Irisin inhibits hepatic gluconeogenesis and increases glycogen synthesis via PI3K/Akt pathway in type 2 diabetic mice and hepatocytes

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Abstract Increased glucose production and reduced hepatic glycogen storage contribute to metabolic abnormalities in diabetes. Irisin, a newly identified myokine, induces the browning of white adipose tissue, but its effects on gluconeogenesis and glycogenesis are unknown. Here, we investigated the effects and underlying mechanisms of irisin on gluconeogenesis and glycogenesis in hepatocytes with insulin resistance, and its therapeutic roles in type 2 diabetic mice. Insulin resistance was induced by glucosamine (GlcN) or palmitate in human hepatocellular carcinoma cells (HepG2) and mouse primary hepatocytes. Type 2 diabetes was induced by streptozotocin/high-fat-diet (STZ/HFD) in mice. In HepG2, irisin ameliorated the GlcN-induced increases in glucose production, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) expressions and glycogen synthase (GS) phosphorylation; it prevented the GlcN-induced decreases in glycogen content, PI3K p110α subunit level and the phosphorylation of Akt, FOXO1 and glycogen synthase kinase-3 (GSK3). These effects of irisin were abolished by the inhibition of PI3K or Akt. The effects of irisin were confirmed in mouse primary hepatocytes with GlcN-induced insulin resistance and in human HepG2 with palmitate-induced insulin resistance. In diabetic mice, persistent subcutaneous perfusion of irisin improved the insulin sensitivity, reduced fasting blood glucose, increased GSK3 and Akt phosphorylation, glycogen content and irisin level, and suppressed GS phosphorylation, PEPCK and G6Pase expressions in liver. Irisin improves glucose homeostasis by reducing gluconeogenesis via PI3K/Akt/FOXO1-mediated PEPCK and G6Pase down-regulation and increasing glycogenesis via PI3K/Akt/GSK3-mediated GS activation. Irisin may be regarded as a novel therapeutic strategy for insulin resistance and type 2 diabetes.

Abbreviations: GlcN, glucosamine; HepG2, human hepatocellular carcinoma cells; STZ, streptozotocin; HFD, high-fat-diet; PEPCK, phosphoenolpyruvate carboxykinase; G6pase, glucose-6-phosphatase; GS, glycogen synthase; PI3K, phosphoinositide 3-kinase; FoxO1, forkhead box transcription factor O1; GSK3, glycogen synthase kinase-3; FNDC5, fibronectin type III domain containing 5; UCP1, uncoupling protein 1; WAT, white adipose tissue; FBS, fetal bovine serum.
INTRODUCTION

Type 2 diabetes is characterized by hyperglycemia and insulin resistance in target tissues. Liver plays a central role in the maintenance of blood glucose by balancing the de novo synthesis (gluconeogenesis), glucose uptake and storage via the glycogen synthesis (glycogenesis) and glucose release via the breakdown of glycogen (glycogenolysis) [1]. Glycogen synthesis is mainly regulated by glycogen synthase kinase 3 (GSK3) and glycogen synthase (GS) [2]. Gluconeogenesis is primarily modulated by phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [3], which are up-regulated in response to hormones during fasting and are robustly down-regulated by insulin and glucose [1,4]. Gluconeogenesis is strongly stimulated during fasting and is aberrantly activated in diabetes [5]. Excessive hepatic glucose production is a major contributor to both the fasting hyperglycemia and the exaggerated postprandial hyperglycemia in both type 1 and type 2 diabetes [5]. Insulin resistance in the liver is an important factor causing fasting hyperglycemia and type 2 diabetes [6].

