Original Paper

VDR Activation Reduces Proteinuria and High-Glucose-Induced Injury of Kidneys and Podocytes by Regulating Wnt Signaling Pathway

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Key Words

Diabetic nephropathy • Proteinuria • Podocytes • Vitamin D receptor • Paricalcitol • Wnt signaling pathway

Abstract

Background: Diabetic nephropathy (DN) is a major cause of end-stage renal disease and proteinuria is one of the most prominent clinical manifestations. The expression of Vitamin D receptor (VDR) in patients with chronic kidney diseases was decreased, while VDR agonists could partially alleviate the proteinuria of DN in animal models. The present study was designed to determine the expression of VDR in renal tissues and its relationship with proteinuria the diabetic model db/db mice. **Methods:** The regulation effects of VDR on the Wnt signaling pathway were analyzed using RNA interference and VDR agonist paricalcitol. **Results:** With the increase in age of the db/db mice, the VDR protein and mRNA levels in renal tissues were decreased, proteinuria increased, and the protein and mRNA levels of GSK-3 β and β -catenin increased. Paricalcitol treatment resulted in the up-regulation of VDR and down-regulation of GSK-3 β and β -catenin, indicating that VDR had a regulatory effect on the Wnt signaling pathway. **Conclusion:** VDR activation could reduce proteinuria of DN mice and alleviate high-glucose-induced injury of kidneys and podocytes by regulating the key molecules of Wnt signaling pathway.

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Published by S. Karger AG, Basel

Introduction

Diabetes poses a major public health problem worldwide, with ever-increasing incidence and prevalence in recent years. Diabetic nephropathy (DN) is one of the most

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common microvascular complications of patients with diabetes, which is characterized by structural and functional changes in the kidneys, leading to end-stage renal disease (ESRD). The major clinical manifestations of DN are proteinuria, hematuria, and progressive chronic kidney disease [1, 2]. Proteinuria is one of the most common clinical manifestations of DN and closely correlates with mesenchymal cell trans-differentiation and barrier functional injury. Podocytes are highly differentiated epithelial cells located in the glomerular basement membrane, forming the outermost layers of the glomerular filtration. There is a relationship between podocyte injury and the occurrence and progression of albuminuria. Proteinuria can cause DN deterioration, developing into ESRD. Therefore, DN is regarded as a “podocyte cell disease” [3, 4]. We and others have found that high glucose stimuli can cause podocyte cells epithelial to mesenchymal cell transition (EMT) [5, 6].

Vitamin D receptor (VDR) belongs to the superfamily of nuclear hormone receptors, which encompasses receptors for steroid and thyroid hormones and retinoic acid [7]. It has been well documented that $1, 25(\text{OH})_2\text{D}_3$ mediates the VDR's biological effects by binding to it and then recruiting cofactors to form a transcriptional complex that binds to vitamin D response elements (VDRE) in the promoter region of target genes to alter transcriptional events within the target cells [8, 9]. Recent studies have shown that VDR-mediated signal transduction pathways play a critical role in the development of DN, such as inflammatory response [10], immune regulation, reduction of proteinuria, inhibition of the Ras system [9, 11], and prevention of fibrosis [12]. VDR can prevent the progression of diabetes by promoting islet cell repair and reducing insulin resistance and glucose tolerance [13].

Paricalcitol (19-nor-1, 25-hydroxy-vitamin D₂), a synthetic vitamin D analog, can reduce urinary albumin in type 2 DM patients [9, 13-15]. As a vitamin D receptor agonist, it has been used for the treatment of hyperparathyroidism. Several studies have indicated that paricalcitol has renoprotective effects, including anti-inflammatory and anti-fibrotic effects in various nephropathy models [17]. However, the mechanism of action for this drug has not been fully elucidated. The Wnt/ β -catenin pathway is now recognized as one of the most important signaling pathways involved in EMT [16] and GSK-3 β plays an important role in the regulation of the β -catenin pathway [17]. We have recently reported that VDR, GSK-3 β , β -catenin are co-localized in podocytes under high glucose conditions [18]. However, the relationships among VDR, GSK-3 β , and β -catenin remain unclear. The present study was designed to investigate the renoprotective effects of paricalcitol on mouse podocytes and reduction of proteinuria in DN mouse models and to explore the Wnt/GSK-3 β / β -catenin signaling pathway in its renoprotective mechanisms.

