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Original Article

Paricalcitol prevents glucose induced-podocyte injury by inhibiting Wnt/β-catenin pathway

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Abstract: Previous reports found that vitamin D analog Paricalcitol can inhibit β-catenin, we tested whether parcialcitol could ameliorate glucose-induced podocyte injury. The podocytes MPC5 cells were treated and divided into 6 groups: low glucose (L), low glucose with mannitol (L+D), high glucose group (H), high glucose + Paricalcitol (H+P), high glucose + losartan (H+losartan) and High glucose + LiCl group (H+Licl). The podocyte injury induced by high glucose and the effect of Paricalcitol on it were evaluated by measuring the cell proliferation and cell apoptosis rates. The nuclear translocation of VDR and β-catenin were evaluated. The upregulated TGF-β and Col IV indicated podocyte injury induced by high glucose, with increased cell apoptosis rate and decreased proliferation rate. After treated with Paricalcitol, elevated WT and nephrin was observed, while the TGF-β and Col IV was down-regulated. It promoted VDR nuclear translocation and blocked β-catenin nuclear translocation. Administration of LiCl, a WNT/β-catenin Signaling pathway agonist, could reverse the effects of Paricalcitol, suggesting that Paricalcitol led to suppression of β-catenin-mediated gene transcription. In response to high glucose, podocytes can undergo a spectrum of changes that may involve apoptosis, slow proliferation, and cell injury. Paricalcitol prevented this podocyte injury by inhibiting Wnt/β-catenin signaling.

Keywords: Paricalcitol, podocyte injury, glucose, Wnt/β-catenin, kidney disease

Introduction

The presence of proteinuria is not only a surrogate marker of kidney damage but also attributes to the development and progression of chronic kidney diseases (CKDs) [1]. Podocytes are highly specialized, terminally differentiated visceral epithelial cells that reside on the glomerular basement membrane outside the glomerular capillaries, which are key components of glomerular filtration barrier. Increasing evidence suggest that podocyte injury is one of the major causes leading to defective glomerular filtration, which results in proteinuria [1]. Despite the importance of podocyte injury in proteinuria is well recognized, the mechanisms and signal pathways leading to podocyte damage in the vast majority of proteinuric kidney disorders remain poorly understood. It was previously shown that Wnt/β-catenin signal played a critical role in promoting podocyte injury, proteinuria and renal fibrosis. Studies indicated that activation of signaling caused podocyte dedifferentiation and mesenchymal transition, which impaired podocyte integrity and disrupted the glomerular filtration barrier, leading to proteinuria [3-5]. These findings revealed that strategies targeted Wnt/β-catenin signal pathway may be feasible approaches for the treatment of CKDs [6, 7].

Wnt/β-catenin is an evolutionarily conserved cellular signaling system that plays an essential role in regulating cell proliferation and differentiation, stem cell maintenance, angiogenesis, inflammation, fibrosis and carcinogenesis [2]. Briefly, Wnt proteins induce a series of downstream signaling events after binding to their

receptors, resulting in β-catenin dephosphorylation and stabilization. This allows β-catenin to translocate into the nuclei, and then to stimulate the transcription of Wnt target genes [3]. On the basis of this canonical pathway of Wnt signaling, it is conceivable that either inhibiting Wnt expression or repressing β-catenin transcriptional activity could be an effective way to control the Wnt/β-catenin signaling. Aberrant regulation of Wnt/β-catenin has been implicated in many types of kidney diseases including obstructive nephropathy, chronic allograft nephropathy, diabetic nephropathy, polycystic kidney disease, focal and segmental glomerulosclerosis, and adriamycin nephropathy [10, 11]. WNT pathway is over-activated in retinas from human patients with diabetic retinopathy and in those from animal models of diabetes [12]. Podocyte-specific and tubule-specific knockout of β-catenin does not cause any overt abnormality, suggesting that the β-catenin protein is functionally dispensable in the kidney under normal physiological conditions [13, 14].

