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Glycyrrhizic Acid Protects Glomerular Podocytes Induced by High Glucose by Modulating SNARK/AMPK Signaling Pathway*

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[Abstract] Objective: Diabetic nephropathy is one of the most important microvascular complications of diabetes, which mainly refers to glomerular capillary sclerosis. Podocytes are an important part of glomerular capillaries. Previous clinical and basic studies have shown that fibrosis is the main factor of diabetic nephropathy. This study aimed to assess the protective mechanism of glycyrrhizic acid (GA) on glomerular podocytes induced by high glucose as we hypothesized that GA may have antifibrotic and anti-inflammatory effects on podocytes through regulation of the adenosine 5′-monophosphate-activated protein kinase (AMPK)/sucrose nonfermenting AMPK-related kinase (SNARK) signaling pathway. **Methods:** SNARK siRNA was used to transfect podocytes. Real-time quantitative polymerase chain reaction and immunofluorescence staining assays were used for molecular and pathological analysis. The expression levels of key pathway proteins (including TGF-β1, α-SMA, SITR1, AMPKα, LKB1, PGC-1α, NF-κB, IL-6, and TNF-α) were verified by Western blotting. The expression of inflammatory factors in podocytes was detected by ELISA. **Results:** We demonstrated that GA decreased the expression of podocyte fibrosis signaling pathway-related factors by upregulating the AMPK pathway and its related factors. However, after transfection of podocytes with SNARK siRNA, there was an increased expression of fibrosis-related factors and inflammation-related factors. **Conclusion:** GA can protect podocytes and alleviate fibrosis and inflammation induced by high glucose, which is related to the AMPK signaling pathway. Meanwhile, knockdown of SNARK protein can inhibit the AMPK signaling pathway, aggravate fibrosis, and increase inflammation.
Key words: podocyte; glomerular fibrosis; glycyrrhizic acid; diabetic nephropathy; AMPK; SNARK

Diabetes mellitus (DM) is a type of endocrine and metabolic disease that seriously affects human health. Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes[1, 2]. The most important pathophysiological change in the progression of DN to end-stage renal disease is irreversible glomerular fibrosis. Transforming growth factor-β1 (TGF-β1) and alpha smooth muscle actin (α-SMA) are key drivers of fibrosis, and their increased expression can lead to fibrosis in DN[3, 4]. Adenosine 5′-monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine kinase. Under physiological and pathological conditions, when cells are subjected to hypoxia, ischemia, glucose loss, and heat stress, the intracellular AMP/ATP ratio increases and AMPK is activated. Activated AMPK regulates cell metabolism and maintains the cell energy supply and cell homeostasis. In addition, there is a close relationship between the AMPK signaling pathway and pathological fibrosis[5]. There is a close relationship between the adenosine 5′-monophosphate-activated protein kinase (AMPK) signaling pathway and fibrosis. The reduction or loss of AMPK signaling pathway activity in tissue cells can promote the occurrence and progression of fibrosis, while the activation of AMPK signaling pathway in tissue cells can inhibit the occurrence or progression of fibrosis to a certain extent[6, 7]. Moreover, AMPK activation decreased TGF-β1 promoter activity. Since the TGF-β/SMAD signaling pathway is a key pathway for the induction of EMT[6], AMPK can block the TGF-β/SMAD signaling pathway and therefore inhibit the EMT process[7]. Sucrose nonfermenting AMPK-related kinase (SNARK) has a role in glucose and lipid homeostasis, similar to AMPK[8]. SNARK was

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identified as the fourth member of the AMPK kinase family in 2001[9]. The catalytic domain of SNARK has significant homology with the catalytic domain of AMPKα1, AMPKα2, and AMPK-related kinase 5[8]. Although the function of SNARK is unknown, mice after SNARK depletion exhibit pathological symptoms such as elevated serum triglyceride concentrations, hyperinsulinemia, glucose intolerance, and impaired contractile stimulated glucose transport, implying that SNARK maintains glucose and lipid homeostasis in a manner similar to AMPK. In recent years, targeted agents have been developed against AMPK in many chronic inflammatory diseases and several cancers, but none have been developed against SNARK. Our next study is to explore whether GA plays a protective and preventive role in the fibrosis of DN by regulating SNARK, that is, whether GA can play a protective role in the kidney by regulating the expression of SNARK/AMPK and downstream factors[10]. To determine whether GA is a specific SNARK receptor regulator, further clarification of the mechanism of GA in the treatment of DN and the discovery of specific therapeutic drugs for DN are necessary[11]. Glycyrrhizic acid (GA) is one of components in licorice, which has anti-inflammatory, anti-oxidation effects[12]. In recent years, cell experiments have shown that GA can alleviate fibrosis and inflammation by regulating mitochondrial function and reducing reactive oxygen species production[13, 14]. Our previous studies have demonstrated that GA can alleviate diabetic nephropathy by activating the AMPK signaling pathway, reducing reactive oxygen species production, inhibiting cell apoptosis, and improving renal fibrosis. Therefore, GA may be a potential therapeutic agent for DKD. However, the mechanism is unclear. This study aimed to explore whether GA plays a protective and preventive role in the fibrosis of DN by regulating SNARK, that is, whether GA protects the kidney by regulating the expression of AMPK/SNARK and downstream factors in DN.

1 MATERIALS AND METHODS

1.1 Materials

Mouse glomerular podocytes were purchased from Zhongqiao Xinzhou Co., Ltd. (China). Trypsin EDTA was from Soleippo Co., Ltd. (China). Fetal bovine serum and D-glucose powder were from Shengong Biological Co., Ltd. (China). All 6-cm dishes, 10-cm dishes, 6-well plates, and 24-well plates were from Corning (USA). BCA protein determination kits were purchased from Keygen (China). GA was bought from Mr. Lai Treasure Company (China). INVI DNA and RNA transfection reagent was from Invigentech (USA). TGF-β1, α-SMA, and sirtuin 1 (SIRT1) antibodies were from Affinity Biosciences (China). Liver kinase B1 (LKB1) and peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α antibodies were purchased from Proteintech (USA). Nuclear factor kappa B (NF-κB) antibodies were purchased from Cell Signaling Technology (USA). Interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) antibodies were purchased from Santa Cruz Biotechnology (USA). ELISA kits were purchased from Jiangsu Enzyme Immunity Biotechnology Co., Ltd. (China). The spectrophotometer used in this study was purchased from BioTek (USA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore Corporation (USA). The FV1000 laser scanning confocal microscope was purchased from Olympus (USA).

