

Glucagon-like peptide-1 protects mouse podocytes against high glucose-induced apoptosis, and suppresses reactive oxygen species production and proinflammatory cytokine secretion, through sirtuin 1 activation *in vitro*

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Abstract. Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone that is considered to be a promising target for the treatment of patients with type 2 diabetes. However, the mechanisms underlying the protective effects of GLP-1 on diabetic nephropathy are yet to be fully elucidated. Sirtuin (SIRT)1 encodes a member of the SIRT family of proteins that serves an important role in mitochondrial function and is reported to be associated with the pathogenesis of chronic kidney disease. The present study treated mouse podocytes with various concentrations of D-glucose to establish a high glucose (HG)-induced model of renal injury. The results of a 2',7'-dichlorodihydrofluorescein diacetate assay, Annexin V/propidium iodide staining and ELISA demonstrated that treatment of podocytes with HG significantly enhanced the production of reactive oxygen species (ROS), promoted cell apoptosis and increased the secretion of proinflammatory cytokines, respectively. The cytokines increased following HG treatment included tumor necrosis factor- α , interleukin (IL)-1 β and IL-6. Notably, treatment with GLP-1 attenuated HG-induced increases in ROS production and podocyte apoptosis, which may occur via downregulation of the expression of caspase-3 and caspase-9, and increased expression of nephrin, podocin and SIRT1, as determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Treatment with GLP-1 led to protective effects in podocytes that were similar to those of resveratrol. Furthermore, SIRT1 knockdown using short hairpin RNA significantly enhanced the expression of caspase-3 and caspase-9 in mouse podocytes, compared with normal mouse podocytes. SIRT1 knockdown with or without

GLP-1 administration significantly decreased the expression of caspase-3 and caspase-9 in mouse podocytes, compared with SIRT1 knockdown mouse podocytes. In conclusion, the results of the present study indicated that GLP-1 may be a promising target for the development of novel therapeutic strategies for HG-induced nephropathy, and may function through the activation of SIRT1.

Introduction

Diabetic nephropathy (DN) is among the most common microvascular complications of type 1 and type 2 diabetes mellitus, and is the major cause of end-stage renal disease globally (1,2). Previous studies have reported that podocyte damage, and reduced nephrin and podocin expression, are apparent even during the early stages of DN pathogenesis, and may be involved in the acceleration of DN development (3,4). The generation of reactive oxygen species (ROS), cell apoptosis and immune-mediated inflammatory responses in the kidneys are involved in the development and progression of DN. Glucose-induced increases in ROS generation have been reported to promote the apoptosis of podocytes (5), and mesangial (6) and tubular cells (7), thus promoting the progression of DN. In addition, increasing evidence indicates an important role for proinflammatory cytokines, including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, in DN (8,9). The apoptosis of renal glomerular cells, particularly podocytes, has been reported in human and experimental DN (5,10); however, to the best of our knowledge, whether podocyte apoptosis may initiate or contribute to the progression of DN is yet to be elucidated. Furthermore, the involvement of podocytes in inflammatory responses in the kidneys is yet to be investigated.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that is secreted by intestinal L-cells, which are enteroendocrine cells present throughout the gastrointestinal tract, from the duodenum to the rectum. GLP-1 has been previously associated with various biological functions (11), including reducing β -cell apoptosis and food intake, decreasing the rate of gastric emptying and secretion, and regulating blood glucose levels; thus, GLP-1 has been successfully used in the treatment of patients with type 2 diabetes mellitus (12). Notably, GLP-1 has been reported to exhibit a direct cytoprotective effect

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against oxidative stress in the aorta of diabetic mice (13). GLP-1 acts directly on glomerular mesangial cells through the GLP-1 receptor (GLP-1R), where it exerts anti-inflammatory effects against advanced glycation end-products (AGEs) by suppressing the expression of the receptor for AGEs (14). GLP-1 has also been reported to protect cardiac microvessels against oxidative stress and apoptosis, and suppress the development of microvascular barrier dysfunction, in diabetes by inhibiting Rho through a cyclic AMP/protein kinase A-mediated signaling pathway (15). Notably, evidence indicates that long-term treatment with the GLP-1R agonist exendin-4 may alleviate DN in rat and mouse models (16,17).

Sirtuins (SIRT1) are proteins that are members of the silent information regulation-2 family and possess nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity. Sirtuin (SIRT1) is involved in the deacetylation of histones and numerous transcriptional regulators, thus regulating diverse biological processes (18,19). It has been reported that SIRT1 expression was increased in an acute kidney injury model (20), while it was reduced in the kidneys of streptozotocin-induced diabetic mice (21). In addition, SIRT1 has been associated with the pathogenesis of DN, including the development of age-associated renal lesions, renal hypoxia, renal interstitial fibrosis and tubular cell apoptosis. Therefore, SIRT1 activation may have therapeutic potential in DN due to its antifibrotic, anti-inflammatory, antiapoptotic and blood pressure-controlling effects (22). These findings indicate that SIRT1 may act as a factor for conferring susceptibility to DN.

In the present study, treatment of podocytes with high glucose (HG) was demonstrated to induce the generation of ROS, promote podocyte apoptosis and enhance the secretion of proinflammatory cytokines, thus indicating that podocyte apoptosis or depletion may represent a novel early mechanism implicated in the pathogenesis of DN. SIRT1 may act as a key mediator of podocyte apoptosis *in vitro* and therefore function as a connection between ROS, podocyte apoptosis and DN.

Materials and methods

Cell culture and treatments. MPC-5 mouse podocytes were obtained from BeNa Culture Collection (Kunshan, China) and were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100X penicillin-streptomycin solution (100 U/ml penicillin and 100 mg/l streptomycin) and 10 U/ml interferon (IFN)- γ (ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA). Cells were maintained in a humidified atmosphere at 33°C with 5% CO₂. Following proliferation to 80% confluence, podocytes were cultured in the aforementioned medium without 10 U/ml IFN- γ and were incubated in a humidified atmosphere at 37°C with 5% CO₂ for 10-14 days.