Earlier studies indicate that vitamin D analogs are able to promote the differentiation of colon carcinoma cells by inhibiting β-catenin signaling. This action of vitamin D appears to be mediated by ligand-activated vitamin D receptor (VDR) competing with transcription factor TCF-4 for β-catenin binding [4]. In glomerular diseases, activation of VDR inhibits expression Wnt/β-catenin target genes such as Snail1 and TRPC6, prevents podocyte injury and reduces proteinuria. The VDR-mediated sequestration of β-catenin might therefore contribute to the renal protection of vitamin D analogues in proteinuric CKD [16, 17]. Consistently, administration of vitamin D analogs is able to reduce proteinuria and promote overall survival in patients with CKD by a mechanism that is independent of serum parathyroid hormone, phosphorus, and calcium levels [18, 19]. Taken together, these results led us to hypothesize that administration of vitamin D analog might be able to effectively prevent podocyte dysfunction, proteinuria, and kidney injury by modulating Wnt/β-catenin signaling.

Until now, little work has been done to investigate the effect of vitamin D analog on podocyte injury in high glucose condition. Here we examined the therapeutic effects of paricalcitol (19-nor-1,25-hydroxy-vitamin D2), a synthetic and active vitamin D analog [20], in podocyte injury caused by high glucose. Our data demonstrated that paricalcitol mitigates podocyte injury by inhibiting Wnt/β-catenin signaling. These studies indicate that blocking Wnt/β-catenin signaling is a plausible strategy for therapeutic intervention of proteinuric kidney disorders.

Materials and methods

Cell culture and treatment

The conditionally immortalized mouse podocyte cell line MPC5 was purchased from Youningwei Biotechnology Company (Guangzhou, China). The cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum and recombinant IFN-γ (Invitrogen, Carlsbad, California). To induce podocyte injury, glucose of high concentrations was added into the culture medium for 24 h. The cells were treated with paricalcitol with different concentrations for 24 h. Cell culture was carried out according to the procedures described previously and treated with paricalcitol. Whole-cell lysates were prepared and subjected to Western blot analyses.

Nuclear protein preparation

Nuclear protein preparation was carried out according to the procedure described previously. Briefly, MPC5 cells after various treatments as indicated were washed twice with cold PBS and scraped off the plate with a rubber policeman. After centrifugation, the cell pellets were resuspended in Buffer A (10 mm HEPES, pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, 0.5% NP-40, and 1% protease inhibitor cocktail [Sigma]) and lysed with homogenizer. The cell nuclei were collected by centrifugation at 5000 rpm for 15 minutes and washed with Buffer B (10 mm HEPES, pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, and 1% protease inhibitor cocktail). The nuclei were lysed in SDS sample buffer. For loading control of nuclear protein, the blots were stripped and reprobed with antibody against the target proteins.

Western blot analysis

Glomeruli were isolated by differential serving technique according to the method described elsewhere. The isolated glomeruli were lysed with radioimmune precipitation assay buffer containing, 0.1% SDS, 100 μg/ml PMSF, 1%

Table 1. Primers used in this work

nephrin-F: 5'	TCAACGTGTTATACCCTCCA
nephrin-R: 5'	TTCTGGCAGTGTAGCTGATA
TGFβ-F: 5'	GAGCAACATGTGGAACTCTA
TGFβ-R: 5'	TGAATCGAAAGCCCTGTATT
wnt7a-F: 5'	CCATCATCGTCATAGGAGAA
wnt7a-R: 5'	CGATAATCGCATAGGTGAAG
wnt7b-F: 5'	GGAAGCTTAATGTCCCATTT
wnt7b-R: 5'	TGTGGGCAGTTGTATTATCA
wnt10a-F: 5'	CATGCCTACCTCCTGTCATC
wnt10a-R: 5'	TGGAGCCCTTAGAGTCACTT
wnt10b-F: 5'	GTGCTGTGTGATGAGTGTAA
wnt10b-R: 5'	GAGCCACGATAAACCCTAGA
β-catenin-F: 5'	CACCATGCAGAATACAAATG
β-catenin-R: 5'	CGTAGAACAGTACAGAATCC
col4a3-F: 5'	GAGCTGAGACATGCAACATC
col4a3-R: 5'	GTGTGCCTATTGAGTGGAGA
wnt4-F: 5'	CGTCTCTGATGTGGATGAAA
wnt4-R: 5'	GGTGCTGAACTAAGTCTACC
VDR-F: 5'	GCAGGTTAGAACTTGTGGTA
VDR-R: 5'	CCGTTTCGTAGGTCTACATT
GAPDH-F: 5'	GGCCTCCAAGGAGTAAGAAA
GAPDH-R: 5'	GGCCTCCAAGGAGTAAGAAA