1.2 Cell Culture and Experimental Grouping

Mouse podocyte cells were cultured in RPMI-1640 complete medium containing recombinant murine interferon gamma (50–100 U/mL). The cells were incubated with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (37°C, 5% CO_2) for 17 h. Then, the cells were divided into the normal glucose (NG) group (5.6 mmol/L glucose), high-glucose (HG) group (30 mmol/L glucose), high-glucose+GA (HG+GA) group (30 mmol/L glucose+100 μmol/L GA), high-glucose+siRNA (HG+SI) group, and high-glucose+GA+siRNA (HG+GA+SI) group and cultured for 48 h. After the cells were inoculated into the petri dish, the cells were transfected until a confluency of 60%%–80% was reached. SNARK-siRNA and transfection reagent were mixed 1:1 into a composite solution, and the mixture was blown 15 times with a pipetting device and incubated for 15 min at room temperature. The compound solution was added to the cells with complete medium and placed in an incubator. After 24 h, the media with high glucose and high glucose plus GA were added for intervention and cultured for 48 h for subsequent experiments. Proteins were extracted from the cells for Western blotting. The cell supernatant was used for ELISA.

1.3 Western Blotting

The expression of AMPK, SNARK, LKB1, p-AMPK, SIRT1, PGC-1α, TGF-β1, α-SMA, TNF-α, IL-6, and NF-κB in the cells was detected by Western blotting. The cells were divided into 5 groups. After 48 h of intervention, the cells were washed with cold phosphate buffer 3 times, and then a whole-cell extraction kit (Keygen Biotech, China) was used to extract protein. The protein concentration was detected by the BCA method (Keygen Biotech, China), and then the protein was repacked. The protein extract was transferred from the SDS-PAGE gel to a PVDF membrane, followed by incubation with the following primary antibodies: anti-AMPK (1:1000), anti-SNARK (1:1000), anti-LKB1 (1:1000), anti-p-AMPK (1:1000), anti-SIRT1 (1:1000), anti-PGC-1α

(1:5000), anti-TGF-β1 (1:5000), anti-α-SMA (1:5000), anti-TNF-α (1:5000), anti-IL-6 (1:5000), anti-NF-κB (1:5000), and anti-GAPDH (1:1000) overnight at 4°C. After incubation with the secondary antibody, ECL luminescent liquid was used in the dark, and the signals were quantified using the Bio-RAD Gel Imaging System (Bio-RAD, USA). Values were normalized with the internal control (GADPH).

1.4 Immunofluorescence Assay

For the immunofluorescence detection of AMPK, SIRT1, TGF-β1, α-SMA, and NF-κB expression, the cells were inoculated on glass slides and treated, respectively, for 48 h after reaching the stable state. After 48 h of the intervention, the cells were fixed with 4% paraformaldehyde and sealed with goat serum. After 1 h, the goat serum was aspirated, and the cells were incubated with anti-AMPK (1:200), anti-SIRT1 (1:200), anti-TGF-β1 (1:200), anti-α-SMA (1:200), and anti-NF-κB (1:200) overnight at 4°C, respectively, followed by incubation with FITC-conjugated secondary antibody (1:250, Abbkine, China). The cells were viewed with an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus, USA). The positive cells were observed under 400-fold magnification. The relative fluorescence intensity was measured by Image J software and averaged. The experiment was repeated 3 times, and statistical analysis was performed. The protein expression and fluorescence intensity of AMPK, SIRT1, TGF-β1, α-SMA, and NF-κB were observed by a laser scanning confocal microscope.

1.5 ELISA

The secretion of NF-κB, IL-6, and TNF-α in the cell supernatant was detected by ELISA. The cells were seeded in a 6-well plate at a density of 1×10^5 cells/well. After the state of the cells was stable for 17 h, the cells were divided into the intervention groups. After 48 h, the supernatant of each group was collected, and the cells were centrifuged at 1500×*g* and 4°C for 20 min. The absorbance (*A*) of each well was measured at a wavelength of 450 nm, according to the instructions provided in the ELISA kit.

1.6 RT-qPCR Assay

The mRNA expression levels of AMPK, SIRT1, TGF-β1, α-SMA, and NF-κB in podocytes were detected by RT-qPCR. Total RNA was extracted from podocytes in 10-cm dishes according to the instructions provided by the TIANGEN Total RNA Extraction Kit (China). RNA was transcribed from degenomic DNA (reaction conditions: 42°C for 2 min, 4°C) to cDNA (reaction conditions: 37°C for 15 min, 85°C for 5 s, 4°C). GAPDH was used as the internal reference. The PCR conditions were as follows: 95°C for 30 s, 95°C for 5 s, 60°C for 31 s, 40 cycles, and the data were collected for melting curve analysis. At the end of the reaction, the expression level of the target gene relative to that of the internal reference GAPDH was calculated by the 2-ΔΔCT method, and the experiment was repeated 3 times.

1.7 Statistical Analysis

SPSS 21.0 software (IBM, USA) was used for statistical analysis. Measurement data were expressed as the mean ± standard deviation, and each independent experiment was repeated 3 times. Univariate analysis of variance was used for the comparison of multiple mean values. The Student-Newman-Keuls test (*q* test) was used for multiple comparisons of multiple mean values. $P\leq0.05$ was considered statistically significant.

