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Original Article

Zhenqing recipe relieves diabetic nephropathy through the SIK1/SREBP-1c axis in type 2 diabetic rats

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Abstract: Background: Salt-inducible kinase 1 (SIK1) plays an important role in lipid metabolism, which inhibits lipid synthesis by directly phosphorylating multiple serine residue sites of sterol regulatory element-binding protein 1c (SREBP-1c). This study examined potential therapeutic effect of a Chinese herbal medicine Zhenqing Recipe (ZQR) and metformin on diabetic nephropathy and investigated whether the SIK1/SREBP-1c axis is involved. Methods: The rat model of type 2 diabetes was developed by high-sucrose plus high fat diet for one month combined with low-dose Streptozocin intraperitoneal injection for three days, after which the presence of hyperglycemia and hyperlipidemia was examined to validate the model. The diabetic rats were then randomly allocated to diabetic groups treated by either ZQR or metformin, and normal rats receiving normal diet were included as a control group. Metabolic parameters, renal function, and renal triglyceride were examined and compared between groups. Results: After a treatment of 12 weeks, ZQR and metformin significantly reduced serum glucose and triglyceride, inhibited diabetic nephropathy and improved renal function. The mRNA level of SIK1 was significantly lower in the diabetic rats than that in the control group, while the expression of SREBP-1c had an opposite pattern. However, after receiving ZQR or metformin treatment, the SIK1 level in diabetic rats increased and the SREBP-1c level was downregulated. Consistent results were observed at the protein level. Conclusion: The data suggested that similar to metformin, ZQR could alleviate diabetic nephropathy through SIK1/SREBP-1c axis.

Keywords: ZQR, type 2 diabetes, SIK1, Srebp-1c

Introduction

Diabetic nephropathy is a microvascular complication which is common in patients with diabetes [1, 2]. The underlying mechanism is related to abnormal lipid metabolism and excessive deposition of lipid in the kidney due to insulin resistance. Excessive lipid deposition thickens the glomerular basement membrane and causes glomerular sclerosis, resulting in impaired renal function [1, 3], and therefore, becomes a potential therapeutic target.

One of the key nuclear transcription factors involved in lipid metabolism is sterol regulatory element-binding protein 1c (SREBP-1c), which directly regulates the genes associated with synthesis of fatty acid and triglyceride (TG) [4, 5]. In addition, it also mediates TG deposition in the kidney partly by regulating the release of cytokines including transforming growth factor-β (TGF-β), plasminogen activator inhibitor-1 (PAI-1), and vascular endothelial growth factor (VEGF) [6]. Increased expression of SREBP-1c has been observed in hyperinsulinemia and hyperglycemia, which promotes fatty acid synthesis and deposition of renal TG [6, 7]. The upregulated expression levels of the cytokines further cause kidney hypertrophy and aberrant proliferation of mesangial cells, leading to glomerular sclerosis and proteinuria. This suggests that SREBP-1c may serve as a molecular target for diabetic nephropathy. It has been reported that the aberrant expression of TGF-β, PAI-1 and VEGF in the kidney, the abnormal accumulation of extracellular matrix, and proteinuria are partly reversed after knocking out SREBP-1c in diabetic mouse model developed

by high-fat diet and Streptozocin injection [8, 9].

Salt-induced kinase 1 (SIK1) is also involved in diabetes and lipid metabolism partly by regulating SREBP-1c [10, 11]. The gene of SIK1 locates on human chromosome 21, and is expressed in several tissues, such as adrenal gland, kidney, heart, and liver [12]. It is a serine/threonine kinase, which is an 86 kDa protein containing three domains, including an N-terminal serine/threonine kinase domain, a following SNF-1 homology domain (SNH), and a C-terminal Protein kinase A (PKA) domain. As a member of AMPK family, SIK1 can directly regulate lipid synthesis mediated by SREBP-1c [10]. Evidence from basic research found that overexpression of SIK1 in liver downregulates the expression of lipid-producing genes, while downregulation of SIK1 upregulates SREBP-1c at protein level and its target genes, which could be reversed by co-expressing the mutated SREBP1-c with phosphorylation defects. These results suggest that the SIK1/SREBP-1c axis is critical for maintaining lipid metabolism homeostasis.

