1,25-$(OH)_2D_3$ and its analogue BXL-628 inhibit high glucose-induced activation of RhoA/ROCK pathway in HK-2 cells

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Abstract. It has previously been reported that 1,25-$(OH)_2D_3$ inhibits high glucose-induced epithelial-to-mesenchymal transition (EMT) in HK-2 cells. However, the mechanism of this renoprotective action remains unclear. Elocalcitol (BXL-628), a vitamin D analog, has been suggested to be effective on the RhoA/Rho associated protein kinase (ROCK) pathway, which serves a crucial role in high glucose-induced EMT. The aim of the present study was to investigate the effect of 1,25-$(OH)_2D_3$ and its analogue BXL-628 on high glucose-induced activation of the RhoA/ROCK pathway in human renal proximal tubular cells. HK-2 cells were co-treated with high glucose and either 1,25-(OH)2D3 or BXL-628. The RhoA expression levels and ROCK activity of the membrane were assessed via western blot analysis or immunofluorescence. α-smooth muscle actin (α-SMA) and epithelial (E)-cadherin were detected using western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), whereas collagen I and fibronectin levels were measured by ELISA and RT-qPCR. The results demonstrated that 1,25-$(OH)_2D_3$ and BXL-628 both significantly downregulated the expression of active RhoA and ROCK activity induced by high glucose ($P<0.05$). Furthermore, the expressions of α-SMA, collagen I, and fibronectin were significantly downregulated at both protein and mRNA ($P<0.05$) levels, whereas the expression of E-cadherin was significantly increased ($P<0.05$) by 1,25-$(OH)_2D_3$ or BXL-628 treatment. In conclusion, the vitamin D receptor agonist 1,25-$(OH)_2D_3$ and its analogue BXL-628 were both able to attenuate high glucose-induced EMT and extracellular matrix accumulation of HK-2 cells by suppressing the RhoA/ROCK signaling pathway *in vitro*.

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Introduction

Diabetic nephropathy (DN), a major microvascular complication of diabetes mellitus, is the leading cause of end-stage renal disease (ESRD) in the western world and the second most common cause of renal failure with increasing morbidity in China (1). Although maintenance of normoglycemia and therapeutic intervention in the renin-angiotensin-aldosterone system are effective treatments (2,3), these interventions only partially delay the progression of DN (4); therefore, identifying new therapeutic strategies remains of great importance. The pathogenesis of DN is complicated; renal fibrosis is the primary pathophysiologic process associated with chronic renal failure caused by DN (5). Epithelial-to-mesenchymal transition (EMT) and extracellular matrix (ECM) accumulation under pathological conditions also occur in mature tubular epithelial cells of the adult kidney, and have been implicated in renal interstitial fibrosis (6,7). It has previously been indicated that a large proportion of interstitial fibrosis cases originate from tubular epithelial cells via EMT in diseased kidneys (8). However, the underlying mechanisms for this remain to be elucidated.

RhoA, which is a member of the Rho guanosine triphosphatases (GTPases) family, is essential in the regulation of cellular functions (9). It cycles between an inactive GDP-bound and an active GTP-bound form, and its intrinsic hydrolytic activity is affected by various Rho regulators (10). Membrane localization via post-translational modification is required for RhoA activation (9). Previous studies have demonstrated that RhoA and its downstream kinase Rho-kinase (ROCK) are able to mediate matrix elaboration via mesangial cells (MCs) in hyperglycemic and DN conditions (4,11), and the RhoA/ROCK signaling pathway has been demonstrated to be associated with fibrosis progression in multiple organs including the kidneys, liver and lungs (4,9,12). Furthermore, RhoA/ROCK was previously reported to be associated with EMT (7,13), which was prevented by inhibition of RhoA activation in rat peritoneal cells (13). A previous study by the present authors also revealed that transforming growth factor (TGF)-β1-mediated RhoA/ROCK activation contributed to the dissolution of tight junctions during EMT in HK-2 cell *in vitro* (14).