2 RESULTS

2.1 Effect of GA on Fibrosis-related Factors in Podocytes Induced by High Glucose

According to our previous data, we set the most significant effect of GA at 100 μmol/L[15]. In order to explore the effect of GA on the expression of TGF-β1 and α-SMA in podocytes, Western blotting analysis was performed. As compared with the NG group, TGF-β1 and α-SMA in the HG group were significantly increased, and fibrosis was relieved after the addition of GA (fig. 1).

2.2 Effect of GA on SNARK/AMPK Pathway-related Factors in Podocytes Induced by High Glucose

Since the SNARK/AMPK pathway plays an important role in the protection of fibrosis, we next conducted Western blotting analysis to investigate

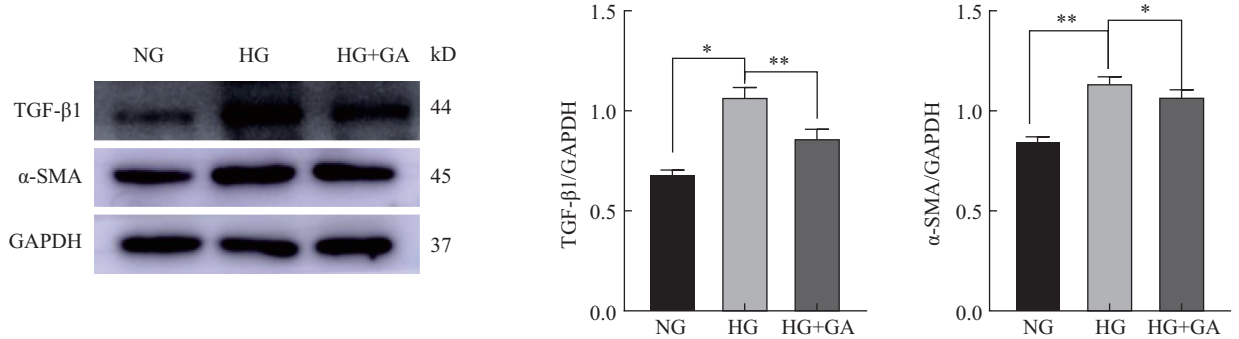

Fig. 1 Glycyrrhizic acid can inhibit the increase of TGF-β1 and α-SMA expression induced by high glucose
NG: control group; HG: high-glucose group; HG+GA: high-glucose+glycyrrhizic acid group. $^*P<0.05$, $^{**}P<0.01$

whether GA can regulate the expression of SNARK/AMPK pathway-related factors. As compared with the NG group, in the HG group, the expression levels of LKB1, p-AMPK, SIRT1, and PGC-1α were significantly decreased, and all were significantly increased after the addition of GA. However, the expression of AMPK did not change significantly among the groups (fig. 2).