However, whether the SIK1/SREBP-1c axis plays a role in the aberrant metabolism of lipid in diabetic nephropathy has not been studied. Metformin is currently widely used for diabetes by activating AMPK activity, inhibiting hepatic gluconeogenesis, and increasing the sensitivity of peripheral tissues to insulin [13-15]. It is unclear whether metformin is also involved in regulating the SIK1/SREBP-1c axis *in vivo*. Zhenqing Recipe (ZQR) is a Chinese herbal formula, which consists of Eclipta prostrata, Fructus Ligustri Lucidi and Dioscorea opposite. We previously found that ZQR downregulates SREBP-1c at protein level and its downstream genes, and therefore reduces lipid deposition in kidney tissues [16], but it is unknown whether ZQR regulates the expression of SREBP-1c via the SIK1/SREBP-1c axis. By employing a rat model of type 2 diabetes, the current study investigated the potential therapeutic effect of ZQR and metformin on diabetic nephropathy and investigated whether the SIK1/SREBP-1c axis is involved.

Materials and methods

Diabetic rat model

Male rats (8-week old) were used to develop diabetic rat model. Before model establishment, the rats were kept in polycarbonate cages under an environment of temperature 23±3°C and humidity 60±5%. Meanwhile, a light cycle of 12 h on/12 h off was provided for one week. They were then randomly assigned to either the normal group (i.e., fed with normal diet, followed by intraperitoneal injection of citrate buffer of the same volume with that in the diabetic group), or the diabetic group (fed with high sucrose and high fat diet for four weeks, followed by intraperitoneal injection of Streptozocin (30 mg/kg; purchased from Sigma-Aldrich Inc. St. Louis, MO, USA, dissolved in citrate buffer with pH 4.5). When the fasting serum glucose level was >12 mmol/L 72 h after Streptozocin injection, the rats were considered to be successfully established with diabetic model. The Ethics Committee of the General Shipping Hospital of Yangtze River approved the study (No. S822).

Preparation for ZQR and metformin

ZQR consisted of Fructus Ligustri Lucidi, Eclipta prostrata, and Dioscorea opposite, which were purchased from Wuhan Longtai Pharmaceutical Co., Ltd (Wuhan, China). The ZQR extract was prepared as previously described [17]. In brief, the herbs were first mixed at a ratio of 1.2: 1.2: 1.5 (w/w/w) respectively, and then soaked in water (1:10, w/v) for 8 h. The herb mixture was further boiled for 2 h twice and then filtered. After being concentrated, 60% ethanol was used to precipitate the extract for 12 h. The extracted solution was then evaporated under reduced pressure and stored at 4°C with a final concentration of 3.0 g/mL. Metformin was purchased from Shenzhen Zhonglian Pharmacy Limited Company (Shenzhen, China), and it was dissolved in water with a final concentration of 30 mg/mL.

Treatment assignment

The study included four treatment groups, including the diabetic group, the ZQR group, metformin group, and control group. Diabetic rats were randomly assigned to one of the groups except for the control group. For the ZQR group and metformin group, the assigned rats were administered with ZQR extract (26 g/kg/d) or metformin suspension (150 mg/kg/d) orally for 12 weeks. While for rats assigned to diabetic group, they only received an equal volume of distilled water. Normal rats receiving

normal diet were assigned to control group, and only received equal volume of distilled water.

Biochemical assays, and measurement of TG in renal cortex

Enzymatic glucose-oxidase kits were used to measure serum glucose, and enzymatic couple colorimetric kits were used to determine TG and total cholesterol (TC) (Kangtai clinical reagent Co. Ltd (Beijing, China)). Urinary creatinine (Ucr) was detected by AU-400 automatic biochemical analyzer, and we calculated the endogenous creatinine clearance rate (Ccr) using the below formula: Ccr (mL/min) = [Ucr (μmol/L) × 24 h urine volume (mL)]/[Scr (μmol/L) × 1440 (min) × body weight (kg)]. Urine albumin (UAE) and fasting plasma insulin (FINS) were determined by radioimmunoassay. Total lipids were extracted from the renal cortex as previously described [17, 18], and TG was determined by the enzymatic method.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

