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Role of Vitamin D in Improvement in Changes of Podocyte P-Cadherin/β-Catenin Complex Induced by Diabetic Conditions

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Introduction. This study aimed to investigate the effect of vitamin D on the pathologic changes of podocyte β-catenin and P-cadherin and podocyte permeability induced by diabetic conditions.
Materials and Methods. We cultured mouse podocytes under normal glucose (5 mM, control); high glucose (HG, 30 mM); advanced glycosylation end products (AGE)-added; and HG plus AGE-added conditions and treated with vitamin D. The distribution of podocyte β-catenin and P-cadherin was shown by confocal microscopy, and protein levels of β-catenin and P-cadherin by Western blotting. Podocytes were incubated with vitamin D at the concentrations of 10 nM and 50 nM for 6, 24, and 48 hours.
Results. The dextran filtration through monolayered podocytes tended to increase in AGE and HG condition compared to that in B5 at 16 hours in permeability assay, which was improved by vitamin D. In confocal imaging, the distribution of β-catenin and P-cadherin were internally concentrated by diabetic conditions, which was ameliorated by vitamin D. In Western blotting, HG and AGE decreased β-catenin protein levels at 6, 24, and 48 hours and vitamin D improved the decreased β-catenin protein levels at 6, 24, and 48 hours. Advanced glycosylation end products also decreased P-cadherin protein amount by 22.9% and 59.1% ($P < .01$) at 24 hours, respectively, which was improved by vitamin D.
Conclusions. Our results suggest that HG and AGE have an influence on the redistribution of β-catenin and P-cadherin and amount of β-catenin protein of podocytes, thereby causing hyperpermeability, which can be reversed by vitamin D.

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INTRODUCTION

Diabetic nephropathy is a major cause of end-stage renal failure in adults, but the pathophysiologic mechanisms remains elusive.[1,2] Diabetic nephropathy is characterized by hyperfiltration and microalbuminuria at an early stage, but can progress to severe proteinuria and kidney failure.[1-3] Hyperglycemia and advanced glycosylation end products (AGE) in diabetic conditions can change renal pathology, such as glomerular hypertrophy, increase of mesangial matrix, thickening of glomerular basement membrane, and effacement of podocyte foot processes, which can result in increase in glomerular permeability.[1,3-5]

Podocytes are highly differentiated epithelial cells, covering the outer layer of the glomerular basement membrane, and the foot processes of podocytes are interdigitated and bridged by a zipper-like structure, the slit diaphragm (SD).[6-8] As a size-selective barrier, the SD is composed of adherens junction proteins, such as nephrin, P-cadherin, NEPH1, and F-actin, with the filtration

slit of 25-nm to 60-nm width and is connected to the actin cytoskeletons by adaptor proteins, such as CD2-associated protein, zonula occludens-1, β-catenin, and podocin.[6-8] β-Catenin is present in podocytes as 2 forms: one is located in the plasma membrane as a cadherin/catenin adhesive complex that stabilizes adherens junctions by maintaining epithelial integrity and the other in the nucleus as a key regulator of gene expression via binding to transcription factors (for example, T-cell factor or lymphoid enhancer-binding factor).[8,9]

Recent reports suggested that vitamin D has an important role in renoprotection in diabetic conditions by multiple mechanisms, such as inhibiting the renin-angiotensin system, inflammatory cascades, Wnt/β-catenin pathway, and pro-apoptotic pathway.[10-14] However, there have been very few data regarding the effects of vitamin D on podocyte SD or adaptor molecules in diabetic conditions.[15,16] Some investigators showed that vitamin D might upregulate the expression of podocyte components, such as podocalyxin or nephrin,[15,16] but limited data were available regarding the effects of vitamin D on other SD or adaptor molecules, such as P-cadherin/β-catenin adhesive complex. The aim of this study was to investigate the effect of vitamin D on the pathologic changes of podocyte P-cadherin and β-catenin and podocyte permeability induced by diabetic conditions.

