

Notoginsenoside R1 attenuates glucose-induced podocyte injury via the inhibition of apoptosis and the activation of autophagy through the PI3K/Akt/mTOR signaling pathway

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Abstract. Injury to terminally differentiated podocytes contributes significantly to proteinuria and glomerulosclerosis. The aim of this study was to examine the protective effects of notoginsenoside R1 (NR1) on the maintenance of podocyte number and foot process architecture via the inhibition of apoptosis, the induction of autophagy and the maintenance of podocyte biology in target cells. The effects of NR1 on conditionally immortalized human podocytes under high glucose conditions were evaluated by determining the percentage apoptosis, the percentage autophagy and the expression levels of slit diaphragm proteins. Our results revealed that NR1 protected the podocytes against high glucose-induced injury by decreasing apoptosis, increasing autophagy and by promoting cytoskeletal recovery. The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target

of rapamycin (mTOR) signaling pathway was further investigated in order to elucidate the mechanisms responsible for the protective effects of NR1 on podocytes. Our data indicated that treatment with NR1 increased the phosphorylation levels of PI3K, Akt and mTOR, leading to the activation of the PI3K/Akt/mTOR signaling pathway in podocytes. To the best of our knowledge, this is the first *in vitro* study to demonstrate that NR1 protects podocytes by activating the PI3K/Akt/mTOR pathway.

Introduction

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease, which ultimately progresses to end-stage renal failure. In early DN, glomerular abnormalities are observed, including the increase in glomerular filtration rate and albuminuria (1,2). Accumulating evidence indicates that podocyte injury contributes to these pathological changes (3,4). Clinical data also demonstrate that podocyte integrity is impaired in individuals with type 1 and 2 diabetes (5,6). It is widely acknowledged that the podocyte slit diaphragm plays a critical role in establishing the selective permeability of the glomerular filtration barrier (7). In our previous study (8), we demonstrated the *in vivo* renal protective role of notoginsenoside R1 (NR1) through the regulation of phosphoinositide 3-kinase (PI3K)/Akt signaling. However, the mechanisms through which NR1 regulates this signaling pathway remain unknown. Therefore in this study, we performed *in vitro* experiments and focused on podocyte slit diaphragm proteins that are important in maintaining podocyte integrity. Podocyte decrement is also the leading cause of these pathological changes. It has been reported that podocytes are highly specialized, terminally differentiated cells with limited replicative capacity (9). In both type 1 and 2 diabetes, the decreased podocyte number is a major pathophys-

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iological precursor to the development of proteinuria (10,11). Taken together, these data indicate that the ability to maintain podocyte integrity and podocyte number seems to be of pivotal importance in protecting against diabetes.

It is acknowledged that the search for more effective therapeutic drugs to prevent podocyte injury and to maintain podocyte number is a great challenge. As herbal medicine has been clinically used in the treatment of DN for centuries in China, in this study, we investigated the role of NR1 (extracted from *Panax notoginseng*) on podocytes and the underlying mechanisms. It has been demonstrated that NR1 attenuates renal ischemia-reperfusion injury in rats (12). It has also been demonstrated that NR1 attenuates the glucose-induced impairment of podocyte adhesive capacity and subsequent podocyte depopulation in rats through the upregulation of $\alpha 3\beta 1$ integrin (13). However, it is not clear whether NR1 has any effect on the number of podocytes by affecting apoptosis and inducing autophagy and podocyte integrity in addition to its effect on podocyte adhesion (13).

Although a large body of evidence has indicated that apoptosis contributes greatly to the podocyte depopulation, autophagy has also been shown to be important in preserving the number of podocytes (14-17). It has also been demonstrated that in renal injury, autophagy plays a protective role by delaying the progression of podocytopathies (18). In our previous *in vivo* study (8), using mice, we reported that NR1 protects podocytes from injury via the PI3K/Akt pathway. However, which site activation/phosphorylation of the PI3K/Akt pathway is regulated by NR1 remains unknown. Among several signaling pathways that are functional in podocytes, it has been reported that decreased the phosphorylation of Akt is associated with podocyte loss in early DN (19). It has been demonstrated that in rats with puromycin aminonucleoside (PAN) nephropathy, the activation of the PI3K/Akt signaling inhibits podocyte apoptosis (20). Furthermore, it has been reported that Na⁺/H⁺ exchanger-1 (NHE-1) attenuates podocyte injury via the PI3K/Akt pathway by inducing the activation of autophagy (21). It is thus possible that the PI3K/Akt pathway may be involved in both apoptosis and autophagy during the process of podocyte injury. Moreover, previous studies have demonstrated that the inhibition of mammalian target of rapamycin (mTOR) protects podocytes from injury and prevents DN through the regulation of autophagy (22,23). Due to the importance of the PI3K/Akt/mTOR pathway in apoptosis and autophagy, in the present study, we investigated the protective effects of NR1 on podocytes, as well as whether and how the PI3K/Akt/mTOR signaling pathway is regulated by NR1. Moreover, we wished to determine whether the PI3K/Akt/mTOR signaling pathway is involved in the protective effects of NR1 on podocytes, and to elucidate the mechanisms through which Akt regulates both apoptosis and autophagy in NR1-treated podocytes.

Materials and methods

Chemicals. NR1 was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the purity of NR1 was >98%. The autophagy inhibitor, 3-methyladenine (3-MA), monodansylcadaverine (MDC), and 2',7'-dichlorofluorescein

diacetate (DCF) were also purchased from Sigma Chemical Co. Small interfering RNAs (siRNAs) against human Beclin 1 and scramble siRNA were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Hoechst 33342, the Click-iT TUNEL Alexa Fluor 488 Imaging Assay kit for microscopy and high-content screening (HCS), SYBR-Green PCR Master Mix, NuPage Novex 10% Bis-Tris gel, NuPage transfer buffer, TRIzol, the High Capacity cDNA Reverse Transcription kit, the Dead Cell Apoptosis kit with Annexin V Alexa Fluor 488 and propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Polyvinylidene fluoride membranes were purchased from Pall Life Sciences (Port Washington, NY, USA). Immobilon Western Chemiluminescent HRP Substrate was purchased from Millipore Corp. (Billerica, MA, USA). The CellTiter-Glo luminescent cell viability assay kit and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay kit were purchased from Promega Corp. (Madison, WI, USA).