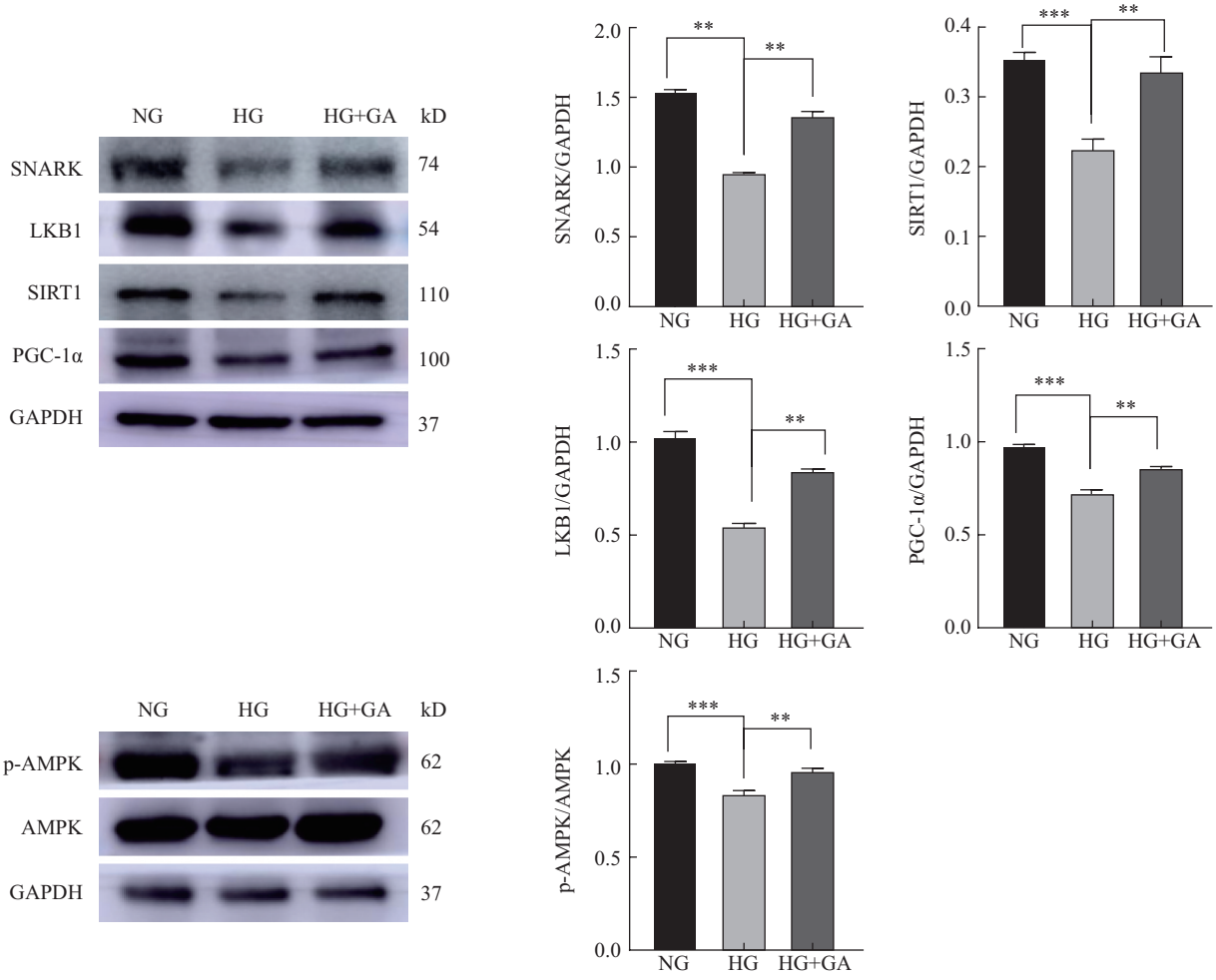

Fig. 2 Glycyrrhizic acid can activate the expression of SNARK/AMPK signaling pathway-related factors in glomerular podocytes induced by high glucose
SNARK, LKB1, p-AMPK, SIRT1, AMPK, and PGC-1α expression in each group was detected by Western blotting. NG: control group; HG: high-glucose group; HG+GA: high-glucose+glycyrrhizic acid group. $^{**}P<0.01$, $^{***}P<0.001$

2.3 Expression of SNARK in Normal Podocyte Group after SNARK Shearing

In order to verify the siRNA shearing effect, 3 kinds of siRNAs were designed and screened. The siRNAs were transfected into podocytes with transfection reagent, and the total protein was extracted from normal and siRNA-transfected (SI-516-SNARK, SI-670-SNARK, and SI-1455-SNARK) cells. The Western blotting results showed that all 3 siRNAs could reduce the expression of SNARK protein, and SI-516-SNARK had the best effect (fig. 3).

2.4 Effect of SNARK-siRNA on Fibrosis-related Factors in Podocytes

In order to further prove how SNARK affects TGF-β and α-SMA at the protein level and mRNA level, we knocked down SNARK with siRNA and added GA for intervention. Western blotting, cell immunofluorescence, and RT-qPCR assays were performed. TGF-β1 and α-SMA were significantly increased in the HG group as compared with NG group. TGF-β1 and α-SMA were also significantly decreased in the HG+GA group as compared with the HG group, TGF-β1 and α-SMA were also significantly decreased in the HG+GA+SI group as compared with the HG+SI group (fig. 4A and 4B). The mRNA expression of TGF-β1 and α-SMA in podocytes was detected by RT-qPCR. It was further proven that decreasing the expression of SNARK protein could increase the expression of podocyte fibrosis-related signaling pathway proteins (fig. 4C).

2.5 Effect of SNARK-siRNA on AMPK Pathway-related Factors in Podocytes

Next, we verified the effect of SNARK on other factors in the SNARK/AMPK pathway. We used siRNA

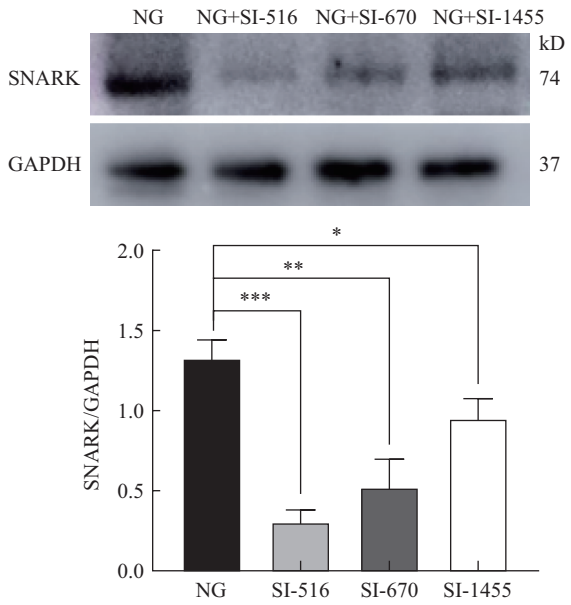

Fig. 3 SNARK siRNA transfection efficiency
After siRNA transfection SNARK was significantly reduced as compared with the NG group, and SI-516 group had the most obvious knockdown effect.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

to knock down SNARK and added GA for intervention. Western blotting, cell immunofluorescence, and RT-qPCR assays were performed. The Western blotting results showed that as compared with the NG group, LKB1, p-AMPK, SIRT1, and PGC-1α were significantly decreased in the HG group. As compared with the HG group, LKB1, p-AMPK, SIRT1, and PGC-1α were also significantly increased in the HG+GA group. LKB1, p-AMPK, SIRT1, and PGC-1α were also significantly increased in the HG+GA+SI group as compared with the HG+SI group (fig. 5A). The mRNA and protein expression levels of AMPK and SIRT1 in podocytes were detected by RT-qPCR and immunofluorescence assays (fig. 5B and 5C).

2.6 Effect of SNARK-siRNA on Inflammation-related Factors in Podocytes

To further prove whether SNARK affects inflammation-related factors at the protein level and mRNA level, we used siRNA to knock down SNARK and added GA for intervention and then carried out Western blotting, cell immunofluorescence, RT-qPCR, and ELISA detection. Western blotting, RT-qPCR, and ELISA showed that as compared with the NG group, NF-κB, TNF-α, and IL-6 were significantly increased in the HG group. As compared with the HG+GA group, NF-κB, TNF-α, and IL-6 were also significantly increased in the HG group. However, as compared with the HG+SI group, NF-κB, TNF-α, and IL-6 in the HG+GA+SI group were significantly decreased (fig. 6A, 6C and 6D; table 1). The expression of NF-κB in podocytes was detected by cellular immunofluorescence. It was further demonstrated that decreasing the expression of SNARK protein increased the protein expression of podocyte inflammation-related factors (fig. 6B).

3 DISCUSSION

DN is a complex inflammatory disease and the main cause of end-stage renal disease (ESRD)[1, 2]. Diabetic kidney disease (DKD) is one of the microvascular complications of diabetes[16, 17]. The main manifestations include an increased glomerular permeability and filtration rate[18]. The glomerular filtration barrier (GFB) destruction is the main pathogenesis, and it is mainly caused by podocyte injury[19–21]. In severe cases, DN can lead to kidney failure[22]. Renal diseases of diabetes include ESRD[23] and chronic kidney disease[24, 25].