Irisin is a cleaved and secreted fragment of fibronectin type III domain containing 5 (FNDC5), which induces uncoupling protein 1 (UCP1) expression and white adipose tissue (WAT) browning. This newly identified myokine enhances energy expenditure and may be involved in the beneficial effects of exercise that have been noted independent of weight loss [7,8]. Adenoviral vectors to express full-length FNDC5 in mice improves glucose tolerance and reduces insulin resistance [7]. Circulating irisin was positively correlated with age, body mass index (BMI), total cholesterol and triglycerides and fasting blood glucose in non-diabetic subjects, and the increased circulating irisin may be an adaptive response to compensate for the decreasing insulin sensitivity and disturbances in metabolism associated with obesity [9]. However, serum irisin levels were similar in pregnant women from the nonobese, obese and gestational diabetes groups, as well as in a separate cohort of nonpregnant lean and obese women. Analysis of the pregnant subjects revealed that there was a significant inverse correlation of BMI with serum irisin [10]. The controversy regarding the serum irisin levels in various studies may be related to the gender difference of irisin levels in various studies [11]. Although some studies show conflicting results, the majority of studies show that serum irisin levels are lower in patients with Type 2 diabetes [12-15]. It is noted that strong irisin immunoreactivity was found in liver [16]. Serum irisin concentrations were inversely associated with the triglyceride contents in the liver and liver enzymes in obese adults [17]. Previous studies have shown that insulin-induced suppression of glycogenolysis and gluconeogenesis was impaired in obesity and type 2 diabetes [18]. Defects in glycogenolysis and gluconeogenesis have been shown to be involved in hepatic insulin resistance in type 2
diabetic humans [19]. However, effects of irisin on hepatic glycogenesis and gluconeogenesis are unknown.

In the present study, the effects of irisin on glycogenesis and gluconeogenesis and related signal pathway were investigated in the human hepatocellular carcinoma cells (HepG2) and mouse primary hepatocytes with insulin resistance; the therapeutical effects of persistent administration of irisin on the glucose metabolic disturbance and insulin resistance were determined in streptozotocin/high-fat-diet (STZ/HFD)-induced type 2 diabetic mice.

MATERIALS AND METHODS

HepG2 cell culture and insulin resistance models

HepG2 cells were obtained from the American Type Culture Collection, and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM glucose, 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To induce insulin resistance, HepG2 cells were incubated with 18 mM GlcN for 18 h or 0.25 mM palmitate for 24 h in serum-free medium [20,21], followed by incubation in medium with 20 nM irisin or vehicle (PBS) for 30 min for measurement of phosphorylated protein or 24 h for other measurements in the presence of GlcN or palmitate. Phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 μM), Akt inhibitor MK2206 (1 μM) were added into the medium 24 hours before administration of irisin or vehicle for assessing the impact of these inhibitors. Cell viability was detected using the Cell Counting Kit-8 (CCK8) assay (Dojindo Laboratory, Japan) according to the manufacturer's instructions.

Isolation of primary hepatocytes

Male C57BL/6J mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Perfusion was made via the portal vein with 50 mL of HEPES buffer containing collagenase II (0.66 mg/mL) at 37°C. The liver was aseptically removed and excised. Cells were dispersed and filtrated via a 70-μm cell strainer into a centrifuge tube. After centrifugation, cells were washed and cell viability was examined by trypan blue dye before planting into six-well plates. Only plates with cell viability greater than 95% were used for subsequent experiments. The hepatocytes were maintained in low glucose DMEM containing 10% FBS with penicillin and streptomycin for 24 h before inducing insulin resistance with GlcN.

Glucose Production
The medium in six-well plates was replaced with 2 ml of glucose production buffer consisting of glucose-free DMEM without phenol red, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. After 3 h incubation, half of the glucose production buffer was collected and the glucose concentration was measured with a glucose oxidase-peroxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The values were normalized to the total protein content determined from the whole-cell extracts [22].

**Glycogen Quantification**

Glycogen levels were tested by Glycogen Assay Kit (BioVision Incorporation, San Francisco, CA, USA) following manufacturer’s instructions. Briefly, cells or tissues were homogenized with 100 μL of ice cold glycogen development buffer for 10 min. Homogenates were spun at 13,000 rpm for 5 min and supernatants were assayed for glycogen content. Glycogen concentration was normalized by cell numbers (10⁹ in each plate) or protein concentration of tissues [23].

**Quantitative real-time PCR**

Total RNA was isolated from cells or tissues using Trizol reagent (Invitrogen, CA, USA). Real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All genes expression levels were normalized by GAPDH levels. The sequences of primers were listed in the online supplemental data (Supplementary Table S1).

**Western blot**

Protein extracts were electrophoresed, blotted, and then incubated with antibodies against Akt, Phospho-Akt (Ser473), PI3 kinase p110α, PI3 kinase p110β, GS, Phospho-GS (Ser641), FOXO1, Phospho-FOXO1 (Ser256), GSK3, Phospho-GSK3 (Ser9), GAPDH (Cell signaling Technology, Danvers, MA, USA), PEPCK (Abcam Ltd Cambridge, UK) and G6Pase (Santa Cruz Dallas, TX, USA) with appropriate secondary HRP-conjugated antibodies, and then developed.

