Original Paper

Notoginsenoside R1 Ameliorates Podocyte Adhesion Under Diabetic Condition Through $\alpha_3\beta_1$ Integrin Upregulation *in Vitro* and *in Vivo*

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Key Words

Notoginsenoside R1 • Podocyte adhesion • Oxidative stress • $\alpha_3\beta_1$ integrin • Diabetic condition

Abstract

Background: Decreased expression of $\alpha_3\beta_1$ integrin may contribute to reduction in podocyte adhesion to glomerular basement membrane (GBM), which represents a novel early mechanism leading to diabetic kidney disease (DKD). Here, we examined the protective effects of Notoginsenoside R1 (NR1) on podocyte adhesion and $\alpha_3\beta_1$ integrin expression under diabetic condition *in vitro* and *in vivo*. **Methods:** Conditionally immortalized mouse podocytes were exposed to high glucose (HG) with 10 and 100 $\mu\text{g}/\text{ml}$ of NR1 for 24 h. Podocyte adhesion, albuminuria, oxidative markers, renal histopathology, podocyte number per glomerular volume, integrin-linked kinase (ILK) activity and $\alpha_3\beta_1$ integrin expression were measured *in vitro* and *in vivo*. **Results:** HG decreased podocyte adhesive capacity and $\alpha_3\beta_1$ integrin expression, the main podocyte anchoring dimer to the GBM. However, NR1 ameliorated impaired podocyte adhesive capacity and partially restored $\alpha_3\beta_1$ integrin protein and mRNA expression. These *in vitro* observations were confirmed *in vivo*. In streptozotocin (STZ)-induced diabetic rats, treatment with NR1 (5 and 10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) for 12 weeks partially restored the number of podocytes per glomerular volume and glomerular $\alpha_3\beta_1$ integrin expression, as well as ameliorated albuminuria, histopathology and oxidative stress. NR1 also inhibited glomerular ILK activity in diabetic rats. **Conclusion:** NR1, a novel antioxidant, ameliorated glucose-induced impaired podocyte adhesive capacity and subsequent podocyte depopulation partly through $\alpha_3\beta_1$ integrin upregulation. These findings might provide a potential new therapeutic option for the treatment of DKD.

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Introduction

Diabetic kidney disease (DKD) is a devastating complication of type 1 and 2 diabetes and the leading cause of end-stage renal disease (ESRD) in the developed countries. Thus, there is a pressing need for the development of novel and definitely effective approaches for preventing, delaying or treating DKD. Podocytes are terminally differentiated epithelial cells that cover and adhere to the outer surface of the glomerular basement membrane (GBM) and play a crucial role in maintaining the structure and function of the GBM. Recent studies have increasingly demonstrated that podocyte injury is a key event in the initiation and progression of DKD [1-3]. Human and experimental studies have documented that podocyte damage occurs at an early stage of DKD, and the cells may ultimately become detached from the GBM [4, 5]. Furthermore, the reduced number of podocytes per glomerulus was the strongest predictor of progression of DKD, where fewer cells predicted more rapid progression [6]. Recent human studies have confirmed that podocyte detachment plays an important role in the development and progression of kidney disease in type 1 and type 2 diabetes [7, 8].

Alterations in adhesive interaction between podocytes and GBM are most likely to be important in podocyte detachment from GBM [9]. One recent report demonstrated that increased glucose concentrations decreased matrix-related cell adhesion in cultured human podocytes, leading to reduced binding to the GBM components [10]. Our previous study also reported that high glucose induced a significant decrease in the adhesion of podocytes to basement membrane protein complex (BMC) *in vitro* [11]. Thus, impaired adhesion to GBM may contribute to the podocyte detachment, which represents a novel early mechanism leading to DKD. The promise of this new insight is the development of novel and effective interventions for the treatment of DKD.

Herbal medicines have traditionally played an important role in the prevention and treatment of diabetes in China for centuries. Panax notoginseng (known as tian qi or san qi) is a well-known medicinal herb for its long history of use in traditional Chinese medicine. Recent studies have reported that Notoginsenoside R1 (NR1), a major component of Panax notoginseng, attenuates renal [12] and intestinal [13] ischemia-reperfusion injury in rats. However, the protective effects of NR1 on podocyte injury in diabetic rats have not been investigated yet. This study aimed to investigate the protective effects of NR1 on podocyte adhesion under diabetic condition *in vitro* and *in vivo*, and then provide a potential new therapeutic option for the treatment of DKD.

Materials and Methods

Drug preparation

Notoginsenoside R1 (NR1, chemical structure $C_{47}H_{80}O_{18}$, molecular weight = 933) was purchased from ShangHai YuanYe Biotechnology Co.,Ltd.(purity above 98%, Shanghai, China).