MATERIALS AND METHODS

Culture of Mouse Podocytes

Mouse podocytes were conditionally immortalized and were provided by Dr Peter Mundel (University of Harvard, Boston, MA, USA). Those were cultured and differentiated as reported previously.[17] In brief, for proliferation of podocytes, cells were cultivated at 33°C in a culture medium supplemented with 10 U/mL of mouse recombinant γ-interferon (Roche, Mannheim, Germany) to induce expression of temperature-sensitive large T antigen (permissive conditions). For differentiation of podocytes, cells were maintained at 37°C without γ-interferon for at least 14 days (nonpermissive conditions).[17]

Culture Additives

Cells were deprived of serum to reduce background 24 hours before each experiment, and then were exposed to glucose or AGE or both. Mouse podocytes were incubated in culture media, which contained either 5 mM (normal glucose) or 30 mM glucose (high glucose, HG) without insulin. Advanced glycosylation end products were produced by the technique previously described by Ha and colleagues.[18] To emulate the long-term diabetic condition, AGE was added (5 μg/mL) and controls were made by using unmodified bovine serum albumin (5 μg/mL). In order to exclude the effect of additionally produced glycated proteins in culture conditions, incubation period was not longer than 48 hours. Fetal bovine serum was decreased to 0.5% in the last media change to reduce background before protein extraction. Advanced glycosylation end products and bovine serum albumin were denoted as 'A' and 'B,' and glucose at 5 mM and 30 mM as '5' and '30', respectively, for identification. Namely, B5 means normal, B30 short-term diabetic condition, A5 long-term normoglycemic or aged condition, and A30 long-term diabetic condition.

For vitamin D treatment, podocytes were incubated with 1,25-dihydroxyvitamin D3 (Sigma) at the concentrations of 10 nM for 6, 24, and 48 hours.

Monolayer Permeability Assay

Podocytes were seeded and confluently grown in a monolayer pattern on the surface of cellulose semi-permeable membranes (Millicell-HA, Millipore Corp, Bedford, MA, USA), which have a pore size of 0.45 μm. After the previously described media was washed completely, fresh media for maintenance without AGE and bovine serum albumin were replaced into each aspect. Then, hydrostatic pressure was continuously applied from lower to apical to the basolateral aspect. Then, 1 mg/mL of fluorescein isothiocyanate-tagged anionic dextran (Invitrogen, Eugene, OR, USA) was added into the apical media and the filtered amounts of dextran at each incubation time (2, 4, 6, 16, 24, and 100 hours) were measured by spectrophotometer at 492 nm.

Confocal Image Analysis

Mouse podocytes were grown on glass cover slips, in which type I collagen were coated and those were incubated for 24 hours and then fixed in 4% paraformaldehyde, permeabilized in phosphate buffer saline, blocked with 10% normal goat serum, and labeled with polyclonal rabbit anti-rat β-catenin (Santa Cruz Biotechnology Inc, Santa Cruz, CA,

USA) or polyclonal rabbit anti-P-cadherin (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Primary antibody-bound specimens were incubated with 1:500 (v/v) Alexa 594 for red conjugates (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and Alexa 488 for green, respective of secondary antibodies, at room temperature for 1 hour. Coverslips were mounted in aqueous mountant and viewed with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Western Blotting

The confluently grown cell layers were incubated with additives during 48 hours for β-catenin and P-cadherin and then various durations for AGE were extracted and protein concentrations were determined as previously described.[18] For the Western blotting of β-catenin, 30 µg of boiled extracts was applied on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Then, the membranes were air-dried and blocked in 3% fat-free milk before incubation with anti-β-catenin antibody. After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), bands were detected by using the enhanced chemiluminescence system (Amersham Biotech Ltd., Bucks, UK). The Western blotting of P-cadherin was also performed with a same protocol. Data on the densitometric analysis of β-catenin/β-tubulin ratio were expressed as mean ± standard deviation. Three independent experiments were performed.

Statistical Analysis

Statistical analysis was performed by the SPSS software (Statistical Package for the Social Sciences, version 12.0, SPSS Inc, Chicago, Ill, USA). The results were shown as mean ± standard deviation and the statistical significance was analyzed by the Student *t* test and nonparametric Kruskal-Wallis test. *P* values of less than .05 were considered significant.