protease inhibitor cocktail, and 1% phosphatase I and II inhibitor cocktail (Sigma) in PBS on ice. The supernatants were collected after centrifugation at 13,000×g at 4°C for 20 minutes. Whole-kidney lysates were prepared using the same procedures. Cultured mouse podocytes were lysed in SDS sample buffer. Protein expression was analyzed by Western blot analysis as described previously [7]. The primary antibodies used were as follows: anti-nephrin (Fitzgerald Industries International), anti-Col V, anti-WT1, anti-VDR (SC-1008), and anti-actin (SC-1616) (Santa Cruz Biotechnology), anti-β-catenin (catalog number 610154; BD Transduction Laboratories, San Jose, California), anti-TGFβ1 (clone 1A4; Sigma), and anti-GAPDH (Ambion, Austin, Texas).

Real-time RT-PCR

Total RNA isolation and real-time RT-PCR were carried out by the procedures described previously. Briefly, the first strand cDNA synthesis was carried out by using a reverse transcription system kit according to the instructions of the manufacturer (Promega, Madison, Wisconsin). Real-time RT-PCR was performed on ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, California) as described previously. The PCR mixture in a 25-μl volume contained 12.5 μl of 2×SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of diluted RT product (1:10), and 0.5 μm sense and antisense primer sets. The sequences of the primer pairs used in real-time PCR were given in **Table 1**. PCR was run by using standard conditions. After sequential incubations at 50°C for 2 minutes and 95°C for 10 minutes, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95°C for 15 seconds and annealing and extension at 60°C for 60 seconds. The standard curve was made from series dilutions of template cDNA. The mRNA levels of various genes were calculated after normalizing with GAPDH.

Cell proliferation and apoptosis assays

To analyze cell proliferation, cells were cultured in a 96-well plate in DMEM with 10% FBS and 100 mg/ml of penicillin/streptomycin for 72 hours. Cell proliferation rates were detected by the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche, USA) according to manufacturer's instructions.

To analyze cell apoptosis, cells were prelabelled with PI and annexin V-FITC, and then measured with an apoptosis detecting kit (Invitrogen, Burlington, Canada) according to manufacture's instructions. Samples were analyzed by a flow cytometry and the results were recorded by matched CellQuest software (Becton Dickinson, San Jose, CA, USA).

Statistical analyses

Statistical analyses of the data were carried out using SigmaStat software (Jandel Scientific, San Rafael, California). Comparison between groups was made using one-way ANOVA followed by a Student-Newman-Kuel's test. $P< 0.05$ was considered significant.

Results

Paricalcitol promotes proliferation and inhibits apoptosis of injured podocytes

After treatment with glucose for 24 h, the cell proliferation rates were measured to evaluate the injury of podocytes MPC5. High glucose (25 mM) resulted in significant slow proliferation