In vitro studies: Cell culture and Cell adhesion assay

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Division of Nephrology, Massachusetts General Hospital, Harvard University) and were conducted as described previously [11]. In brief, podocytes were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, USA), 100U/ml penicillin and 100µg/ml streptomycin. Cells were grown at 33°C with 10 U/ml mouse recombinant interferon-γ (IFN-γ, Sigma Chemical Corporation, USA). At confluence podocytes were incubated at 37°C on collagen-coated dishes for 14 days deprived of IFN-γ to allow differentiation. Differentiated podocytes were cultured for 24h in RPMI 1640 medium and 1% FCS before being exposed to various experimental conditions. The cells were divided into the following groups: (1) normal glucose group (NG) as controls incubated in RPMI 1640 containing 5 mM glucose, (2) high glucose group (HG) incubated in RPMI 1640 containing 30 mM glucose, (3) NR1 group incubated in HG medium treated with 10 and 100 µg/ml of NR1 for 24 h. All the glucose used in the present study was D-glucose. All experimental groups were cultured in quadruplicate.

The cell adhesion assay was performed by centrifugation method as described in our previous study [11]. Tissue culture polystyrene 96-well plates (Corning 3595, Corning, NY, USA) were coated with basement membrane protein complex (BMC) in deionized H₂O for 1 hour at room temperature. Podocytes under different experimental conditions were carefully aspirated to remove undetached cells and refilled with fresh PBS-dextrose for an initial count to determine the density of cells before detachment. The cells were observed under a microscope (Olympus IX70, Japan) equipped with a Hoffman Modulation Contrast system and recorded with a DVC-1310C Magnafire digital camera (Optronics). The lid was removed, and the plate was covered with sealing tape and centrifuged upside down for 10 min at 1500 ×g on a Beckman Allegra centrifuger (GH 6.8 rotor) to detach the cells. The wells were carefully aspirated and refilled with fresh PBS-dextrose for the count under the same conditions to determine the density of remaining adherent cells. Podocyte adhesion was measured on quadruplicate wells by counting attached cells in three different fields and was evaluated in a blind fashion. The adhesive capacity was determined as the ratio of adherent cells/cells before detachment. All the adhesion assays were performed in quadruplicate and the experiment was repeated three times.

Animal studies

All the animal procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. All the animal protocols were approved by the Animal Ethics Committee of Fudan University, Shanghai, China. Healthy male Sprague-Dawley rats weighing 180 to 200g, purchased from Experimental Animal Center, Fudan University, Shanghai, China, were housed in an air-conditioned room at 23 ± 1°C on a 12:12-h light-dark cycle. Animals were fed a standard diet and given water ad libitum. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ) dissolved in 0.1M citrate buffer (pH 4.5) at 65 mg/kg, while the normal control rats received the 0.1M citrate buffer solution. Forty-eight hours after injection of STZ, the blood glucose level was measured from the tail vein. Rats with a blood glucose level over 300 mg/dl were considered to be diabetic and included in the study. Diabetic rats were then randomly divided into three groups (n=9/each group): (1) STZ-induced diabetic rats (DN); (2) STZ-induced diabetic rats treated with low dose of NR1 at 5 mg·kg⁻¹·d⁻¹ (NRL) and (3) STZ-induced diabetic rats treated with high dose of NR1 at 10 mg·kg⁻¹·d⁻¹ (NRH). Normal Sprague-Dawley rats were chosen as control (NC). NR1 was started at 2 weeks after STZ injection and was administered once daily by oral gavage for 12 weeks. The normal control (NC) and diabetic control (DN) rats received the equal volume of saline within the same time. Rats were kept in individual metabolic cages for 24 h urine collection at the end of 12 weeks of treatment. Urine was centrifuged at 800g for 10 min at 25°C. Whole urine was stored at -70°C and thawed just before use. Urinary albumin concentrations were measured using an ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Urine creatinine concentrations were detected by an automatic biochemistry analyzer (Hitachi Model 7600, Japan). At the end of 12 weeks of treatment, blood samples obtained from the tail vein were assayed for blood glucose level. Rats were then anesthetized with pentobarbital sodium and the blood samples were taken through the abdominal aorta for measuring oxidative stress markers, as well as biochemical parameters, including blood urea nitrogen (BUN) and creatinine (Cr) by an automatic biochemistry analyzer (Hitachi Model 7600, Japan). Animals were then killed and the kidneys were harvested immediately.

Histological examination

That of the right kidneys was fixed with 10% buffered formalin and embedded in paraffin for histological evaluation. The kidneys were cut into 4 μm sections and stained with hematoxylin and eosin. The sections were then examined by light microscopy in a blind fashion. Mesangial matrix expansion was evaluated as described previously [14]. Relative mesangial expansion was described as the fold change from the normal control group.