In recent years, the protective effect of vitamin D (VitD)/VitD receptor (VDR) in patients with DN has been illustrated (15,16). Clinical treatment with active VitD is

typically accompanied by hypercalcemic side effects (17), therefore, VitD analogs with fewer side effects may provide therapeutic benefits. A previous clinical study demonstrated that treatment with paricalcitol, which is a VitD analog, was able to safely reduce residual albuminuria in patients with DN (18). Treatment with 1,25-$(OH)_2D_3$ (also known as calcitriol) or its analogs is able to repress the expression of α-SMA and collagen I in a unilateral ureteral occlusion model and cultured HK-2 cells (19,20). The renoprotective role of 1,25-$(OH)_2D_3$ and its analogs have been demonstrated to be associated with anti-inflammation, renin-angiotensin system (RAS) inhibition and prevention of EMT (17,20-22). By binding to the VDR, 1,25-$(OH)_2D_3$ recruits cofactors to form a transcriptional complex which subsequently binds to VitD response elements in the promoter region of target genes, altering transcriptional events within target cells (17,20,23). However, the underlying mechanism of this renoprotective effect remains largely unknown at present.

Morelli *et al* (24) illustrated that treatment with elocalcitol (BXL-628), which is a VitD analog and VDR agonist, impaired RhoA membrane translocation and inhibited RhoA/ROCK activation in bladder smooth muscle cells, repressing RhoA-mediated cell migration and cytoskeleton remodeling (24,25). However, whether the anti-EMT and anti-fibrosis role of 1,25-$(OH)_2D_3$ and analogs is associated with the RhoA/ROCK signaling pathway remains to be elucidated.

The aim of the present study was to investigate the effect of the endogenous VDR ligand 1,25-$(OH)_2D_3$ and its analog BXL-628 on high glucose-induced activation of the RhoA/ROCK pathway in human renal proximal tubular cells. The results of the preset study may potentially elucidate a mechanism of the protective effect of VitD in DN.

Materials and methods

Cell culture and treatment. Human renal proximal tubular epithelial cells, HK-2 (ATCC, Manassas, VA, US), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), streptomycin (100 μg/ml) and penicillin (100 U/ml) in a humidified atmosphere containing 5% CO_2 at 37˚C. When cells reached 80% confluence, they were treated with high glucose (HG group; 30 mmol/l; Henyang Kaixin Chemical Reagent Company, Ltd., Hunan, China) for 0, 0.5, 1, 2, 4, 8 and 12 h to verify whether high glucose conditions activated RhoA. To determine the time at which high glucose induced EMT, HK-2 cells were treated with high glucose for 0, 12, 24, 48 and 72 h at 37˚C respectively; mannitol (30 mmol/l; Henyang Kaixin Chemical Reagent Company, Ltd.) was used as negative control. For further analysis, HK-2 cells were cultured in high glucose medium (supplemented DMEM with 30 mmol/l glucose) at 37˚C, and co-treated with 1,25-$(OH)_2D_3$ (VD3 group; 100 nM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), or BXL-628 (BXL group; 10 nM; Molecular Devices, LLC, Sunnyvale, CA, USA) at 2 or 48 h, respectively, according to the results of the previous experiment. Low glucose (5.6 mmol/l) was used as the normal control (NC) group.

Western blotting. Total protein was extracted from indicated cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and membrane protein was extracted using a Plasma Membrane Protein Extraction kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol. A total of 50 μg total protein or membrane protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat milk, subsequently rinsed and incubated overnight at 4˚C with anti-RhoA (1:1,000; #2117; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-α-SMA (1:3,000; sc-32251; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-epithelial (E)-cadherin (1:3,000; #3195; Cell Signaling Technology, Inc.), anti-ATPase Na^+/K^+ (1:4,000; ab7671; Abcam, Cambridge, MA, USA), anti-phosphorylated-myosin-binding subunit (MBS) (1:1,000; sc-514261; Santa Cruz Biotechnology, Inc.), anti-ROCK (1:1,000; sc-17794; Santa Cruz Biotechnology, Inc.) and anti-β-actin antibodies (1:5,000; PA1-183; Invitrogen; Thermo Fisher Scientific, Inc.). Membranes were subsequently washed with TBST and incubated with the appropriate secondary antibody (goat anti-rabbit or goat anti-mouse; CW0114S and CW0102S, respectively; 1:3,000; Kangwei Biotech Company, Beijing, China) for 2 h at room temperature. Enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) was used to detect the signal on the membrane. Data was analyzed using densitometry with Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and normalized to β-actin or ATPase Na^+/K^+ expression.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde (pH 7.4) at room temperature for 30 min, permeabilized for 10 min with PBS containing 0.1% Triton X-100, rinsed with PBS and subsequently incubated with blocking buffer (normal goat serum; CW0130S; Kangwei Biotech Company) at room temperature for 30 min. Immunostaining was performed as previously described (24) using anti-pan-cadherin (1:100; ab195203; Abcam) and anti-RhoA antibodies (1:100; ab54835; Abcam), followed by tetramethylrhodamine-conjugated AffiniPure (red) goat anti-mouse (1:200; SA00007-1; Proteintech Group, Inc., Chicago, IL, USA) and fluorescein isothiocyanate-conjugated AffiniPure (green) goat anti-rabbit (1:200; SA00003-2; Proteintech Group, Inc.) antibodies, respectively. A fluorescent microscope (BX61 Automated Fluorescent Microscope; Olympus Corporation, Tokyo, Japan) was use to observe the cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total mRNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 2 μg RNA was used for cDNA synthesis with a ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. Total RNA was treated with DNase I prior to transcription according the kit manual. A total reaction volume of 20 μl was used for PCR with a Taq DNA polymerase-based 2x master mix for real-time PCR (SYBR®-Green Real-time PCR Master Mix, Toyobo Co., Ltd.) using the iQ5 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