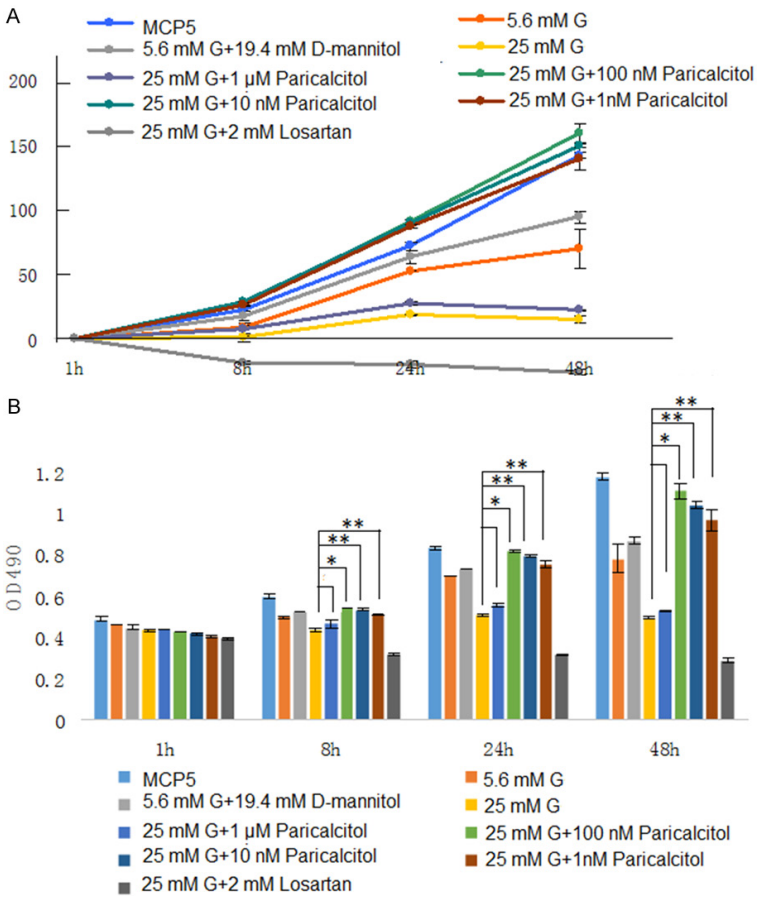

Figure 1. Effects of Paricalcitol on MPC5 cell proliferation. A. Cell proliferation rates of MPC5 cells by an MTT assay in the first 2 days; B. The cell proliferation values of MPC5 cells in the first 2 days. The cells were treated with glucose for 24 h, and then D-mannitol, or Paricalcitol, or Losartan was added for incubation for another 24 h. At last, the cell medium was changed with fresh DMEM supplemented with 10% FBS for further culture. *P<0.05, **P<0.01.

rate compared to low glucose (5.6 mM) (**Figure 1A**). The cell injury caused by low glucose was almost the same as the addition of D-mannitol. Different concentrations of Paricalcitol were administrated to high-glucose injured MPC5 cells, and it was found a dose-dependent improved proliferation in cells. The cell proliferation with 1 μM Paricalcitol treatment was almost as high as that in control (**Figure 1B**). In order to confirm the specific effect of Paricalcitol on podocyte injury caused by high glucose, Losartan was chosen to treat the podocyte injury. Previous report demonstrated that Losartan can treat podocyte injury induced by Ang II via downregulation of TRPC6. It was found here that Losartan induced severer cell injury with slowest cell proliferation rate among all of the groups, instead of ameliorating the cell injury caused by high glucose (P<0.05). These results indicated that Paricalcitol can significantly increase the cell proliferation and ameliorate the cell injury caused by high glucose in a dose-dependent manner, and the underlying molecular mechanism was different from Losartan involved pathway.

Meanwhile, the cell apoptosis was analyzed to confirm the effect of Paricalcitol. The percentages of early cell apoptosis (right lower quadrant) and late cell apoptosis (right upper quadrant) were increased in the high-glucose treated MPC5 cells (8.10% in low-glucose *VS* 11.02% in high-glucose) (**Figure 2A**). However, an obvious decrease of proportion of apoptotic cells was seen in Paricalcitol (5.93%) and LiCl (5.19%) treated cells; while the Losartan (7.46%) did not markedly reverse the cell apoptosis induced by high-glucose. In order to investigate whether Paricalcitol affects cell cycle of MPC5 cells, we detected the cell cycle by FACS (**Figure 2B**). High glucose (25 mM) would decrease the percentage of cells in S phase (57.80% vs 45.29%) and increase the percentage of cells in G1 phase (42.12% vs 53.8%) compared to MCP5 cells. This suggested that high glucose would arrest cells in G1 phase. However, Paricalcitol could reverse the arrest in some extend, as cells in G1 phase (45.32%) were less than that in 25 mM glucose (53.8%), but the difference was not significant. These results suggested that Paricalcitol could ameliorate the apoptosis of MPC5 cells caused by high-glucose, and the Wnt pathway was involved here, as for the effect of Wnt agonist LiCl.