Materials and Methods

All the experimental procedures were performed in accordance with guidelines and regulations of the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

siRNA

Based on the sequence of mouse full-length GSK-3 β gene (GenBank NO. NM 019827.6), the siRNA against GSK-3 β was designed as 5'-CCA CTC AAG AAC TGT CAA GTA-3' and the scramble siRNA control was 5'-UUC UCC GAA CGU GUC ACG UTT-3'. The GSK-3 β and scramble siRNAs were transfected into the differentiated podocytes in the presence of Lipofectamine 2000 (Invitrogen, San Diego, CA, USA).

Cell culture and treatments

Undifferentiated mouse podocytes were provided by Prof. Peter Mundel (Mount Sinai Medical School, New York, NY, USA) and were cultured in RPMI1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 5.6mmol/L of glucose, and 10U/mL of recombinant mice interferon- γ , in an incubator at 33°C with 5% CO₂. After differentiation, the podocytes were cultured at 37°C in RPMI1640 medium without interferon- γ . The podocytes were cultured for 12-14 days for the subsequent experiments. For the siRNA experiments, the podocytes were incubated in RPMI-1640 medium with normal

glucose and divided into three groups: (1) normal control group (NG, 5.6 mmol/L); (2) scramble siRNA control group (scrsiRNA): treated with 100pmol/L of scramble siRNA; and (3) VDRsiRNA group (vdrsiRNA): treated with 100pmol/L of VDRsiRNA. For the study of the effects of paricalcitol, the podocytes were divided into six groups: (1) the normal control (5.6 mmol/L of glucose) group (NG); (2) the NG plus DMSO (0.1 μmol/L) group (NG+D); (3) the NG plus paricalcitol (0.1 μmol/L) group (NG+P); (4) the high glucose (25 mmol/L of glucose) group (25G); (5) the HG plus DMSO (0.1 μmol/L) group (25G+D); and (6) the HG plus paricalcitol (0.1 μmol/L) group (25G+P). For the study of the role of GSK-3β, the podocytes were divided into six groups: (1) normal glucose (5.6 mmol/L of glucose) group (NG); (2) NG plus 100 pmol/L of scramble siRNA group (scramble); (3) NG plus 100 pmol/L of GSK-3β siRNA group (GSKsiRNA); (4) NG plus Paricalcitol (0.1 μmol/L) group (PAR); (5)

Paricalcitol (0.1 μmol/L) plus 100pmol/L of scramble siRNA group (PAR+scramble), and (6) Paricalcitol (0.1 μmol/L) plus 100pmol/L of GSK-3β siRNA group (PAR+ GSKsiRNA).

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen Inc.), according to the manufacturer's instructions. The RNA purity was determined using absorbance at 260 and 280 nm (A260/280) and the cDNA was synthesized using 2 μg of RNA and the First StcDNA Synthesis Kit (Fermentas, Burlington, Canada) using the M-MuLV Reverse Transcriptase and random primers. The sequences of specific primers used in the present study are shown in Table 1. The cDNA (1 μL) was mixed with the forward and reverse primers (0.5 μL each), 2×PCR MasterMix (12.5 μL; Life Technologies Co., Grand Island, NY, USA) and deionized water (in total volume of 25 μL); the reaction was performed in a thermocycler (BD Diagnostics, Sparks, MD, USA) under the conditions of cycle shown in Table 2. After completion of the reaction to confirm the amplification curve and melting curve, the CT values of the corresponding index was calculated as $\Delta\Delta CT$ by the following formula: $\Delta\Delta CT = (CtA2 - CtB2) - (CtA1 - CtB1)$. Wherein A represents the target gene Ct values, B for reference gene Ct values, 1 and 2 represent different treatment groups. The experiment was repeated three times, and the average values were reported. The results calculated by the target gene treatment group with 2- $\Delta\Delta CT$ times higher than the target gene were expressed. The PCR products were separated by 2.0% agarose gel electrophoresis. The Image J image software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the grayscale values.