To establish the diabetic injury model, mouse podocytes (1x10⁵ cells/well) were exposed to normal glucose (5.5 mM) or HG (15, 30 and 50 mM) for 48 h. Cells maintained in normal glucose (5.5 mM) were used as the control group. In addition to HG induction, mouse podocytes were simultaneously treated with GLP-1 (10, 100 and 500 nM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or 10 μ M resveratrol (RSV;

Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China) for 48 h.

ROS detection. A 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe combined with flow cytometric analysis was used to detect alterations in the ROS levels generated in podocytes 48 h after the various treatments, as described previously (23). Briefly, podocytes were resuspended in PBS and the density was adjusted to 1x10⁵ cells/well. The podocytes were subsequently incubated with 10 μ M DCFH-DA (Beyotime Institute of Biotechnology, Shanghai, China) for 20 min in the dark at 37°C and subjected to flow cytometric analysis (BD Biosciences, Franklin Lakes, NJ, USA) using a BD Accuri C6 flow cytometer and BD Cell Quest software version 1.0.264.21 (BD Biosciences).

Cell apoptosis assay. Cell apoptosis was analyzed 48 h after the various treatments using flow cytometry and an Annexin V-FITC Apoptosis Detection kit (C1062; Beyotime Institute of Biotechnology). Briefly, mouse podocytes were plated in 6-well plates at a density of 1x10⁵ cells/well and incubated with 195 μ l Annexin V-fluorescein isothiocyanate (FITC) and 5 μ l propidium iodide (PI) for 15 min in the dark at 4°C. Analysis of cell apoptosis was subsequently performed using a flow cytometer. The early apoptotic cells are presented in the lower right quadrant of the fluorescence-activated cell sorting (FACS) histograms, and the late apoptotic cells, which were stained with FITC and PI, emitted red-green fluorescence and are presented in the upper right quadrant of the FACS histograms. FACS was performed on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (version 9.0.2; FlowJo LLC, Ashland, OR, USA).

ELISA. TNF- α , IL-6 and IL-1 β levels present in the culture supernatants of mouse podocytes 48 h after the various treatments were determined using Mouse Interleukin 1 β (IL-1 β) ELISA kit (PI301; Beyotime Institute of Biotechnology), Mouse Interleukin 6 (IL-6) ELISA kit (KMC0061; Thermo Fisher Scientific, Inc.), Mouse Tumor necrosis factor α (TNF- α) ELISA kit (PT512; Beyotime Institute of Biotechnology), respectively, according to the manufacturer's protocol.

Caspase activity assays. The activity of caspase-3 and caspase-9 was analyzed using Caspase-3 and Caspase-9 Colorimetric Assay kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer's protocol. Briefly, mouse podocytes (1x10⁵ cells/well) 48 h after the various treatments were collected, resuspended in 50 μ l chilled cell lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) and incubated on ice for 10 min. Following centrifugation for 1 min at 400 x g at 4°C, the supernatants were transferred to a fresh tube and the protein concentration was assessed using a bicinchoninic acid protein assay kit (PICPI23223; Thermo Fisher Scientific, Inc.). 100 μ g protein was diluted in 50 ml cell lysis buffer for each assay. The absorbance of each sample was measured at 405 nm using a Multiskan EX microplate reader (Thermo Fisher Scientific, Inc.).

SIRT1 knockdown in mouse podocytes. The pLKO.1 lentiviral vector, psPAX2 packaging plasmid and pMD2G envelope

plasmid were obtained from Addgene, Inc. (Cambridge, MA, USA). The SIRT1-targeting short hairpin (sh)RNA (position 516-534: GCGGATAGGTCCATATACT; forward: CCGGGC GGATAGGTCCATATACTTTCTCGAGGAATACCTCAT CTTTCCTCTTTTTTTC; reverse: AATTGAAAAAAGAG GAAAGATGAGGTATTCCTCGAGAAAGTATATGGACC TATCCGCGGCCT) or a scramble shRNA sequence (AGA GCTATCGGCATCATGT; forward: CCGGAGAGCTAT CGGCATCATGTTTCTCGAGGAATACCTCATCTTTCC TCTTTTTTTC; reverse: AATTGAAAAAAGAGGAA AGATGAGGTATTCCTCGAGAAACATGATGCCGATAG CTCTGGCCT; Sangon Biotech Co., Ltd., Shanghai, China) was cloned into the pLKO.1 lentiviral vector using Age I and Eco I restriction enzymes. The pLKO.1 lentiviral vector with scramble shRNA was used as the negative control (NC). 293T cells (American Type Culture Collection, Manassas, VA, USA) at the density of 1×10^5 cells/well cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) were seeded in 60-mm culture dishes 37°C and, after 24 h, they were co-transfected with $1 \mu\text{g}$ pLKO.1-SIRT1-shRNA or pLKO.1-NC-shRNA, and $0.1 \mu\text{g}$ psPAX2 and $0.9 \mu\text{g}$ pMD2G, using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent, according to the manufacturer's protocol. The recombinant lentiviral particles were collected 48 h post-transfection and were used to infect mouse podocytes. Mouse podocytes (1×10^5 cells/well) cultured in RPMI-1640 medium 10% fetal bovine serum were infected with $2 \mu\text{l}$ lentivirus at a multiplicity of infection of 20 in the presence of $8 \mu\text{g/ml}$ polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4-6 h at 37°C . Sequencing was performed by Shanghai Majorbio Pharmaceutical Technology Co., Ltd. (Shanghai, China) to confirm the recombinant virus.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from podocytes 48 h after the various treatments using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA using an AMV reverse transcriptase kit (Fermentas; Thermo Fisher Scientific, Inc.) for 60 min at 37°C , 5 min at 85°C and 5 min at 4°C . qPCR was performed on cDNA using SYBR-Green 10X Supermix (Takara Biotechnology Co., Ltd., Dalian, China) on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. The sequences of the primers that were used in the present study were: SIRT1 forward, 5'-TGACGCTGTGGCAGATTG-3' and reverse, 5'-CAAGGC GAGCATAGATACCG-3'; nephrin forward, 5'-GGACCC ACACTACTACTC-3' and reverse, 5'-CTCTCCACCTCGTCA TAC-3'; podocin forward, 5'-TTGTTTCCTGGCTCCTTC-3' and reverse, 5'-TGCCTTGGGACTACTTTC-3'; caspase-3 forward, 5'-CTGACTGGAAAGCCGAAAC-3' and reverse, 5'-GCAAAGGGACTGGATGAAC-3'; caspase-9 forward, 5'-GTGAAGAAGACGCTGACTG-3' and reverse, 5'-GCA TCCATCTGTCCCATAG-3'; and GAPDH forward, 5'-ATC ACTGCCACCCAGAAG-3' and reverse, 5'-TCCACGACG

GACACATTG-3'. GAPDH was used the internal control for normalization. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (24). The experiment was repeated three times.