mRNA	Primer sequences	Primer length (bp)
α-SMA	Forward: 5'-TTCAATGTCCCAGCCATGTA-3' Reverse: 5'-GAAGGAATAGCCACGCTCAG-3'	125
E-cadherin	Forward: 5'-TGCCCAGAAAATGAAAAAGG-3' Reverse: 5'-GTGTATGTGGCAATGCGTTC-3'	120
Collagen I	Forward: 5'-CCAAATCTGTCTCCCCAGAA-3' Reverse: 5'-TCAAAAACGAAGGGGAGATG-3'	136
Fibronectin	Forward: 5'-GCTTCCTGGCACTTCTGGTC-3' Reverse: 5'-CTACATTCGGCGGGTATGGT-3'	136
β-actin	Forward: 5'-CATCCTGCGTCTGGACCTGG-3' Reverse: 5'-TAATGTCACGCACGATTTCC-3'	107

SMA, smooth muscle actin; E, epithelial.

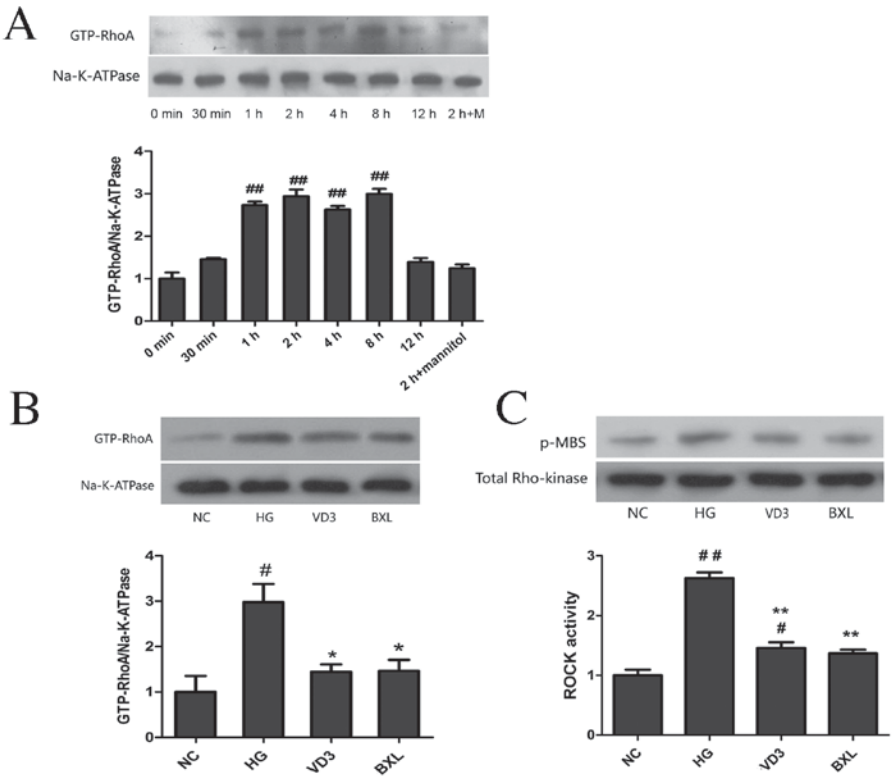

Figure 1. Effect of 1,25-$(OH)_2D_3$ and BXL-628 on GTP-RhoA and active ROCK expression. (A) GTP-RhoA expression in HK-2 cells exposed to high glucose for 0, 0.5, 1, 2, 4, 8 and 12 h. (B) Expression of GTP-RhoA protein in HK-2 cells from NC, HG, VD3 and BXL groups following 2 h treatment. (C) ROCK activity in HK-2 cells from NC, HG, VD3 and BXL groups following 2 h treatment. ROCK activity was assessed p-MBS. Data are expressed as the mean ± standard error of the mean (n=3). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. NC group; *P<0.05, **P<0.01 vs. HG group. GTP-RhoA, activated RhoA; ROCK, Rho associated protein kinase; NC, normal control group; HG, high glucose group; VD3, 1,25-$(OH)_2D_3$ group; BXL, BXL-628 group; p-MBS, phosphorylated myosin-binding subunit.

The sequence detector was programmed for the following PCR conditions: 50˚C for 2 min, 95˚C for 10 min, 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The relative expressions of α-SMA, collagen I, fibronectin and E-cadherin mRNA were expressed relative to β-actin as an internal control, using the $2^{-\Delta\Delta Cq}$ method (26). Oligo 6.0 software (Molecular Biology Insights, Inc., Cascade, CO, USA) was used to design PCR primers (Table I), which were synthesized by Shanghai Sangong Pharmaceutical Co., Ltd. (Shanghai, China).

ELISA assay. The concentrations of collagen I and fibronectin secreted from cultured cells in medium were determined using an ELISA kits (CSB-E13445 h and CSB-EL005721HU; Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocol. The final absorbance at 450 nm was measured using TECAN Infinite M200 microplate reader (Thermo Fisher Scientific, Inc.). Concentrations of collagen I and fibronectin were calculated using a standard curve constructed in the same plate, and expressed relative to the cell protein concentration.