Western blotting analysis

The total cellular proteins were obtained from each group using the RIPA lysis buffer (Dingguo Changsheng Biotech Co., Ltd., Beijing, China). Proteins were quantified using a BCA protein assay kit (Dingguo Changsheng Biotech Co., Ltd.). The samples with equal amount of total proteins (20 μg) were separated using 10% SDS-PAGE and were transferred on PVDF membranes (Roche, Mannheim, Germany). The membranes were blocked using 5% powdered milk diluted in TBS containing 0.05% Tween 20 (TBST) for 1h. The primary

Table 1. The sequences of primers used in the present study

Name	Sequences
VDR	Upstream: 5'-TCCTTCCTCTGCTGGTAT-3' Downstream: 5'-CTCCTTGTTAGTGTGGTAG-3'
nephrin	Upstream: 5'-ACTGGAGGAATGTAGGTAATG-3' Downstream: 5'-TGTGTTCTTGCTTCTGTGA-3'
podocin	Upstream: 5'-AGAAGAGGAGAAGGAGTT-3' Downstream: 5'-TTGGAGTTGAATGGTGT-3'
GSK-3β	Upstream: 5'-CCACCATCACCATTAAAGAGTT-3' Downstream: 5'-CGTAATCCTTCAATCCACCTT-3'
β-catenin	Upstream: 5'-ATGGAGGAGATAGTAGAA-3' Downstream: 5'-AATGGAATGGTATTGAGT-3'
α-SMA	Upstream: 5'-AACTGTGAATGTCCTGTG-3' Downstream: 5'-CATAGGTAACGAGTCAGAG-3'
MMP9	Upstream: 5'-ACACGACATCTTCCAGTA-3' Downstream: 5'-CACCTTGTTCACCTCATT3'
GAPDH	Upstream: 5'-AGTGGCAAAGTGGAGATT 3' Downstream: 5'-GTGGAGTCATACTGGAACA-3'

Table 2. Conditions of qRT-PCR

Step	Cycle	Temperature	Time
Pre denaturation	1	95°C	5min
Cycle of reaction	45		
denaturation		95°C	30sec
annealing		60°C	30 sec
extension		72°C	60 sec
melt curve	1		
		95°C	15 sec
		60°C	60 sec
		95°C	15 sec

antibody was incubated overnight at 4°C. After washing with PBS, the secondary antibody was incubated at 37°C for 2h. An ECL chemiluminescence reagent kit (CoWin Biotech, Beijing, China) was used to view the protein bands. The blots were analyzed using the ImageJ software. The antibodies used

Table 3. The antibodies used in the present study

Protein	Antibody	Vendor	Dilution information
GAPDH	mouse anti mouse	Shanghai Kang Cheng biological engineering Co., Ltd, China	1:1000
VDR	mouse anti mouse	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:1000
β-catenin	mouse anti mouse	Abcam, Cambridge,UK	1:1000
GSK-3β	mouse anti mouse	Abcam, Cambridge,UK	1 ab3239 1:2000
α-SMA	mouse anti mouse	Abcam, Cambridge,UK	, ab 7817 1:2000
nephrin,	rabbit anti mouse	Abcam, Cambridge,UK	, ab 58968, 1:1000
podocin,	rabbit anti mouse	Abcam, Cambridge,UK	, ab 50993, 1:1000
MMP9	rabbit anti mouse	Abcam, Cambridge,UK	1:1000

in the present study are shown in Table 3. And the secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG (1:2000) was obtained from Dingguo Changsheng Biotech Co., Ltd. (Beijing, China).

Animals and treatments

All the animal experimental procedures were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The C57BL/KsJ db/db, six-week, male mice and age-matched db/m mice were purchased from Nanjing Center for Experimental Animals (Nanjing, China). The mice were housed at 25°C and 50–60% relative humidity, in a 12h/12h light/dark cycle, with free access to food and water. The mice were randomly assigned to four groups (20 mice per group): 1) db/m normal control group (group C, untreated), 2) db/db normal control group (group N, untreated), 3) db/db vehicle-treated group (group D, 0.3μg/kg, by intraperitoneal injection, i. P.), and 4) db/db paricalcitol-treated group (group P, equal amounts of dimethylsulfoxide, i.p.). The treatments began at the age of 10 weeks and ended at 22 weeks old; all the treatments were performed three times a week (Days 1, 3, and 5 each week). Some of these mice were sacrificed at Weeks 10, 14, 18, and 22, respectively. All the mice were killed under 10% chloral hydrate anesthesia and the kidneys were then isolated.

Measurements of renal function, blood glucose, and serum lipids

For 24-h complete urine collection, the mice were placed individually in metabolic cages with free access to food and water. Urine albumin and creatinine were measured by the Kidney Lab of First Affiliated Hospital of Zhengzhou University. The blood samples were collected from the tail veins after fasting for 8 h (during experiments), and by cardiac puncture (at the end of experiments). Serum levels of glucose (GLU), creatinine, urea nitrogen, uric acid, triglyceride (TG), cholesterol (TC), glycated serum protein (GSP) and albumin/creatinine ratios were all assayed using an Automatic Biochemistry Analyzer CS-400 (Changchun Dirui Medical Technology Co Ltd, Changchun, China).