Paricalcitol ameliorate podocyte injury by promoting VDR

Because glucose injury would lead to the decrease of podocyte density, we initially investigated the expression of podocyte-specific

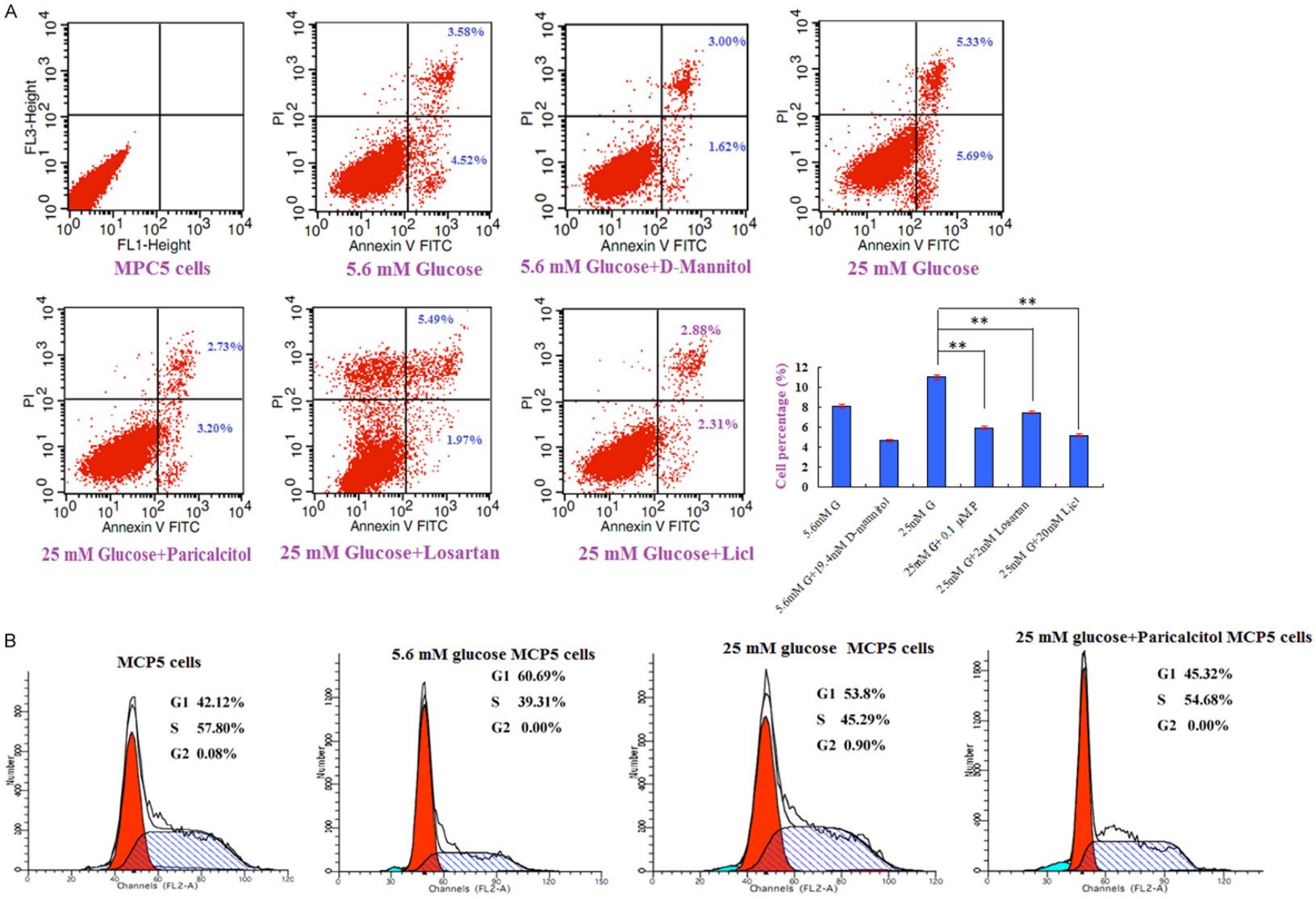

Figure 2. Effects of Paricalcitol on MPC5 cell apoptosis and cell cycle. Cell apoptosis and cell cycle of MPC5 cells treated by different drugs by flow cytometry. **P<0.01.