RESULTS

Increased Permeability by High Glucose and Advanced Glycosylation End Products

The dextran filtration of monolayered podocytes tended to increase in AGE (A30) than in B5 at 16 hours in permeability assay (Figure 1). Increased podocyte permeability by AGE (A30) was improved by vitamin D (Figure 1). These results indicated that vitamin D might improve podocyte hyperpermeability induced by AGE in diabetic conditions.

Changes of β-Catenin and P-Cadherin in Confocal Imaging

Changes of β-catenin and P-cadherin on confocal imaging in diabetic conditions are shown in (Figure 2). In confocal imaging, staining for β-catenin and P-cadherin was most intense in the cell membrane. In merged views, the distribution of β-catenin and P-cadherin were colocalized. In diabetic conditions, β-catenin moved toward the cytoplasm from the cell membrane at B30, A5, and A30, compared

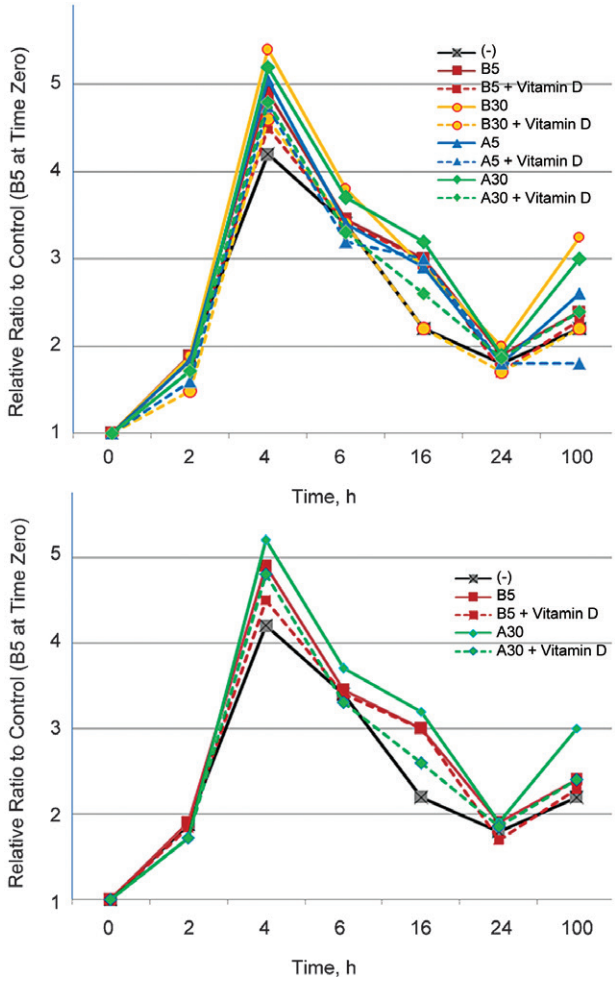

Figure 1. Effects of vitamin D on the increased monolayered podocyte permeability in diabetic conditions. Podocyte permeability tended to increase in advanced glycosylation end products and high glucose condition (A30) as compared to that in normal condition with 5 mM glucose (B5 at 16 hours), which was improved by vitamin D.