Podocytes are an important component of the glomerular basement membrane, and the combination of the glomerular basement membrane with glomerular endothelial cells maintains the filtration capacity of the kidney[26–28]. When podocytes are injured in DKD, podocytes separate from the basement membrane of the glomeruli, resulting in compensatory hypertrophy of the remaining podocytes[29, 30], an increased podocyte foot process width, GFB destruction, and an increased glomerular filtration rate[31, 32]. Although hyperglycemia is thought to be a driving force in the development of DKD, some studies suggest that hyperglycemia may not be a major culprit of DKD[33, 34]. Evidence has shown that HG affects metabolic stress activation[35, 36], including hypoxia, DNA damage, and oxidative stress[37]. In addition, HG can activate the phosphorylation level of AMPK by adenylate, leading to oxidative stress of glomerular podocytes[38]. Our previous studies have demonstrated that GA can alleviate diabetic nephropathy by activating the AMPK signaling pathway, reducing reactive oxygen species production, inhibiting cell apoptosis, and improving renal fibrosis. Therefore, GA may be a potential therapeutic agent for DKD. However, the mechanism is unclear.

GA is one of the most important active components in licorice, which has anti-inflammatory, and anti-oxidation effects[12]. In recent years, cell experiments have shown that GA can alleviate fibrosis and inflammation by regulating mitochondrial function and reducing reactive oxygen species production[13]. GA can significantly improve hyperglycemia, hyperlipidemia, and related oxidative stress[14]. According to our previous data, we set the most significant effect of GA at 100 μmol/L[15]. GA can enhance podocyte activity at this concentration.

TGF-β1, as a potential key driver of fibrosis[39], can lead to fibrosis in DN[3, 4]. Studies have revealed that TGF-β1, which was originally found in cancer

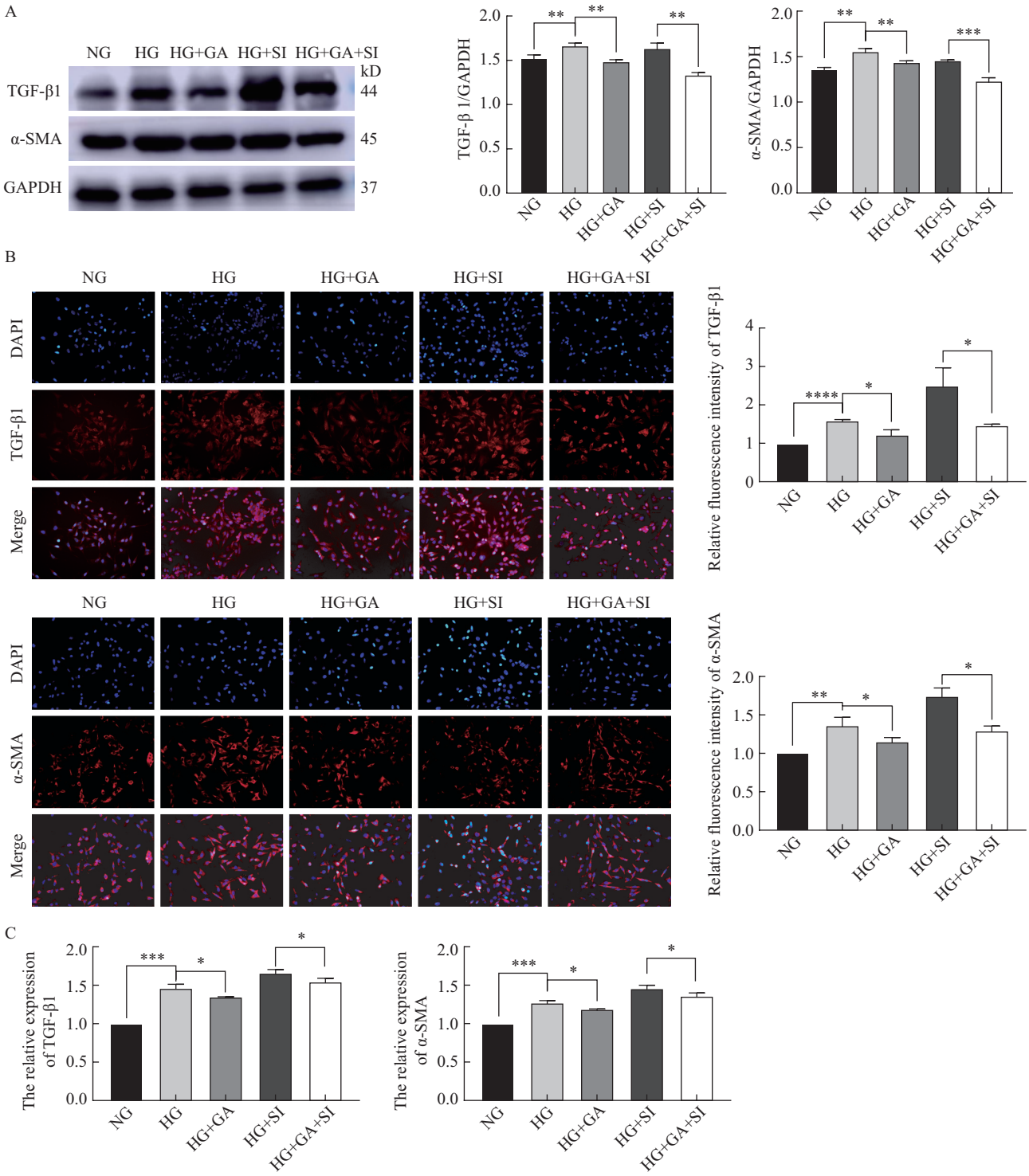

Fig. 4 SNARK-siRNA transfection can increase the expression of fibrosis-related factors