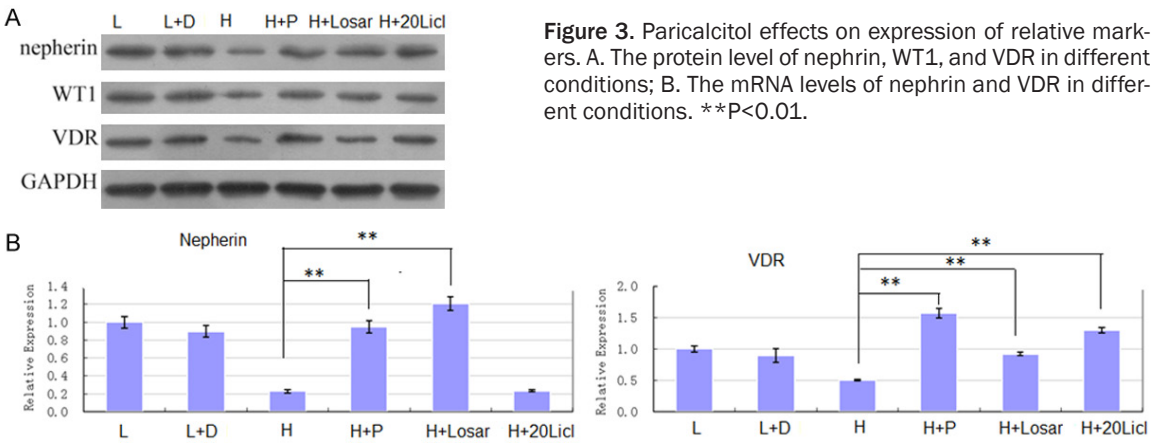

Figure 3. Paricalcitol effects on expression of relative markers. A. The protein level of nephrin, WT1, and VDR in different conditions; B. The mRNA levels of nephrin and VDR in different conditions. **P<0.01.

markers such as nephrin and WT1. As shown in **Figure 3A**, western blot demonstrated that both nephrin and WT1 protein levels were decreased in high-glucose treated MPC5 cells, and Paricalcitol significantly abrogated their induction. As know that Paricalcitol, a vitamin D analog, can bring the vitamin D receptor (VDR) translocates to the nucleus, therefore we analyzed the expression of VDR in nucleic. High glucose resulted in the low expression of VDR, and Parcalcitol can reverse the effect and elevate significantly expression of VDR. Meanwhile, Losartan and Licl also can increase the VDR induced by high-glucose injury. The mRNA expression of nephrin and VDR was just similar with protein expression rules (**Figure 3A**). Taken these, Paricalcitol can ameliorate significantly the glucose injury through promoting expression of VDR.

Paricalcitol suppresses expression of Wnts and β-catenin nuclear translocation

Because of the decreased expression of VDR in case of high glucose, which can inactivate the Wnt/β-catenin signaling, we next investigated the important markers involved in Wnt/β-catenin signaling, including Wnts and β-catenin. A comprehensive analysis of 5 Wnt genes has demonstrated that numerous Wnts were up-regulated in the podocyte injury caused by high glucose. We found that paricalcitol could specifically inhibit expression of multiple Wnts, including Wnt4, Wnt7a and Wnt7b (**Figure 4A**). However, paricalcitol seemed not to suppress Wnt10a and wnt10b expression. We also investigated the expression of downstream genes β-catenin, TGF-β, Col IV. It was found that the high expression of β-catenin and Col IV induced by high glucose was inhibited by Paricalcitol at protein level (**Figure 4B**). These results suggested that paricalcitol was able to selectively suppress some WNTs expression induced by high glucose. Paricalcitol induces up-regulated VDR expression to activate Wnt/β-catenin pathway and prevents podocyte damage in cell culture.