**G6Pase and PEPCK activity analysis**

G6Pase and PEPCK activity was measured as previously described [24]. Briefly, G6Pase activity was assayed by quantifying the released phosphate. Phosphoenolpyruvate was carboxylated by PEPCK to form oxaloacetate, which was then converted into malate using malic dehydrogenase. This conversion was monitored by a decrease in NADH absorbance at 340 nm.
Mice model of type 2 diabetes

Six-week-old male C57BL/6J mice were used for inducing type 2 diabetes. Experiments were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication, 8th edition, 2011). The mice were caged in an environment under controlled temperature and humidity with free access to water and diet under a 12 h light/dark cycle. Combination of low-dose streptozotocin (STZ) and high-fat diet-fed (HFD)-treated rat or mouse is identified as an ideal animal model of type 2 diabetes, which simulates natural disease progression and metabolic characteristics typical of type 2 diabetes [25,26]. This model is appropriate for testing anti-diabetic agents for the treatment of type 2 diabetes [27]. After a week of adaptation in living environment, the mice were randomly divided into three groups (n=7 for each group). One group of mice (Ctrl) received an intraperitoneal injection of vehicle and were fed with normal diet (14.7 kJ/g, 13% of energy as fat) throughout the experiment. Another two groups of mice combined into one group (STZ/HFD) to induce type 2 diabetes mellitus. These mice were subjected to 4-hour’s fasting followed by intraperitoneal injection of low-dose of STZ (120 mg/kg body weight in 10 mmol/L citrate buffer, pH 4.0). After 3 weeks, the mice were fed with HFD (21.8 kJ/g, 60% of energy as fat; D12492, Research Diets, New Brunswick, NJ, USA) instead of previously used normal diet. Eight weeks after injection of STZ, the mice were re-divided into two groups, which respectively received subcutaneous perfusion of saline or irisin with micro-osmotic pumps for 2 weeks, and were maintained with HFD feeding (Supplementary Figure S1).

Subcutaneous perfusion of irisin

Mice were anaesthetized with intraperitoneal injection of pentobarbital (50 mg/kg) for the surgery of pump implantation. A micro-osmotic pump (model 1002; Alzet, Palo Alto, CA, USA) was implanted into the mid-scapular region of each mouse under aseptic manipulation. The pump delivered saline or irisin (0.24 nmol/μL, 1.44 nmol/day) for 14 days.

Measurement of insulin and irisin levels

Serum insulin level was determined with a mouse insulin ELISA kit (ALPCO, Salem, NH, USA). Serum and liver irisin levels were measured with an irisin human, rat, mouse and canine EIA kit (EK-067-16, Phoenix Pharmaceuticals, Burlingame, CA, USA). For the anti-irisin antibody used in this study, minimum detectable concentration of irisin is 6.8 ng/ml. The cross-reactivity is 100% with irisin or irisin (42-112), and 9% with FNDC. No cross-reactivity is found with FNDC5 (165-212), FNDC5 (162-209) or irisin (42-95). The recovery is 104%, 82%
and 88.7% for spiked irisin at 5, 10 and 20 ng/ml, respectively. Intra- and inter-assay variation are <10% and <15%, respectively. The accuracy and validity have been checked [28]. The interobserver and intraobserver variabilities of measurements with the kits were 6.2% and 5.9%, respectively [29]. We conducted a validation of irisin (spiking and recovery) for the ELISA kit, and found that the recovery was 101.8%, 98.0% and 88.2% for spiked irisin at 5, 10 and 20 ng/ml, respectively.

**Insulin tolerance test (ITT) and glucose tolerance test (GTT)**

For insulin and glucose tolerance tests, mice were fasted for 6 hours and overnight, respectively, before intraperitoneal injection of insulin (0.75 units/kg body weight) or glucose (2.0 g/kg body weight) [30]. Glucose levels in tail-vein blood samples were measured with a blood glucometer (One Touch, Milpitas, CA, USA) at 15, 30, 60 and 120 min after the injection.

**Detection of glycogen deposits in liver**

Periodic acid schiff (PAS) staining was used to visualize the glycogen deposits in livers. Liver tissue was fixed with 4% paraformaldehyde and then mounted in paraffin and sectioned. Sections were oxidized in periodic acid for 20 minutes at room temperature and rinsed three times in deionized H₂O (dH₂O). After the treatment with 0.5% sodium bisulfite in 0.05 N HCl for 10 min, sections were counterstained with hematoxylin-eosin staining for 1 min, rinsed in dH₂O, and observed for the red staining parts as the presence of glycogen using a light microscope.