TRIZOL reagent was used to homogenize rat kidney tissue (1 mL per 100 mg tissue) according to instructions provided by the manufacturer. In brief, chloroform phase separation removed DNA and protein. Isopropanol was then used to precipitate RNA in the aqueous phase, which was then re-suspended in DEPC-treated water. The absorbance at 260 nm and 280 nm was determined respectively to evaluate the quantity and quality of the isolated RNA. Single-strand cDNA was first synthesized by reverse transcription, and then PCR was performed. The primers used were: SREBP-1c: sense 5'-GGAGCCATGGATTGCACATT-3', antisense 5'-AGGAAGGCTTCCAGAGAGGA-3'; SIK1: sense 5'-CACTTTGCGGCTATTTACTACCT-3', antisense 5'-GCTGAAGCGAACTGTGGAGAT-3'; beta-actin: sense 5'-CGTTGACATCCGTAAAGACCTC-3', antisense 5'-TAGGAGCCAGGGCAGTAATCT-3'.

PCR product was visualized by 2% agarose gel electrophoresis, photo-graphed and quantified by Gel Documentation and Analysis System (ModelJS-300; Shanghai Peiqing Science and Technology Co. Ltd, Shanghai, China). The mRNA expression of beta-actin was used to normalize the relative mRNA level of the target genes in each sample.

Western blot

Lysing buffer with PMSF (1 mL: 10 μL) was used to homogenize kidney samples (50 mg), followed by incubation on ice for 30 min. The homogenates were then centrifuged at 12,000 × g for 30 min at 4°C in order to collect the supernatants. The BCA protein assay was used to examine protein concentrations. After that, protein samples were first denatured at 95°C in loading buffer and then subjected to electrophoresis on pre-cast 10% SDS-PAGE gels. Samples were then electroblotted onto nitrocellulose membranes (0.2 μm pore) after electrophoresis. The 5% (w/v) milk powder in Tris buffered saline containing 0.2% Tween 20 (TBST) was used to block the membranes for 2 h at room temperature, followed by incubation with SIK1 (mouse source, obtained from Santa crus) or SREBP-1c mono-antibody (rabbit source, obtained from Santa crus) at 4°C overnight. We used horseradish peroxidase-conjugated anti-IgG as a secondary antibody. After rinsing, chemiluminescence kit was used to detect the reactive bands, and the Quantity one imaging processing system (BIO-RAD, Hercules, California, USA) was sued to quantitate immunoblot intensities by densitometry.

Co-immunoprecipitation

To examine potential interaction between SIK1 and SREBP-1c, co-immunoprecipitation was performed. Similar to the procedure described above, kidney samples were lysed and then the supernatants of the lysates were incubated with anti-SIK1 antibody (or IgG, which was used as negative control) for 45 minutes at room temperature, after which protein A-Sepharose beads were added and incubated for an additional 30 min. Western blot was then performed as described above for detecting SIK1 and SREBP-1c.

Statistical analysis

Results were expressed as the mean ± SEM (standard error of mean). We used Student's t test for the comparisons between two groups and one-way ANOVA for multiple-group comparison. A $P<0.05$ was considered as statistically significant. All the statistical analyses were performed by SPSS (Version 13, SPSS Inc., USA), and GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) was used for statistical plots.

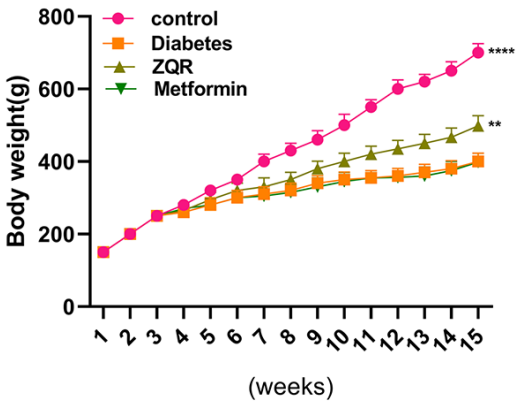

Figure 1. Body weight of rats in different groups. Body weight was monitored weekly. Data are presented as means ± SEM (n = 10 for control group, n = 8 for each of the other groups). **P<0.01 vs. Diabetic group. ****P<0.0001 vs. Diabetic group. Abbreviations: ZQR, Zhenqing Recipe; SEM, standard error of mean.