Discussion

Proteinuria, the clinical manifestation of defective glomerular filtration, is an early pathologic feature of CKDs. It not only serves as an alternative marker for the progression and prognosis of kidney injury but also is an important pathogenic mediator triggering subsequent inflammatory and fibrotic responses in renal parenchyma [21, 22]. The results presented in this study proves that Paricalcitol, a synthetic, vitamin D analog, possesses a satisfied renal protective efficacy in podocyte injury induced by high glucose. The beneficial effects of Paricalcitol are likely mediated by its ability to inhibit Wnt expression and to block β-catenin-mediated downstream gene. These studies underscore that vitamin D is a potent endogenous, natural antagonist of Wnt/β-catenin signaling. Our results also indicate that targeting this signaling could be an effective way to mitigate podocyte injury in high glucose stimulation.

Diabetes-associated kidney injury is characterized by hypertrophy and accumulation of matrix proteins culminating in kidney fibrosis [5]. The mechanisms leading to increment in matrix protein content include an increase in synthesis

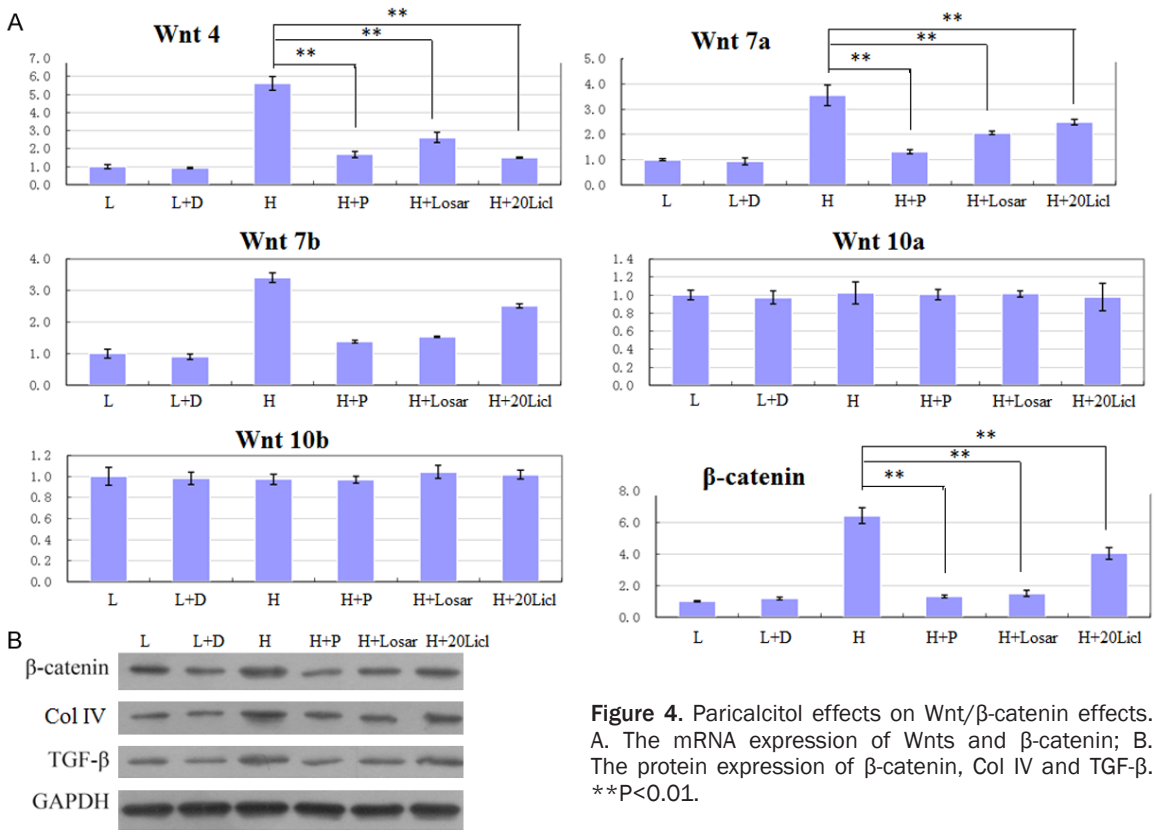

Figure 4. Paricalcitol effects on Wnt/β-catenin effects. A. The mRNA expression of Wnts and β-catenin; B. The protein expression of β-catenin, Col IV and TGF-β. **P<0.01.

and inhibition of degradation. High glucose-induced synthesis of matrix proteins can be independently regulated at the levels of transcription and mRNA translation. Elaborate signaling pathways regulate both transcription and translation in the kidney in diabetic mice [24]. High glucose causes glomerular cell injury, including mesangial cells, podocytes, and endothelial cells, leading to loss of renal function and eventual development of end-stage renal disease. Podocyte loss and dysfunction in renal microenvironments are important features in the pathogenesis of diabetic nephropathy (DN) [25, 26].

The studies reported here likely offer significant, mechanistic insights into the mechanism by which vitamin D analogs protect podocytes from injury. Previously, it was shown that activation of the canonical pathway of Wnt/β-catenin signaling plays an imperative role in mediating podocyte dysfunction [6]. Modulation of this signal system *in vivo* by an array of genetic and pharmacologic maneuvers evidently influences the development and severity of podocyte damage and proteinuria. Notably, the importance of β-catenin in mediating podocyte injury is recently confirmed by an independent study [3, 14]. Therefore, it is not surprising that targeting Wnt/β-catenin signaling by Paricalcitol ameliorates