Histology and immunofluorescence

The removed kidneys were weighed and the cortices were then separated. The left cortices were snap-frozen for Western blotting and real-time PCR analyses, and the right cortices were used for pathological analyses. The renal tissues were fixed in Bouin's solution, embedded in paraffin, cut into 5-μm sections, and stained with periodic acid-Schiff (PAS) and H&E staining. For immunofluorescence analysis, the renal cortex of 1 mm × 1 mm × 1 mm was fixed in 2.5% glutaraldehyde and the remaining kidney tissues were fixed in 4% paraformaldehyde.

The podocytes were stimulated by 25 mmol/Lof glucose for 36h, and fixed in 4°C 4% paraformaldehyde for 20 min and 0.6% Tween-20 for 15min, and then blocked with 5% BSA blocking solution for 20 min at room temperature. The cells were exposed to antibodies against VDR, Nephrin, Podocin, α-SMA, and MMP9 overnight. The cells were washed three times with PBS, and then incubated with the Alexa Fluor 488 goat anti-rabbit IgG (H+L) (green) and the Alexa Fluor 594 donkey anti-mouse IgG (H+L) (red) (Invitrogen Inc., Carlsbad, CA, USA) at 37°C for 1 h. The fluorescence was observed under a fluorescence microscope (Olympus, CX31-32RFL, Japan).

Statistical analysis

The data are expressed as means $\pm$ SD. SPSS 17.0 software (Chicago, IL, USA) was used for the statistical analysis. Two-way analysis of variance (ANOVA) was used for the multi-group comparisons, and Least Significant Difference (LSD) Test was performed for pair-wise comparison. $P < 0.05$ was considered statistically significant.

Results

Effects of VDR expression level on gene expression and barrier function of podocytes

In order to determine the relationship between the VDR expression and phenotype and barrier function of podocytes, we used a siRNA to knock down the expression of VDR. As shown in Fig. 1, Western blotting analyses demonstrated that, compared with control groups, VDR siRNA resulted in the down-regulation of the protein levels of VDR, nephrin, and podocin ($P < 0.05$) and the up-regulation of the protein levels of α -SMA and MMP9 ($P < 0.05$). The results of qRT-PCR analyses indicated that (Fig. 2), the mRNA expression levels of VDR, nephrin, and podocin were all down-regulated, compared with the control groups ($P < 0.05$) and the mRNA expression levels of α -SMA and MMP9 were up-regulated ($P < 0.05$). The results of the albumin flow indicated that VDR siRNA treatment resulted in an increase in the albumin flow of the podocytes ($P < 0.05$).

Effects of paricalcitol on gene expression of the Wnt signaling pathway

Our previous experimental results have found that, under high glucose conditions, the expression of GSK-3 β and β -catenin is increased, indicating that the activity of Wnt signaling pathway is increased. In the present study, Western blotting analyses indicated that, compared with the NG group, the expressions of α -SMA, MMP9, GSK-3 β , and β -catenin

Fig. 1. Western blotting analysis for protein expression after VDR siRNA knock out. *: compared with NG group and scr siRNA group, $p < 0.05$ for VDR; #: compared with NG group and scr siRNA group, $p < 0.05$ for GSK-3 β ; &: compared with NG group and scr siRNA group, $p < 0.05$ for β -catenin; α : compared with NG group and scr siRNA group, $p < 0.05$ for podocin; β : compared with NG group and scr siRNA group, $p < 0.05$ for nephrin; γ : compared with NG group and scr siRNA group, $p < 0.05$ for α -SMA; δ : compared with NG group and scr siRNA group, $p < 0.05$ for MMP9.

Fig. 2. qRT-PCR analysis for gene expression after VDR siRNA knock out.*: compared with NG group and scr siRNA group, $p < 0.05$ for VDR; #: compared with NG group and scr siRNA group, $p < 0.05$ for GSK-3β; &: compared with NG group and scr siRNA group, $p < 0.05$ for β-catenin; α: compared with NG group and scr siRNA group, $p < 0.05$ for podocin; β: compared with NG group and scr siRNA group, $p < 0.05$ for nephrin; γ: compared with NG group and scr siRNA group, $p < 0.05$ for α-SMA; δ: compared with NG group and scr siRNA group, $p < 0.05$ for MMP9.

