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1,25(OH)$_2$ D$_3$ attenuates indoxyl sulfate–induced epithelial-to-mesenchymal cell transition via inactivation of PI3K/Akt/β-catenin signaling in renal tubular epithelial cells

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ABSTRACT

Objectives: Indoxyl sulfate (IS), a uremic toxin, has been shown to promote the epithelial-to-mesenchymal transition (EMT) of human proximal tubular cells and to accelerate the progression of chronic kidney disease (CKD). Despite the well-known protective role of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$ D$_3$] in EMT, the effect of 1,25(OH)$_2$ D$_3$ on IS-induced EMT in human proximal tubular epithelial cells and the underlying mechanism remain unclear. The aim of this study was to determine whether IS (0-1 mM) dose-dependently inhibited the protein expression of E-cadherin and increased the protein expression of alpha-smooth muscle actin, N-cadherin, and fibronectin.

Methods: This study investigated the molecular mechanism by which 1,25(OH)$_2$ D$_3$ attenuates IS-induced EMT. HK-2 human renal tubular epithelial cells was used as the study model, and the MTT assay, Western Blotting, siRNA knockdown technique were used to explore the effects of 1,25(OH)$_2$ D$_3$ on EMT in the presence of IS.

Results: Pretreatment with 1,25(OH)$_2$ D$_3$ inhibited the IS-induced EMT-associated protein expression in HK-2 cells. IS induced the phosphorylation of Akt (S473) and β-catenin (S552) and subsequently increased the nuclear accumulation of β-catenin. Pretreatment with 1,25(OH)$_2$ D$_3$ and LY294002 (phosphoinositide 3-kinase [PIK3] inhibitor) significantly inhibited the IS-induced phosphorylation of Akt and β-catenin, nuclear β-catenin accumulation, and EMT-associated protein expression.

Conclusions: Results from the present study revealed that the anti-EMT effect of 1,25(OH)$_2$ D$_3$ is likely through inhibition of the PI3K/Akt/β-catenin pathway, which leads to down-regulation of IS-driven EMT-associated protein expression in HK-2 human renal tubular epithelial cells.

Introduction

Chronic kidney disease (CKD) is characterized by the progressive deterioration of renal function that ultimately leads to end-stage renal disease, requiring dialysis or kidney transplantation. Regardless of the primary cause, CKD is represented by progressive scarring and irreversible fibrosis that accelerates kidney dysfunction. More recently, research on CKD has focused on the glomerular pathologic injury inherent to the disease [1,2]. Studies have reported that tubular injury plays an important role in the progression of CKD, and evidence suggests that the epithelial-mesenchymal transition (EMT)

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of tubular epithelial cells (TECs) is one of the leading causes of renal fibrosis in CKD [3,4].

EMT is a crucial process during embryonic development, wound healing, and tissue regeneration but also has been implicated in pathophysiologic processes, including tumor metastasis [5] and organ fibrosis [6]. During EMT, cells lose their adhesive properties through down-regulation of E-cadherin and display mesenchymal properties such as an increase in the expression of α-smooth muscle actin (α-SMA), N-cadherin, fibronectin, and vimentin in human renal TECs [7,8]. In in vitro studies, several signaling pathways, such as Wnt/β-catenin [5], toll-like receptor 4/nuclear factor-κB [8], RhoA/Rho associated protein kinase (ROCK) signaling pathway [9], participate in the regulation of EMT-associated protein expression under different experimental conditions.

Indoxyl sulfate (IS), one of the circulating uremic toxins, is known to induce glomerular sclerosis and interstitial fibrosis, which gradually leads to renal injury and dysfunction [10]. During the progression of CKD, the expression of transforming growth factor (TGF)-β1, tissue inhibitors of metalloproteinase (TIMP)-1, and α-SMA induced by IS causes EMT [3,11]. Although that study indicated that IS-treated cells showed significantly reduced protein expression of E-cadherin and zonula occluden-1 (ZO-1) and significantly increased protein expression of α-SMA in HK-2 cells and in the kidneys of hypertensive rats [11], the molecular mechanisms of the induction of EMT by IS are not fully elucidated.