The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): nephrin (sc-377246), desmin (sc-65983), pro-caspase-3 (sc-7148), pro-caspase-9 (sc-56073), cleaved caspase-9 (sc-22182), Bax (sc-493), Bid (sc-135847), Bcl-2 (sc-492), Bcl-xL (sc-8392), poly(ADP-ribose) polymerase (PARP; sc-7150) and β -actin (sc-47778). Besides, rat anti-mouse podocalyxin (PCX) antibody (MAB1556) was purchased from R&D Systems (Minneapolis, MN, USA). In addition, antibodies against podocin (ab50339), CD2AP (ab84829), active caspase-3 (ab49822), Beclin (ab62557), PI3K p85 (ab189403), phosphorylated (p-)Beclin 1 (S234; ab183335), mTOR (ab2732), p-mTOR (S2448; ab109268), p-mTOR (S2481; ab137133), p-PI3K p85 (Y607; ab182651) were from Abcam (Cambridge, UK). Antibodies to light chain 3 (LC3; #12741), Akt (pan; #2920), cleaved caspase-9 (#9509), p-Akt (T308; #9275), p-Akt (S473, #4060), p-p70S6K (Thr389; #9206) and p-eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; T37/46; #2855) were all purchased from Cell Signaling Technology, Inc. Goat anti-rabbit IgG antibody conjugated with HRP (#11-035-003), goat anti-mouse IgG antibody conjugated with HRP (#115-035-003), and goat anti-rabbit IgG antibody conjugated with Cy3 (#111-165-144) were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

Cell culture and treatment with NR1. Conditionally immortalized human podocytes were kindly provided by Dr Guisen Li (Division of Nephrology, from the Sichuan Provincial People's Hospital) and were cultured as previously described (24). In brief, in order to maintain an undifferentiated proliferative state, podocytes were cultured at 33°C with RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). Before use, when the cells had reached 70-80% confluence, they were cultured at 37°C to reach differentiation. The podocytes were divided into the following groups: i) the low glucose (LG) group: the cells were incubated in RPMI-1640 containing 5 mM D-glucose; ii) the high glucose (HG) group: the cells were incubated in RPMI-1640 containing 30 mM D-glucose; iii) the NR1-treated (NR1) group: the cells were incubated in

RPMI-1640 containing 30 mM D-glucose and then treated with 20 μ M NR1 for 24 h.

Cell viability and cell morphology. To ensure that NR1 can abolish the growth inhibitory effects of high glucose on podocytes, podocyte viability was measured within 24 h of exposure to a series of NR1 concentrations (0, 1, 5, 10, 20, 50 and 100 μ M). The number of viable cells was assessed by CellTiter-Glo luminescent cell viability assay kit according to the manufacturer's instructions in triplicate. Cell morphology was also observed under a light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Hoechst staining. The podocytes were grown to 80% confluence on 6-well plates under growth-restricted conditions and treated with low glucose, high glucose, or a combination of both high glucose and 20 μ M NR1 for 24 h. Hoechst 33342 was added to achieve a final concentration of 10 μ M and the cells were analyzed after 5 min of Hoechst staining. Apoptotic cells with condensed chromatin were manually counted for a minimum of 20 high-powered fields per experimental condition by two blinded technicians. The number of apoptotic nuclei was expressed as a percentage of the total number of apoptotic nuclei.

Annexin V-PI assay. Following the experimental treatments, the differentiated human podocytes were dispersed from 6-well culture plates, collected in PBS, washed with FACS buffer, and stained using the Dead Cell Apoptosis kit with Annexin V-PI following the manufacturer's instructions. Cells (10^4) were acquired using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The number of apoptotic cells was analyzed using CellQuest software 3.3 (Becton-Dickinson) and was expressed as a percentage of the number of total cells.

TUNEL assay. Podocyte apoptosis was analyzed by TUNEL assay according to the manufacturer's recommendations. Following fixation in 4% buffered paraformaldehyde (PFA), the cells were incubated with terminal deoxynucleotidyl transferase using FITC-labeled nucleotides. DAPI served as a nuclear counterstain (blue). TUNEL-positive cells (green) in 15-20 randomly selected fields were manually counted out of the total number of cells.

Caspase assay. Podocyte apoptosis was also assessed by measuring the activity of caspase-3 using the caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. In brief, the cells were lysed, and the supernatant was collected, quantified and incubated with the caspase-3-specific color substrate Ac-DEVD-pNA. Caspase-3 activity was determination by measuring optical density at OD400 nm.

Immunofluorescence staining. The podocytes were grown in triplicate on type I collagen-coated glass coverslips, fixed with 4% PFA, and permeabilized with Triton X-100. Following the removal of the remaining Triton X-100, the cells were incubated with anti-active caspase-3 antibodies overnight, followed by Cy3-conjugated secondary antibody. For nuclear staining, the cells were stained with DAPI. For the negative contro, PBS was

used instead of the primary antibody. The total cell population and the number of cleaved caspase-3-positive cells were manually counted.

MDC assay and Beclin knockdown. Following incubation with 20 μ M NR1 for the indicated periods of time, the podocytes were cultured with 0.05 mM MDC at 37°C for 60 min. The fluorescence intensity of the cells was analyzed by flow cytometry. The cells were respectively transfected with Beclin 1 siRNA and scramble siRNA at 33 nM using DharmaFect (#T-2004; Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions (25). The transfected cells were used for subsequent experiments 24 h later.

Western blot analysis. The cells were lysed and the protein lysates were centrifuged at 12,000 rpm for 30 min. Supernatants were collected and loaded on a SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for electrophoresis. Following electrophoresis, the proteins were transferred onto PVDF membranes in transfer buffer. The membranes were blocked with 5% non-fat dry milk in TBS for 1 h. The blots were incubated overnight at 4°C with primary antibodies at the dilutions recommended by the manufacturer, followed by incubating with horseradish peroxidase (HRP)-conjugated secondary antibody, and then visualized by Immobilon Western Chemiluminescent HRP substrate (26).