Western blot analysis. Total proteins were isolated from mouse podocytes 48 h after the various treatments in radio-immunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing 0.01% protease and phosphatase inhibitor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The protein concentration was assessed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Proteins ($20\text{-}30 \mu\text{g}$) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Roche Diagnostics GmbH, Mannheim, Germany). Following blocking with 5% skimmed milk at 4°C overnight, the membranes were incubated at 4°C overnight with the following primary antibodies: Anti-SIRT1 (ab28170; 1:1,000), anti-nephrin (ab58968; 1:500), anti-podocin (ab181143; 1:2,000), anti-caspase-3 (ab44976; 1:500), anti-caspase-9 (ab2013; 1:1,000) and anti-GAPDH (5174; 1:2,000). All primary antibodies were purchased from Abcam (Cambridge, MA, USA). The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (A0208, A0181, A0216; 1:1,000; Beyotime Institute of Biotechnology) for 1 h at 37°C . Protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and blots were semi-quantified by densitometry using Quantity One software version 4.5.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation of three independent experiments. Statistical analysis was performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance followed by Tukey's post-hoc test was performed to statistically analyze the differences among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GLP-1 attenuates oxidative stress induced by HG in mouse podocytes. It is established that ROS are implicated in DN (25). To investigate whether GLP-1 may attenuate HG-induced oxidative stress in mouse podocytes, the present study assessed ROS production by mouse podocytes *in vitro*. As presented in Fig. 1A, HG (15, 30 and 50 mM) treatment enhanced ROS production in mouse podocytes following 48 h incubation compared with cells maintained in normal glucose conditions. Notably, GLP-1 (10, 100 and 500 nM) was revealed to suppress ROS production in a dose-dependent manner in 30 mM HG-stimulated mouse podocytes (Fig. 1B).

GLP-1 suppresses HG-induced apoptosis in mouse podocytes. To determine the effect of GLP-1 on HG-induced apoptosis in mouse podocytes, flow cytometry was performed. Treatment with HG (15, 30 and 50 mM) induced a significant dose-dependent increase in apoptotic cells (Fig. 2A), compared with cells maintained in normal glucose conditions. Compared with podocytes cultured in 30 mM HG,