In humans, the main source of vitamin D is from photochemical synthesis by ultraviolet irradiation of 7-dehydrocholesterol in the skin. An additional source is from dietary intake. Vitamin D, an inactive precursor, is further metabolized to 25-hydroxyvitamin D [25(OH)D_3] and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3] in liver and kidney, respectively. Although the half-life of 1,25$(OH)_2$ D_3 in serum is shorter than that of 25(OH)D_3, the physiologic activity of 1,25$(OH)_2$ D_3 is greater than that of 25(OH)D_3 [12].

In addition to maintaining calcium homeostasis and bone health [13], 1,25$(OH)_2$ D_3 also possesses antitumor [14], anti-inflammatory [15], immunomodulation [16], cardiovascular protective [17], and renoprotective properties [18]. Severe vitamin D deficiency is common in patients with CKD and in those receiving dialysis [19]. Although use of vitamin D supplementation is of great benefit to protect against CKD and renal fibrosis [20], the precise mechanism is not well understood. In the present study, therefore, we investigated the inhibition of EMT by 1,25$(OH)_2$ D_3 in IS-induced HK-2 cells and the possible mechanisms involved.

Materials and methods

Reagents

Phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 1,25$(OH)_2$ D_3 were from Sigma-Aldrich (St. Louis, MO, USA); Dulbecco's Modified eagle Medium (DMEM), fetal bovine serum (FBS), 25% trypsin-EDTA, and penicillin–streptomycin solution were from GIBCO/BRL (Grand Island, NY, USA); SP600125 (JNK inhibitor II), PD98059 (ERK inhibitor), and SB203580 (p38 inhibitor) were from TOCRIS (Ellisville, MO, USA); SB431542 (TGF-β type 1 receptor inhibitor) was from Abcam (Cambridge, MA, USA); antibodies against E-cadherin (GTX100443), N-cadherin (GTX127345), and fibronectin (GTX112794) were from GeneTex Inc (Irvine, CA, USA); antibodies against α-SMA (#14968) and β-catenin Ser552 (#5651) were from Cell Signaling Technology (Beverly, MA, USA); antibody against Akt/PKB (#05-591), β-catenin (#06734), β-actin (#MAB1501) were from Millpore (Billerica, MA, USA). Antibody against poly (ADP-ribose) polymerase (PARP; 11 835 238 001) was from Roche (Darmstadt, Germany). Antibody against phosphor-Akt Ser473 (STJ97726) was from St John's Laboratory (London, UK).

Cell culture

HK-2 cells purchased from Bioresources Collection and Research Center (BCRC, Hsin-Chu, Taiwan) were maintained in DMEM/F12 supplemented with 10% FBS, insulin-transferrin-selenium, 100 U/mL penicillin, and 100 mg/mL streptomycin and were incubated at 37°C in a 5% carbon dioxide humidified incubator.

Cell viability assay

HK-2 cells were grown to 70% to 80% confluence and were then treated with or without various concentrations of 1,25$(OH)_2$ D_3 for 24 h. Afterward, cell viability assays were performed according to a previous study [21].

Cytosolic and nuclear extracts and whole cell protein preparation

After each experiment, cells were washed twice with cold phosphate-buffered saline (PBS) and were then scraped from the dishes with PBS. Cell homogenates were centrifuged at 2000*g* for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 350 μL of hypotonic extraction buffer containing 10 mM HEPES, 10 mM potassium chloride (KCl), 1 mM magnesium chloride ($MgCl_2$), 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 7000*g* for 15 min, the resulting supernatant was used as the cytosolic fraction for Western blotting and the pellets containing nuclei were extracted by gentle mixing with 50 μL of hypertonic extraction buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol at 4°C for 30 min. The samples were then centrifuged at 10 000*g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at −80°C until Western blotting was performed. For whole cell protein preparations, cells were washed twice with cold PBS and were harvested in 100 μL of 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9000*g* for 30 min at 4°C. The supernatant was used as whole cell proteins for Western blotting.