Results

Effect of ZQR and metformin on body weight

The rats in the control group showed expected increase in weight and rapid response, while the rats in the diabetic group gradually became wilted and dull, without significant increase in weight. Increased amounts of water intake, food intake, and urine output were observed in the diabetic group. The rats in the ZQR group and the metformin group were more responsive, sensitive, and shiny compared to those in the diabetic group. The body weight of the rats in the ZQR group was relatively stable with a slight increase, but the weight of the rats in the metformin group showed the same trend as that in the diabetic group (**Figure 1**).

Effect of ZQR and metformin on metabolic parameters

After 4 weeks of high-sucrose and high-fat diet, there was no significant difference in fasting plasma glucose (FBG) between the diabetic group and the control group (P>0.05), but the levels of FINS, serum TG, and TC were significantly higher than those in the control group (P<0.05, P<0.05, and P<0.01, respectively), while the insulin sensitivity index (ISI) was significantly lower than that in the control group (P<0.05, Supplementary Table 1). Three days after Streptozocin injection, the FBG of the diabetic group was significantly higher than that of the control group (P<0.01), but the level of FINS was lower than that before the Streptozocin injection, which was not significantly higher than that in the control group (P>0.05, Supplementary Table 2). After 12 weeks of treatment, the FBG and serum TG in the ZQR group and the metformin group were significantly lower than those in the diabetic group (P<0.01 for ZQR group, P<0.05 for metformin group), but the serum TC did not change significantly (P>0.05). Compared with the metformin group, ZQR showed a better therapeutic effect (**Table 1**).

Effect of ZQR and metformin on renal function

Compared with the diabetic group, the KI and UAE in the ZQR group and the metformin group were significantly lower (P<0.05 for KI, and P<0.01 for UAE), while the level of Ccr was significantly higher (P<0.05) (**Table 2**). Compared with the control group, the TG content in the renal cortex significantly increased in the diabetic group (P<0.01), which significantly decreased after receiving ZQR or metformin treatment (P<0.01) (**Table 3**).

Effect of ZQR and metformin on renal histomorphology changes

There was a clear structure of glomeruli and renal tubules and normal glomerular capsule cavity in the control group. Compared with the control group, larger glomerular tuft area and higher mesangial matrix index were observed in the diabetic group (**Figure 2A** and **2D**), indicating glomerular hypertrophy and mesangial matrix expansion. These pathological changes were attenuated in the ZQR group and the metformin group (**Figure 2B** and **2C**).

Effect of ZQR and metformin on the levels of SIK1 and SREBP-1c in renal tissue

Compared with the control group, both the mRNA level and the protein level of SREBP-1c in the renal tissue of the diabetic group was significantly higher (P<0.01), while SIK1 showed a significantly lower expression level (P<0.01). After 12 weeks' treatment, compared with the diabetic group, ZQR significantly increased the mRNA level and the protein level of SIK1 (P<0.01) but decreased the expression of SREBP-1c (P<0.01) in the kidney tissue (**Figures 3** and **4**; the original, full-length gel and blot

Table 1. Effect of ZQR and metformin on FBG, TG and TC

Group	n	FBG (mmol/L)	TG (mmol/L)	TC (mmol/L)
Control group	10	5.21±0.53	0.66±0.17	2.13±0.19
Diabetic group	8	16.67±4.33	1.33±0.33	4.35±1.91
P value (vs. Control group)	-	<0.001	0.008	<0.001
(Diabetic group +) ZQR	8	10.94±3.33	0.79±0.27	4.44±0.87
P value (vs. Control group)	-	<0.001	0.023	<0.001
P value (vs. Diabetic group)	-	<0.001	0.027	0.557
(Diabetic group +) Metformin	8	12.86±3.57	1.02±0.31	4.37±0.64
P value (vs. Control group)	-	<0.001	<0.001	<0.001
P value (vs. Diabetic group)	-	0.016	0.027	0.480

Note: Results are presented as mean ± SEM for each group. Abbreviations: ZQR, Zhenqing Recipe; FBG, fasting plasma glucose; TG, triglyceride; TC, total cholesterol; SEM, standard error of mean.