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted using the RNeasy micro kit (Qiagen, Hilden, Germany) and reverse transcribed [1 μ g total RNA, 1 μ l random primer (50 μ mol/l; Applied Biosystems, Foster City, CA, USA), 1X reverse transcriptase buffer, and 10 units reverse transcriptase (Promega Corp.) in a total volume of 20 μ l]. The RNA and primer were heated to 72°C and slowly cooled before reverse transcription at 42°C for 1 h. The room temperature reaction was then diluted to 100 μ l with RNase-free water (Applied Biosystems) as previously described (27). For quantitative PCR (qPCR), 2.5% of the total room temperature reaction was used as input for PCR using SYBR-Green Master Mix (Applied Biosystems) and templates were subjected to 36 rounds of PCR (94°C for 30 sec; 56°C for 30 sec; 72°C for 1 min) on an ABI 7900 Real-Time PCR system. As previously described (28-30), the following primers were used: podocin forward, 5'-GGCTGTGGAGGCTGAAGC-3' and reverse 5'-CTCAGAAGCAGCCTTTTCCG-3'; nephrin forward, 5'-CGGTACAGGATCTGGCTGTT-3' and reverse 5'-CTCTCACCCTCGTCATACA-3'; podocalyxin forward, 5'-CTACGGACTCATCTAACAAA-3' and reverse, 5'-AGATAACC GATGACGTA-3'; desmin forward, 5'-CAGAATTGAA TCTCTCAACG-3' and reverse, 5'-CTTCAGAAATGTT CTTAGCC-3'; CD2AP forward, 5'-AACAGATACCGAA GGTA AAA-3' and reverse, 5'-ATCAAATGGATTACT CCCC-3' and GAPDH forward, 5'-TGGTCCACCAGGGCT GCTT-3' and reverse, 5'-AGCTTCCCGTTCTCAGCCTT-3'. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative amount of the target RNAs, as previously described (31).

Statistical analysis. Data are expressed as the means \pm SEM from at least 3 independent experiments. Statistical significance was determined by one-way ANOVA with Bonferroni correc-

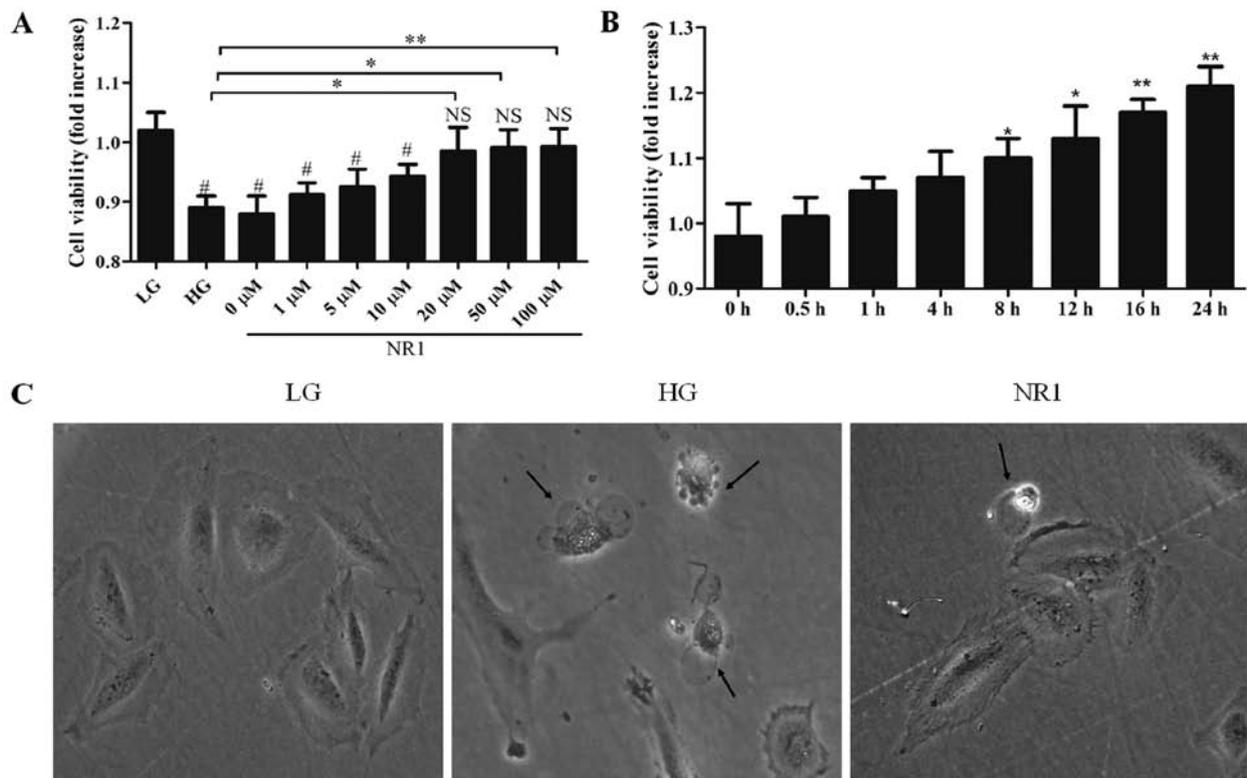


Figure 1. Notoginsenoside R1 (NR1) increases the viability of podocytes cultured under high glucose conditions. (A) Dose-dependent effect of NR1 on the viability of podocytes. (B) Time-course analysis of the effects of NR1 on cell viability. (C) Cell morphological changes. All images are at $\times 400$ magnification, and the arrows indicate membrane blebbing. Data are shown as the means \pm SEM from at least 3 independent experiments, * $p < 0.05$, ** $p < 0.01$, # $p < 0.05$ vs. LG group; NS, no significant difference vs. LG group.

tion for the comparison of 3 groups or the Student's t-test for the comparison of 2 groups.

Results

NR1 protects against high glucose-induced podocyte apoptosis. Accumulating evidence has indicated that podocyte apoptosis is an early step in the progression of DN (15-17). We thus hypothesized that NR1 may exert protective effects on podocytes by inhibiting apoptosis. Cell viability was measured to determine the dose response of podocytes to NR1 (Fig. 1A). NR1 at the concentration of $20 \mu\text{M}$ was proven to be effective in protecting the podocytes, as evidenced by a significant increase in cell viability compared to the group exposed to high glucose. Subsequent time-course experiments using $20 \mu\text{M}$ NR1 revealed that NR1 treatment increased cell viability significantly after 8 h and resulted in a 22.7% increase at 24 h (Fig. 1B). NR1 restored cell viability in the HG-treated podocytes, indicating its protective effects on podocytes. To induce significant changes in cell viability, NR1 treatment at $20 \mu\text{M}$ for 24 h of the HG-treated podocytes was used in all subsequent experiments.