Fig. 3. Western blotting analysis for the effects of paricalcitol on protein expression.*: compared with NG group, $p < 0.05$ for VDR; #: compared with NG group, $p < 0.05$ for GSK-3β; &: compared with NG group, $p < 0.05$ for β-catenin; α: compared with NG group, $p < 0.05$ for podocin; β: compared with NG group, $p < 0.05$ for nephrin; γ: compared with NG group, $p < 0.05$ for α-SMA; δ: compared with NG group, $p < 0.05$ for MMP9.*p: compared with 25G group, $p < 0.05$ for VDR; #p: compared with 25G group, $p < 0.05$ for GSK-3β; &p: compared with 25G group, $p < 0.05$ for β-catenin; αp: compared with 25G group, $p < 0.05$ for podocin; βp: compared with 25G group, $p < 0.05$ for nephrin; γp: compared with 25G group, $p < 0.05$ for α-SMA; δp: compared with 25G group, $p < 0.05$ for MMP9.

were up-regulated and the levels of VDR, nephrin, and podocin were decreased in the high glucose group, which was reversed by paricalcitol (Fig. 3). The qRT-PCR results indicated that, compared with the NG group, the mRNA expressions of α-SMA, MMP9, GSK-3β and β-catenin were up-regulated and the expression levels of VDR, nephrin, and podocin were decreased in the high glucose group, which was reversed by paricalcitol treatment (Fig. 4).

Effects of GSK-3β siRNA on gene expressions of the Wnt signaling pathway

We next determined the influence of VDR on β-catenin under the condition of siRNA knockdown of GSK-3β. The results of Western blotting analyses indicated that, compared with control groups, GSK-3β siRNA resulted in the up-regulation of VDR, nephrin, and podocin ($P < 0.05$) and the down-regulation of α-SMA, MMP9, GSK-3β, and β-catenin ($P < 0.05$, Fig. 5), which was not affected by paricalcitol treatment. The qRT-PCR analyses indicated that, compared with control groups, GSK-3β siRNA treatment resulted in the increased mRNA expression of VDR, nephrin, and podocin and the decreased mRNA levels of α-SMA, MMP9, GSK-3β, and β-catenin ($P < 0.05$), which was not affected by paricalcitol treatment (Fig. 6).

Fig. 4. qRT-PCR analysis for the effects of paricalcitol on gene expressions. *: compared with NG group, $p < 0.05$ for VDR; #: compared with NG group, $p < 0.05$ for GSK-3 β ; &: compared with NG group, $p < 0.05$ for β -catenin; α : compared with NG group, $p < 0.05$ for podcin; β : compared with NG group, $p < 0.05$ for nephrin; γ : compared with NG group, $p < 0.05$ for α -SMA; δ : compared with NG group, $p < 0.05$ for MMP9. *p: compared with 25G group, $p < 0.05$ for VDR; #p: compared with 25G group, $p < 0.05$ for GSK-3 β ; &p: compared with 25G group, $p < 0.05$ for β -catenin; α p: compared with 25G group, $p < 0.05$ for podcin; β p: compared with 25G group, $p < 0.05$ for nephrin; γ p: compared with 25G group, $p < 0.05$ for α -SMA; δ p: compared with 25G group, $p < 0.05$ for MMP9.

Fig. 5. Western blotting analysis for the role of GSK-3 β in protein expression. *: compared with NG group, $p < 0.05$ for VDR; #: compared with NG group, $p < 0.05$ for GSK-3 β ; &: compared with NG group, $p < 0.05$ for β -catenin; α : compared with NG group, $p < 0.05$ for podcin; β : compared with NG group, $p < 0.05$ for nephrin; γ : compared with NG group, $p < 0.05$ for α -SMA; δ : compared with NG group, $p < 0.05$ for MMP9.

Effects of paricalcitol on proteinuria and kidney injury in DN mice

We next investigated whether paricalcitol was effective in ameliorating proteinuria in mice. The body weight, absolute body fat, food intake, water intake, and urine volume of db/db mice were consistently increased with age, compared with the normal control mice (Table 4). The 6-week old db/db diabetic mice also exhibited significantly higher blood glucose levels, which was consistently increased throughout the study period, compared with the normal control animals (Table 5). Early renoprotection effects of paricalcitol were examined by measuring the 24-h urinary albumin excretion (UAE) in the db/db mice, compared with the controls. We treated the db/db mice with paricalcitol starting at 10 weeks old and demonstrated that paricalcitol significantly ameliorated urinary albumin excretion in db/db mice after only four weeks of treatment (Table 6). The 24-h urine protein levels of Scr and BUN were increased significantly in group D and group N compared with group C, while decreased in group P compared with groups D and N (Table 6). This reduction of urinary albumin was maintained throughout the duration of the study. Collectively, the above observations revealed that an early stage of DN occurred at 6-week old. In comparison with groups D and N, the treatment did not affect serum levels of triglycerides, cholesterol, glucose, and glycated