Western blotting

The protein content in each sample was quantified by use of the Coomassie Plus Protein Assay Reagent Kits (Pierce Chemical Company, Rockford, IL, USA). Protein aliquots were denatured and separated on 7.5% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes (New Life Science Product, Inc., Boston, MA, USA). The nonspecific binding sites in the membranes were blocked with 5% nonfat dry milk in 15 mM Tris-150 mM sodium chloride buffer (pH 7.4) at 4°C overnight. After blocking, the membrane was incubated with anti–E-cadherin (1:1000), anti–N-cadherin (1:1000), anti–α-SMA (1:1000), anti-fibronectin (1:1000), anti-PARP (1:1000), anti–β-catenin (1:1000), and anti–phospho-β-catenin (Ser552; 1:1000) or anti-Akt (1:1000), anti–phospho-Akt (Ser473; 1:1000), and anti–β-actin (1:4000) antibodies at 4°C overnight. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated antirabbit (1:6000) or antimouse immunoglobulin G (1:6000) at 37°C for 1 h, and the immunoreactive bands were developed by use of the Western Lightning Plus-ECL kit (PerkinElmer, Waltham, MA, USA). Band intensities was calculated using AlphaEaseFC software version 6.0.0 (Alpha Innotech, San Leandro, CA, USA).

Measurement of extracellular TGF-β concentrations

The cells were pretreated with 50 nM 1,25$(OH)_2$ D_3 for 1 h and then incubated with 1 mM IS for indicated time. Extracellular TGF-β concentrations were measured by using the TGF-β enzyme immunoassay kit (Invitrogen, Carlsbad, CA, USA).

RNA interference by small interfering RNA of β-catenin

Small interfering RNA (siRNA) for Twist1 was predicted and synthesized by MDbio Inc. (Taipei, Taiwan). The cells were grown to 60% to 70% confluence in 35-mm plates and were transfected with β-catenin siRNA or non-targeting siRNA (negative control) by use of Lipofectamine RNAi MAX Transfection Reagent (Invitrogen). β-catenin siRNA or negative siRNA was diluted in 50 μL Opti-Minimal Essential Medium (MEM), respectively, and mixed with 2 μL of transfection reagent diluted in 98 μL of Opti-MEM. After incubation for 30 min at room temperature, the mixture was added to 800 μL of Opti-MEM and applied to the cells (1 mL/plate). After 8 h of transfection, the transfection reagent-containing medium was replaced with 10% FBS-containing DMEM/F12 medium for another 24 h, and then the cells were treated as indicated in the experimental design.

Statistical analysis

Data are shown as mean ± SD. Statistical significance among group means was determined by one-way analysis of variance followed by Tukey's test, and the difference between mean values was determined by Student's *t* test (version 10; SPSS, Chicago, IL, USA). $P<0.05$ were taken to be statistically significant.