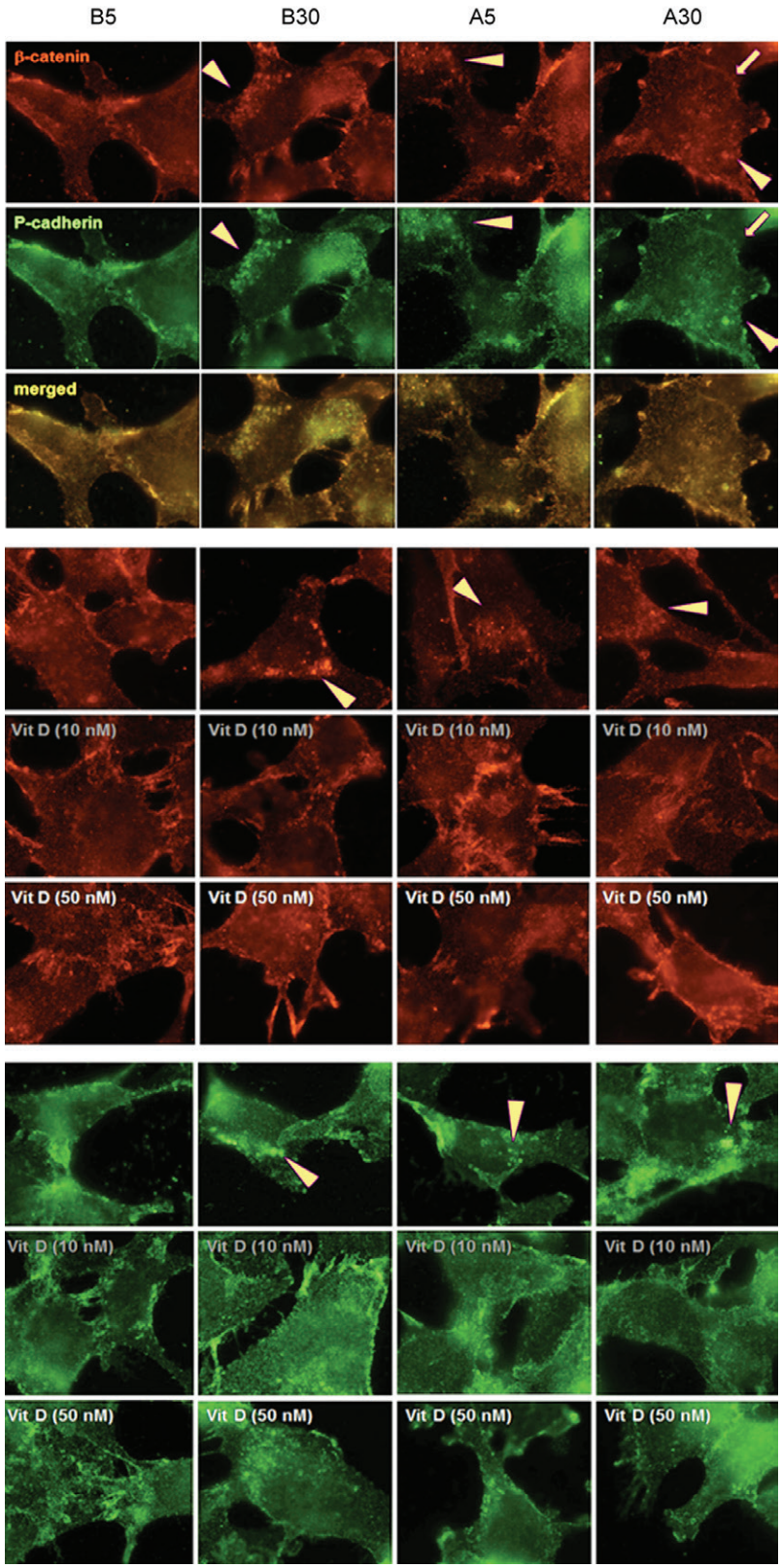

Figure 2. Changes in distribution of β-catenin and P-cadherin in diabetic conditions and vitamin D treatment (× 1000). β-Catenin was colocalized with P-cadherin at cell membrane areas. **Top,** Diabetic conditions (B30, A5, and A30) decreased the immunostainings and disrupted the distributions of β-catenin and (arrows). **Middle and Bottom,** Changes of β-catenin and P-cadherin were improved by vitamin D.

with B5 and were internally concentrated (arrows, Figure 2 Top). Moreover, intercellular staining of β-catenin was decreased. A similar redistribution of P-cadherin was also observed in podocytes with internal concentration and decreased intercellular staining. In merged views, β-catenin and P-cadherin were distributed at the cell membrane at B5 but redistributed into the cytoplasm at B30, A5, and A30, suggesting that high glucose and AGE might have an impact on the redistribution of β-catenin and P-cadherin in podocytes (Figure 2 Top).