Immunohistochemical analysis

Sections of kidneys from the experimental rats were immunostained for α₃ integrin, β₁ integrin and Wilms' tumor gene-1 (WT-1) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining procedures were performed according to the manufacturer's instructions. Paraffin-embedded sections (4 μm-thick) were deparaffinized with xylene and rehydrated through a descending ethanol gradient. Primary antibodies

were diluted in PBS containing 1% bovine serum albumin (BSA). All antibodies were incubated during 45 minutes at room temperature. The sections incubated with PBS, instead of the primary antibody, served as the negative controls. The amount of α_3 integrin and β_1 integrin was analyzed using the Medical Image Analysis System (Beijing University of Aeronautics & Astronauts, Beijing, China). In each glomerulus, the percentage of $\alpha_3\beta_1$ integrin-positive area within the glomerular area was calculated. All slides (20 glomeruli per section and $n = 6$ per group) were observed independently by two blinded investigators.

At least 12 sequential glomerular sections were analyzed and we also measured the glomerular volume using the Medical Image Analysis System (Beijing University of Aeronautics & Astronauts, Beijing, China). The mean area of each glomerular profile was measured by manually encircling the glomerular area and calculating that area by computerized morphometry using the Image Measurement System (Beijing University of Aeronautics & Astronauts, Beijing, China). The number of podocytes per glomerulus was determined by counting the number of WT-1-expressing nuclei per glomerular volume. The glomerular volume/podocyte (GV/P, μm^3) were calculated as determined in the morphometric analysis of podocyte counting methods according to the previous study [15]. For quantitative analysis, WT1-positive cells were quantified by counting five different fields at 400 $\times$ magnification with two independent investigators who were blinded to the treatment that the animals had received.

Determination of levels of oxidative stress markers

The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as malondialdehyde (MDA) level in serum and kidney cortex were determined using the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instruction. The activities of CAT, SOD and GPx, as well as the content of MDA in kidney tissues were also determined with the above commercial kits. The mauve product (nitrite) produced by the oxidation of hydroxylamine has an absorbance at 550 nm. One unit of SOD activity was calculated as the amount that reduced the absorbance at 550 nm by 50 % and the SOD activity was expressed as U/mg protein. Kidney CAT activity was measured as the decrease in H_2O_2 concentration by recording the absorbance at 240 nm. Results were expressed as U/mg protein. GPx activity in the kidney was assayed by a coupled assay using H_2O_2 and dithio-bis-nitrobenzoic acid (DTNB). One unit of enzyme activity of the enzymes unit represents a decrease in GSH concentration of 1 $\mu\text{mol/L/min}$ after subtraction of non-enzymatic mode. Results were normalized as U/mg protein. MDA content in kidney tissues was determined with the above commercial kit. The red product has an absorbance at 532 nm according to the manufacturer's instructions and the results were expressed as nmol/mg protein.

ILK activity assay

ILK activity was assayed by detecting phosphorylation of Akt using Western blot analysis. Isolated glomeruli were lysed in an ice-cold lysis buffer. Cell lysates were then incubated with anti-ILK antibody (Upstate Biotechnology, Inc., Lake Placid, NY, USA). A kinase assay was performed by incubating the immune complexes with Akt/PKB (Upstate Biotechnology Inc., Lake Placid, NY, USA) and ATP. The kinase reaction was terminated by addition of sodium dodecyl sulfate (SDS) sample buffer. The supernatants were resolved by SDS/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were then probed with phospho-Akt (ser-473) antibody (Cell Signaling Technology, Beverly, MA, USA), followed by incubation with HRP-conjugated secondary antibody. Chemiluminescence detection was performed with the KC™ Detection kit (KC-420, KangChen Biotechnology, Shanghai). Equality of loading was ensured by using a monoclonal antibody to anti-total Akt antibody (Cell Signaling Technology, Inc., Beverly, USA). Densitometric quantitation was performed using a Bio-Rad VersaDoc imaging system model 5000 with Bio-Rad Quantity One software. Relative ILK activity was described as the fold change from the normal control group.

Western blotting

Isolated rat glomeruli or cultured podocytes under different experimental conditions were lysed in lysis buffer. The lysates were clarified by centrifugation at 12,000 $\times g$ for 15 min. Protein estimation was performed by Coomassie brilliant blue. The tissue protein was separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with PBS containing 5% skim milk. Membranes were

then incubated overnight with α_3 integrin and β_1 integrin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); Negative controls were performed without primary antibody. After washing, the secondary antibody was added and incubated 1h at room temperature. Chemiluminescence detection was performed with the KC™ Detection kit (KC-420, KangChen Biotechnology, Shanghai). Equality of loading was ensured by using a monoclonal antibody to GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometric quantitation was performed using a Bio-Rad VersaDoc imaging system model 5000 with Bio-Rad Quantity One software. Protein expression was quantified as the ratio of specific band to GAPDH. Relative protein expression was described as the fold change from the normal control group.