**Chemicals**

STZ, collagenase II and palmitate were purchased from Sigma (St Louis, MO, USA). Glucose and glucosamine were obtained from Beyotime Institute of Biotechnology (Shanghai, China). LY294002 and MK2206 were bought from Selleck Chemicals LLC (Boston, MA, USA). Irisin was bought from ChinaPeptides Co., Ltd (Shanghai China), which was derived from E.Coli, and was purified and identified. The quality and quantity of purified irisin was analyzed with a SDS-PAGE gel electrophoresis. Irisin was treated with Pierce high-capacity endotoxin removal resin (Thermo Scientific, MA, USA) to remove endotoxin.

**Statistics**

Values were expressed as mean±SE. One-way or two-way ANOVA followed by post hoc Bonferroni test was used for the multiple comparisons. Student’s t test was used for the comparisons between two groups. A value of $P<0.05$ was considered statistically significant.
RESULTS

Gluconeogenesis and glucose production in hepatocytes

GlcN or palmitate is used to induce insulin resistance in vitro in HepG2 and primary hepatocytes [20,21]. PEPCK and G6Pase are two key hepatic gluconeogenesis enzymes [3]. GlcN treatment increased PEPCK and G6Pase mRNA levels \((P=0.011 \& 0.012)\), protein expression \((P=0.004 \& 0.049)\) and activity \((P=0.024 \& P=0.037)\) as well as glucose production \((P=0.023)\) in HepG2. These findings were not seen when the cells were incubated in the presence of irisin (Figure 1). Similar effects of irisin on PEPCK and G6Pase mRNA expressions (Supplementary Figure S2) and glucose production (Supplementary Figure S3) were found in GlcN-treated mouse primary hepatocytes. Furthermore, effects of irisin on glucose production were confirmed in palmitate-treated HepG2 (Supplementary Figure S4). These results indicate that irisin rectifies the enhanced gluconeogenesis and glucose production in the hepatocytes with insulin resistance.

Glycogen synthesis and glycogen content in hepatocytes

GSK3 inhibits glycogen synthesis via phosphorylating GS, a key enzyme that catalyzes the last step in glycogen synthesis [31]. Insulin is known to increase the GSK3 phosphorylation and thereby activates glycogen synthase and causes the conversion of glucose to glycogen [32]. GlcN treatment reduced GSK3 phosphorylation \((P=0.042)\) and glycogen content \((P=0.020)\) but increased GS phosphorylation \((P=0.003)\), which were ameliorated by irisin in human HepG2 cells \((P=0.032, 0.039 \& 0.047)\) (Figure 2). Similar effects of irisin on glycogen synthesis (Supplementary Figure S5) and glycogen content (Supplementary Figure S6) were observed in GlcN-treated mouse primary hepatocytes. Furthermore, effects of irisin on glycogen content were confirmed in palmitate-treated HepG2 (Supplementary Figure S4). The findings provide ample evidence that irisin prevents the reduced glycogen synthesis and glycogen content in the hepatocytes with insulin resistance.

PI3K/Akt/FOXO1 pathway in hepatocytes

The enzyme activity of PI3K is mediated by a group of catalytic and regulatory subunits [33]. FOXO1 promotes the expression of PEPCK and G6Pase, and the phosphorylation of FOXO1 causes its inactivation in hepatocytes [34]. Insulin suppresses FOXO1 activity through activation of the PI3K/Akt signaling pathway [35]. We found that both p110α and p110β subunits of PI3K were down-regulated in GlcN-treated HepG2 cells \((P=0.017 \& 0.009)\), but irisin only prevented the reduced p100α subunit expression \((P=0.013)\) rather than p100β subunit expression \((P=0.153)\) (Figure 3A and B). Moreover, the phosphorylation of Akt and FOXO1 was reduced in GlcN-treated HepG2 cells \((P=0.040 \& 0.031)\), which were eliminated.
by irisin ($P=0.003$ & $0.045$) (Figure 3C and D). These results indicate that irisin causes the FOXO1 phosphorylation and thereby inhibits the FOXO1 activity via activation of the PI3K/Akt signaling pathway. It is well known that insulin inhibits gluconeogenesis and increases glycogen synthesis via PI3K/Akt pathway [36,37]. We found that irisin showed similar phosphorylation effects on Akt and FOXO1 to insulin in control HepG2 (Figure 3F and G), suggesting irisin and insulin share the PI3K/Akt pathway.