1,25-dihydroxyvitamin D3 (10 nM and 50 nM) improved the decreased and internally concentrated β-catenin and P-cadherin immunostaining to be as those in B5 (Figure 2 Middle and Bottom). These findings indicated that glucose and AGE might affect the amount and redistribution of β-catenin and P-cadherin of podocytes, thereby causing hyperpermeability, which could be reversed by vitamin D.

Western Blotting of β-Catenin in Podocytes

In Western blotting (Figure 3), density values for β-catenin protein of representative immunoblots from each group revealed that decreased β-catenin protein levels in A30 at 6, 24, and 48 hours ($P < .01$). A30 condition decreased β-catenin protein amount by 32.5% at 6 hours, 31.8% at 24 hours, and by 26.7% at 48 hours incubation ($P < .01$; Figure 3). High glucose (B30) condition also decreased β-catenin protein amount by 24.7% at 6 hours and 31.8% at 24 hours incubation, but it was not statistically significant (Figure 3A). 1,25-dihydroxyvitamin D3 (10 nM) treatment in A30 significantly improved the decreased β-catenin protein levels at 6, 24, and 48 hours incubation times (Figure 3B).

A5 and A30 condition decreased P-cadherin protein amount by 22.9% ($P < .05$) and 59.1% ($P < .01$) at 24 hours, respectively, which was improved by 1,25-dihydroxyvitamin D3 (10 nM, $P < 0.01$ and 50 nM, $P < 0.05$) treatment (Figure 3C).