Real-time quantitative RT-PCR

Total RNA was extracted from samples of isolated rat glomeruli or cultured podocytes by the Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Invitrogen). Then, 2 μ g of total RNA was reverse transcribed using the SuperScript RT kit according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). The following sequence-specific primers for mouse podocytes were used: α_3 integrin: sense 5'-CCCTCGCTTTGTAGGTTA-3' and anti-sense 5'-GTCCCTGTCAGC CTCCAC-3'; β_1 integrin: sense 5'-GTTCCATGCGTAGCGACAA-3' and anti-sense 5'-TTCTCC-CTGCTTCCACTTTAG-3'. GAPDH: sense 5'-CTCATGACCACAGTC CATGC-3' and anti-sense 5'-CACATTGGGGGTAGGAACAC-3'. The following sequence-specific primers for rat kidney tissue were used: α_3 integrin: sense 5'-TACTACTTCTGAACGAAAGAGGAGG-3' and antisense 5'-CAGGTCTGGGTAT AAGTTTTCATC-3'. β_1 integrin: sense 5'-GACCTGCCTTGGTGTCTG TGC-3' and antisense 5'-GGCAACC ACGCCTGCTACAAT-3'. GAPDH: sense 5'-GGAAAGCTG TGGCGTGAT-3' and antisense 5'-AAGGTGGAAGAATGGGAGTT-3'. Quantitative RT-PCR was performed using the ABI PRISM7900 Sequence Detection System (Applied Biosystems) with SYBR Green Master Mix. Each reaction was amplified in triplicate and ratio results were calculated based on the $2^{-\Delta\Delta CT}$ method as described according to the previous study [16]. Gene expression was normalized to GAPDH mRNA levels as an endogenous control and described as the fold change from the normal control group.

Statistical analysis

All data were expressed as means $\pm$ standard deviation (SD). The significance of differences among experimental groups was determined by one-way ANOVA analysis followed by Dunnett's multiple range test. A *P* value < 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS 13.0 software.

Results

Effects of NR1 on HG-stimulated adhesion of podocytes to BMC

As shown in cell adhesion assay, exposure to HG for 24 h resulted in a significant decrease in the adhesion of podocytes to BMC compared with incubation in NG (Fig. 1A). However, treatment with 10 and 100 μ g/ml of NR1 for 24 hours significantly increased the adhesion of podocytes to BMC (Fig. 1A). Taken together, treatment with NR1 resulted in increased podocyte adhesion and this effect was evident at a dose as low as 10 μ g/ml.

Effects of NR1 on HG-stimulated protein and mRNA expression of $\alpha_3\beta_1$ integrin subunits in podocytes

Compared with incubation in NG, exposure to HG for 24 h reduced the mRNA expression of both α_3 and β_1 integrin subunits in podocytes. Podocytes treated with NR1 showed a significant increase in α_3 and β_1 integrin mRNA expression and a maximal increase was obtained at a concentration of 100 μ g/ml (Fig. 1B). Likewise, exposure to HG for 24 h significantly decreased the protein levels of both α_3 and β_1 integrin subunits. However, the HG-induced decrease in the protein levels of both α_3 and β_1 integrin subunits was partially restored by NR1 treatment and this effect was evident at a dose as low as 10 μ g/ml (Fig. 1C). These results indicated that NR1 attenuated the HG-induced reduction in $\alpha_3\beta_1$ integrin expression in podocytes.

Fig. 1. Effects of Notoginsenoside R1 (NR1) on cell adhesion and $\alpha_3\beta_1$ integrin expression in cultured mouse podocytes. Podocytes were exposed to NG, HG, HG with 10 $\mu\text{g}/\text{ml}$ (NL) and 100 $\mu\text{g}/\text{ml}$ (NH) of NR1 for 24 h. The adhesion of podocytes to BMC was assessed by centrifugation cell adhesion assay. The $\alpha_3\beta_1$ integrin protein and mRNA expression was examined by western blot and real-time PCR, respectively. NG, normal glucose (5 mM glucose); HG, high glucose (30 mM glucose); NL, HG with 10 $\mu\text{g}/\text{ml}$ of NR1; NH, HG with 100 $\mu\text{g}/\text{ml}$ of NR1. Results were expressed as the means $\pm$ SD ($n=4$). * $P<0.05$ vs NG; # $P<0.05$ vs HG.

Table 1. Metabolic and biochemical parameters. Metabolic and biochemical parameters in diabetic rats at the end of 12 weeks of NR1 treatment. UAE, Urinary albumin excretion; ACR, albumin/creatinine ratio; BG, blood glucose; BUN, blood urea nitrogen; SCr, serum creatinine; NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR (5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR (10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n=9$). * $P<0.05$ vs NC group. # $P<0.05$ vs DN group