To determine whether NR1 protects podocytes from apoptosis, cell morphology was assessed under a light microscope (Fig. 1C). The podocytes in the HG group exhibited marked apoptotic morphological changes, including membrane blebbing, cell volume reduction and rounding. These morphological changes were attenuated in the podocytes in the NR1 group (Fig. 1C). Moreover, the percentage apoptosis was measured by Hoechst 33342 staining (Fig. 2A and B). As shown

in Fig. 2A, considerably greater DNA fragmentation in the nuclei was observed in the podocytes in the HG group than in those in the LG group, and normal, round and homogeneously stained nuclei were observed in the NR1-treated podocytes. Statistical analysis also indicated that exposure to high glucose resulted in increased apoptosis events that were attenuated by NR1 (Fig. 2B). There was no statistically significant difference in the percentage apoptosis between the cells in the LG group and those in the NR1 group. In order to validate the results obtained by Hoechst 33342 staining, Annexin V-PI staining and FACS analysis were performed as a second independent method to measure apoptosis (Fig. 3). The FACS data revealed similar results as the Hoechst staining. In addition, TUNEL staining was performed as a third independent method to measure apoptosis (Fig. 4A), and the numbers of TUNEL-positive cell percentages were manually calculated (Fig. 4B). Consistent with the other two methods, the TUNEL staining results also demonstrated that high glucose induced the apoptosis of podocytes and that this effect was reversed by NR1. Taken together, these data convincingly demonstrated that NR1 inhibits podocyte apoptosis *in vitro*.

Effects of NR1 on foot process effacement. It has been previously demonstrated that the slit diaphragm bridges adjacent foot processes and functions as the ultimate molecular size filter (32). Given the clinical significance of the slit diaphragm proteins, we further analyzed the expression levels of podocin, nephrin and CD2AP. As shown in Fig. 5A, the expression levels of these proteins were significantly decreased under high glucose

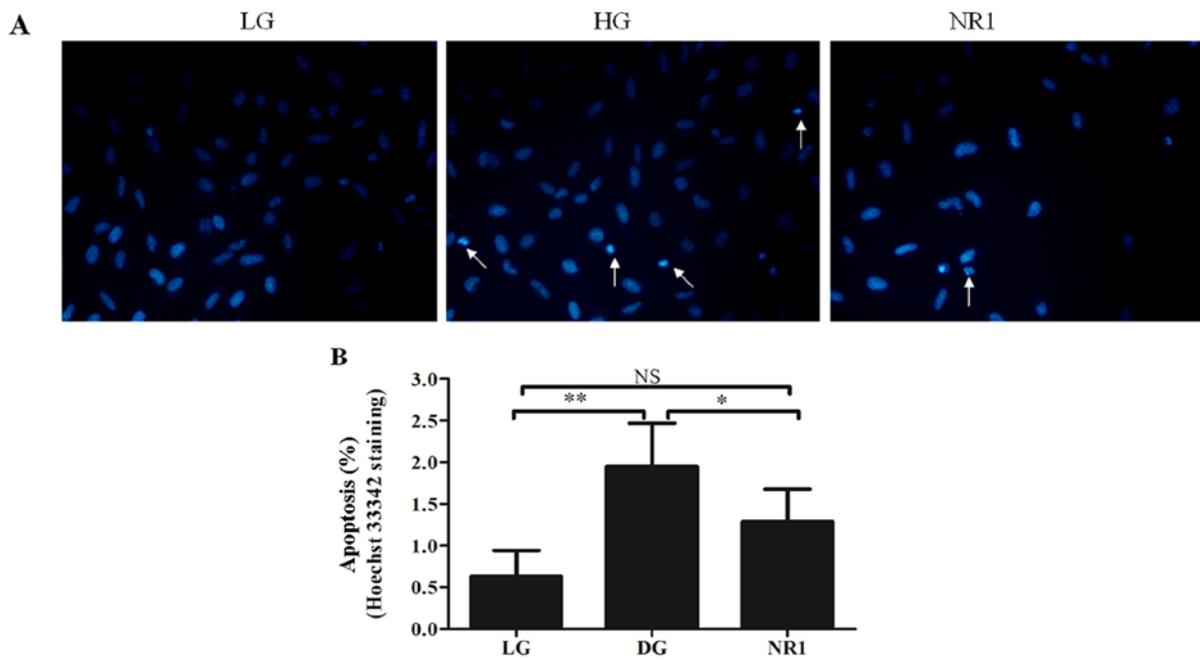


Figure 2. Assessed by Hoechst 33342 staining, notoginsenoside R1 (NR1) alleviates high glucose-induced podocyte apoptosis. (A) Hoechst 33342 staining of podocytes, and (B) apoptosis percentages quantified by Hoechst 33342 staining. All images are at x100 magnification, and the arrows indicate the apoptotic nuclei. Data are shown as the means \pm SEM from at least 3 independent experiments, * p <0.05; ** p <0.01; NS, no significant difference.