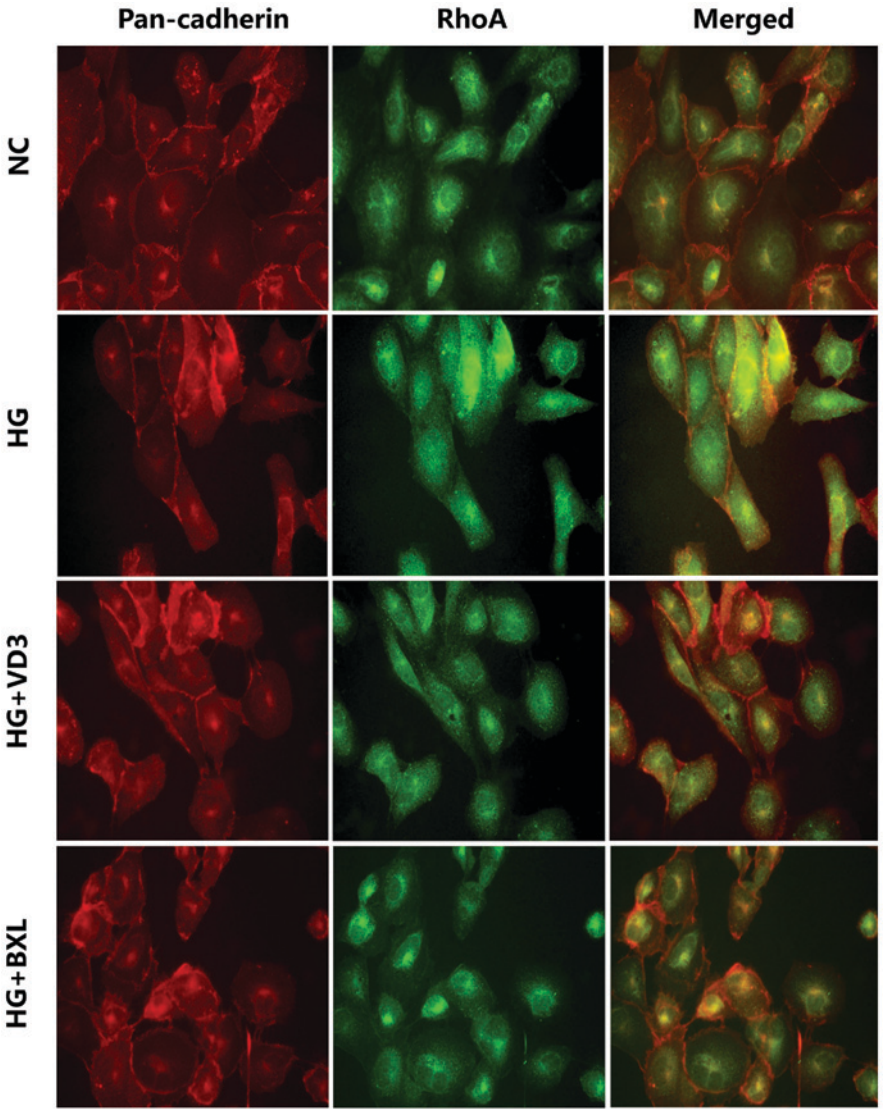

Figure 2. 1,25-$(OH)_2D_3$ and BXL-628 inhibit RhoA translocation from the cytosol to the plasma membrane. Confocal immune localization by dual labelling of pan-cadherin (red, tetramethylrhodamine) and RhoA (green, fluorescein isothiocyanate) in HK-2 cells from NC, HG, VD3 and BXL groups following 2 h treatment. Yellow indicates co-localization of RhoA and pan-cadherin at plasma membrane level. Cells were analyzed by confocal microscopy and individual and merged stains are shown. Magnification, x400. ROCK, Rho associated protein kinase; NC, normal control group; HG, high glucose group; VD3, 1,25-$(OH)_2D_3$ group; BXL, BXL-628 group.

Statistical analysis. All experiments were performed at least 3 times. GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for the analysis and graphics presentation. One-way analysis of variance followed by Tukey's multiple comparisons test or Student's t-test was used depending on the experimental condition. The data were expressed as mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

1,25-$(OH)_2D_3$ and BXL-628 decreases RhoA activation and ROCK activity in HK-2 cells. Using western blot analysis, it was demonstrated that high glucose conditions induced RhoA activation in a time-dependent manner, which was significantly increased from 1 to 8 h ($P<0.01$) compared with the control group, reaching a peak at 2 h and returning to the control level at 12 h post-administration (Fig. 1A). The effects of 1,25-$(OH)_2D_3$ and its analog BXL-628 on HK-2 cells treated with high glucose for 2 h were also investigated. As illustrated in Fig. 1B, active RhoA protein expression in the HG group was significantly upregulated compared with the NC group ($P<0.05$), and treatment with 1,25-$(OH)_2D_3$ or BXL-628 significantly decreased the expression of active RhoA protein (both $P<0.05$). Additionally, ROCK activity was evaluated by measuring the phospho-MBS level (Fig. 1C). It was revealed that ROCK activity was significantly lower in the VD3 and BXL groups compared with the high glucose group ($P<0.01$).

Indirect immunofluorescence also revealed that expression of active RhoA protein was markedly reduced in the VD3 and BXL groups compared with the NC group (Fig. 2). The intracellular localization of RhoA was monitored by staining with a specific monoclonal antibody and compared with pan-cadherin immune reactivity as a cell membrane marker. The merged