Fig. 6. qRT-PCR analysis for the role of GSK-3 β in gene expression. *: compared with NG group, $p < 0.05$ for VDR; #: compared with NG group, $p < 0.05$ for GSK-3 β ; &: compared with NG group, $p < 0.05$ for β -catenin; α : compared with NG group, $p < 0.05$ for podocin; β : compared with NG group, $p < 0.05$ for nephrin; γ : compared with NG group, $p < 0.05$ for α -SMA; δ : compared with NG group, $p < 0.05$ for MMP9.

serum proteins in group P. Additionally, paricalcitol did not affect body weight gain.

We further examined the effects of paricalcitol on the expression of VDR in renal tissues of the db/db mice and found that with the increase of week-age of db/db mice, the expression of VDR was gradually decreased, which was reversed by paricalcitol (Figures 7 and 8). With the increase of week-age of db/db mice, the expression of GSK-3 β and its downstream molecule β -catenin, the key molecules in Wnt signaling pathway, were gradually increased, which was reversed by paricalcitol (Fig. 9). In kidney tissues of the db/db mice, with the increase of week-age, the expressions of nephrin and podocin, the marker proteins of podocyte, were gradually decreased, and

the expressions of α -SMA and MMP9, the marker proteins of mesenchymal, were gradually increased, which were reversed by paricalcitol (Figures 10 and 11). Under the electron microscope, glomerular basement membrane in the renal tissues of db/db mice and db/db-D mice showed irregular thickening, had not deposited electron dense but with visible

Table 4. Comparison of body weights (g) from different groups at different time points, *compare with Group C at same week time, $P < 0.05$ ($n = 5$)

	6week	10 week	14 week	18 week	22 week
Group C	20.0 $\pm$ 1.86	21.58 $\pm$ 1.42	23.03 $\pm$ 2.05	24.3 $\pm$ 1.34	25.8 $\pm$ 2.05
Group N	34.36 $\pm$ 2.23*	46.11 $\pm$ 2.15*	54.6 $\pm$ 2.32*	59.6 $\pm$ 1.78*	60.4 $\pm$ 2.31*
Group D	34.67 $\pm$ 2.11*	47.21 $\pm$ 2.45*	55.67 $\pm$ 3.21*	60.4 $\pm$ 2.45*	61.50 $\pm$ 2.75*
Group P	35.01 $\pm$ 1.78*	46.21 $\pm$ 2.40*	55.07 $\pm$ 1.68*	58.5 $\pm$ 2.31*	60.7 $\pm$ 1.87*

Table 5. Comparison of blood glucose levels (mmol/L) among different groups at different time points, *compare with Group C at same week time, $P < 0.05$ ($n = 5$)

	6 week	10 week	14 week	18 week	22 week
Group C	7.96 $\pm$ 1.56	8.17 $\pm$ 1.92	8.24 $\pm$ 1.87	8.89 $\pm$ 1.23	8.88 $\pm$ 1.56
Group N	15.62 $\pm$ 2.10*	18.34 $\pm$ 1.50*	18.22 $\pm$ 1.70*	25.60 $\pm$ 2.23*	22.67 $\pm$ 2.56*
Group D	16.66 $\pm$ 2.01*	18.79 $\pm$ 1.8*	18.48 $\pm$ 1.2*	24.30 $\pm$ 3.4*	26.8 $\pm$ 3.8*
Group P	16.22 $\pm$ 1.5*	18.34 $\pm$ 1.1*	18.05 $\pm$ 1.7*	24.7 $\pm$ 2.6*	26.3 $\pm$ 2.9*

Table 6. Comparison of 24-h urinary albumin excretion (mg/L) among different groups at different time points, *: compare with Group C at same week time, $P < 0.05$; Δ : compare with Group N at same week time, $P < 0.05$; #: compare with Group D at same week time, $P < 0.05$ ($n = 5$)