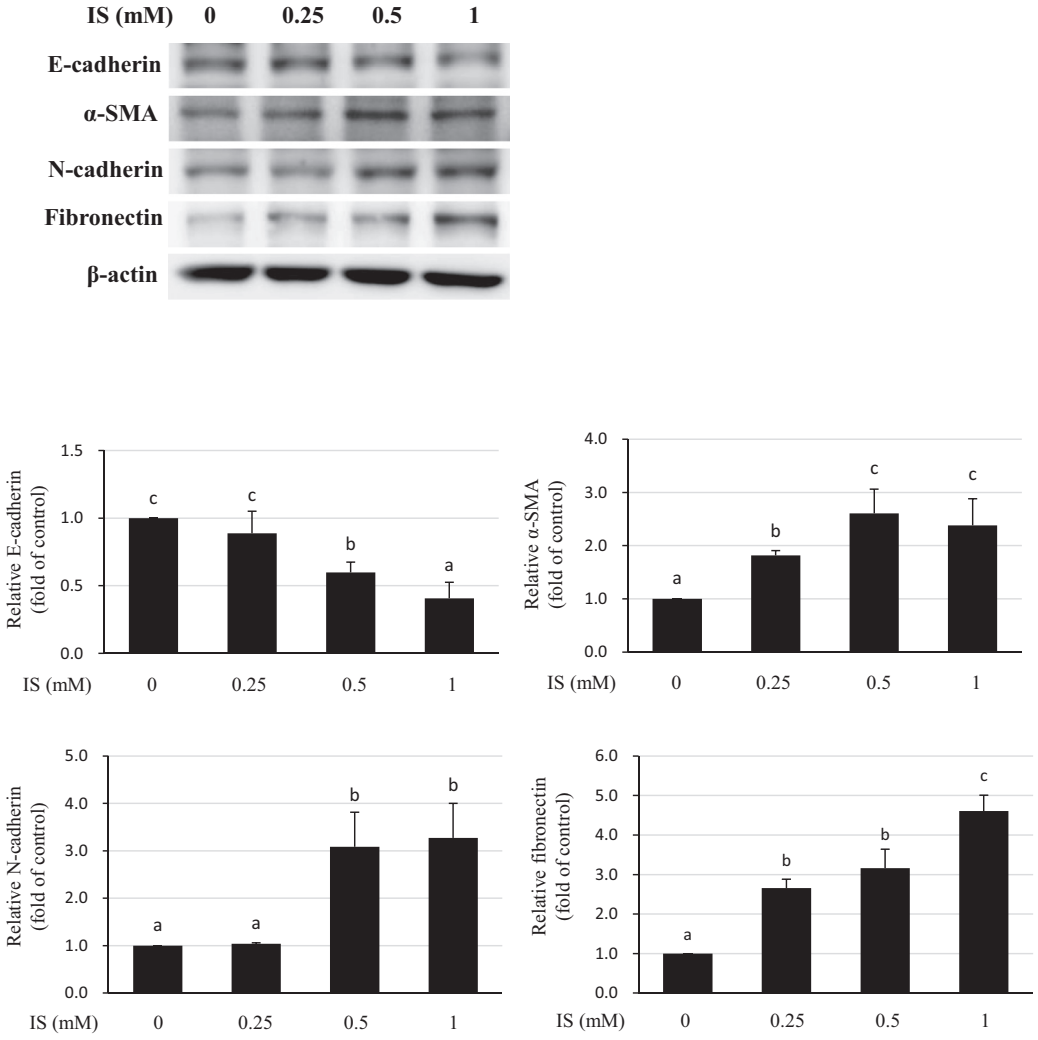

Fig. 1. Effect of IS on expression of E-cadherin, α-SMA, N-cadherin, and fibronectin in HK-2 cells. Cells were treated with indicated concentrations of IS for 24 h. Cells were harvested and aliquots of whole cell protein (10 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown. β-actin was used as internal loading control for whole cell protein. Bands were quantified and normalized to loading control bands on the same blot. Values are means ± SD, n = 3. Bars with different letters are significantly different ($P < 0.05$). α-SMA, alpha-smooth muscle actin; IS, indoxyl sulfate.

Results

Effect of IS on E-cadherin, α-SMA, N-cadherin, and fibronectin expression in HK-2 cells

As measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cell viabilities of HK-2 cells treated with IS alone and IS plus 1, 10, and 50 nM 1,25 $(OH)_2$ D_3 were 101.2% ± 1.9%, 102.3% ± 5%, 99.3% ± 0.3%, and 96.4% ± 1.4%, respectively, compared with the unstimulated controls (100%). These results indicated that there were no adverse effects on the growth of cells up to a concentration of 50 nM 1,25$(OH)_2$ D_3 in the presence of 1 mM of IS. In the following experiments, therefore, 1 mM of IS was used to induce EMT-associated protein expression and the highest concentration of 1,25$(OH)_2$ D_3 was set at 50 nM.