Group	UAE ($\mu\text{g}/24\text{h}$)	ACR ($\text{mg}/\text{g Cr}$)	BG (mmol/L)	BUN(mmol/L)	SCr($\mu\text{mol}/\text{L}$)
NC	180.2 $\pm$ 29.5	17.4 $\pm$ 2.4	5.3 $\pm$ 0.8	5.7 $\pm$ 0.7	30.0 $\pm$ 4.3
DN	1200.1 $\pm$ 231.9 *	82.5 $\pm$ 11.9 *	26.4 $\pm$ 4.3 *	6.1 $\pm$ 0.9	34.1 $\pm$ 4.8
NRL	711.3 $\pm$ 145.3 #	55.6 $\pm$ 7.0 #	25.5 $\pm$ 4.1 *	5.9 $\pm$ 0.8	33.7 $\pm$ 4.4
NRH	495.6 $\pm$ 85.6 #	34.9 $\pm$ 5.1 #	24.5 $\pm$ 3.8 *	5.5 $\pm$ 0.6	32.1 $\pm$ 4.1

Effects of NR1 on serum and urine levels of biochemical markers in diabetic rats

The levels of albuminuria, blood glucose (BG), blood urea nitrogen (BUN) and creatinine (Cr) were shown in Table 1. The STZ-induced diabetic rats showed severe albuminuria (expressed as the $\mu\text{g}/24\text{h}$ and $\text{mg}/\text{g Cr}$) when compared with the normal control rats ($P<0.05$). NR1 treatment significantly reduced urinary albumin excretion (UAE) and albumin/creatinine ratio (ACR) in diabetic rats. This protective effect was dose-dependent, which was evident at a dose as low as $5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (NRL group) and reached the peak effect at $10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (NRH group) (Table 1). Moreover, blood glucose (BG) level was significantly higher in diabetic rats compared with nondiabetic animals. However, no differences in levels of BUN and Cr were observed between NR1 treated and untreated STZ-induced diabetic rats, which indicated that NR1 did not cause apparent toxicity to the kidney.

Fig. 2. Effects of NR1 treatment on mesangial expansion in diabetic rats. Representative hematoxylin and eosin (HE) staining of kidney sections (A). Semiquantitative analyses of mesangial expansion (B). NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR1 ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n = 6$). * $P < 0.05$ vs NC group. # $P < 0.05$ vs DN group.

Fig. 3. Effects of NR1 treatment on the number of podocytes per glomerular volume in diabetic rats. Representative WT1 immunostaining in the glomeruli of kidney sections (A). Quantitative analyses of changes in the number of podocytes per glomerular volume (B). The number of podocytes per glomerulus was determined by counting the number of WT-1-expressing nuclei per glomerular volume. The glomerular volume/podocyte (GV/P, μm^3) were calculated as determined in the morphometric analysis of podocyte counting methods. NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR1 ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n = 6$). * $P < 0.05$ vs NC group. # $P < 0.05$ vs DN group.

Effects of NR1 on renal histopathology and podocyte number in diabetic rats

At 14 weeks after STZ injection, the diabetic rats showed focal mesangial matrix expansion when compared with normal control rats. However, treatment with NR1 significantly attenuated mesangial expansion compared to the untreated STZ-induced diabetic rats (Fig. 2). To assess podocyte detachment, the tissue sections were immunostained WT1 and the number of podocytes per glomerulus was determined by counting the number

Fig. 4. Effects of NR1 on $\alpha_3\beta_1$ integrin expression in diabetic glomeruli detected by immunohistochemical staining. Representative photomicrographs of immunostaining for $\alpha_3\beta_1$ integrin in the glomeruli of kidney sections (A). Semiquantitative analyses of immunostaining for α_3 integrin (B) and β_1 integrin (C) per glomerulus. NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR1 ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n = 6$). * $P < 0.05$ vs NC group. [#] $P < 0.05$ vs DN group.

of WT-1-positive nuclei per glomerular volume. At 14 weeks after STZ injection, the diabetic rats showed a severe reduction in podocyte number per glomerular volume when compared with the normal control rats ($P < 0.05$). However, daily treatment with NR1 for 12 weeks had a substantial normalizing effect on podocyte number per glomerular volume in diabetic rats. The podocyte number per glomerular volume in NR1-treated rats was significantly greater than that in untreated diabetic rats. This protective effect was observed at $5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of NR1, and the maximal increase was achieved by $10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of NR1 (Fig. 3). The above results indicated that NR1 significantly ameliorated podocyte depopulation in diabetic rats.