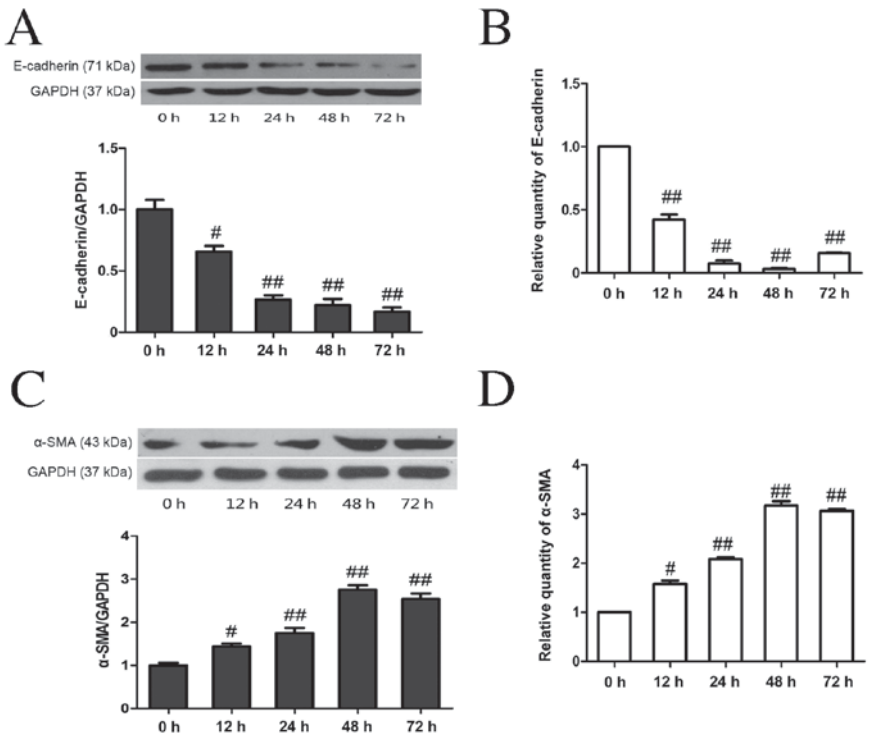

Figure 3. Effect of high glucose on E-cadherin and α-SMA expression. (A) E-cadherin expression in HK-2 cells exposed to high glucose for 0, 12, 24, 48 and 72 h. (B) Relative expression of E-cadherin mRNA. (C) α-SMA expression in HK-2 cells exposed to high glucose for 0, 12, 24, 48 and 72 h. (D) Relative expression of α-SMA mRNA. Data are expressed as the mean ± standard error of the mean (n=3). $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. 0 h. E, epithelial; SMA, smooth muscle actin.

images obtained by dual labeling of RhoA and pan-cadherin immune staining confirmed that high glucose stimulation increased RhoA localization at the plasma membrane, whereas treatment with 1,25-$(OH)_2D_3$ or BXL-628 treatment reduced RhoA membrane expression.

Effects of 1,25-$(OH)_2D_3$ and BXL-628 on the expression of E-cadherin and α-SMA. As shown in Fig. 3, high glucose stimulation resulted in a significant time-dependent decrease in E-cadherin protein levels (P<0.05 at 12 h, P<0.01 from 24-72 h; Fig. 3A) and mRNA (P<0.01; Fig. 3B) compared with the NC group, whereas high glucose stimulation resulted in a significant time-dependent increase in α-SMA at both the protein and mRNA levels (both P<0.05 at 12 h, P<0.01 from 24-72 h; Fig. 3C-D). Treatment with either 1,25-$(OH)_2D_3$ or BXL-628 attenuated the downregulation in E-cadherin expression at the protein (P<0.05; Fig. 4A) and mRNA levels (P<0.01; Fig. 4B) compared with the HG group. Both 1,25-$(OH)_2D_3$ and BXL-628 treatments significantly inhibited the expression of α-SMA at the protein (P<0.05; Fig. 4C) and mRNA (P<0.01; Fig. 4D) levels compared with the HG group (Fig. 4B and D).