E-cadherin, α-SMA, N-cadherin, and fibronectin are recognized to play crucial roles in the EMT of TECs during renal fibrosis [8]. To clarify the role of IS in the induction of EMT, we treated HK-2 human renal tubular cells with increasing concentrations of IS (0−1 mM) for 24 h. As shown in Figure 1, IS dose-dependently suppressed the protein expression of E-cadherin, but increased the protein expression of mesenchymal markers, such as α-SMA, N-cadherin, and fibronectin. The highest concentration of IS for induction of EMT-associated proteins used in the present study was 1 mM.

1,25$(OH)_2$ D_3 suppresses IS-induced EMT-associated protein expression in HK-2 cells

A previous study showed that 1,25$(OH)_2$ D_3 can inhibit the EMT in human bronchial epithelial cells [18]. We used various concentrations of 1,25$(OH)_2$ D_3 to demonstrate the role of 1,25$(OH)_2$ D_3 in protection against IS-induced EMT in HK-2 cells. As noted in Figure 2, 1 mM of IS significantly inhibited E-cadherin expression and induced protein expression of α-SMA and fibronectin, and this induction was significantly reversed by pretreatment with 10 and 50 nM 1,25$(OH)_2$ D_3. However, treatment with 1,25$(OH)_2$ D_3 did not inhibit IS-induced N-cadherin protein expression in HK-2 cells (Fig. 2).

1,25$(OH)_2$ D_3 and β-catenin siRNA abolish IS-induced accumulation of nuclear β-catenin and EMT-associated protein expression in HK-2 cells

β-catenin is necessary for the induction of α-SMA and fibronectin [22,23]. In this study, the nuclear accumulation of β-catenin