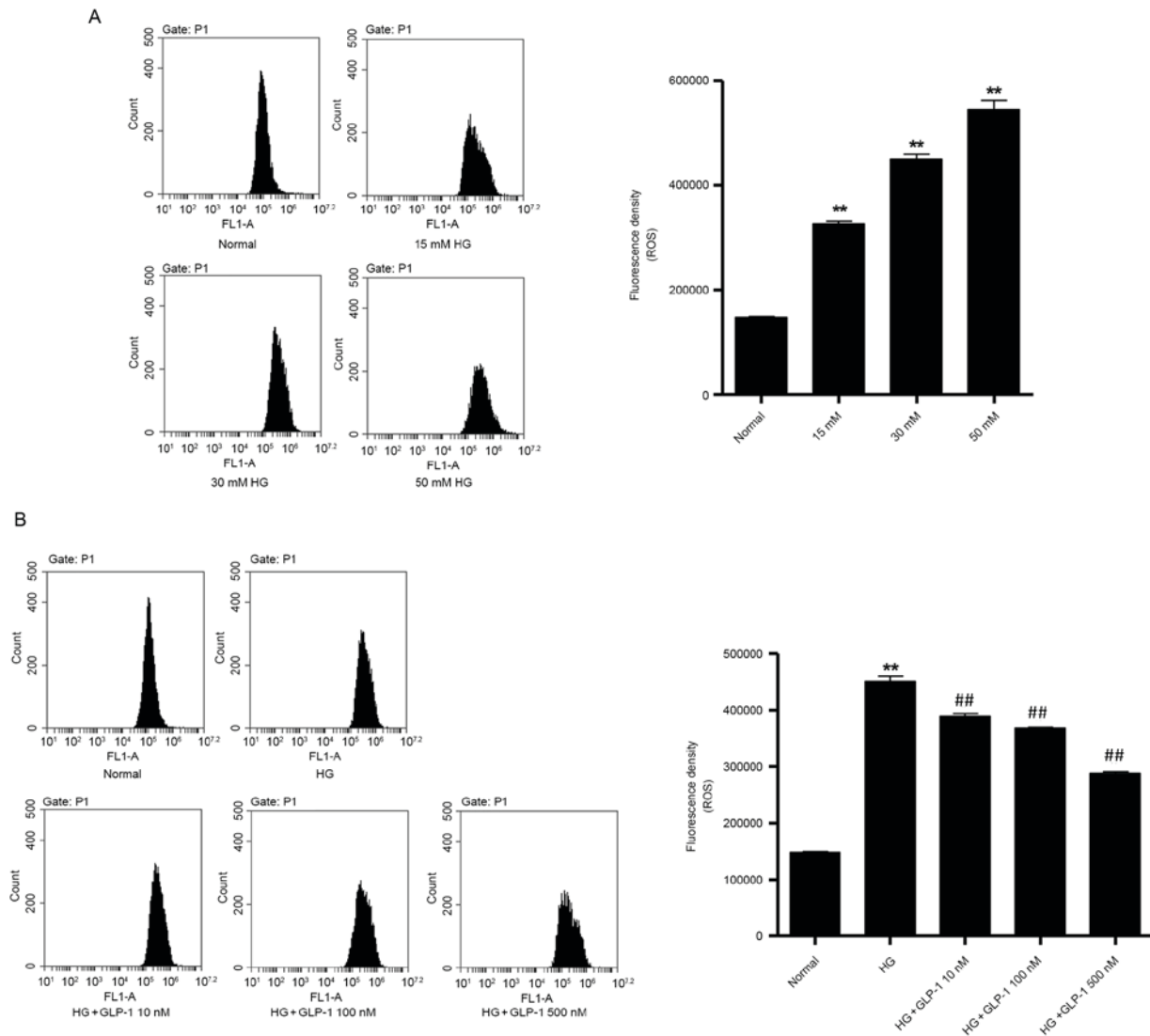


Figure 1. Effects of GLP-1 on oxidative stress in HG-treated mouse podocytes. (A) Mouse podocytes were treated with various concentrations of glucose and the ROS levels were measured using the DCFH-DA fluorescent probe combined with flow cytometry. (B) Mouse podocytes were treated with 30 mM glucose in the absence or presence of various concentrations of GLP-1 and the ROS levels were measured using the DCFH-DA fluorescent probe combined with flow cytometry. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. HG group. GLP, glucagon-like peptide; HG, high glucose; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate.

the GLP-1 (10, 100 and 500 nM)-treated groups exhibited a significant decrease in cell apoptosis ($30.6 \pm 0.26\%$ in 10 nM GLP-1 group, $23.9 \pm 0.23\%$ in 100 nM GLP-1 group and $15.8 \pm 0.56\%$ in 500 nM GLP-1 group vs. $36.8 \pm 0.06\%$ in HG group; $P < 0.01$; Fig. 2B).

GLP-1 attenuates HG-induced downregulation of SIRT1 expression in mouse podocytes. To investigate whether GLP-1 may counteract HG-induced alterations in SIRT1 expression in mouse podocytes, RT-qPCR and western blot analysis was performed. The results demonstrated that 30 mM glucose induced a significant decrease in the mRNA and protein expression of SIRT1, compared with cells maintained under normal glucose conditions (Fig. 3A and B). However, compared with podocytes cultured in HG, GLP-1 (10, 100 and 500 nM)-treated cells exhibited increases in the mRNA and protein expression levels of SIRT1 (Fig. 3A and B).

GLP-1 attenuates HG-induced depletion of podocyte markers in mouse podocytes. To determine whether HG treatment may induce podocyte depletion, the expression of podocyte-specific markers, including nephrin and podocin, was assessed using RT-qPCR and western blot analysis. As presented in Fig. 4A-C, the mRNA and protein expression of nephrin and podocin was significantly suppressed following treatment of mouse podocytes with 30 mM glucose, compared with podocytes cultured in normal glucose, thus indicating that exposure to HG may induce podocyte depletion. However, compared with podocytes cultured in HG, cells treated with 500 nM GLP-1 exhibited a significant increase in the mRNA and protein expression of nephrin and podocin (Fig. 4A-C). Notably, treatment with GLP-1 was revealed to mimic the protective effects of 10 μ M RSV administration in HG-induced mouse podocytes (Fig. 4A-C). Consistent with the aforementioned results for SIRT1 expression, 500 nM GLP-1 significantly attenuated