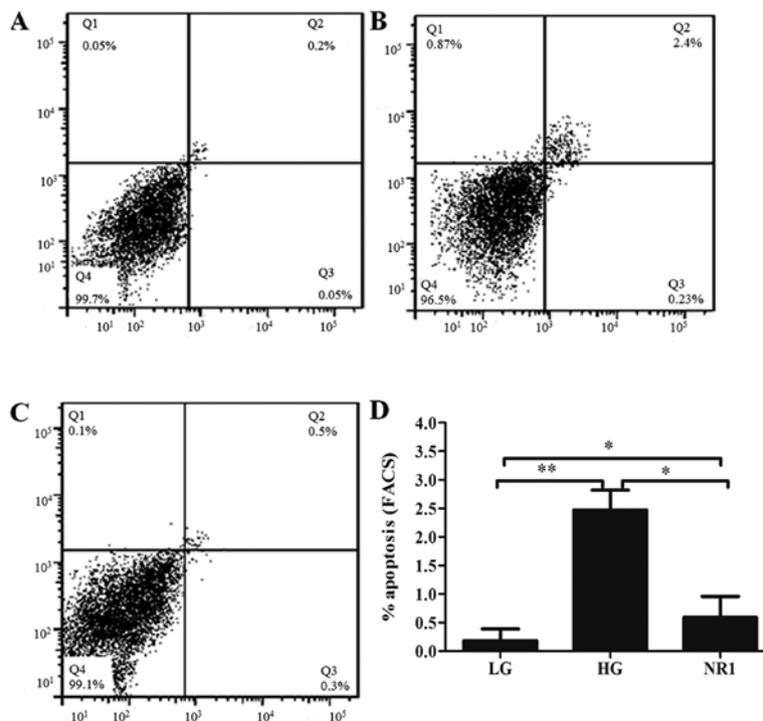


Figure 3. Annexin V-propidium iodide (PI) staining results demonstrating that notoginsenoside R1 (NR1) protects podocytes from apoptosis. Podocytes treated with (A) low glucose, (B) high glucose and (C) NR1 were fixed and then stained with Annexin V-PI. (D) Statistical analysis by flow cytometry. * p <0.05; ** p <0.01.

conditions and NR1 reversed this effect. Consistent with the results of western blot analysis, NR1 also restored the mRNA levels of podocin, nephrin and CD2AP in the HG-treated podocytes (Fig. 5B). As PCX inhibits cell-matrix interactions (33), and desmin is a marker of podocyte injury (34,35), the protein expression of PCX and desmin was assessed by western blot

analysis. Exposure to high glucose enhanced the expression levels of PCX and desmin in podocytes, while NR1 inhibited these effects (Fig. 5).

NR1 inhibits apoptosis via the caspase-dependent pathway. To further determine how NR1 inhibits podocyte apoptosis,

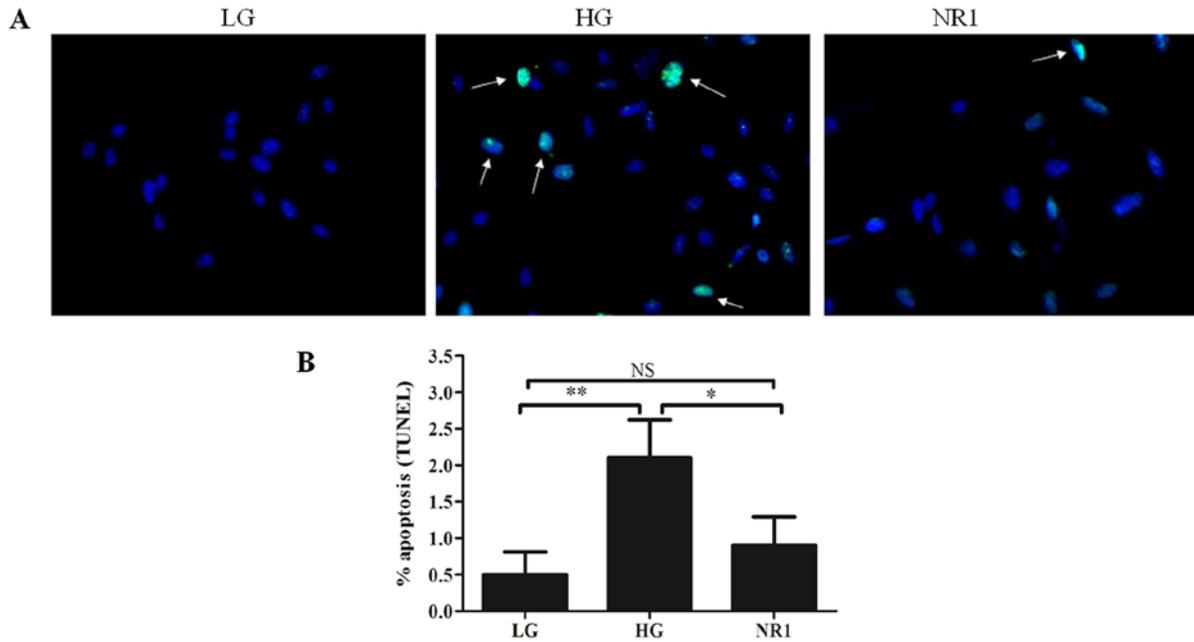


Figure 4. (A) TUNEL staining of podocytes and (B) statistical analysis demonstrating that notoginsenoside R1 (NR1) protects podocytes from apoptosis. Arrows indicate the apoptotic cells. All images are at x100 magnification. Data are shown as the means ± SEM from at least 3 independent experiments. *p<0.05; **p<0.01; NS, no significant difference.

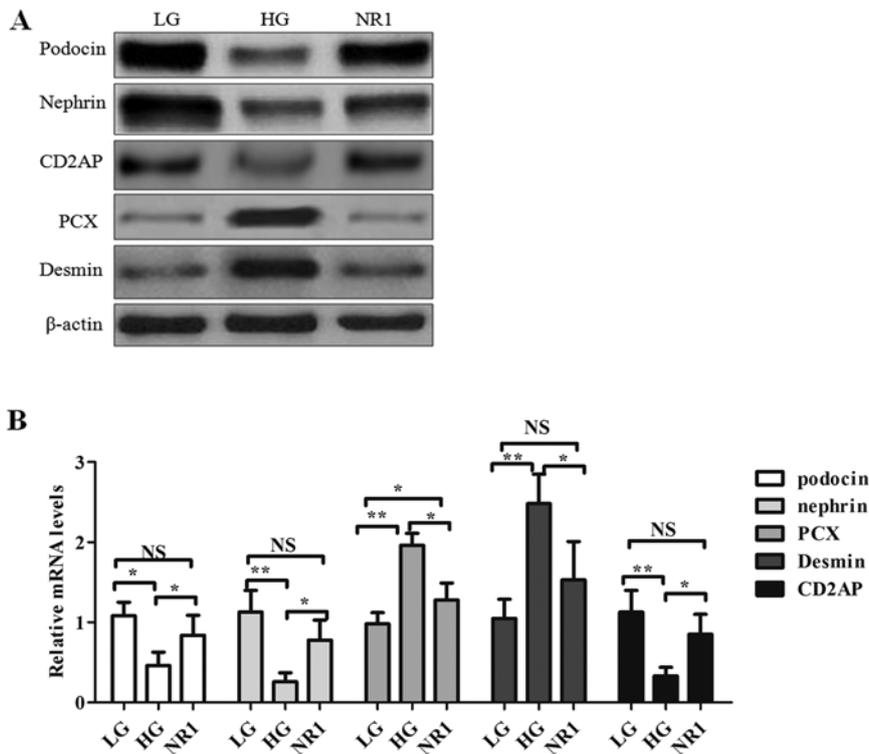


Figure 5. Notoginsenoside R1 (NR1) protects podocytes by restoring the expression of slit diaphragm proteins. (A) Western blot analysis of podocyte slit diaphragm proteins. Protein bands shown are representative of more than 3 independent experiments with similar results. (B) mRNA expression levels of podocin, nephrin, CD2AP, podocalyxin (PCX), and desmin quantified by RT-qPCR. Data are shown as the means ± SEM from 3 independent experiments. *p<0.05; **p<0.01; NS, no significant difference.