Table 2. Effect of ZQR and metformin on renal function

Group	n	KI (%)	UAE (mg/24 h)	Ccr (mL/min)
Control group	10	0.58±0.04	1.82±0.43	3.92±0.88
Diabetic group	8	0.85±0.07	12.33±2.45	1.60±0.18
P value (vs. Control group)		0.003	<0.001	0.009
(Diabetic group +) ZQR	8	0.63±0.05	7.60±3.35	2.98±0.46
P value (vs. Control group)		0.829	<0.001	0.033
P value (vs. Diabetic group)		0.017	0.005	0.019
(Diabetic group +) Metformin	8	0.65±0.06	9.07±2.39	2.81±0.23
P value (vs. Control group)		0.149	<0.001	0.036
P value (vs. Diabetic group)		0.022	0.003	0.031

Note: Results are presented as mean ± SEM for each group. Abbreviations: ZQR, Zhenqing Recipe; KI, kidney index; UAE, urine albumin; Ccr, creatinine clearance rate; SEM, standard error of mean.

Table 3. Effect of ZQR and metformin on renal TG

Group	n	TG (mmol/L)
Control group	10	0.163±0.499
Diabetic group	8	0.291±0.078
P value (vs. Control group)	-	0.003
(Diabetic group +) ZQR	8	0.178±0.075
P value (vs. Control group)	-	0.202
P value (vs. Diabetic group)	-	0.004
(Diabetic group +) Metformin	8	0.171±0.069
P value (vs. Control group)	-	0.392
P value (vs. Diabetic group)	-	0.004

Note: Results are presented as mean ± SEM for each group. Abbreviations: ZQR, Zhenqing Recipe; TG, triglyceride; SEM, standard error of mean.

images for **Figures 3** and **4** are provided in the Supplementary Figures 1 and 2). Result of co-immunoprecipitation suggested there was no direct interaction between SIK1 and SREBP-1c (Supplementary Figures 3 and 4).

Discussion

We have previously indicated that ZQR helped to decrease blood glucose and blood lipids in diabetic rats and improve the renal function [17]. However, although it was proved that ZQR works through downregulating Srebp-1c, it remains unclear about the underlying mechanism. In the present study, we examined the protective effect of ZQR against diabetic nephropathy with metformin as a positive control, and further evaluated the molecular mechanism, especially the role of the SIK1/SREBP-1c axis.

We established rat model with type 2 diabetes by high-fat diet with low dose intraperitoneal injection of Streptozocin. The diabetic rats showed a lower body weight, higher levels of TC, TG, FBG, and FINS than those of normal control rats. Compared with diabetic rats, the body weight of ZQP treated rats increased significantly. The effect of ZQP on FBG and TG was also evaluated in our study. It has been proved that the three herbs in ZQP could improve glucose and lipid metabolism separately [19, 20]. Consistent with our previous study, the levels of FBG, TG and TC decreased after 12 weeks of ZQP treatment, which was similar in the metformin group. These results demonstrated the protective effect of ZQP on hyperglycemia and hyperlipidemia.

SREBP-1c mediates abnormal lipid metabolism and renal TG deposition, which are involved in the development of diabetic nephropathy. Studies have shown that Streptozocin-induced type 1 diabetic rats can increase renal tissue TG deposition and upregulate the expression of

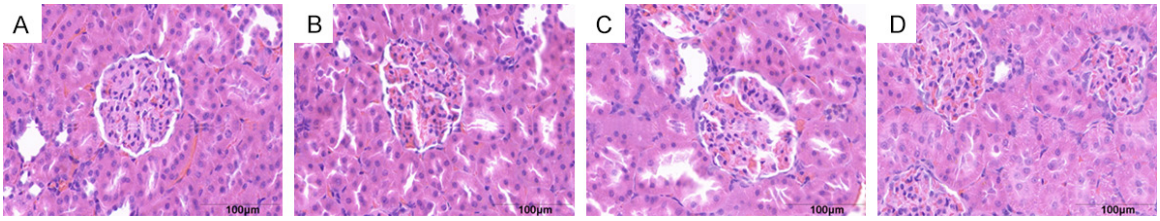

Figure 2. HE staining of kidney section. ZQR attenuated glomerular hypertrophy and mesangial matrix expansion. Control group (A), ZQR group (B), Metformin group (C), and Diabetic group (D). Original magnification: × 200. Abbreviations: HE, Hematoxylin and eosin; ZQR, Zhenqing Recipe.

