

Vitamin D₃ Ameliorates High Glucose-Induced Podocyte Apoptosis

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Abstract: Objective: Diabetic kidney disease (DKD) is a severe kidney disease characterized by podocyte apoptosis, injury, and accumulation of extracellular matrices that ultimately lead to end-stage renal disease. Vitamin D₃ was reported to provide renal protection in DKD through the Vitamin D receptor (VDR), however there was limited data on whether it has a protective effect on podocytes of DKD induced by high glucose. **Methods:** The experimental subjects were conditionally immortalized mouse podocytes (MPC-5), which were divided into 4 groups: NG (normal glucose) group, HM (Hypertonic) group, HG (high glucose) group, VD₃ (HG +1,25-dihydroxyvitamin D₃ (1,25-D₃)) group. Western-blot analysis was performed to detect the expression of Bcl-2, Bax and Caspase-3 in podocytes. The mRNA Expression of Bcl-2, Bax and Caspase-3 in the three groups were detected by RT-PCR method. **Results:** Compared with the NG group, the expression of apoptosis proteins Caspase-3 and Bax in the podocytes of HG group increased, and the expression of Bcl-2 decreased. Pretreatment with vitamin D₃ attenuated these abnormalities in podocytes in HG group. **Conclusion:** Vitamin D can alleviate the apoptosis of podocytes induced by high glucose in vitro.

Keywords: Diabetic Nephropathy, Vitamin D₃, Podocyte, Apoptosis

Introduction

DKD is part of the most common chronic complications of diabetes mellitus, which can also lead to end-stage renal disease (ESRD) with clinical manifestation of progressively worsening albuminuria and a declining glomerular filtration rate¹. Podocytes are terminally differentiated glomerular visceral epithelial cells which are critical components of the glomerular filtration barrier to prevent loss of urinary protein². Several investigations have shown that in podocytes interfered with high glucose, Ca²⁺-dependent calcineurin was activated and reactive oxygen species (ROS) was increased, which stimulate TRPC6 to induce podocyte apoptosis through the RhoA/ROCK pathway^{3,4}. In addition, there is increasing evidence that podocyte apoptosis is an important determinant of proteinuria and glomerular sclerosis in diabetic patients^{5,6}. High glucose induces podocyte apoptosis through the activation and regulation of Bax, BCL2 and Caspase3⁷. Traditionally the vitamin D endocrine system was believed to play an important role in bone metabolism, calcium homeostasis and contribute to cell differentiation, cell growth inhibition and immune regulation⁸. Vitamin D₃ is absorbed into the gastrointestinal tract or synthesized by the effect of sunlight on the skin, and then it can be converted into 25-hydroxyvitamin D₃ in the liver, subsequently, and converted to 1,25-D₃ in the proximal tubule of the kidney⁹. 1,25-D₃ which has various physiological functions is an activated form of the endocrine

hormone vitamin D. A recent clinical trial shows that paricalcitol, an activated vitamin D analog, can reduce proteinuria in DKD patients¹⁰. 1,25-D₃ prevents puromycin aminonucleoside from apoptosis of glomerular podocytes by activating the Phosphatidylinositol 3-Kinase/Akt-Signaling Pathway¹¹. No previous literature reports that 1,25-D₃ could affect podocyte apoptosis in diabetic nephropathy. In this study, high glucose stimulates podocytes to mimic the environment of diabetic nephropathy. Then we study the expression of apoptotic proteins to evaluate vitamin D₃ improve the apoptosis of podocytes induced by high glucose or not, in order to provide new treatment idea for preventing DKD.

Materials and methods

Cell Culture

The conditionally immortalized mouse podocyte cell line (MPC5) were obtained from The Central Laboratory of Shandong University and grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 10-50 U/ml recombinant mouse γ -IFN, 100 U/ml penicillin and 100 μ g/ml streptomycin in a 5% CO₂ incubator at 33°C. And then the podocytes were changed to an incubator at 37 °C for growth (without γ -IFN). We divided the podocytes into 4 groups: NG group (5.5mmol/L), HM group (NG+mannitol 25 mmol/L), HG group (30mmol/L), VD₃ group (HG +1,25-D₃, 100nmol/L).