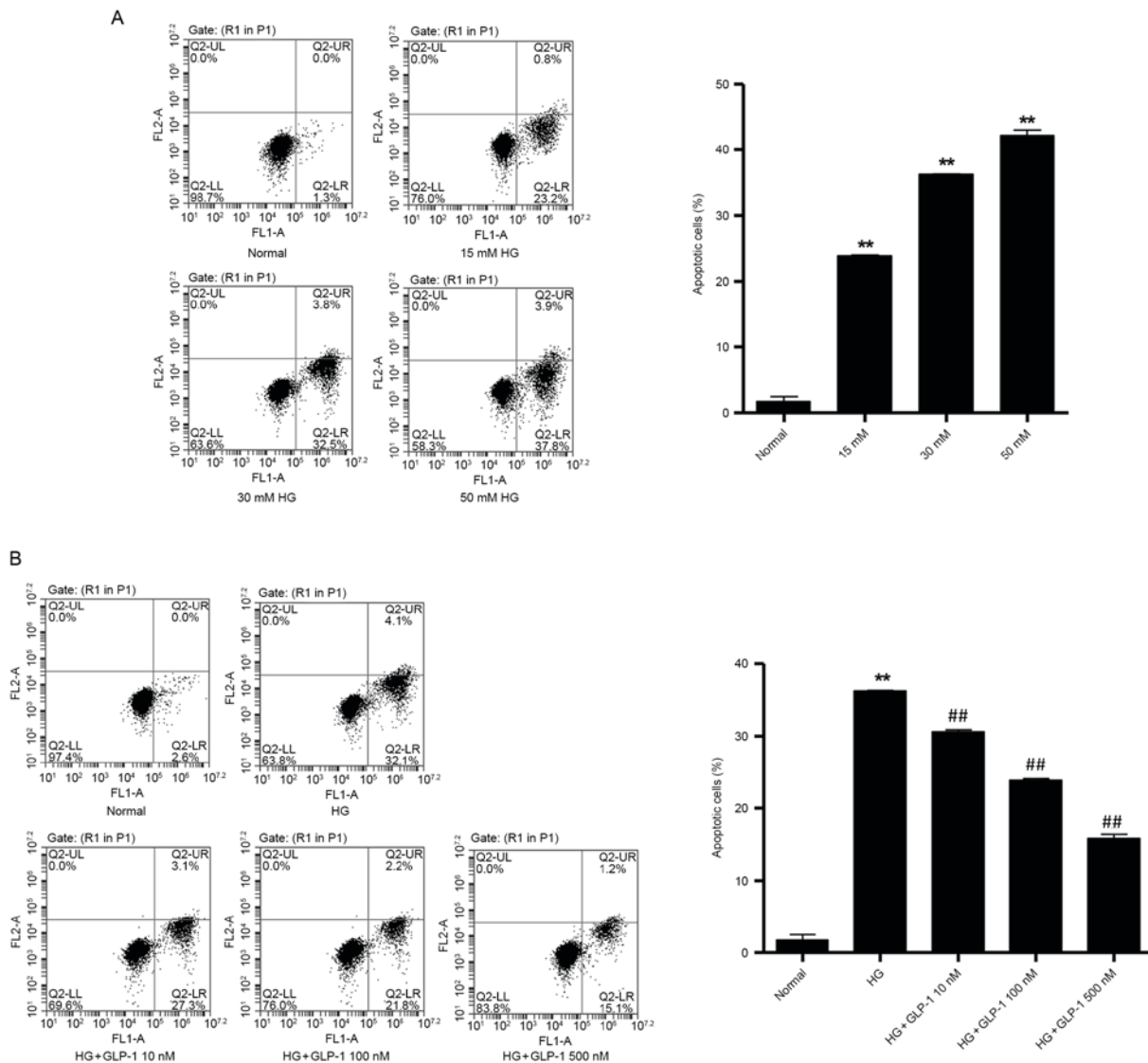


Figure 2. Effects of GLP-1 on HG-induced apoptosis in mouse podocytes. (A) Mouse podocytes were treated with various concentrations of glucose and cell apoptosis was assessed using Annexin V/PI staining followed by flow cytometry. (B) Mouse podocytes were treated with 30 mM glucose in the absence or presence of various concentrations of GLP-1 and cell apoptosis was assessed using Annexin V/PI staining followed by flow cytometry. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. HG group. GLP, glucagon-like peptide; HG, high glucose; PI, propidium iodide.

the HG-induced decrease in SIRT1 expression in mouse podocytes (Fig. 4A-C).

GLP-1 attenuates HG-induced upregulation of caspase expression and activity in mouse podocytes. RT-qPCR and western blot analysis were performed to assess the mRNA and protein expression of caspase-3 and caspase-9 in podocytes. The results demonstrated that 30 mM glucose significantly enhanced the mRNA and protein expression of caspase-3 and caspase-9, compared with cells maintained under normal glucose (Fig. 4C-E). However, GLP-1 treatment was demonstrated to significantly suppress the mRNA and protein expression of caspase-3 and caspase-9, which was induced by HG in mouse podocytes (Fig. 4C-E). Furthermore, the results of caspase activity assays demonstrated that, compared with cells treated with normal levels of glucose, 30 mM glucose significantly increased the activity of caspase-3 and caspase-9 (Fig. 4F and G). Consistent with mRNA and

protein results, compared with podocytes cultured in HG, 500 nM GLP-1-treated cells exhibited a significant decrease in the activity of caspase-3 and caspase-9, which was similar to the protective effects produced by RSV administration in HG-cultured mouse podocytes (Fig. 4F and G). Taken together, these results indicate that GLP-1 may exert an antiapoptotic effect in mouse podocytes cultured in HG conditions.

GLP-1 suppresses HG-induced secretion of proinflammatory cytokines in mouse podocytes. To determine the effect of GLP-1 on HG-induced inflammatory responses in mouse podocytes, ELISA assays were performed to detect the levels of proinflammatory cytokines secreted by podocytes. The results demonstrated that treatment with 30 mM glucose induced a significant increase in the levels of TNF- α , IL-1 β and IL-6 secreted by mouse podocytes (Fig. 5). Compared with HG-cultured podocytes, cells treated with 500 nM GLP-1

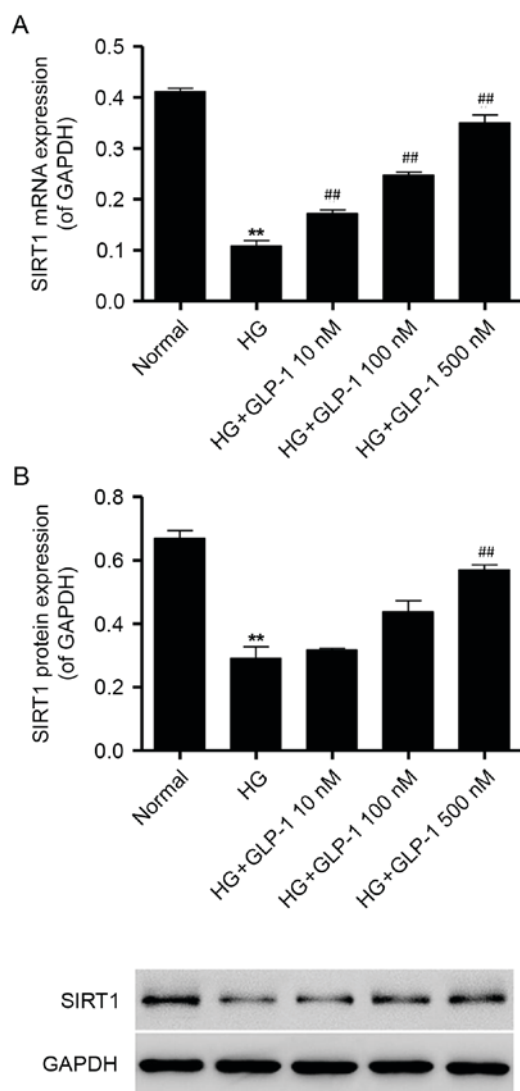


Figure 3. Effects of GLP-1 on SIRT1 expression in HG-treated mouse podocytes. Mouse podocytes were treated with 30 mM glucose in the absence or presence of various concentrations of GLP-1 and the (A) mRNA and (B) protein expression levels of SIRT1 were assessed using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. HG group. GLP, glucagon-like peptide; SIRT, sirtuin; HG, high glucose.

exhibited a significant decrease in the secretion of TNF- α , IL-1 β and IL-6; the effects of GLP-1 were similar to the protective effects induced by RSV administration in HG-stimulated mouse podocytes (Fig. 5).