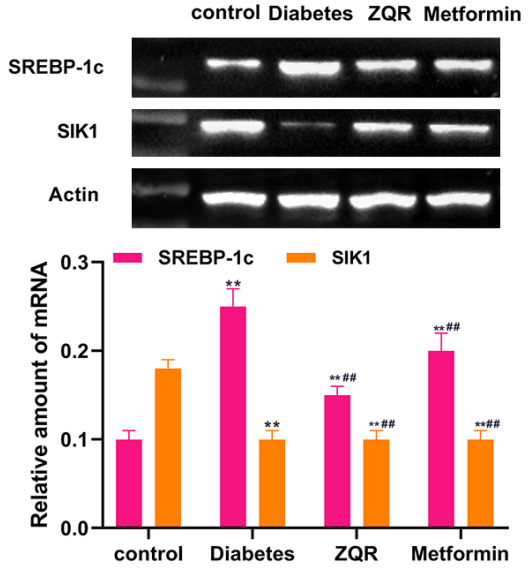

Figure 3. The mRNA levels of SREBP-1c and SIK1 in rat kidney examined by RT-PCR. n = 10 in each group. **P<0.01 vs. Control group. $^{\#\#}$P<0.01 vs. Diabetic group. Abbreviations: SREBP-1c, sterol regulatory element-binding protein 1c; SIK1, salt-inducible kinase 1; RT-PCR, reverse transcription-polymerase chain reaction; ZQR, Zhenqing Recipe.

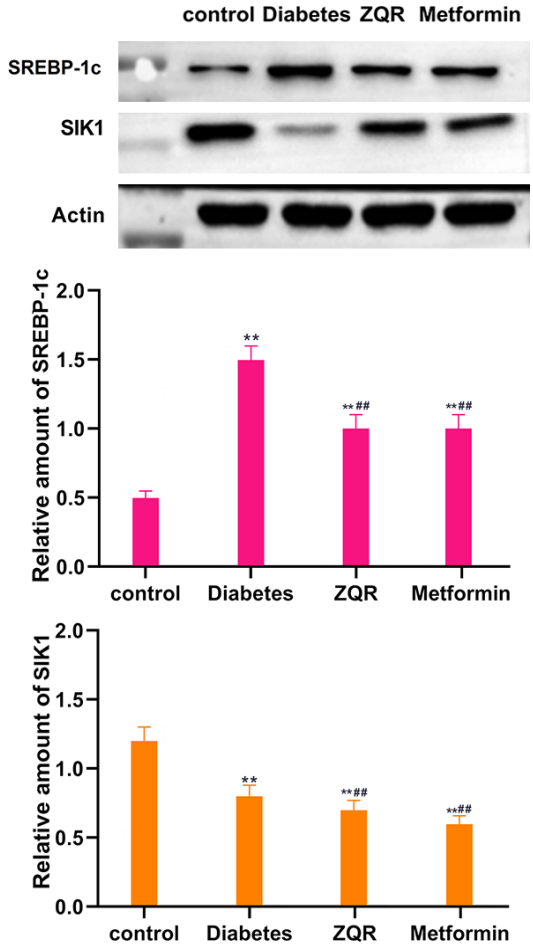

Figure 4. The protein levels of SREBP-1c and SIK1 in kidney. n = 10 in control group and n = 8 in each of the other groups. **P<0.01 vs. Control group. $^{\#\#}$P<0.01 vs. Diabetic group. There was no significant difference in the protein levels of both SREBP-1c and SIK1 between the ZQR and the Metformin group (P all >0.05). Abbreviations: SREBP-1c, sterol regulatory element-binding protein 1c; SIK1, Salt-inducible kinase 1; ZQR, Zhenqing Recipe.

SREBP-1c [8]. In our study, the diabetic rats showed an increased mRNA and protein levels of SREBP-1c in the kidney and increased TG content in renal tissue. IN addition, impaired renal function and renal pathological changes were observed during a follow-up of twelve weeks, which was consistent with other studies. We found that FBG, TG, UAE, renal weight index, and renal TG content in diabetic rats were significantly decreased after ZQR or metformin treatment, with an increased Ccr and improved renal tissue morphology, suggesting a renal protection effect of ZQR and metformin.