Effects of 1,25-$(OH)_2D_3$ and BXL-628 on the expression of ECM in HK-2 cells. To further analyze the effects of 1,25-$(OH)_2D_3$ and its analog BXL-628 on ECM accumulation induced by high glucose, the expressions of collagen I and fibronectin in the culture supernatant were detected using ELISA. Cellular mRNA expression was evaluated using qPCR. The results indicated that high glucose stimulation significantly increased the expression of collagen I protein (P<0.05; Fig. 5A) and mRNA (P<0.01; Fig. 5B) compared with the NC group. High glucose stimulation also induced a significant increase in fibronectin protein (P<0.05; Fig. 5C) and mRNA (P<0.01; Fig. 5D) compared with the NC groups. Notably, both 1,25-$(OH)_2D_3$ and BXL-628 significantly decreased the expression of collagen I and fibronectin compared with the HG group (P<0.01 for collagen protein, collagen mRNA and fibronectin mRNA; P<0.05 for fibronectin protein; Fig. 5A-D).

Discussion

The present study demonstrated that 1,25-$(OH)_2D_3$ and its analog BXL-628 contribute to renoprotection by preventing high glucose-induced EMT and ECM accumulation via inactivating the RhoA/ROCK signaling pathway *in vitro*. Hyperglycemia is one of the most important risk factors in diabetes and its complications, including DN (1,5). Hyperglycemic conditions activate a number of important intracellular signaling pathways, including the TGF-β/SMAD, nuclear factor-κB and Rho/ROCK pathways (12,15,27,28), which serve crucial roles in the diabetic renal inflammation response, RAS activation, EMT and fibrosis (3,8,19,29). VitD and VDR have previously been demonstrated to have renal protective functions via their regulative role in pathways associated with the prevention of EMT and fibrosis (17,20,30,31). Peng *et al* (4) illustrated that high glucose was able to activate RhoA/Rho-kinase in MCs, leading to downstream activator protein-1 activation and fibronectin induction (4). In accordance with the studies mentioned above, the results of the present study also demonstrated that high glucose-induced activation of the RhoA/ROCK pathway subsequently resulted in EMT and ECM accumulation in HK-2 cells. Furthermore, treatment