GLP-1 reduces SIRT1 shRNA-induced upregulation of caspase-3 and caspase-9 expression in mouse podocytes. To investigate the roles of SIRT1 in the protective effects of GLP-1 in mouse podocytes, SIRT1 expression was silenced in mouse podocytes following infection with a pLKO.1-SIRT1-shRNA recombinant lentiviral vector. The results demonstrated that the mRNA and protein expression of SIRT1 in mouse podocytes following transduction with the pLKO.1-SIRT1-shRNA vector was significantly decreased by 81.1 ± 0.03 and $60.3 \pm 0.03\%$, respectively (Fig. 6A-C), thus confirming successful knockdown of SIRT1 following

transduction. In addition, SIRT1-shRNA transduction was demonstrated to enhance the protein and mRNA expression of caspase-3 and caspase-9, compared with the cells maintained in normal glucose and the NC shRNA group, which was similar to the effect of HG exposure in mouse podocytes (Fig. 6D-F). Notably, western blotting and RT-qPCR results demonstrated that treatment of podocytes with 500 nM GLP-1 significantly suppressed the expression of caspase-3 and caspase-9, and upregulated the expression of SIRT1, in pLKO.1-NC-shRNA and pLKO.1-SIRT1-shRNA lentiviral infected mouse podocytes (Fig. 6D-F). These results indicate that GLP-1 may inhibit the HG-induced apoptosis of mouse podocytes through the activation of SIRT1 *in vitro*.

Discussion

In the present study, the protective effects of GLP-1 against cellular damage were investigated in HG-treated mouse podocytes *in vitro*, in order to determine the potential roles of GLP-1 in the pathogenesis of DN. The results of the current study demonstrated that knockdown of SIRT1 expression enhanced cell apoptosis in mouse podocytes, while treatment with GLP-1 counteracted these effects. A previous study reported that SIRT1 exerted its cytoprotective effects through a number of mechanisms, including via antiapoptotic, antioxidative and anti-inflammatory functions, and its involvement in the regulation of mitochondrial biogenesis and autophagy (26).

One of the major biomarkers for the prediction of DN progression is a reduced density of podocytes (27); however, the molecular pathways and pathological mechanisms that are associated with podocyte depletion in DN are yet to be fully elucidated. Nephron and podocin are podocyte-specific protein markers that are critical for the establishment of the podocyte slit diaphragm structure and for the maintenance of the intact filtration barrier (28). In the present study, the expression of nephrin and podocin was detected in mouse podocytes, and the results revealed that HG exposure significantly suppressed nephrin and podocin expression; notably, GLP-1 treatment was demonstrated to enhance the expression of nephrin and podocin in HG-treated cells.

Based on *in vitro* evidence presented in the current study, it may be hypothesized that the exposure of podocytes to HG leads to cytotoxic effects, as indicated by the increased ROS production and enhanced cell apoptosis following HG treatment. These results are consistent with previous reports that demonstrated that glucose caused impairments in the mitochondrial membrane potential and increased ROS generation in podocytes (29), and the HG-induced ROS increase initiated podocyte apoptosis and podocyte depletion *in vitro* and *in vivo* (27), which was alleviated following GLP-1 treatment. In cardiac microvascular endothelial cells, the protective effects of GLP-1 have been proposed to be mediated through the inhibition of Rho/Rho-associated protein kinase activation, which may subsequently reduce HG-induced oxidative stress and apoptosis (15). Members of the caspase family, particularly caspase-3 and caspase-9, have been reported to serve important roles in the HG-induced apoptosis of proximal tubular epithelial cells (30). In addition,