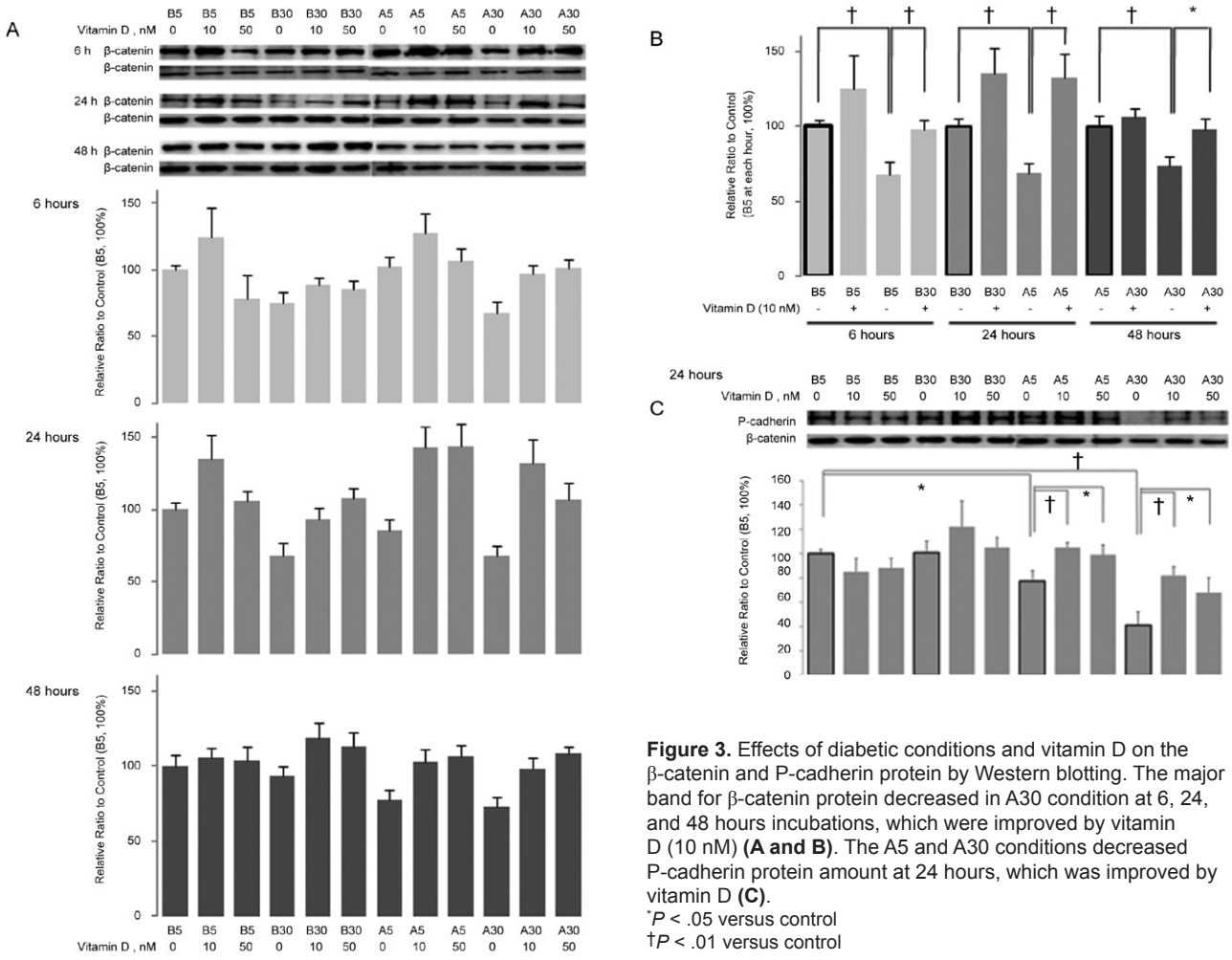

Figure 3. Effects of diabetic conditions and vitamin D on the β-catenin and P-cadherin protein by Western blotting. The major band for β-catenin protein decreased in A30 condition at 6, 24, and 48 hours incubations, which were improved by vitamin D (10 nM) **(A and B)**. The A5 and A30 conditions decreased P-cadherin protein amount at 24 hours, which was improved by vitamin D **(C)**.
*$P < .05$ versus control
†$P < .01$ versus control

DISCUSSION

Regardless of the underlying causes, the presence of proteinuria suggest ultrastructural changes in podocytes, such as with retraction and effacement of the interdigitating foot processes, which were accompanied by the alterations of SD and associated molecules.[6,8,19,20]

Among various adaptor proteins of podocytes, β-catenin has dual functions according to the subcellular localization. β-Catenin is a component of the P-cadherin/β-catenin complex which stabilizes podocyte adherens junctions for maintenance of SD integrity and it is linked to the podocyte cytoskeletons.[6,8,19,20] However, β-catenin also regulates gene expression by binding to transcription factors such as T-cell factor and lymphoid enhancer-binding factor in the nucleus and this canonical pathway, which regulates β-catenin involves glycogen synthase kinase-3β/Wnt signaling.[8,21]

Previous studies have mainly focused on the changes of Wnt/β-catenin signaling, because it has been regarded as an important player in the pathogenesis of podocyte dysfunction and albuminuria.[9,10] Dai and coworkers[10] reported that overexpression of Wnt1 in vivo activated glomerular β-catenin and aggravated albuminuria and adriamycin-induced suppression of nephrin expression, whereas blockade of Wnt signaling with Dickkopf-1 ameliorated podocyte lesions, suggesting that the hyperactive Wnt/β-catenin signaling is a crucial mediator in inducing podocyte injury. However, there have been scarce data regarding the effects of diabetic conditions on podocyte β-catenin as a P-cadherin/ β-catenin adhesive complex. Recent studies showed that the SD is a modified adherens junction rather than a tight junction, at which P-cadherin is localized.[7] Cadherins are a superfamily of glycoproteins that mediate calcium-dependent, homotypic cell-cell adhesion in solid tissues and there are E (epithelial)-, N (neural)- and P (placental)-cadherins.[22] They are known to play important roles in maintaining the structural integrity of epithelial tissues,[22] and P-cadherin is known to serve as a basic scaffold for the SD.[7]

Our results showed that HG and AGE had an influence on the redistribution of β-catenin and P-cadherin and amount of β-catenin protein of podocytes, thereby causing hyperpermeability, which can be reversed by vitamin D. Although rare, previous reports have also shown that the expression of P-cadherin or β-catenin was altered by diabetic conditions.[23-25] Xu and colleagues[23] reported that HG significantly reduced P-cadherin mRNA and protein expression in cultured podocytes and glomerular P-cadherin mRNA and protein expressions were significantly lower in diabetic rats than in control rats. Ha and coworkers[24] showed that high glucose with or without AGE suppressed the production of P-cadherin at the transcriptional level and that these changes might explain the functional changes of SD in diabetic conditions. Recently, we demonstrated that AGE and hyperglycemia could inhibit the production of β-catenin at the transcriptional and posttranslational levels in rat glomerular epithelial cells and mouse podocytes.[25]