	6week	10 week	14 week	18 week	22 week
Group C	1.21 $\pm$ 0.57	2.23 $\pm$ 0.68	2.01 $\pm$ 0.21	2.56 $\pm$ 0.78	2.07 $\pm$ 0.45
Group N	5.3 $\pm$ 2.71*	12.64 $\pm$ 2.2*	14.32 $\pm$ 1.44*	16.56 $\pm$ 3.34*	18.9 $\pm$ 2.67*
Group D	5.41 $\pm$ 2.21*	13.43 $\pm$ 3.11*	13.89 $\pm$ 2.67*	17.23 $\pm$ 2.45*	18.32 $\pm$ 3.12*
Group P	6.1 $\pm$ 2.34*	12.9 $\pm$ 1.08*	11.8 $\pm$ 1.77* Δ #	9.34 $\pm$ 1.22* Δ #	8.67 $\pm$ 1.56* Δ

Fig. 7. Western blotting analysis of the expression of VDR in kidney tissues from mice with different ages and different treatments. A, B: different week-old mice kidney tissue VDR Western Blotting results and analysis; C: Different Diets mouse kidney organization VDR qRT-PCR analysis; D, E: 22 weeks of different treated mice kidney tissue VDR Western Blotting results and analysis, F: 22 weeks of different treated mice kidney tissue VDR qRT-PCR analysis. (Note: B, C in *: Compared with db / m $P < 0.05$; #: and 10w db/db compared $P < 0.05$; Δ: with 14w db/db compared $P < 0.05$; π: and 18w db/db compared to $P < 0.05$. E, F * in: the db / m compared to $P < 0.05$; #: P compared with db/db group and db/db-D group < 0.05).

foot process fusion; paricalcitol treatment improved the morphology: glomerular basement membrane was homogeneous smooth without thickening and foot process fusion was no visible. Paricalcitol alleviated the foot process fusion of the podocytes in the db/db mice (Fig. 12).

We also examined whether this protection was associated with renal function in the db/db mice. A decline in glomerular filtration rate (GFR) was observed in mice of 17-weeks or older; as a result, an elevation in serum creatinine (SCR) was frequently reported at 16 weeks or older (Table 7). Paricalcitol significantly reduced the SCR levels in the db/db mice, indicating an improvement in the renal function.

Discussion

Vitamin D and VDR could provide possible renoprotection against DN[12]. More fibronectin and less nephrin are expressed in the VDR knockout mice compared to diabetic wild-type mice. In the present study, down-regulation of VDR by siRNA decreased the expression of nephrin and podocin and increased the expression of α -SMA and MMP9.

Fig. 8. Immunofluorescence analysis for the expression of VDR in kidney tissues from mice at different ages and with different treatment.

Fig. 9. Comparison of the expression of GSK-3 β and β -catenin in kidney tissues of the mice at different ages and with different treatments. A, B: Western Blot results of GSK-3 β and β -catenin of db/db mice in different ages; C: qRT-PCR results of GSK-3 β and β -catenin of db/db mice in different ages; D, E: Western Blot results of GSK-3 β and β -catenin of mice in different treatment groups on the 22-week; F: qRT-PCR results of GSK-3 β and β -catenin of mice in different treatment groups on the 22-week (Note: in B and C: *: compared with db / m $P < 0.05$; #: and 10w db/db phase ratio $P < 0.05$; Δ : with 14w db/db compared to $P < 0.05$; π : and 18w db/db compared to $P < 0.05$. in E and F: *: the db / m compared to $P < 0.05$; #: and db/db group and db/db-D group compared to $P < 0.05$).

Fig. 10. Comparison of expressions of nephrin, podocin, α -SMA, and MMP9 of db/db mice at different ages. A: Western Blot results of nephrin, podocin, α -SMA and MMP9 of db/db mice in different ages; B: Western Blot results of nephrin and podocin; C: qRT-PCR results of nephrin and podocin; D: Western Blot results of α -SMA and MMP9; E: qRT-PCR results of α -SMA and MMP9. (Note: *: and db / m compared to $P < 0.05$; #: and 10w db/db compared to $P < 0.05$; Δ : Compared with 14w db/db $P < 0.05$; π : Compared with 18w db/db $P < 0.05$).

The Wnt signaling pathway plays an important role in the regulation of differentiation of cellular morphology and function and β -catenin is a multifunctional protein and is a key factor involved in classic Wnt signaling. Under normal physiological conditions, the Wnt signaling pathway in kidney is “static”, axin, APC, and GSK-3 β combine with β -catenin, making β -catenin phosphorylated [19-21]. In our previous experiment, the expressions of GSK-3 β

Fig. 11. Comparison of expression of nephrin, podocin, α -SMA and MMP9 of mice with different treatments. A: Western Blot results of nephrin/podocin, α -SMA and MMP9 of mice indifferent treatment groups; B: Western Blot results of nephrin and podocin; C: qRT-PCR results of nephrin and podocin; D: Western Blot results of α -SMA and MMP9; E: qRT-PCR results of α -SMA and MMP9. (Note: *: compared with db/m $P < 0.05$; #: P compared with db/db group and db/db-D Group < 0.05).