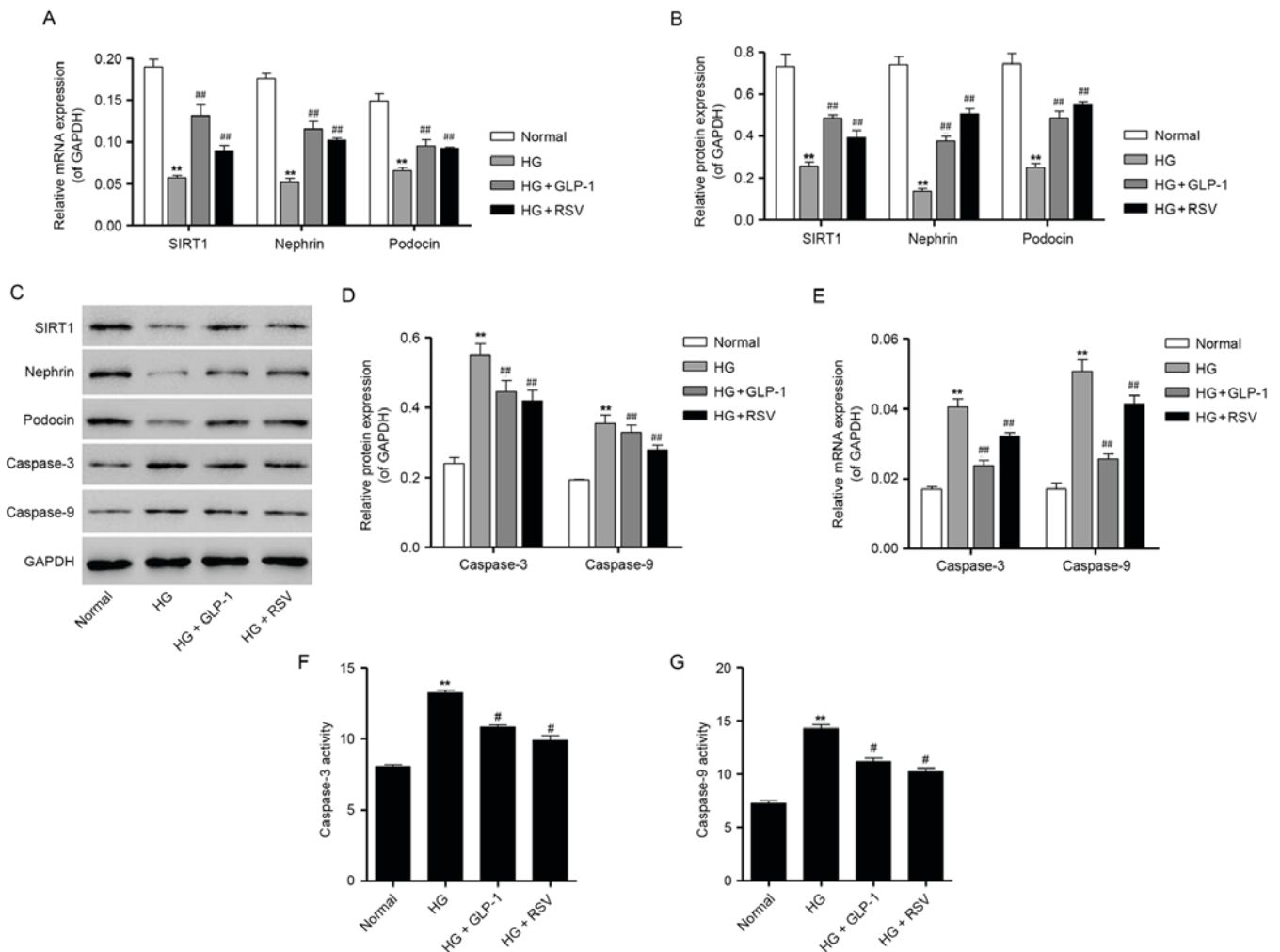


Figure 4. Effects of GLP-1 on the expression of podocyte-specific markers and apoptosis-associated proteins in HG-treated mouse podocytes. (A) Mouse podocytes were treated with 30 mM HG in the absence or presence of 500 nM GLP-1 or 10 μ M RSV, and the mRNA expression of SIRT1, nephrin and podocin was determined by RT-qPCR. (B) Protein expression levels of SIRT1, nephrin and podocin were quantified by densitometric analysis following treatment with 30 mM HG in the absence or presence of 500 nM GLP-1 or 10 μ M RSV. (C) Representative western blot bands for SIRT1, nephrin, podocin, caspase-3 and caspase-9 are presented. GAPDH was employed as the loading control. (D) Protein expression levels of caspase-3 and caspase-9 were quantified by densitometric analysis. (E) RT-qPCR results for caspase-3 and caspase-9 mRNA expression following treatment with 30 mM HG in the absence or presence of 500 nM GLP-1 or 10 μ M RSV. The activity of (F) caspase-3 and (G) caspase-9 in HG-treated mouse podocytes was also measured using colorimetric biochemical assays. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. HG group. GLP, glucagon-like peptide; HG, high glucose; RSV, resveratrol; SIRT, sirtuin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

high levels of glucose promoted ROS generation through NAD phosphate oxidase and mitochondrial mechanisms, which in turn activated caspase-3 in podocytes *in vitro* (27). In the present study, treatment of podocytes with HG significantly increased the mRNA and protein expression levels of caspase-3 and caspase-9 in podocytes, compared with in normal glucose-treated cells. GLP-1 treatment was revealed to counteract the effects of HG in podocytes, as indicated by the significant downregulation of the mRNA and protein expression of caspase-3 and caspase-9. In accordance with the present study, GLP-1 has been reported to suppress caspase-3 activation in pancreatic β cells (31) and reverse the increase in apoptotic cells induced by chronic hyperglycemia in the kidneys of Zucker diabetic fatty rats (1).

Inflammatory responses are an important factor contributing to renal injury in HG-induced DN (32). In the present study, HG treatment was revealed to induce cytotoxic and oxidative stress responses, as well as inflammatory

responses, in mouse podocytes. The results of the current study demonstrated that the levels of TNF- α , IL-1 β and IL-6 secreted by mouse podocytes were increased in response to HG, as indicated using ELISA. These results are consistent with those of a previous study, which reported increased TNF- α , IL-1 β and IL-6 levels in the kidneys of diabetic rats (1). Notably, in the present study, GLP-1 treatment significantly suppressed the secretion of TNF- α , IL-1 β and IL-6, which was consistent with a previous report that demonstrated that the GLP-1 agonist exendin-4 reduced the infiltration of macrophages and the secretion of inflammatory cytokines, including TNF- β , IL-6 and IL-1 β , by macrophages (33).

The present study demonstrated that SIRT1 expression was downregulated in HG-treated mouse podocytes, while it was upregulated following GLP-1 treatment in a dose-dependent manner. RSV has been reported to exert protective effects against numerous diseases, including diabetes, neurodegenerative disorders, cognitive disorders,

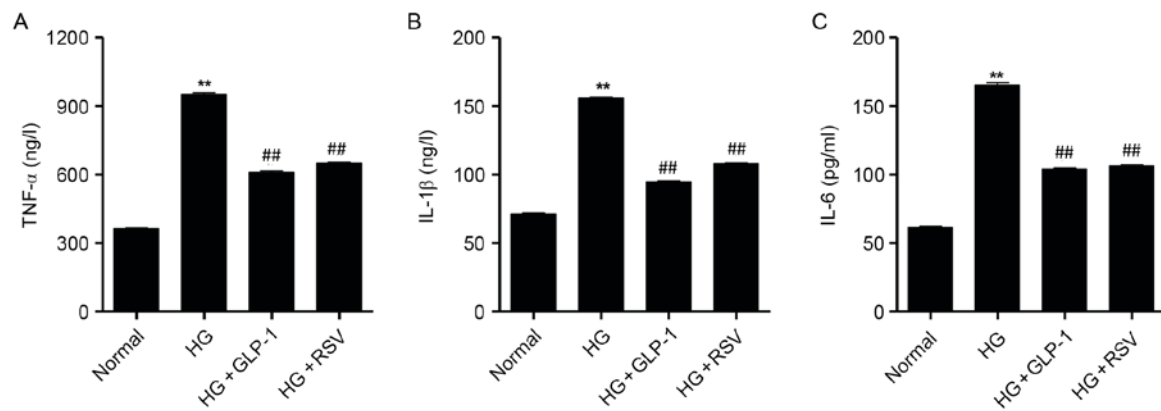


Figure 5. Effects of GLP-1 on inflammatory responses in HG-treated mouse podocytes. Mouse podocytes were treated with 30 mM glucose in the absence or presence of 500 nM GLP-1 or 10 μ M RSV, and the levels of (A) TNF- α , (B) IL-1 β and (C) IL-6 secreted by mouse podocytes were measured using ELISA. Data are presented as the mean \pm standard deviation. ** P <0.01 vs. normal group; ## P <0.01 vs. HG group. GLP, glucagon-like peptide; HG, high glucose; RSV, resveratrol; TNF, tumor necrosis factor; IL, interleukin.