proteinuria. In the injured podocyte, the expression of several Wnts is up-regulated. Regardless of what specific Wnt is induced, however, β-catenin, the common downstream mediator of the canonical Wnt signaling, is induced, indicating a robust activation of the canonical pathway of Wnt signaling in this model [27, 28]. Interestingly, this Wnt/β-catenin signaling is virtually blocked after Paricalcitol treatment, underscoring that the vitamin D analog is able to constrain the activity of Wnt/β-catenin signaling *in vitro*.

Our results indicate that paricalcitol prevents the development of DN after high glucose injury. These findings are quite significant and obviously have clinical relevance. It is conceivable that Paricalcitol could inhibit cell injury caused by high glucose by two different mechanisms. On one hand, Paricalcitol selectively suppresses the expression of multiple Wnt genes includ-

ing Wnt4, Wnt7a, Wnt7b, whose expression is up-regulated after glucose injury. This action presumably prevents Wnt induction and β-catenin activation in the injured podocyte after glucose addition in cell culture. On the other hand, paricalcitol apparently has the ability to inhibit β-catenin-mediated gene transcription by inducing VDR binding to active, nuclear β-catenin. This leads to the sequestration of the β-catenin transcriptional activity in the nuclei.

The therapeutic efficacy of paricalcitol in DN is impressive, which could involve multiple mechanisms. In addition to modulating Wnt/β-catenin signaling, we cannot exclude the possibility that Paricalcitol may elicit its beneficial activities by other routes as well. In that regard, Paricalcitol has been shown to promote cell proliferation and suppress cell apoptosis of podocytes. Similar to Paricalcitol, Losartan could treat podocyte injury induced by Ang II via downregulation of TRPC6. However, Losartan can not ameliorate the high-glucose injury, which suggested that Paricalcitol would regulate another molecular mechanism for treatment. The underlying mechanism would be investigated further.

Conclusion

In summary, our study demonstrated that high-glucose-induced downregulation of VDR plays an important role in the reduction of podocyte injury. The Wnt/β-catenin signaling pathway mediates the VDR effects. Paricalcitol can induce up-regulated VDR expression to activate Wnt/β-catenin pathway and prevents podocyte damage in cell culture. These findings may help develop a new therapeutic strategy for the management of podocyte injury in diabetes.

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Disclosure of conflict of interest

None.

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