In our study, the distribution of β-catenin and P-cadherin were colocalized in the cell membrane of podocytes, but intercellular staining of them was decreased and internally concentrated and β-catenin protein levels were decreased in diabetic conditions, suggesting that high glucose and AGE might have an impact on the amount and redistribution of P-cadherin/β-catenin adhesive complex in podocytes.

In our study, we also showed that podocyte permeability was increased by AGE. Recently, Ha and colleagues[26] reported that both HG- and AGE-added condition could induce phosphoinositide 3-kinase and its downstream mediators (Akt) and the distributional change and suppression of zonula occludens-1 protein, one of the adaptor proteins in podocytes, thereby causing hyperpermeability of podocytes. Therefore, we speculate that diabetic conditions might induce the activation of phosphoinositide 3-kinase/Akt and then the changes in downstream pathways, such as the zonula occludens-1 and P-cadherin/β-catenin adhesive complex and cell integrity, causing hyperpermeability subsequently in podocytes.

Vitamin D might be important for maintaining podocyte health in the kidney by preventing epithelial-to-mesenchymal transformation, suppressing renin gene expression and inflammation.[11] Wang and colleagues[12] reported that 1,25-dihydroxyvitamin D suppressed high-glucose-induced apoptosis of podocytes by blocking p38- and ERK-mediated proapoptotic pathways and Sanchez-Niño and colleagues[13] showed that

vitamin D receptor activation by calcitriol and paricalcitol reduced monocyte chemoattractant protein-1, and interleukin-6 in podocytes and tubular cells as well as glomerular infiltration by macrophages, glomerular cell NF-κB activation, apoptosis, and extracellular matrix deposition. Deb and colleagues[14] demonstrated that angiotensinogen mRNA expression in the kidney was markedly increased in mice lacking the vitamin D receptor, compared with wild-type mice, and angiotensinogen induction in diabetic mice was suppressed by treatment with a vitamin D analog, indicating that vitamin D suppresses hyperglycemia-induced angiotensinogen expression by blocking NF-κB-mediated pathway.

Nevertheless, there have been scarce data regarding the effects of vitamin D on podocyte SD molecules or adaptor proteins in diabetic conditions. Verouti and colleagues[15] reported that vitamin D analogues reactivate the expression of specialized podocyte components, including podocalyxin, and Deb and coworkers[16] showed that vitamin D stimulates nephrin expression in podocytes by acting on a vitamin D response elements in the proximal nephrin promoter. Moreover, there have been no data regarding the effects of vitamin D on podocyte P-cadherin/β-catenin adhesive complex in diabetic conditions, and our results demonstrated that 1,25-dihydroxy vitamin D3 (10 nM) treatment in A30 condition significantly improved the decreased β-catenin protein levels at 6, 24, and 48 hours incubation times and also decreased podocyte permeability, indicating that vitamin D might improve podocyte hyperpermeability in diabetic conditions by restoring β-catenin.

Because the podocyte structure can be compromised by high glucose in vitro and diabetic nephropathy in vivo, and vitamin D has few side effects, vitamin D could be used in clinical practice, considering an important role in preserving structural integrity of the podocyte by acting on P-cadherin/β-catenin complex. However, there is a limitation of our study, because we performed in vitro study and the results cannot be fully applied to in vivo models. Therefore, our results should also be validated in mouse and human models of diabetic nephropathy in the future.

CONCLUSIONS

The results of our study suggest that diabetic conditions induce the distributional change and suppress the production of β-catenin in podocytes, which can be improved by vitamin D. Therefore, vitamin D may be helpful to prevent the pathological changes of podocytes in diabetic nephropathy.

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CONFLICT OF INTEREST

None declared.

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