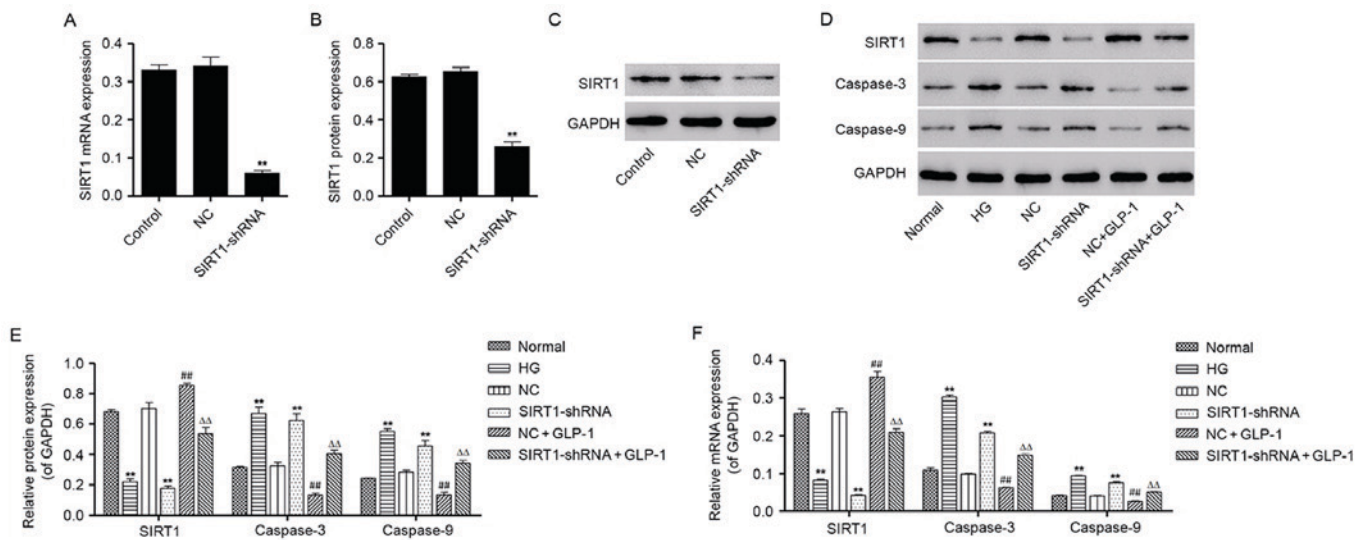


Figure 6. Effects of GLP-1 on caspase-3 and caspase-9 expression in mouse podocytes following SIRT1 knockdown. (A) SIRT1 mRNA expression in mouse podocytes transduced with pLKO.1-SIRT1-shRNA lentiviral vectors was determined by RT-qPCR. (B) Protein levels of SIRT1 in mouse podocytes transduced with pLKO.1-SIRT1-shRNA lentiviral vectors were determined by western blot analysis and quantified by densitometric software. (C) Representative western blot bands for SIRT1 protein expression in control cells and mouse podocytes transduced with NC- or SIRT1-shRNA. (D) Western blot analysis was performed to measure the protein expression of SIRT1, caspase-3 and caspase-9 following transduction of mouse podocytes with NC- or SIRT1-shRNA lentiviral vectors with or without 500 nM GLP-1. (E) Densitometric analysis was performed to quantify the protein expression in different groups. (F) RT-qPCR was performed to measure the mRNA expression of SIRT1, caspase-3 and caspase-9 in NC- or SIRT1-shRNA-transduced mouse podocytes with or without treatment with 500 nM GLP-1. Data are presented as the mean \pm standard deviation. For parts A and B, ** P <0.01 vs. NC group; for parts E and F, ** P <0.01 vs. normal group, ## P <0.01 vs. NC group and $\Delta\Delta P$ <0.01 vs. SIRT1-shRNA group. GLP, glucagon-like peptide; SIRT, sirtuin; shRNA, short hairpin RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Control, untransduced cells; NC, negative control; HG, high glucose; NC, NC-shRNA-transduced cells.

cancer, kidney diseases and cardiovascular diseases, through the activation of SIRT1 (18,34). In the present study, RSV was used as a positive control to compare the effect of GLP-1 on SIRT1 expression. The results of the present study demonstrated that RSV inhibited the HG-induced increase in the ROS generation and apoptosis of mouse podocytes (data not shown). SIRT1 activation by RSV has been reported to suppress the urinary excretion of albumin and reduce the apoptosis of proximal tubule epithelial cells *in vitro* and *in vivo* (35). Furthermore, in the present study, knockdown of SIRT1 expression resulted in the upregulation of caspase-3 and caspase-9 expression in mouse podocytes, thus

indicating that SIRT1 knockdown may promote the progression of DN. Notably, treatment with GLP-1 was revealed to inhibit the SIRT1 knockdown-induced increase in caspase-3 and caspase-9 expression, which indicates that GLP-1 may protect mouse podocytes against HG-induced damage and apoptosis through the activation of SIRT1 *in vitro*.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that GLP-1 may exert protective effects against HG-induced damage in mouse podocytes, as indicated by the suppression of HG-induced ROS production, apoptosis and inflammatory responses, through the activation of SIRT1 *in vitro*. The results of the current study may provide

novel insights into the roles of SIRT1 in DN, and indicate that the modulation of SIRT1 expression may have potential as a novel therapeutic strategy to alleviate podocyte injury induced by HG in patients with diabetes.

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