podocytes in the LG, HG and NR1 groups were stained with cleaved caspase-3 antibody. The results revealed that high glucose contributed to podocyte apoptosis by increasing cleaved

caspase-3 expression, while NR1 significantly inhibited the expression of cleaved caspase-3 (Fig. 6A). Furthermore, caspase-3 activity assay (Fig. 6B) revealed that high glucose enhanced

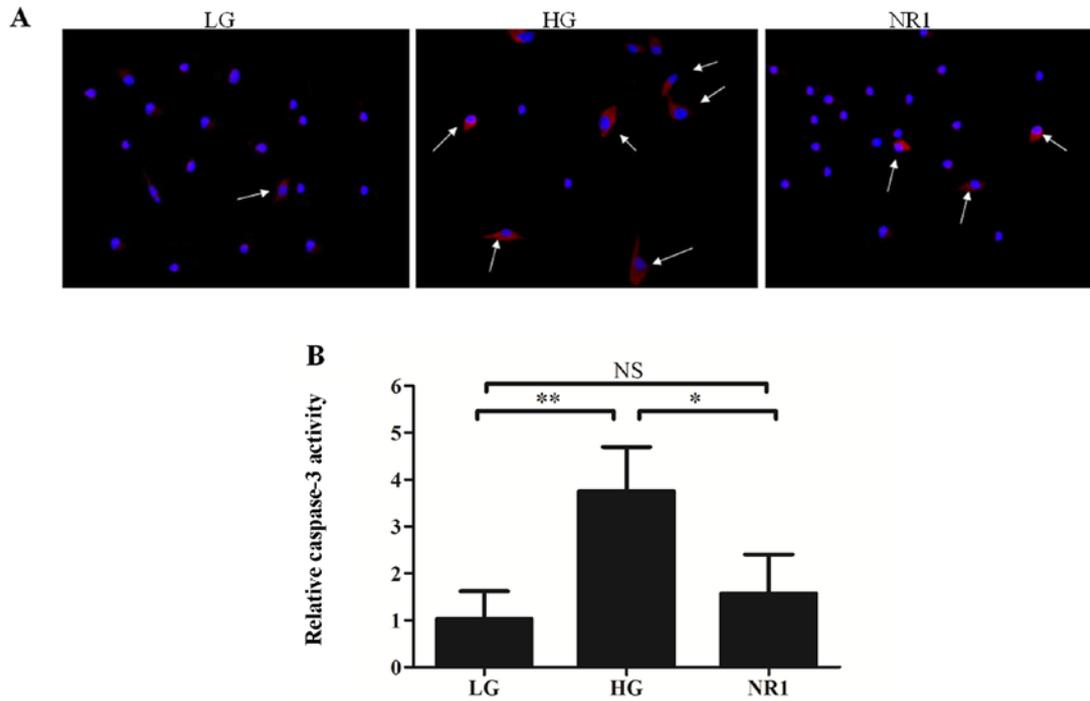


Figure 6. Notoginsenoside R1 (NR1) inhibits caspase-3 activity. (A) Immunofluorescence staining of cleaved caspase-3. White arrows indicate the cells stained positive for cleaved caspase-3. (B) Caspase-3 activity. Data are shown as the means \pm SEM from 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; NS, no significant difference.

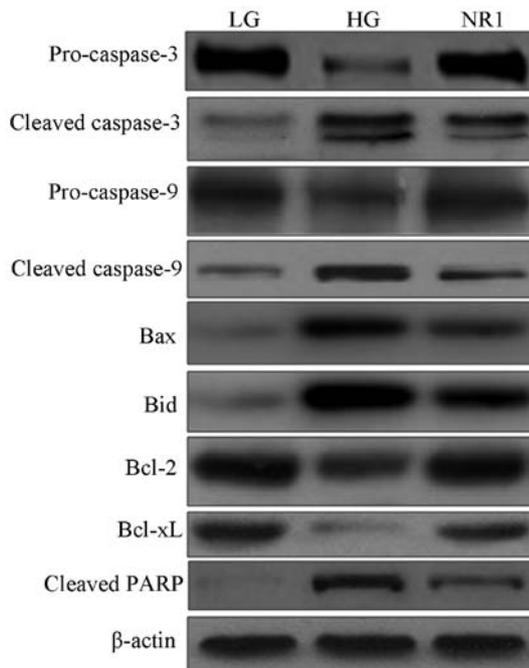


Figure 7. Western blot analysis of proteins in the caspase pathway indicating that notoginsenoside R1 (NR1) inhibits podocyte apoptosis in a caspase-dependent manner. Protein bands shown are representative of more than 3 independent experiments with similar results.

caspase-3 activity in the podocytes, while NR1 significantly attenuated this effect. Western blot analysis of proteins involved in the apoptotic pathway (Fig. 7) revealed decreased expression levels of pro-caspase-3 and -9, and increased expression levels of cleaved caspase-3 and -9 in the HG group podocytes, indi-

cating the activation of caspase-9 and caspase-3. However the cleavage of caspase-3 and -9 was partially inhibited by NR1. In addition, the upregulation of the levels of pro-apoptotic Bax and Bid proteins, as well as the downregulation of the anti-apoptotic Bcl-2 and Bcl-xL proteins were observed in the podocytes in the HG group; however, NR1 downregulated the expression levels of Bax and Bid, and upregulated the expression levels of Bcl-2 and Bcl-xL in the HG-treated podocytes. Further experiments of the PARP cleavage product indicated that the exposure of the podocytes to HG increased the cleavage of PARP, and treatment with NR1 resulted in a substantial decrease in the cleavage of PARP. Taken together, our data demonstrate that NR1 significantly attenuates HG-induced podocyte apoptosis via the caspase-dependent pathway.