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Western blot analysis

Proteins were extracted from MPC5 using RIPA lysis buffer. The BCA kit (Solarbio, CHINA) was used to detect protein concentrations. Equal amounts of protein (30µg) were separated by SDS-PAGE on a 15% acrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking the PVDF membrane with blocking solution for 1 h at room temperature, it was incubated in a refrigerator at 4 ° C overnight with the following rabbit anti-mouse primary antibody: anti-Bax (Abcam, UK), anti-Bcl-2 (Abcam, UK), anti-Caspase-3 (Abcam, UK). The membrane was incubated with goat anti-rabbit IgG secondary antibody at room temperature after washing well. The antigens were visualized using the chemiluminescent gel imaging system (UVP, USA).

Real-Time PCR(RT-PCR) Analysis

Total RNA was extracted from podocytes using TRIZOL reagent (TaKaRa, Japan). RNA concentration and purity were detected by an ultraviolet spectrophotometer. We had use a reverse transcription kit (TaKaRa, Japan) to synthesize the cDNA. According to the manufacturer's protocol, Real-Time PCR was performed in an ABI PRISM 7000 HT (11744-100; Applied Biosystems, Invitrogen Life Technologies, USA) using SYBR Premix Ex Taq (TaKaRa). The relative gene expression comparisons were performed using the comparative CT method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Primer sequences targeting Bax, Bcl-2 and Caspase-3 are presented in table 1. The statistical results analyzed at least three independent experimental results.

Annexin V and propidium iodide staining assay

Apoptotic cells in different groups were determined using an Annexin V/PI apoptosis detection kit according to manufacturer's protocol (Solarbio, China). Briefly, the cell pellet was resuspended in 1 binding buffer followed by incubation with 5 µl Annexin V/Alexa Fluor 647 and 5µl PI in the dark for 15 min. Cell fluorescence was then analyzed using a Cell Lab Quanta SC Flow cytometer (BD, FACSCAI IBUR, USA). Cells positive for Annexin V-FITC and negative for PI were considered apoptotic.

Statistical analysis

The results were expressed as the mean ± standard error of mean (SEM) and analyzed with SPSS 22.0. Statistical differences among different groups were determined by one-way analysis of variance (ANOVA). Statistical significance was defined as P<0.05

Results

RT-PCR detected the expression of Bcl-2, Bax and Caspase-3 in rats

Compared NG group and HG group, the mRNA expression level of Caspase-3 in the HG group increased, but the mRNA expression level of Bcl-2/Bax showed the opposite trend, which was shown in

Figure 1. Compared with the HG group, the mRNA expression level of Caspase-3 in VD group decreased, but the mRNA expression level of Bcl-2/Bax increased, as shown in Figure 1. There was no significant difference in the mRNA expression of Caspase-3 and the ratio of Bcl-2/Bax between the HM group and the NG group.

The Western-blot detected the expression of Bcl-2, Bax and Caspase-3 in rats

Changes in Western-blot expression of Bcl-2, Bax and Caspase-3 were basically consistent with changes in mRNA expressions as shown by RT-PCR in rats. Compared NG group and HG group, the protein expression levels of Caspase-3 and BCL-2/Bax showed the opposite trend, as described in Figure 1. Compared with the HG group, the protein expression level of Caspase-3 in VD group decreased, but the protein expression level of Bcl-2/Bax increased, as shown in Figure 1. Compared with the NG group, there was no significant difference in the protein expression of Caspase-3 and the ratio of Bcl-2/Bax between the HM group and the NG group.

The decrease of Bcl-2/Bax induces the activation of Caspase and the apoptosis of downstream cells, which plays an important role in the regulation of apoptosis. In this study, high glucose inhibited Bcl-2 expression and increased Bax expression. However, 1,25-D₃ increases the Bcl-2/Bax ratio and inhibits the activation of caspase-3 at the same time. Our results showed that 1,25-D₃ can improve the expression of Bcl-2/Bax and Caspase-3 in podocyte apoptosis in a high glucose environment.