**Effects of PI3K and Akt inhibitors in hepatocytes**

PI3K inhibitor LY294002 prevented the roles of irisin in reducing the PEPCK and G6Pase mRNA levels ($P=0.026$ & $0.067$), activity ($P=0.041$ & $0.032$) and protein expressions ($P=0.012$ & $0.001$) as well as the glucose production ($P=0.002$) in GlcN-treated HepG2 cells. Akt inhibitor MK2206 showed a similar role in preventing these effects of irisin in GlcN-treated HepG2 cells (Supplementary Figure S7 and S8, Figure 4A and D). Inhibition of PI3K or Akt abolished the roles of irisin in increasing the phosphorylation of FOXO1 ($P=0.0001$ & $0.0001$) in GlcN-treated HepG2 cells (Figure 4F), indicating that irisin prevents the increased gluconeogenesis and glucose production in hepatocytes with insulin resistance via PI3K/Akt-mediated FOXO1 inactivation and subsequent inhibition in PEPCK and G6Pase mRNA expression. On the other hand, LY294002 or MK2206 abolished the roles of irisin in promoting the GSK3 phosphorylation ($P=0.022$ & $0.010$) (Figure 4B), reducing the GS phosphorylation ($P=0.002$ & $0.004$) (Figure 4C) and increasing the glycogen content ($P=0.004$ & $0.094$) (Figure 4E) in GlcN-treated HepG2 cells, indicating that irisin normalizes the reduced glycogen synthesis and glycogen content in hepatocytes with insulin resistance via PI3K/Akt-mediated GSK3 inactivation and subsequent GS activation. The findings were further confirmed in GlcN-treated mouse primary hepatocytes in which LY294002 or MK2206 abolished the roles of irisin in reducing glucose production (Supplementary Figure S9) and increasing glycogen content (Supplementary Figure S10).

**Irisin levels, body weight and food intake in mice**

The effects of subcutaneous perfusion of irisin with micro-osmotic pumps on glucose metabolism and insulin resistance were identified in mice with type 2 diabetes induced by STZ/HFD. The serum and liver irisin levels were lowered in STZ/HFD mice ($P=0.035$ & $0.047$), and irisin perfusion increased serum and liver irisin levels in STZ/HFD mice ($P=0.004$ & $0.020$) (Figure 5A and B), confirming the effectiveness of perfusion of irisin with osmotic micro-pumps. However, subcutaneous perfusion of irisin had no significant effect on body weight, food intake and systolic blood pressure (Figure 5C-F).

**Glucose metabolism in mice**

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STZ/HFD mice showed much higher fasting blood glucose level than that in control mice ($P=0.020$). STZ/HFD mice did not show significant change in serum insulin level ($P=0.166$), and irisin perfusion had no significant effect on serum insulin level in STZ/HFD mice ($P=0.084$) (Figure 6A). Irisin perfusion reduced the increased fasting blood glucose level ($P=0.032$) in STZ/HFD mice (Figure 6B). Liver glycogen content was reduced in STZ/HFD mice ($P=0.0001$), which was attenuated by the irisin treatment ($P=0.007$) (Figure 6C). Similarly, liver sections with PAS-staining showed that the reduced glycogen in STZ/HFD mice was rectified by irisin perfusion (Figure 6D). GTT and ITT were used for evaluating glucose tolerance and insulin resistance. In the GTT, STZ/HFD mice manifested significantly elevated glucose excursions following glucose challenge compared with control mice. The glucose excursion was reduced in irisin-treated STZ/HFD mice (Figure 6E). In the ITT, the efficiency of insulin was quantified by its ability in reducing blood glucose level. Insulin was less effective in STZ/HFD mice than that in control mice, suggesting impaired insulin sensitivity in STZ/HFD mice. Irisin improved insulin sensitivity in STZ/HFD mice (Figure 6F). These results indicate that persistent administration of irisin reduces blood glucose level, increases liver glycogen synthesis and attenuates insulin resistance in mice with type 2 diabetes.