NR1 induces autophagy in podocytes. Increasing evidence indicates that podocytes have a higher level of constitutive autophagy than other intrinsic renal cells do, and we thus investigated the expression levels of autophagy-related proteins. Western blot analysis (Fig. 8A) revealed that HG increased the expression of LC3-II, and decreased the expression of LC3-I and Beclin 1 in podocytes. However, NR1 significantly reversed these effects on these proteins in the HG-treated cells to levels comparable to those of the LG group. To further investigate the role of autophagy in NR1 treatment, Beclin 1 was knocked down by siRNA (Fig. 8B). By measuring the MDC fluorescence intensity in the presence of Beclin 1 siRNA, we found that the silencing of Beclin 1 resulted in reduced autophagy in the NR1-treated podocytes (Fig. 8C). To further evaluate whether NR1 induces autophagy, the chemical autophagy inhibitor, 3-MA, was added to the medium of podocytes in each group. The MDC fluorescence intensity of the podocytes treated

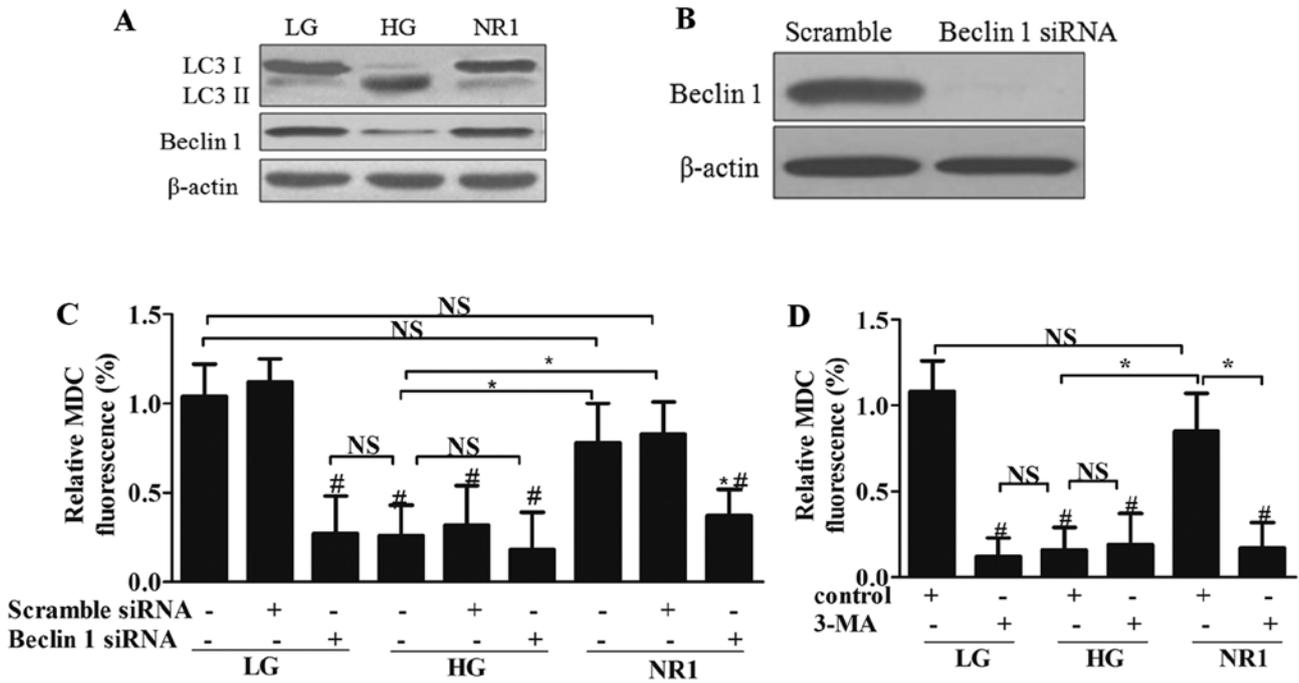


Figure 8. Notoginsenoside R1 (NR1) induces autophagy. (A) Western blot analysis of autophagy-related proteins. (B) Western blot analysis of the efficiency of Beclin 1 siRNA knockdown. The autophagy percentages were determined with either (C) Beclin siRNA or (D) with 3-MA. #p<0.05 vs. LG group; *p<0.05; NS, no significant difference.

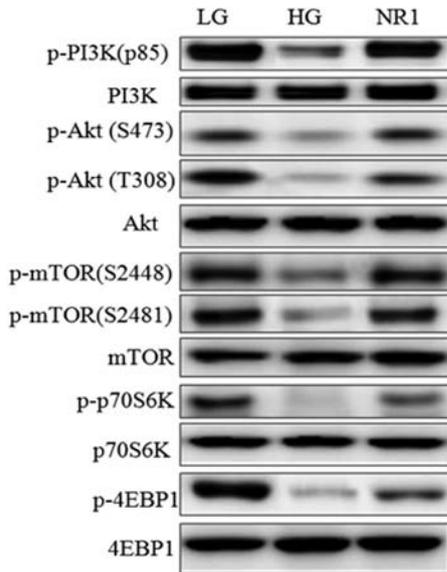


Figure 9. Western blot analysis of proteins in the PI3K/Akt/MTOR pathway indicating that notoginsenoside R1 (NR1) inhibits apoptosis and increases autophagy via the PI3K/Akt/MTOR pathway. Protein bands shown are representative of more than 3 independent experiments with similar results.

with 3-MA also revealed that 3-MA inhibited NR1-induced autophagy, while 3-MA had no effect on the HG-treated podocytes (Fig. 8D). These observations suggested that NR1 increased the basal autophagy that was inhibited by HG.