Effects of NR1 on $\alpha_3\beta_1$ integrin expression in diabetic rats

By immunohistochemical staining, we observed that the expression of α_3 integrin and β_1 integrin between the GBM and the podocyte was markedly reduced in the renal tissue from STZ-induced diabetic rats when compared with the normal control rats. However, NR1 treatment dose-dependently expended the positive area of α_3 integrin and β_1 integrin expression in diabetic rats (Fig. 4). The results of Western blot analysis further confirmed these findings (Fig. 5A). The protein expression of α_3 integrin and β_1 integrin was decreased in kidneys from diabetic rats when compared with the normal control rats. Diabetic rats revealed a significant decrease in protein levels of α_3 integrin and β_1 integrin while the rats received NR1 showed a marked increase in protein production of α_3 integrin and β_1 integrin (Fig. 5A). Moreover, changes in mRNA expression of α_3 integrin and β_1 integrin were quantified by real-time quantitative RT-PCR. Similarly, glomerular mRNA expression of α_3 integrin and β_1 integrin were markedly reduced in diabetic rats, which was partially abrogated by treatment with NR1. This effect was evident at a dose as low as $5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ and the peak effect was observed at $10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of NR1 (Fig. 5B). The above results demonstrated that the protective effect of NR1 on podocyte adhesion and subsequent podocyte depopulation in diabetic rats was associated with the upregulation of $\alpha_3\beta_1$ integrin expression.

Fig. 5. Effects of NR1 on protein and mRNA expression of $\alpha_3\beta_1$ integrin in diabetic glomeruli. Representative immunoblots stained for $\alpha_3\beta_1$ integrin in glomeruli (A). Real-time quantitative RT-PCR of $\alpha_3\beta_1$ integrin in glomeruli (B). NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR1 ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n = 4$). * $P < 0.05$ vs NC group. # $P < 0.05$ vs DN group.

Fig. 6. Effects of NR1 on ILK activity in isolated glomeruli from diabetic kidneys. ILK activity was assayed by detecting phosphorylated Akt (p-Akt) by Western blot analysis. Each band was scanned and subjected to densitometry and expressed as a fold increase relative to total Akt. NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$); NRH, DN rats treated with NR1 ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Results were expressed as the means $\pm$ SD ($n = 4$). * $P < 0.05$ vs NC group. # $P < 0.05$ vs DN group.

Effects of NR1 on ILK activity in diabetic rats

Since ILK has been shown to mediate cell adhesion, ILK kinase assay was performed. Glomerular ILK activity was elevated in diabetic rats (Fig. 6). However, NR1 treatment for 12 weeks markedly inhibited ILK activation in diabetic glomeruli. As little as $5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of NR1 decreased the activity of ILK, and the maximal inhibition was achieved by $10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of NR1 (Fig. 6).

Effects of NR1 on oxidative stress in serum and kidney tissues from diabetic rats

Compared with the normal control rats, there was a significant increase in serum level of MDA and a marked decrease in serum activities of CAT, SOD and GPx, in STZ-induced diabetic rats, respectively. Treatment with NR1 reduced the serum level of MDA while increased the serum activities of CAT, SOD and GPx in STZ-induced diabetic rats (Fig. 7). Likewise, diabetic rats presented a remarkable increase in MDA levels and a dramatical reduction in activities of CAT, SOD and GPx in renal cortex when compared to the normal control rats. All of these

Fig. 7. Effects of NR1 on serum activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), as well as content of malondialdehyde (MDA) in diabetic rats. Serum activities of GPx (A), SOD (B) and CAT (C), as well as serum levels of MDA (D) in rats of each group. NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 (5mg·kg⁻¹·d⁻¹); NRH, DN rats treated with NR1 (10 mg·kg⁻¹·d⁻¹). Results were expressed as the means ± SD (n = 6). *P < 0.05 vs NC group. #P < 0.05 vs DN group.

Fig. 8. Effects of NR1 on activities of GPx, SOD and CAT, as well as content of MDA in diabetic kidneys. Activities of GPx (A), SOD (B) and CAT (C), as well as MDA level (D) in renal cortex from rats of each group. NR1 treatment was started 2 weeks after STZ injection and lasted 12 weeks. NC, normal control rat; DN, STZ-induced diabetic rat; NRL, DN rats treated with NR1 (5mg·kg⁻¹·d⁻¹); NRH, DN rats treated with NR1 (10 mg·kg⁻¹·d⁻¹). Results were expressed as the means ± SD (n = 6). *P < 0.05 vs NC group. #P < 0.05 vs DN group.

abnormalities were partially restored by NR1 treatment (Fig. 8). These results indicated that NR1 significantly decreased the amount of a product of lipid peroxidation (MDA) while increased the activities of antioxidant enzymes (CAT, SOD and GPx) in diabetic rats.

Discussion

As the number of patients with diabetes increases, DKD is rapidly becoming a worldwide public health problem. There is no cure once the disease is diagnosed, but early treatment at a sub-clinical stage can prevent or at least delay the progression of DKD. A reduction in podocyte adhesive capacity via decreased expression of $\alpha_3\beta_1$ integrin, the main podocyte anchoring dimer to the GBM, may represent a novel early mechanism leading to DKD [1, 5, 17, 18]. The novel finding of this study was that NR1, a major component of Panax notoginseng, protected against impaired podocyte adhesion under diabetic condition *in vitro*