Hepatic gluconeogenesis and glycogen synthesis and Akt phosphorylation in mice

Irisin perfusion attenuated the up-regulation of PEPCK and G6Pase mRNA levels ($P=0.045$ & 0.042), protein expression ($P=0.049$ & 0.002) and activity ($P=0.028$ & 0.013) in the liver in STZ/HFD mice (Figure 7A-C). The GSK3 phosphorylation was reduced, while GS phosphorylation was increased in STZ/HFD mice, which were normalized by irisin perfusion ($P=0.002$ & 0.023) (Figure 7D and E). Furthermore, the Akt phosphorylation was inhibited in STZ/HFD mice, which was recovered by irisin perfusion ($P=0.005$) (Figure 7F). The in vivo experiments confirmed our in vitro findings that irisin prevents the enhanced gluconeogenesis and decreased glycogen synthesis via the Akt-mediated regulation of key enzymes of gluconeogenesis and glycogen synthesis in diabetes.

DISCUSSION

Liver is primarily responsible for the maintenance of blood glucose level by its ability to produce glucose from gluconeogenesis or glycogen breakdown and to store glucose as glycogen [1]. Increased endogenous glucose production and reduced hepatic glycogen storage contribute to the metabolic abnormalities in diabetes [6]. The primary novel findings in the present study are that irisin reduces gluconeogenesis via PI3K/Akt/FOXO1-mediated PEPCK and G6Pase down-regulation and increases glycogenesis via PI3K/Akt/GSK3-mediated GS activation in hepatocytes with insulin resistance. Another important finding is that persistent administration of irisin effectively reduces fasting blood glucose level, hepatic
gluconeogenesis and glucose production, increases hepatic glycogen synthesis and storage, and improves insulin resistance in mice with STZ/HFD-induced type 2 diabetes.

It is well known that increased endogenous glucose production, primarily of hepatic origin, is a major determinant of fasting hyperglycemia in type 2 diabetes [3]. Gluconeogenesis is enhanced in diabetes, and hepatic insulin resistance in diabetes is linked with a reduced ability of insulin in inhibiting gluconeogenesis and glucose production in the liver [5]. Reduction of hepatic glucose production can be considered as a therapeutic target in diabetes [2,18]. Type 2 diabetes is strongly associated with a decrease in insulin-stimulated GS activity and glycogen synthesis [38]. The present study provides evidence that irisin inhibited hepatic gluconeogenesis and endogenous glucose production, and increased hepatic glycogen synthesis and storage not only in human HepG2 cells and mice primary hepatocytes with insulin resistance, but also in mice with type 2 diabetes. The decreased gluconeogenesis and increased glycogenesis at least partially contribute to the irisin-induced reduction in fasting blood glucose level and the improvement in glucose homeostasis and insulin resistance in type 2 diabetes. These results suggest that irisin may be an effective therapeutic strategy for type 2 diabetes.

Hepatic glucose production is mainly regulated by PEPCK and G6Pase, which are important target genes of FOXO1 and catalyse the rate-limiting steps in gluconeogenesis [39]. Activation of FOXO1 in liver induces gluconeogenesis via increasing PEPCK and G6Pase expressions, and insulin suppresses hepatic gluconeogenesis via activating the PI3K/Akt pathway and subsequent FOXO1 phosphorylation and inactivation [34,40]. In the present study, we found that irisin reduces gluconeogenesis via PI3K/Akt/FOXO1-mediated PEPCK and G6Pase down-regulation, which is similar to the signal pathway of insulin in the suppression of gluconeogenesis.

GS is known to catalyse the rate-limiting step for insulin-mediated glycogen synthesis, and GSK3 inhibits glycogen synthesis by suppressing GS via inhibitory phosphorylation [32]. GSK3 has been implicated to mediate the development of insulin resistance, mainly by inhibiting glycogen synthesis [38]. Insulin activates PI3K/Akt signaling cascade and subsequent GSK3 phosphorylation and inhibition. This GSK3 inhibition leads to the GS activation and thereby glycogen synthesis [38]. GSK3 inhibitors may have a therapeutic potential in the treatment of diabetes and insulin resistance [41]. In the present study, we found that irisin increases liver glycogen synthesis via PI3K/Akt/GSK3-mediated GS activation in insulin resistance and type 2 of diabetes, which is similar to the signal pathway of insulin in promoting glycogen synthesis.
It is very interesting that irisin and insulin share the similar downstream signal pathway in reducing gluconeogenesis and increasing glycogen synthesis in insulin resistance and type 2 of diabetes. The present in vitro and in vivo studies indicate irisin is effective in preventing excess gluconeogenesis and reduced glycogen synthesis in insulin resistance and type 2 of diabetes. More importantly, persistent administration of irisin reduces fasting blood glucose and improves insulin resistance in mice with STZ/HFD-induced diabetes. The greatest strength is that the irisin is effective for treating diabetes with insulin resistance. Although insulin and irisin may share the same pathway, the side effect of weight gain may be avoided if irisin is used as a therapeutic agent in type 2 diabetes.