Discussion

DKD, a severely disabling complication of diabetes, is characterized by decreased glomerular filtration rate and progressive increase in urinary albumin, can lead to ESRD¹². However, the cellular mechanisms of DKD that initiate and maintain this disorder remains unclear. Therefore, we should pay more attention to the prevention and treatment of DKD. The podocyte apoptosis critically contributes to pathologically enhance DKD processing and the pathogenesis of proteinuria¹³. Podocytes are terminally differentiated glomerular visceral epithelial cells which are fundamental components of the glomerular filtration barrier to prevent loss of urinary protein^{2,13}. Given that podocytes cannot be divided, the apoptosis of podocytes aggravates the expansion of mesangial matrix and the excretion of urinary protein which suggest that podocyte apoptosis is an early pathological mechanism during DKD progression^{14,15}. High glucose induces podocyte apoptosis through the activation and regulation of Bax, BCL2, Caspase3⁷. The podocytes apoptosis are the central link in DKD pathological conditions¹¹. In addition, the apoptosis and structural changes of podocytes are considered to be predictors of DKD progression¹⁶. Therefore, The regulation of

podocyte apoptosis is a promising therapeutic approach for diabetic nephropathy³.

The Vitamin D intervention in type 2 diabetic mice can protect glomeruli from diabetic nephropathy¹⁸. Vitamin D₃ is absorbed into the gastrointestinal tract or synthesized by the effect of sunlight on the skin, and then it can be converted into 25-hydroxyvitamin D₃ in the liver, subsequently, and converted to 1,25-D₃ in the proximal tubule of the kidney⁹. Vitamin D receptors are widely distributed throughout the body, suggesting that the role of vitamin D₃ is not limited to the regulation of calcium and phosphorus metabolism⁸. Studies have shown that it acts on multiple systems, including the cardiovascular system, the immune system, glycolipid metabolism, and the reproductive systems¹⁹. Meanwhile, Vitamin D₃ has therapeutic and prophylactic effects on oxidative stress, liver, pancreas and kidney damage in alloxan-induced diabetic rats²⁰. Vitamin D analogues combined with AT1 receptor antagonists can slow down the progression of type 2 diabetic nephropathy, suggesting that vitamin D has protective effect on DKD²¹. In a word, there is an urgent need for drugs targeting podocytes for DKD proteinuria and pathological changes. Therefore, the research on the inhibition of podocyte apoptosis calls for more attention.

In this experimental study, we found that high glucose induced the podocyte apoptosis, increased the mRNA and protein expression of Caspase-3 significantly and decreased the mRNA and protein expression of Bcl-2 / Bax remarkably. The Bcl-2 / Bax family is known to play an important role in the regulation of apoptosis, and a decrease in the ratio of Bcl-2 / Bax can triggers Caspase-3 activation leading to apoptosis⁷. There was no significant change in the HM group, which indicating that then changes of apoptotic protein and mRNA expression in the HG group were not caused by high osmotic pressure. The protein and mRNA expression levels of Caspase-3, Bcl-2/Bax could be affected by 1,25-D₃ on the premise of high glucose induction, thus reduce the apoptosis of podocyte. Therefore, we could conclude that Vitamin D₃ restrain podocyte apoptosis in high glucose environment. However, the current clinical treatment for kidney disease is nonspecific and not concentrated on podocytes.

In conclusion, the results of this experiment indicate that 1,25-D₃ improves podocyte apoptosis induced in high glucose environment through inhibiting caspase/Bax/Bcl-2 signaling pathway in vitro. These findings broaden new insights into the development of renal protective drugs. What we're going to focus on is the molecular mechanism. It helps to identify the target of drug therapy for DKD. Meanwhile, the mechanism of vitamin D₃ involved in DKD and other renal diseases remains to be explored.

