islets were both intact in normal group. Most of pancreatic islets were round or oval. The morphous of α, β cells was normal and the arrangement was regular. Compared with control group, the numbers of

gland alveolus were strikingly decreased in diabetic group. Islets were broken partly. Most of β cells were atrophied and vacuolization. Treatment of berberine for alloxan–induced diabetic mice could not improve the damage of pancreatic islets and β cells significantly. In addition, we have analyzed the islet numbers in tissue sections of pancreas in different groups. Compared with control group (27.75±1.71/per tissue section), the islet numbers of model (16.75±1.71) and berberine treated group (18±2.16) both decreased significantly while there was no difference between the two groups. In agreement with a recent study, berberine lowers hyperglycemia and improves impaired glucose tolerance rather than increase insulin release and synthesis in diabetic mice [35]. We presume that berberine lowers hyperglycemia mostly through activation of Akt signaling but not by ameliorating the damage of pancreas in diabetic mice.

In conclusion, our study demonstrated that berberine enhanced the activity of Akt and GCK, inhibited phosphorylation of GSK-3β and increased glycogen synthesis in diabetic liver, suggesting that the activation of Akt signal cascade might be one of the hypoglycemic mechanisms of berberine in the treatment of diabetes.

Conflict of Interest

The authors have no conflicts of interest to declare.

Acknowledgement

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