In vitro studies revealed that SREBP-1c was a direct substrate for SIK1 [10]. SIK1, as a member of the AMPK family, is a heterotrimeric protein. AMPK is an energy regulator in the body that promotes glucose reabsorption, glycolysis

and fatty acid oxidation [21]. SIK1 directly phosphorylates the multiple serine residue sites of SREBP-1c to inhibit lipid synthesis [22, 23]. We found that the expression level of SIK1 was significantly lower in the diabetic rats compared to that of the control group. Consistently, the expression of SREBP-1c is higher in the control group compared with diabetic rats, indicating that SIK1 was directly or indirectly involved in the regulation of SREBP-1c expression in diabetic rats. We also found that the mRNA and protein levels of SREBP-1c in the renal cortex of the ZQR group and metformin group significantly decreased compared to the diabetic group, while the mRNA and protein levels of SIK1 significantly increased. Therefore, it indicates that ZQR may protect the kidneys of diabetic rats through the SIK1/SERBP-1c axis.

In conclusion, the present study revealed that ZQR decreased hyperglycemia and hyperlipidemia, and improved renal function in diabetic rats. The observed therapeutic effect of ZQR could be related to its upregulation of SIK1 which further inhibits the SREBP-1c mediated TG synthesis. More studies are needed to clarify the underlying mechanisms of ZQR, which may disclose novel therapeutic targets for diabetic nephropathy.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Effect of 1-month high-sucrose/fat diet on metabolic parameters

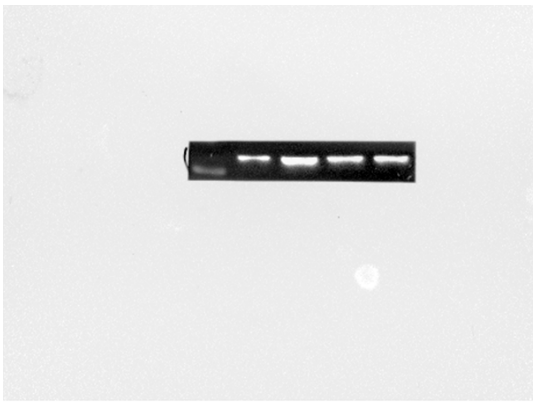
Group	n	FBG (mmol/L)	TG (mmol/L)	TC (mmol/L)	FINS (mU/L)	ISI
Diabetic group	30	6.38±1.24	1.14±0.25	2.84±0.43	29.39±0.68	-5.66±0.35
Control group	10	5.14±0.64	0.70±0.18	1.91±0.22	19.07±0.34	-4.07±0.51
P value	-	0.294	0.004	<0.001	<0.001	0.036

Note: Results are presented as mean ± SEM for each group. Abbreviations: FBG, fasting plasma glucose; TG, triglyceride; TC, total cholesterol; FINS, fasting plasma insulin; ISI, insulin sensitivity index; SEM, standard error of mean.

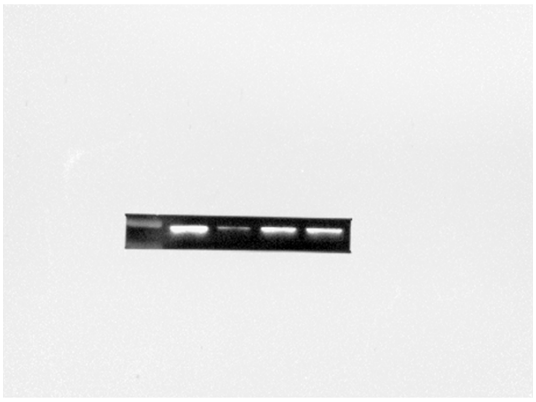
Supplementary Table 2. Effect of Streptozocin on FBG and FINS

Group	n	FBG (mmol/L)	FINS (mU/L)
Diabetic group	30	14.34±1.0	21.74±1.01
Control group	10	4.47±0.48	19.85±0.43
P value	-	<0.001	0.093

Note: Results are presented as mean ± SEM for each group. Abbreviations: FBG, fasting plasma glucose; FINS, fasting plasma insulin; SEM, standard error of mean.