Fig. 12. TEM analysis of the renal tissues of db/db mice and db/db-D mice. Glomerular basement membrane showed irregular thickening, had not deposited electron dense and the foot process fusion was visible; compared with db/db mice, the lesions of renal tissue in db/db-P mouse was improved. Glomerular basement membrane in renal tissue of db/m mice was homogeneous smooth without thickening, and foot process fusion was no visible.

and β -catenin were increased under the high glucose condition, and consequently the expression of mesenchymal marker proteins were increased, and barrier function of podocytes was destroyed. And the expression of VDR and β -catenin were co-localized in the nuclei. The expression of VDR was decreased after using GSK-3 β inhibitor to interfere the expression of GSK-3 β in the renal tissues of db/db

Table 7. Comparison of serum creatinine ($\mu\text{mol/L}$) among different groups at different time points, *: compare with Group C at same week time, $P < 0.05$; Δ : compare with Group N at same week time, $P < 0.05$; #: compare with Group D at same week time, $P < 0.05$ ($n = 5$)

	6 week	10 week	14 week	18 week	22 week
Group C	26.71 $\pm$ 3.14	26.88 $\pm$ 2.87	28.32 $\pm$ 2.22	28.12 $\pm$ 2.34	28.69 $\pm$ 3.45
Group N	27.12 $\pm$ 3.03	40.77 $\pm$ 2.12*	48.77 $\pm$ 3.56*	51.89 $\pm$ 2.79*	55.67 $\pm$ 3.27*
Group D	27.32 $\pm$ 2.73	40.11 $\pm$ 3.21*	48.44 $\pm$ 4.32*	52.49 $\pm$ 3.89*	56.05 $\pm$ 2.33*
Group P	27.45 $\pm$ 1.67	41.56 $\pm$ 4.15*	42.7 $\pm$ 3.67* Δ [#]	37.67 $\pm$ 4.15* Δ [#]	35.4 $\pm$ 4.05* Δ [#]

mice, but the interaction of VDR with GSK-3 β and β -catenin remains unclear. The present study showed that with down-regulation of GSK-3 β expression, the expression of β -catenin was decreased and the expression of VDR increased.

In the present study, the db/db mice at six weeks of age exhibited hyperglycemia and albuminuria. With increasing age, the disease progressed and kidney function deteriorated further, resulting in a significant rise in albuminuria and renal fibrosis. Though there were some studies about the roles of some potential chemicals or drugs on diabetic kidney injury, our study showed the new role and mechanism of VDR agonist [22, 23]. The results from the present study demonstrated that paricalcitol possessed an impressive renal protective efficacy in DN. Considering the pathologic sequences of this model, it is conceivable that the reno-protective effect of paricalcitol may be primarily attributable to its prevention of podocyte injury. This notion was further substantiated by the observations that paricalcitol prevented the loss of podocyte-specific nephrin, podocin. Interestingly, this anti-proteinuric action of this vitamin D analogs has also been reported in several clinical studies in patients with chronic renal insufficiency, as well as in other animal models of proteinuric kidney diseases [24]. Therefore, it is becoming clear that paricalcitol is quite effective in alleviating podocyte injury and proteinuria under different circumstances [25, 26].

Hyperglycemia leads to nephropathy by various mechanisms, such as increased endothelial cell permeability to albumin, hypertrophy, thickening of the basement membrane and renal fibrosis [27, 28]. In the present study, our data demonstrated that the expressions of GSK-3 β , β -catenin, α -SMA, and MMP9 were increased under high glucose condition, and these changes were attenuated by paricalcitol pretreatment. Additionally, paricalcitol could reverse the decrease in the levels of VDR, nephrin, and podocin caused by high glucose, further confirming its effects on prevention of renal injury in DN. These results may provide a basis for further rationale use of this drug in the clinic to treat and prevent DN.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 81400726, 81670663 and 81570690), the National Key Research and Development Program (No. 2016YFC1305404) and the key program of Joint Funds of the National Natural Science Foundation of China (No. U1604284).

Disclosure Statement

The authors have declared none conflict of interests.

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