and *in vivo*, which was involved in upregulation of $\alpha_3\beta_1$ integrin expression and inhibition of oxidative stress. This conclusion was based upon the following findings: (i) NR1 significantly attenuated HG-induced impaired podocyte adhesion and this effect was associated with upregulation of $\alpha_3\beta_1$ integrin protein and mRNA expression; (ii) NR1 partially restored the number of podocytes per glomerular volume and glomerular $\alpha_3\beta_1$ integrin expression, as well as ameliorated albuminuria, histopathology in diabetic rats; (iii) NR1 significantly reduced the amount of a product of lipid peroxidation (MDA) while elevated the activities of antioxidant enzymes (CAT, SOD and GPx) in diabetic rats. These results strongly indicated that NR1, a novel antioxidant, might increase $\alpha_3\beta_1$ integrin expression, ameliorate podocyte adhesion to GBM and subsequent podocyte depopulation, and therefore ultimately delay the progression of DKD.

Podocytes adhere tightly to the underlying GBM and cell-matrix adhesions involve extracellular ligands within the GBM, adhesion molecules and intracellular linker proteins that couple to the podocyte cytoskeleton [19]. Podocyte detachment from the GBM has been confirmed to be associated with the development of proteinuria and glomerulosclerosis in DKD [7] and urinary podocyte loss is a more specific marker of ongoing renal injury than proteinuria [20]. Thus, podocyte injury and depletion play key roles in the pathogenesis of DKD and development of podocyte-specific drugs may provide novel therapeutic approaches for DKD. Our methods of podocyte counting relied on accurately identifying podocyte nuclei. WT-1 is constitutively expressed in podocyte nuclei within glomerulus and is used as a specific marker for podocytes [21, 22]. Thus, we used WT-1 immunohistochemistry staining for podocyte nuclei and we also measured the podocyte number per glomerular volume to exactly estimate the changes in numbers of podocytes per glomerulus. In this study, the diabetic rats showed a significant reduction in podocyte number per glomerular volume, which was in agreement with other study [5]. However, daily treatment with NR1 for 12 weeks had a substantial normalizing effect on podocyte number per glomerular volume in diabetic rats. NR1 also ameliorated proteinuria and renal histopathology in diabetic rats. These results clearly demonstrated that NR1 significantly ameliorated podocyte adhesion to GBM and subsequent podocyte depopulation, as well as the structural and functional abnormalities in diabetic rats.

To reveal the mechanisms underlying the action of NR1 on podocyte detachment, we investigated the effects of NR1 on $\alpha_3\beta_1$ integrin expression and oxidative stress in diabetic rats. Firstly, we examined the effects of NR1 on $\alpha_3\beta_1$ integrin expression in renal cortex by immunohistochemical analysis, western blotting and real-time quantitative RT-PCR, respectively. Podocyte detachment from the underlying GBM may result from the alteration of $\alpha_3\beta_1$ integrin, the principal adhesion molecular that attaches the podocyte to the GBM [9, 23-25]. The $\alpha_3\beta_1$ integrin, localized exclusively to the basal membrane domains in podocyte, mediates cell adhesion and regulates glomerular permeability by maintaining the shape and adhesion of the podocytes [25, 26]. Specific blocking of the $\alpha_3\beta_1$ integrin inhibits adherence of podocytes to a type IV collagen matrix [27]. Decreased expression of $\alpha_3\beta_1$ integrin expression in podocytes may serve to foot process detachment, podocyte loss and proteinuria in humans with primary FSGS and chronic PAN-treated rats [28]. Previous study reported that downregulation of $\alpha_3\beta_1$ integrin expression, by mechanical forces or TGF β 1, was *per se* sufficient to decrease podocyte adhesion *in vitro*, which contributed to the elucidation of the mechanisms of the podocyte detachment from the GBM observed in diabetes and other glomerular diseases [29]. *In vitro* study has demonstrated that high glucose reduces expression of $\alpha_3\beta_1$ integrin in cultured human glomerular epithelial cells, accompanied by decreased binding of these cells to type IV collagen [10]. Our previous experiments also showed decreased expression of $\alpha_3\beta_1$ integrin in mouse podocytes cultured in high glucose media [11]. *In vivo* studies further confirmed that expression of $\alpha_3\beta_1$ integrin was decreased in diabetic rats [17, 18]. Therefore, decreased expression of $\alpha_3\beta_1$ integrin may serve to podocyte detachment from GBM, which represents a novel early mechanism leading to DKD. This leads us to find a new therapeutic target in the treatment of podocyte injury and proteinuria. In this study, diabetic rats showed reduced expression of $\alpha_3\beta_1$ integrin in

kidneys, which consisted with previous studies [17, 18]. However, NR1-treated rats showed a significant increase in protein and mRNA expression of $\alpha_3\beta_1$ integrin. Immunohistochemical analysis further confirmed that renal expression of $\alpha_3\beta_1$ was reduced in diabetic rats, which was partially restored by NR1. These results indicated that NR1 up-regulated the expression of $\alpha_3\beta_1$ integrin in podocytes, thus maintaining the adhesion of podocytes to GBM. Taken together, the regulatory effects of NR1 on $\alpha_3\beta_1$ integrin are likely to be accountable for its action of protecting against podocyte detachment.