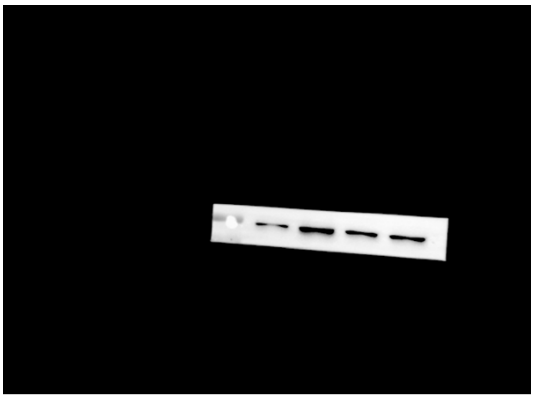
SREBP-1c mRNA



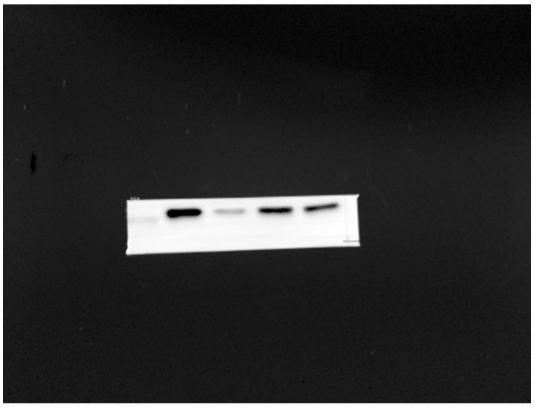
SIK1 mRNA

Actin mRNA

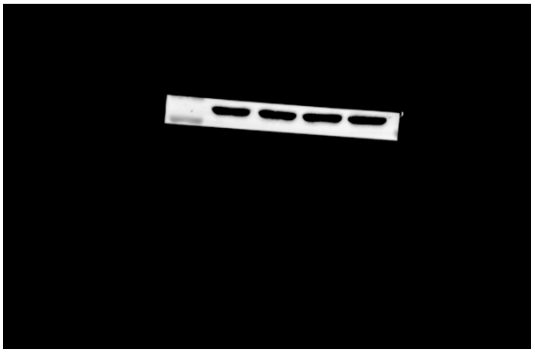
Supplementary Figure 1. Original, full-length gel images for **Figure 3**.



SREBP-1c protein

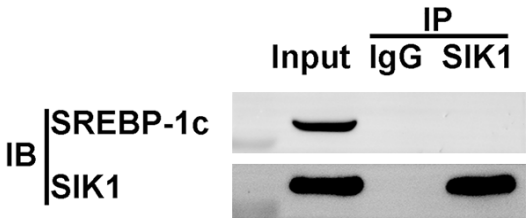


SIK1 protein

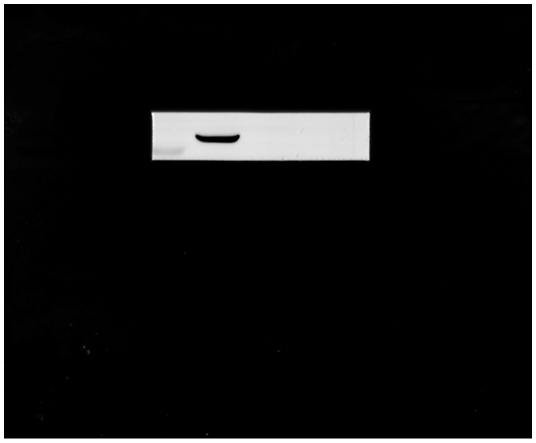


Actin protein

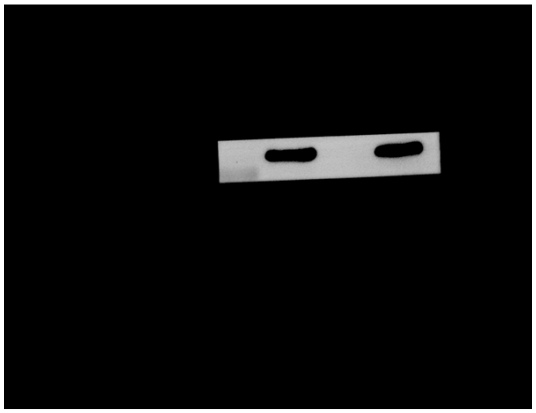
Supplementary Figure 2. Original, full-length blot images for **Figure 4**.



Supplementary Figure 3. Co-immunoprecipitation between SIK1 and SREBP-1c.



SREBP-1c



SIK1

Supplementary Figure 4. Original, full-length blot images for **Supplementary Figure 3**.