A: Western blotting results of TGF-β1 and α-SMA; B: cell immunofluorescence results of TGF-β1 and α-SMA; C: RT-qPCR results of TGF-β1 and α-SMA. NG: control group; HG: high-glucose group; HG+GA: high-glucose+glycyrrhizic acid group; HG+SI: high-glucose+SNARK-siRNA group; HG+GA+SI: high-glucose+glycyrrhizic acid+SNARK-siRNA group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

cell lines, can promote fibronectin production and collagen accumulation in epithelial and mesenchymal cells as well as achieve fibrosis through transcriptional activation[40, 41]. The reduction or loss of AMPK signaling pathway activity in tissue cells can promote the occurrence and progression of fibrosis, while activation of the AMPK signaling pathway in tissue cells can inhibit it to a certain extent[42, 43]. Previous studies have shown that pulmonary fibrosis in rats can be prevented by inducing AMPK phosphorylation/activation[44]. Inhibiting SMAD2/3 phosphorylation and nuclear translocation in the TGF-β/SMAD2/3 signaling

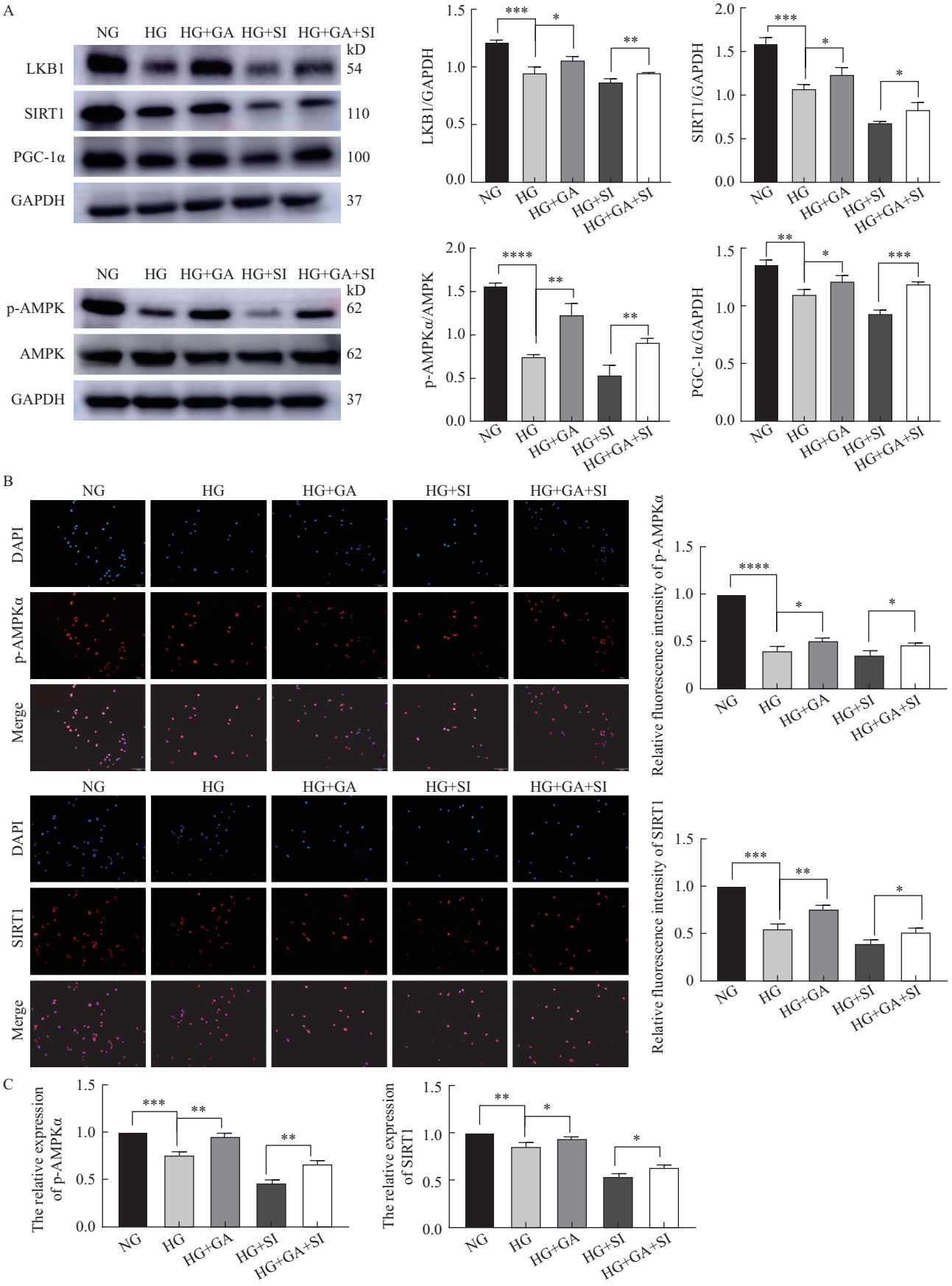

Fig. 5 SNARK-siRNA transfection can decrease the expression of SNARK/AMPK signaling pathway-related factors

A: Western blotting results of LKB1, p-AMPK, SIRT1, AMPK, and PGC-1α; B: cell immunofluorescence results of p-AMPK and SIRT1; C: RT-qPCR results of p-AMPK and SIRT1. NG: control group; HG: high-glucose group; HG+GA: high-glucose+glycyrrhizic acid group; HG+SI: high-glucose+SNARK-siRNA group; HG+GA+SI: high-glucose+glycyrrhizic acid+SNARK-siRNA group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