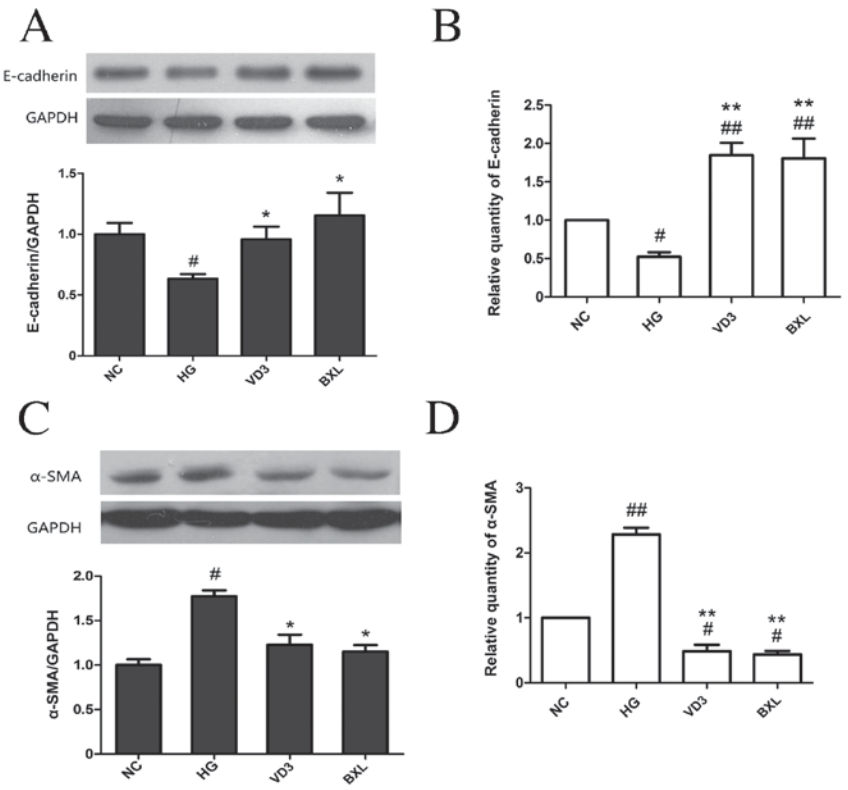

Figure 4. The effect of 1,25-$(OH)_2D_3$ and BXL-628 on E-cadherin and α-SMA expression. (A) Expression of E-cadherin protein in HK-2 cells from NC, HG, VD3 and BXL groups following 48 h treatment. (B) Relative expression of E-cadherin mRNA. (C) Expression of α-SMA protein in HK-2 cells from NC, HG, VD3 and BXL groups following 48 h treatment. (D) Relative expression of α-SMA mRNA. Data are expressed as the mean ± standard error of the mean (n=3). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. NC group; $^{*}P<0.05$, $^{**}P<0.01$ vs. HG group. E, epithelial; SMA, smooth muscle actin; NC, normal control group; HG, high glucose group; VD3, 1,25-$(OH)_2D_3$ group; BXL, BXL-628 group.