Insulin regulates gluconeogenesis and glycogen synthesis by insulin receptors (IR)-mediated insulin receptor substrate (IRS) phosphorylation, and thereby activates PI3K/Akt signal pathway [42]. However, the molecular mechanisms of irisin in inducing the PI3K/Akt activation remain unknown. A limitation of the present study is that the irisin receptors and the interaction of irisin and insulin are not determined, which is worthy of further investigation. It is noted that irisin had no significant effect on serum insulin level, indicating that the effect of irisin is not caused by the change in insulin level. Moreover, the effects of irisin on glycogenolysis, glucose uptake of cells and lipid metabolism were not investigated in the present study. Those effects may contribute to the beneficial effects of irisin in treating diabetes.

It has been found that circulating irisin correlated positively with body mass index (BMI) in non-diabetic individuals [43,44]. Circulating irisin level was higher in morbidly obese individuals than the normal weight and anorexic patients [45]. Weight-loss induced by bariatric surgery reduced circulating irisin level [44]. The increased circulating irisin may be an adaptive response to compensate for the decreasing insulin sensitivity and disturbances in metabolism associated with obesity [46]. It is noteworthy that the circulating irisin levels were reduced in patients with type 2 diabetes [12-15]. In the present study, reduced serum and liver irisin levels were found in mice with STZ/HFD-induced type 2 diabetes. Administration of irisin improved the glucose metabolism in type 2 diabetic mice and in hepatocytes with insulin resistance. These results indicate that exogenous irisin is effective in ameliorating glucose metabolic derangements in diabetic mice with insulin resistance.

It has been shown that most previously published assays based on commercial ELISAs were reporting unknown cross-reacting proteins, and the ELISAs for irisin do not measure irisin calling into question the role of irisin. It was found that commercial anti-irisin antibodies can measure recombinant non-glycosylated irisin, but not recombinant glycosylated irisin [47]. So far, however, all previous studies showed that irisin is a bioactive molecule, and no study showed that irisin play its role via its glycosylation. It is possible that
glycosylation block the binding site of antibody to the glycosylated irisin. Albrecht E et al made a comparison of ELISA data with Western blot analyses in 4 of 5 commercial antibodies except EK-067-16 [47], and thus their conclusion is not completely applicable to EK-067-16. Most importantly, the cross-reactivity of the anti-irisin antibody (EK-067-16) used in the present study is known, and its accuracy and validity are checked by the company and independent researchers [28,29]. The spiking and recovery test in our lab support the validation of this commercial irisin ELISA kit.

In conclusion, irisin decreases gluconeogenesis by the PI3K/Akt/FOXO1 pathway-mediated down-regulation of PEPCK and G6Pase, and increases glycogenesis by the PI3K/Akt/GSK3 pathway-mediated GS activation in human HepG2 cell line, mouse primary hepatocytes and mouse model of type 2 diabetes. Importantly, long-term subcutaneous administration of irisin attenuates hyperglycemia and insulin resistance in mice with type 2 diabetes. The effects of irisin on gluconeogenesis and glycogen synthesis at least partially contribute to the reduced glucose production, increased glycogen accumulation and the improved glucose homeostasis in type 2 diabetes. Irisin may be taken as effectively therapeutic strategy for type 2 diabetes.

CLINICAL PERSPECTIVES

(1) Increased glucose production and reduced hepatic glycogen storage contribute to metabolic abnormalities in diabetes. The newly identified myokine irisin induces the browning of white adipose tissue and increases energy expenditure. However, effects of irisin on hepatic glycogenesis and gluconeogenesis are unknown.

(2) In the present study, irisin reduces gluconeogenesis via the PI3K/Akt/FOXO1 pathway-mediated down-regulation of PEPCK and G6Pase, and increases glycogenesis via the PI3K/Akt/GSK3 pathway-mediated GS activation in human HepG2 cell line and mouse primary hepatocytes with insulin resistance as well as in type 2 diabetic mice. Moreover, long-term subcutaneous administration of irisin attenuates hyperglycemia and insulin resistance in mice with type 2 diabetes.

(3) This study provides evidence that irisin inhibits hepatic gluconeogenesis, increases glycogen synthesis and improves insulin resistance. Irisin may be regarded as a novel therapeutic strategy for insulin resistance and type 2 diabetes.