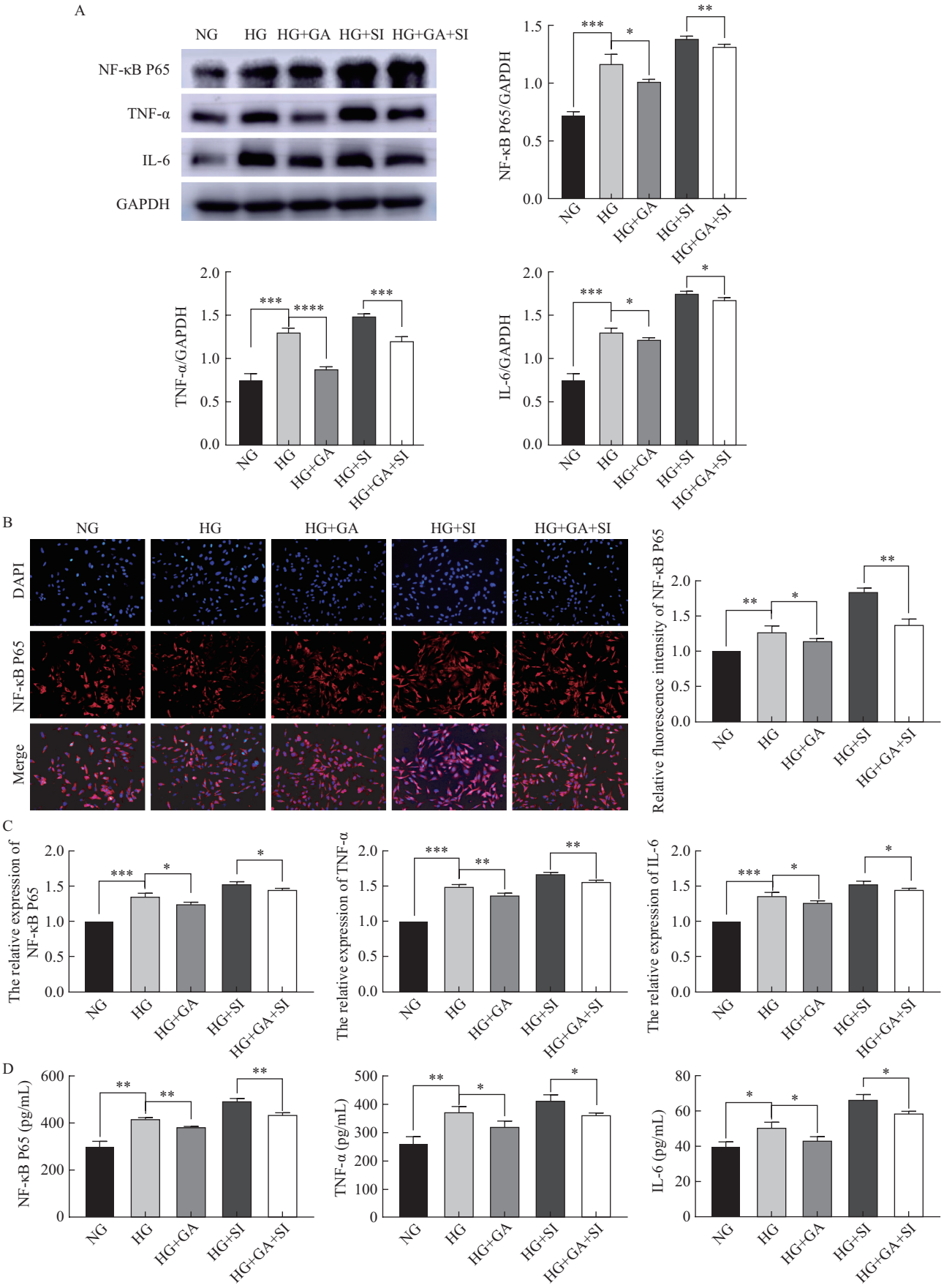

Fig. 6 SNARK-siRNA transfection can increase the expression of inflammation-related factors

A: Western blotting results of NF-κB, TNF-α, and IL-6; B: cell immunofluorescence results of NF-κB; C: RT-qPCR results of NF-κB, TNF-α, and IL-6; D: ELISA results of NF-κB, TNF-α, and IL-6. NG: control group; HG: high-glucose group; HG+GA: high-glucose+glycyrrhizic acid group; HG+SI: high-glucose+SNARK-siRNA group; HG+GA+SI: high-glucose+glycyrrhizic acid+SNARK-siRNA group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

Table 1 Effect of inflammatory factor expression in glomerular podocytes ($\bar{x}\pm s$, n=3)

Factors	NG	HG	HG+GA	HG+SI	HG+GA+SI
NF-κB (pg/mL)	296.21±25.99**	413.64±8.79$^{\#\#}$	380.03±5.22	491.56±12.18$^{\Delta\Delta}$	435.15±8.10
TNF-α (pg/mL)	260.02±15.47**	373.51±10.98$^{\#}$	321.30±11.76	414.09±12.06$^{\Delta}$	363.51±4.06
IL-6 (pg/mL)	39.77±1.52*	50.36±1.89$^{\#}$	43.36±1.11	66.38±1.65$^{\Delta}$	58.40±0.69

GA, glycyrrhizic acid; HG, high glucose; IL-6, interleukin 6; NG, normal group; NF-κB, nuclear factor kappa B; SI, SNARK siRNA; TNF-α, tumor necrosis factor alpha. $^{*}P$<0.05, $^{**}P$<0.01 *vs*. HG group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. HG+GA group; $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 *vs*. HG+GA+SI group

pathway by activating AMPK phosphorylation can improve the epithelial-to-mesenchymal transition (EMT)[45]. In addition, LKB1 expression inhibited the phosphorylation of SMAD2/3 induced by TGF-β1, and siRNA enhanced the effect of TGF-β1 after knockdown of LKB1 or the AMPKα1 subunit. These results indicate that AMPK inhibits the transcription of TGF-β1 as well as the EMT process. In this study, the expression levels of TGF-β1 and α-SMA were detected by Western blotting. The results showed that compared with the control group, the expression levels of TGF-β1 and α-SMA were significantly increased in the high-glucose environment, and the GA intervention reduced their expression. These results indicate that GA could improve the expression of fibrosis-related factors in glomerular podocytes and alleviate the dysfunction of glomerular podocytes. After the addition of SNARK siRNA, the fibrosis-related factors were significantly increased as compared with the HG group and HG+GA group. Furthermore, immunofluorescence assays showed that the fibrosis-related factors had an obvious rising trend in the high-glucose environment. GA could reduce their expression, and with SNARK siRNA intervention, the fibrosis-related factors were significantly increased compared to the HG group and the HG+GA group. The RT-qPCR assay showed that the expression trend of fibrosis-related factors was the same as that of the above experiments. Therefore, GA and SNARK may be important factors for relieving podocyte fibrosis under the effect of high glucose.