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Table 1. Primers used in the in vitro study using mouse podocytes

Primer	Sequence
Bax	Forward : 5'-TTGCCCTCTTCTACTTTGCTAG-3'
	Reserve : 5'-CCATGATGGTTCTGATCAGCTC-3'
Bcl-2	Forward : 5'-GATGACTTCTCTCGTCGCTAC-3'
	Reserve : 5'-GAACTCAAAGAAGGCCACAATC-3'
Caspase-3	Forward : 5'-GAAACTCTTCATCATTCCAGGCC-3'
	Reserve : 5'-GCGAGTGAGAATGTGCATAAAT-3'
GAPDH	Forward : 5'-CTCATGACCACAGTCCATGC-3'
	Reserve : 5'-CACATTGGGGGTAGGAACAC-3'

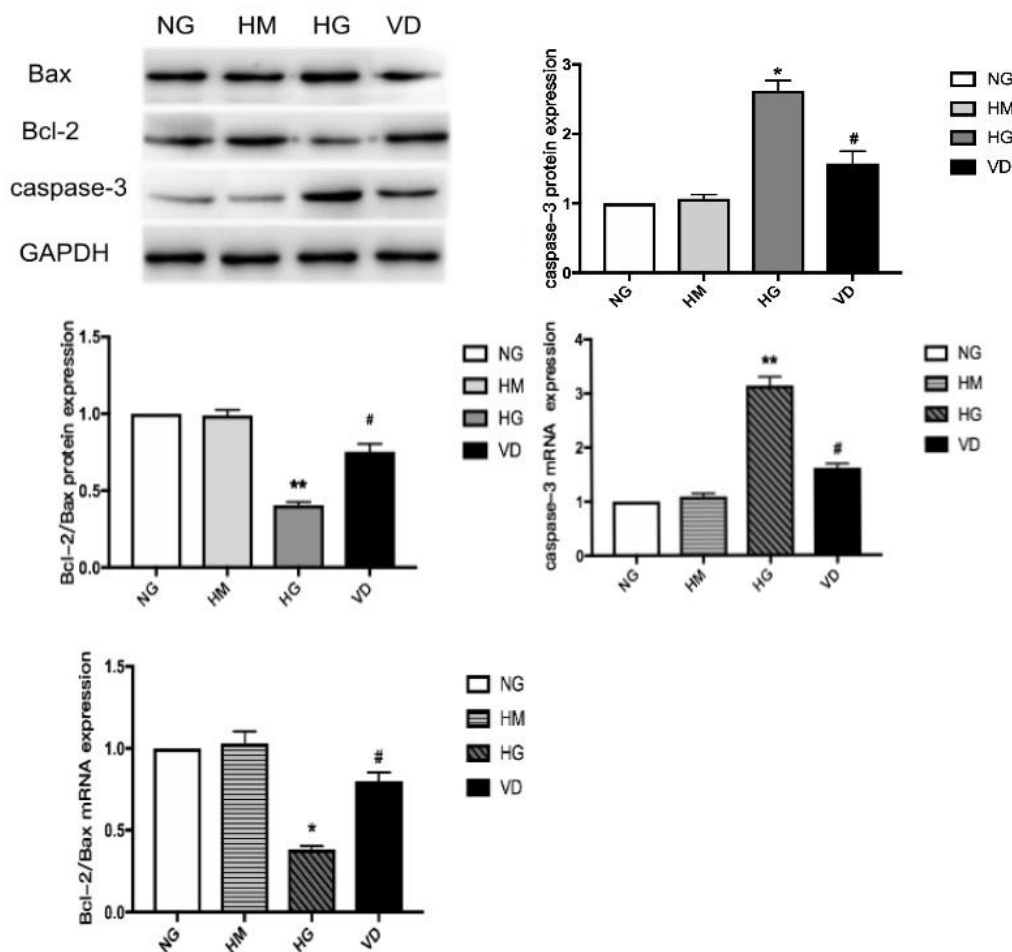


Figure 1, Effect of 1,25-D₃ on Caspase-3 and Bcl-2/Bax expression in podocyte in podocytes interfered with high glucose. The Bcl-2 and Bax expression is described as Bcl-2/ Bax ratio. Caspase-3 and Bcl-2/Bax protein expression is determined by western-blot (A,C,E).mRNA expression is determined by RT-PCR (B,D). Data are presented as mean ± SEM. *P* < 0.05, ** *P* < 0.01, compared to NG group; # *P* < 0.05, compared to HG group.