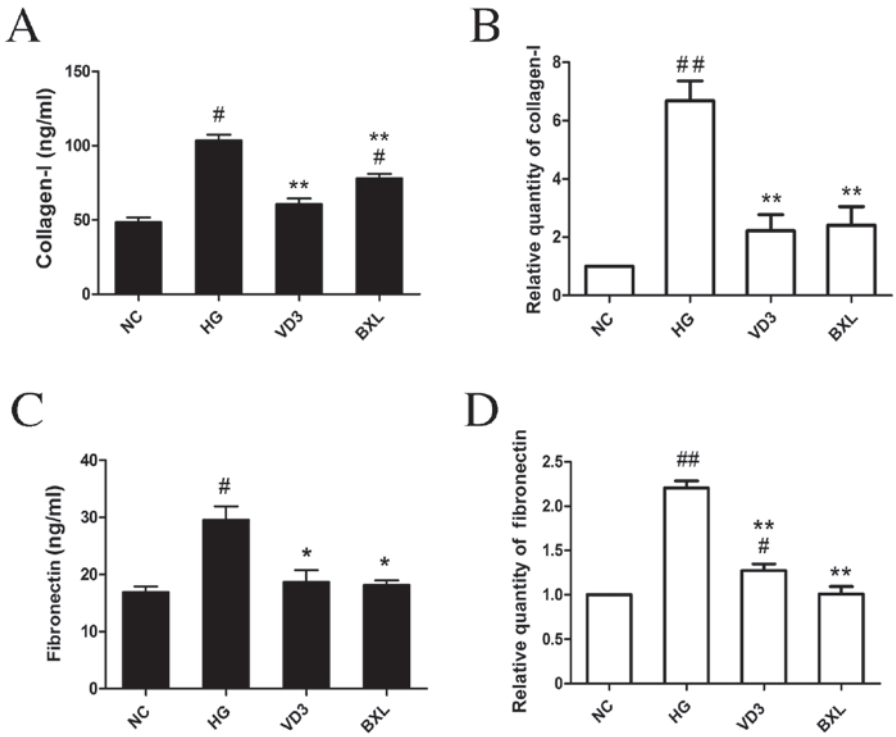

Figure 5. The effect of 1,25-$(OH)_2D_3$ and BXL-628 on collagen I and fibronectin expression. (A) Protein level of collagen I in the culture supernatant of HK-2 cells from NC, HG, VD3 and BXL groups following 48 h treatment. (B) Relative expression of collagen I mRNA. (C) Protein level of fibronectin in the culture supernatant of HK-2 cells from NC, HG, VD3 and BXL groups following 48 h treatment. (D) Relative expression of fibronectin mRNA. Data are expressed as the mean ± standard error of the mean (n=4). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. NC group; $^{*}P<0.05$, $^{**}P<0.01$ vs. HG group. NC, normal control group; HG, high glucose group; VD3, 1,25-$(OH)_2D_3$ group; BXL, BXL-628 group.

with 1,25-$(OH)_2D_3$ or BXL-628 significantly inactivated the RhoA/ROCK pathway and attenuated high glucose-induced EMT and ECM accumulation in HK-2 cells. The present study found that 1,25-$(OH)_2D_3$ and BXL-628 treatment was able to decrease the high glucose-stimulated upregulation of α-SMA and downregulation of E-cadherin. Additionally, fibronectin and collagen I accumulation were significantly suppressed in both the VD3 and BXL groups compared with the HG group.

VDR acts as a transcriptional factor and a nongenomic activator of the RhoA/ROCK and p38MAPK/mitogen and stress activated kinase (MSK)1 pathways, which are required for the biofunction of 1,25-$(OH)_2D_3$ in colon carcinoma cells (32). By binding to VDR, 1,25-$(OH)_2D_3$ induces an influx of Ca^{2+} into cytoplasm and subsequently activates the RhoA/ROCK and p38MAPK-MSK1 kinase pathways (32). This indicates that the rapid modulation of ion content and cytosolic GTPases and kinases is associated with EMT and ECM accumulation (33,34). However, the present study demonstrated that 1,25-$(OH)_2D_3$ acts as an inhibitor of RhoA/ROCK and its role may depend on cell types. In accordance with the results of the present study, it has previously been demonstrated that BXL-628 is able to inhibit RhoA activation in a dose-dependent manner without altering RhoA expression, and ameliorates excessive hyperplasia and aberrant differentiation in bladder smooth muscle cells (24,25). In the present study, 1,25-$(OH)_2D_3$ exerted a similar effect to BXL-628 at a much higher concentration. This suggests that BXL-626 may be a more efficient pharmaceutical choice, although further beneficial value must be investigated through more clinical studies and fundamental research. 1,25-$(OH)_2D_3$ and BXL-628 do not alter the expression of RhoA and ROCK, however they do increase their membrane translocation and binding to GTP (24,35).

In conclusion, the results of the present study demonstrate that 1,25-$(OH)_2D_3$ and BXL-628 treatment repress the high glucose-activated RhoA/ROCK pathway in HK-2 cells. Additionally, activation of the VitD/VDR pathway by treatment with 1,25-$(OH)_2D_3$ or BXL-628 decreased the expression of α-SMA, collagen I and fibronectin, and increased E-cadherin expression; this is suggestive of an anti-EMT and anti-fibrosis role of 1,25-$(OH)_2D_3$ and BXL-628. These results indicate that the renal protective effects of 1,25-$(OH)_2D_3$ and its analog are mediated, at least in part, by the RhoA/ROCK signaling pathway.

Acknowledgements

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