Novel roles for SNARK in the context of TGF-β signaling have recently been identified[46]. TGF-β ligand binding to its respective receptor leads to the activation of SMAD2/SMAD3 as well as subsequent complex formation and nuclear translocation, ultimately resulting in the transcriptional induction of SNARK[47]. In addition, the knockout of SNARK leads to the downregulation of SMAD3, indicating that the stability of SMAD3 is increased by SNARK, which supports TGF-β signal transduction[46, 47]. In this study, the expression levels of p-AMPKα, AMPKα, LKB1, SIRT1, PGC-1α, and SNARK were detected by Western blotting. The results showed that the SNARK/AMPK pathway and its related factors, except for AMPKα, showed a significant downward trend in the high-glucose environment and that GA could enhance its expression. After the intervention of SNARK siRNA, the fibrosis-related factors were significantly reduced compared with the HG group and the HG+GA group. High glucose, GA, and siRNA did not significantly change the expression of AMPKα, suggesting that these interventions are regulated by phosphorylated AMPK, not by the regulation of total AMPK protein. Moreover, immunofluorescence assays showed that the factors related to SNARK/AMPK pathway showed a significant downward trend in the high-glucose environment and that GA could increase their expression. After the intervention of SNARK siRNA, the expression levels of fibrosis-related factors were significantly reduced compared with those in the HG group and the HG+GA group. Furthermore, the RT-qPCR assay showed the same expression trend of the SNARK/AMPK pathway-related factors. These experiments suggest that GA and SNARK siRNA can improve the expression of SNARK/AMPK pathway-related proteins in glomerular podocytes and may improve the fibrosis of glomerular podocytes through this pathway.

It has been reported that SIRT1 regulates the transcriptional activation of target genes through protein deacetylation[48]. Conditional deletion of SIRT1 in podocytes of diabetes dB/db mice leads to acetylation of NF-κB subunit p65 and signal transducer and activator of transcription 3 (STAT3)[49], which may increase the urinary protein level. Compared with db/db mice without gene deletion, kidney injury is more serious in mice with STRT1 deletion. Additionally, in human podocytes, advanced glycation end products[50] induce acetylation of p65 and STAT3[51]. AMPK activation also inhibits IL-1β[52]. Stimulated chemokine (C-X-C motif) ligand 10 secretion is related to the decreased phosphorylation of IL-1 receptor associated kinase-4 (IRAK4) and the downregulation of mitogen-activated protein kinase kinase 4 (MKK4)/c-jun N-terminal kinase (JNK) and IkappaB kinase (IκB)/NF-κB signal transduction pathways[53]. AMPK activation inhibits TNF-α-stimulated IκB/NF-κB signal transduction, but it has no effect on JNK phosphorylation. The Janus kinase (JAK)/STAT3 pathway is also inhibited by AMPK after IL-6 stimulation and during adipogenesis[54]. AMPK activation *via* inhibition of NF-κB p65 acetylation translocation from the cytoplasm to the nucleus and IκB and IκB phosphorylation reduce proinflammatory

expression and secretion stimulated by cytokines[55] as well as reduce IL-1β and TNF-α expression[56]. In addition, increased phosphorylation of JNK and STAT3 was observed in adipose tissue of mice lacking AMPKα1, indicating that these different proinflammatory signaling pathways are negatively regulated by AMPK in adipose tissue *in vivo*[57]. TNF-α and IL-1β trigger the proinflammatory effect by simultaneously participating in the intracellular NF-κB[58, 59] and multiple mitogen activated protein kinase (MAPK) signaling pathways[60]. IL-1β interacts with IRAK4 in the IRAK family. IRAK4 phosphorylates itself and activates downstream kinases. The activation of IRAK further stimulates the formation of signal bodies, including TNF receptor associated factor-6 (TRAF-6)[61], TGF-β-activated kinase-1 (TAK1), and IκB, and IκB stimulates the phosphorylation of the inhibitor of NFκB as well as other proinflammatory cytokines and chemokines[62]. TNF-α and IL-1β stimulate the activation of proinflammatory MAPKs, such as the JNK pathway, which is parallel to the activation of NF-κB. IL-6 activates the stimulating JAK-mediated STAT3[50, 63] transcription factor by binding to the coreceptor gp130 (classical signaling) or to soluble IL-6 receptor alpha that binds to gp130 (trans signaling)[64].

This experimental study showed that the expression levels of inflammation-related factors were detected by Western blotting assays, and the results showed that the inflammatory factors had an obvious upward trend in the high-sugar environment and that GA could reduce their expression levels. After the addition of SNARK siRNA, the fibrosis-related factors were significantly increased compared with the HG group and the HG+GA group. In addition, immunofluorescence assays demonstrated that inflammation-related factors showed an obvious upward trend in the high-sugar environment and that GA could reduce their expression. Moreover, the RT-qPCR experiment showed the same expression trend of the inflammation-related factors. These results indicate that GA could improve the inflammatory response in glomerular podocytes and then relieve the fibrosis of glomerular podocytes.

To sum up, GA can alleviate podocyte fibrosis and inflammation induced by high glucose by regulating the SNARK/AMPK signaling pathway. The results of this study provide technical support for the application of GA in the field of DN treatment, but its specific mechanism of action needs further research.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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