AUTHOR CONTRIBUTION
Tong-Yan Liu, Chang-Xiang Shi, Run Gao, Hai-Jian Sun, Xiao-Qing Xiong and Lei Ding performed the experiment. Tong-Yan Liu, Qi Chen, Yue-Hua Li, Jue-Jin Wang, Yu-Ming Kang and Guo-Qing Zhu made contributions to the conception and design. Tong-Yan Liu and Jue-Jin Wang performed the analysis. Tong-Yan Liu and Guo-Qing Zhu wrote the manuscript. All authors contributed to the discussion and revision of the manuscript.

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FIGURE LEGENDS

Figure 1 Effects of irisin on the key enzymes of gluconeogenesis (PEPCK and G6Pase) and glucose production in glucosamine (GlcN)-treated human HepG2 cells. A, relative values of PEPCK and G6Pase mRNA; B, relative values of PEPCK and G6Pase proteins; C, PEPCK and G6Pase activity; D, glucose production; E, cell viability; F, representative images of Western Blot showing PEPCK and G6Pase proteins. Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs. Vehicle; n=4 for each group.

Figure 2 Effects of irisin on the key enzymes of glycogenesis (GSK3 and GS) and glycogen level in glucosamine (GlcN)-treated human HepG2 cells. A, phosphorylation of GSK3; B, phosphorylation of GS; C, glycogen levels; D, cell viability; E, representative images of Western Blot showing GSK3 and GS proteins. Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs. Vehicle; n=4 for each group.

Figure 3 Effects of irisin on the PI3K p110α subunit and p110β subunit expression, and the phosphorylation of Akt and FOXO1. A and B, PI3K p110α subunit and p110β subunit protein expression in glucosamine (GlcN)-treated HepG2 cells; C and D, phosphorylation of Akt and FOXO1 in GlcN-treated HepG2 cells; E, cell viability; F and G, comparison of the effects of irisin and insulin on the phosphorylation of Akt and FOXO1 in Ctrl HepG2 cells. Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs. Vehicle; n=4 for each group.

Figure 4 PI3K inhibitor LY294002 or Akt inhibitor MK2206 prevented the effects of irisin on PEPCK, G6Pase, GSK3, glucose production, glycogen and FOXO1 in glucosamine (GlcN)-treated human HepG2 cells. A, relative values of PEPCK and G6Pase proteins; B, GSK3 phosphorylation; C, GS phosphorylation; D, glucose production. E, glycogen levels; F, FOXO1 phosphorylation; G, cell viability; H, representative images of Western Blot. Values are mean±SE. * P<0.05 vs. Vehicle+Vehicle; † P<0.05 vs. Vehicle+Irisin. n=4 for each group in A-C; n=6 for each group in D.

Figure 5 Effects of irisin on irisin levels, body weight, food intake and blood pressure in Ctrl and STZ/HFD mice. A, serum irisin levels; B, liver irisin levels. C, body weight; D, accumulated food intake (g) in 2 weeks; E, accumulated food intake (KJ) in 2 weeks; F, systolic blood pressure; Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs. STZ/HFD-Saline, n=7 for each group.

Figure 6 Effects of irisin on glucose metabolism and insulin resistance in Ctrl and STZ/HFD mice. A, serum insulin levels; B, fasting blood glucose level; C, liver glycogen content; D, representative images of periodic acid Schiff staining (PAS) of liver sections.
showing that the decreased glycogen in STZ/HFD mice was prevented by irisin treatment. red: glycogen; purple: nuclei of liver cells; E, glucose tolerance test (GTT); F, insulin tolerance test (ITT). Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs.STZ/HFD-Saline; n=7 for each group.

Figure 7 Effects of irisin on PEPCK, G6Pase, GSK3, GS and Akt in the livers of Ctrl and STZ/HFD mice. A, relative values of PEPCK and G6Pase mRNA in liver; B, relative values of PEPCK and G6Pase proteins in liver; C, PEPCK and G6Pase activity in liver; D, GSK3 phosphorylation; E, GS phosphorylation; F, Akt phosphorylation; G, representative images of Western Blot showing PEPCK, G6Pase, GSK3, GS and Akt proteins. Values are mean±SE. * P<0.05 vs. Ctrl; † P<0.05 vs. STZ/HFD-Saline; n=4 for each group.

Figure 8 Schematic diagram showing the downstream signal mechanism of irisin in modulating gluconeogenesis and glycogen synthesis in hepatocyte. Grey arrows represent the changes caused by irisin.