NR1 protects podocytes from injury via the PI3K/Akt/mTOR pathway. In order to further explore the mechanisms through which NR1 inhibits apoptosis and increases autophagy, we

investigated whether the PI3K/Akt/mTOR signaling pathway is involved in these mechanisms. As shown in Fig. 9, the phosphorylation levels of PI3K (p85), Akt, mTOR and p70S6K and 4E-BP1, which are downstream genes of mTOR, were markedly decreased in the podocytes in the HG group. However, NR1 increased the phosphorylation levels of these proteins, indicating that the activation of PI3K/Akt/mTOR pathway may be involved in NR1 induced podocyte protection. These data suggested that the NR1-induced protective effects on podocytes were dependent on the phosphorylation and activation of the PI3K/Akt/mTOR pathway.

Discussion

In the present study, we demonstrated that NR1 preserved the podocyte number by inhibiting apoptosis and inducing autophagy. NR1 increased cell viability in a dose- and time-dependent manner (Fig. 1A and B). NR1 significantly attenuated high glucose induced podocyte apoptosis (Fig. 1), mainly through the caspase-dependent pathway (Figs. 6 and 7). NR1 also protected podocytes from high glucose-induced damage by restoring the expression of proteins involved in podocyte slit diaphragm (Fig. 5), which was dependent on the activation of autophagy by NR1 (Fig. 8). Additionally, we found that NR1 activated the PI3K/Akt/mTOR signaling pathway, which in turn inhibited apoptosis and enhanced autophagy (Fig. 9). Taken together, our studies suggest that NR1 protects podocytes from high glucose-induced injury by inhibiting apoptosis and promoting autophagy via the PI3K/Akt/mTOR pathway.

Podocytes, terminally differentiated cells, have a very limited capacity for division and replacement. Therefore, podocyte damage and decreased podocyte number contribute to proteinuria and to the development of glomerulosclerosis.

Emerging evidence indicates that the podocyte slit diaphragm contributes significantly to the size-selective filtration barrier in the kidneys (36,37). Moreover, the disruption of the cytoskeleton leads to foot process effacement in podocytes (38). Herein, to elucidate the mechanisms responsible for the protective effects of NR1 against high glucose-induced podocyte damage, we performed western blot analysis of slit diaphragm proteins and found that NR1 restored the expression levels of these proteins (Fig. 5A). In addition, the results of RT-qPCR (Fig. 5B) also demonstrated that NR1 reduced the expression of desmin and increased the expression of slit diaphragm proteins, which are closely linked to the pathogenesis of foot process effacement under hyperglycemia.

The inhibition of basal autophagy has also been reported to be detrimental to the podocyte architectural structure (22). Maintaining podocyte number plays a pivotal role in the treatment of DN; therefore, in our study, we focused on the anti-apoptotic and autophagy-inducing effects of NR1. Accumulating clinical and experimental data have demonstrated that increased apoptosis and the inhibition of autophagy are important causes of decreased podocyte number (14,39). Therefore, therapies targeting apoptosis and autophagy pathways may stabilize podocyte number and delay the progression of chronic glomerular disease. Our study demonstrated that NR1 preserves the podocyte number by inhibiting apoptosis and inducing autophagy. Firstly, we found that NR1 increased cell viability of HG-treated podocytes in a dose- and time-dependent manner. Furthermore, NR1 protected the HG-treated podocytes from apoptosis, verified by 3 independent methods. Secondly, we found that NR1 was capable of inducing autophagy in HG-treated podocytes, and this effect was inhibited by Beclin siRNA or 3-MA (an autophagy inhibitor). Moreover, the enhancement of autophagy induced by NR1 resulted in elevated expression levels of slit diaphragm proteins and restored the cytoskeleton structure. These data are consistent with the hypothesis that autophagy plays an important role in maintaining podocyte number and function.

To explore the mechanisms underlying the anti-apoptotic effects of NR1, we focused on the caspase pathway. There is evidence to indicate that caspase-3 is involved in many forms of apoptosis (40). It has been demonstrated that caspase-3 is dispensable for certain forms of apoptosis (41). Therefore, whether caspase pathway is involved in the NR1-induced inhibition of podocyte apoptosis gained our attention. It has been demonstrated that in podocytes, PA caused activation of caspase-3 (42,43). Furthermore, previous studies have indicated that caspase-3 is activated in podocytes under high glucose conditions, and the activation of caspase-3 is caused by Bcl-2 insufficiency (44,45). However, to the best of our knowledge, no studies to date have focused on whether Bcl-xL and other proteins in the caspase pathway are involved in the effects of NR1. One of the major findings of our study was that NR1 suppressed apoptosis in a caspase-dependent manner. The results of western blot analysis revealed that Bcl2, Bcl-xL and other proteins in the caspase pathway were involved in the NR1-induced inhibition of apoptosis. In addition, immunofluorescence staining of cleaved caspase-3 and caspase-3 activity assay also demonstrated that caspase-3 activity was inhibited in the NR1-treated podocytes. Taken together, by three independent methods,

our study demonstrates that the caspase pathway is involved in the NR1-induced inhibition of the apoptosis of podocytes.

Among a wealth of signaling pathways in podocyte protection, the PI3K/Akt signaling pathway is one of the main anti-apoptotic pathways. A growing body of evidence supports the notion that the PI3K/Akt pathway controls the remodeling of the actin cytoskeleton and cell viability (46,47). Studies on podocytes, isolated from diabetic mice, have demonstrated that the insulin-dependent phosphorylation of Akt is impaired, and the dysregulation of Akt phosphorylation is associated with podocyte apoptosis (19). Moreover, *in vivo* studies have indicated that PI3K p85 interacts with nephrin and CD2AP to form the slit diaphragm protein complex that was associated with a strong activation of Akt (48). Therefore, the PI3K/Akt pathway is involved in both the control of cell number and the maintenance of podocyte biology. In this study, we provide evidence showing that the PI3K/Akt pathway is inactivated after podocyte injury by HG, and is then activated following treatment with NR1. We also proved that NR1 induced podocyte autophagy by the phosphorylation of Akt and mTOR.

In conclusion, our study suggests that NR1 protects podocytes from injury, whose damage and decrement account for proteinuria. NR1 plays an anti-apoptotic and autophagy-inducing role in cultured podocytes through the PI3K/Akt/mTOR signaling. Moreover, NR1 protects slit diaphragm proteins in podocytes under high glucose conditions. Taken together, these findings provide insight into the possible mechanisms through which NR1 treatment may preserve the overall podocyte number and prevent podocyte injury, thereby attenuating the development of glomerulosclerosis and many forms of chronic kidney disease.

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