Moreover, the effect of NR1 on ILK activity in diabetic rats was evaluated. ILK, a recently identified integrin cytoplasmic-binding protein, plays a crucial role in cell adhesion. ILK expression has been reported to be dramatically increased in diabetic glomeruli [30]. In this study, glomerular ILK activity was increased in STZ-induced diabetic rats. Our previous study has shown that HG elevates ILK activity in cultured mouse podocytes [11]. These findings indicated that ILK activation caused impaired cell-matrix adhesion in podocytes under diabetic condition. Several studies have demonstrated a close relationship between the integrin β_1 -ILK cascade dysregulation and diabetic nephropathy in human and experimental diabetic animal models [18, 30, 31]. This study demonstrated that NR1 significantly inhibited the ILK activation in isolated glomeruli from diabetic kidneys. Thus, the regulatory effects of NR1 on integrin-ILK system are likely to be accountable for its protective effects on podocyte adhesion.

We next investigated the effects of NR1 on oxidative stress markers in diabetic rats. It has been reported that high glucose increases reactive oxygen species (ROS) generation in podocytes [32]. There was mounting evidence demonstrating that ROS induced podocyte injury and proteinuria under a variety of circumstances [33]. Previous study demonstrated that glucose-induced ROS caused podocyte depletion at the onset of DKD [34]. Furthermore, downregulation of $\alpha_3\beta_1$ integrin and alterations in cell-matrix adhesion in podocytes might depend on extracellular ROS [35]. Treatment of animals with phosphatidylcholine-bound superoxide dismutase decreased proteinuria and preserved the α_3 integrin expression in podocytes [36]. Thus, hyperglycemia-induced ROS might reduce $\alpha_3\beta_1$ integrin expression and ultimately result in podocyte detachment from GBM. Consistent with previous reports [37, 38], the present study showed increased MDA content and decreased activities of CAT and SOD and GPx in STZ-induced diabetic rats. More recently, NR1 has been found to have antioxidant effects both *in vivo* and *in vitro* [39, 40]. In this study, the serum and tissue activities of antioxidant enzymes (CAT, SOD and GPx) were significantly increased and the MDA content decreased in NR1-treated rats, suggesting a significant antioxidant effect of NR1. Moreover, NR1 up-regulated $\alpha_3\beta_1$ integrin expression, as well as attenuated impaired podocyte adhesion, albuminuria and renal histopathology in diabetic rats. Thus, the protective effect of NR1 on podocyte adhesive capacity and its regulatory effect on integrin-ILK system may be associated with its antioxidant action.

Oxidative stress played an important role in the pathogenesis of podocyte apoptosis and depletion at the onset of diabetic nephropathy [34]. It has been reported that NR1 has antioxidant effects both *in vivo* and *in vitro* [39, 40]. In this study, we also demonstrated that NR1 reduced oxidative stress both in serum and kidney cortex from STZ-induced diabetic rats. Thus, we reasoned that the antioxidant NR1 might have protective effect on podocyte apoptosis under hyperglycaemic conditions and this antiapoptotic effect needs to be investigated in the further study.

In our preliminary study in rats, we chose the doses of AS-IV at 2.5, 5, 10 and 20 mg·kg⁻¹·d⁻¹ and we found that these doses (5 and 10 mg·kg⁻¹·d⁻¹) could effectively reduce proteinuria and did not cause any toxicity. So these doses were chosen in our study.

There are limitations in this study. First, we only selected two doses of NR1 in this study, and the dose-dependent effects of NR1 should be examined in the further study. Second, we did not measure the compound in the blood of the animals and this research should be performed in the further study. Third, we cannot be certain that the results from the rodent model can be translated to humans. Therefore, the applicability of NR1 in DKD needs to be further investigated in clinical trial.

In conclusion, this study clearly demonstrated that NR1, a novel antioxidant, ameliorated podocyte adhesion and subsequent podocyte depopulation under diabetic condition partly through $\alpha_3\beta_1$ integrin upregulation *in vitro* and *in vivo*. These findings might provide a potential new therapeutic option for treatment of DKD and other renal diseases affecting podocytes.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (81000305 to Dingkun Gui and 81270824 to Niansong Wang) and Traditional Chinese Medicine Foundation of Shanghai Municipal Commission of Health and Family Planning (2014JP018A to Dingkun Gui). This work was funded by Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (PWZxq2014 - 07 to Li Wei) and Joint Research Project of Pudong Health Bureau of Shanghai (PW2013D-5 to Dingkun Gui). This work was also supported by grant from Outstanding Young Talents Program in East campus, Shanghai Jiao Tong University Affiliated Sixth People's Hospital to Dingkun Gui.

Disclosure Statement

The authors have declared that no competing interests exist.

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