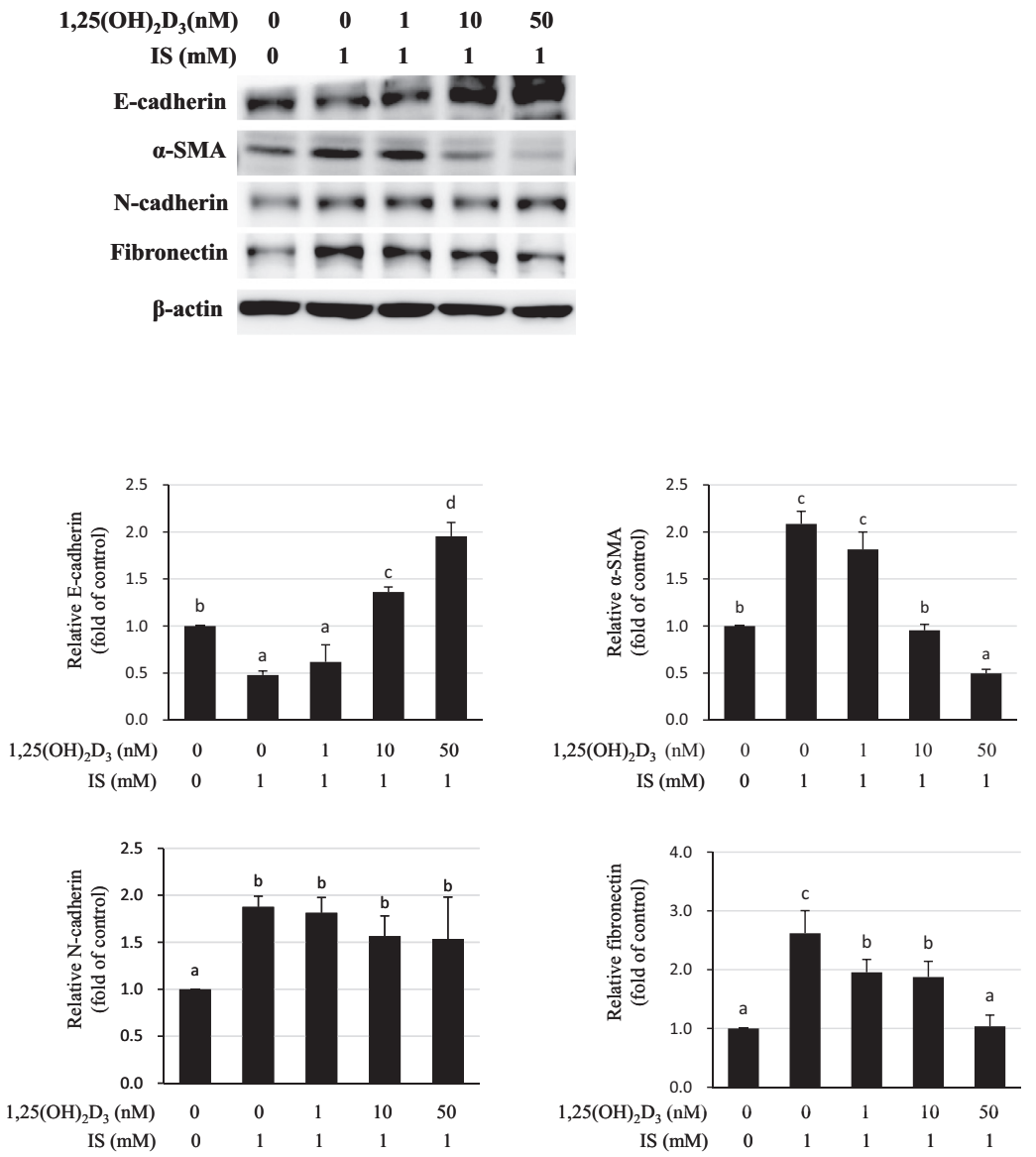

Fig. 2. Effect of 1,25(OH)$_2$ D$_3$ on IS-modulated expression of E-cadherin, α-SMA, N-cadherin, and fibronectin in HK-2 cells. After attachment, cells were pretreated with or without 1, 10, or 50 nM 1,25(OH)$_2$ D$_3$ for 1 h before the addition of IS. Twenty-four hours after IS addition, cells were harvested and aliquots of whole cell protein (10 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown. β-actin was used as internal loading control for whole cell protein. Bands were quantified and normalized to loading control bands on the same blot. Values are means ± SD, n = 3. Bars with different letters are significantly different ($P < 0.05$). 1,25(OH)$_2$ D$_3$, 1,25-dihydroxyvitamin D$_3$; α-SMA, alpha-smooth muscle actin; IS, indoxyl sulfate.

was significantly increased by IS in a time-dependent manner, and the maximum induction of the nuclear β-catenin level appeared at 8 h after IS treatment (Fig. 3A). When cells were pretreated with 1,25(OH)$_2$ D$_3$, the IS-induced increase in the nuclear β-catenin level was dose-dependently attenuated (Fig. 3B). After knockdown of β-catenin expression by siRNA transfection, the IS-induced expression of α-SMA and fibronectin was eliminated and the inhibition of E-cadherin by IS was reversed (Fig. 3C).

1,25(OH)$_2$ D$_3$ attenuated the IS-induced phosphorylation of β-catenin at Ser552 in HK-2 cells

Phosphorylation of β-catenin at Ser552 (-Ser522-β-catenin) has been found to promote translocation of β-catenin into the nucleus, resulting in transcriptional upregulation of downstream genes [24]. As noted, the phosphorylation of β-catenin at Ser552 significantly increased by IS in a time-dependent manner (Fig. 4A), and the induction of p-Ser522-β-catenin was dramatically attenuated by 1,25(OH)$_2$ D$_3$ (Fig. 4B).

1,25(OH)$_2$ D$_3$ and PI3 K inhibitor abolish IS-induced phosphorylation of Akt, accumulation of β-catenin, and EMT-associated protein expression in HK-2 cells

Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity [25]. Phosphorylation of Akt at Ser473 was clearly increased at 30 min after IS treatment (Fig. 5A). Treatment with 1,25(OH)$_2$ D$_3$ dose-dependently inhibited the IS-induced phosphorylation of Akt (S473; Fig. 5B). In the presence of LY294002, the IS-induced nuclear accumulation of β-catenin (Fig. 5C) as well as the expression of α-SMA and fibronectin, was eliminated and the inhibition of E-cadherin by IS was reversed (Fig. 5D).

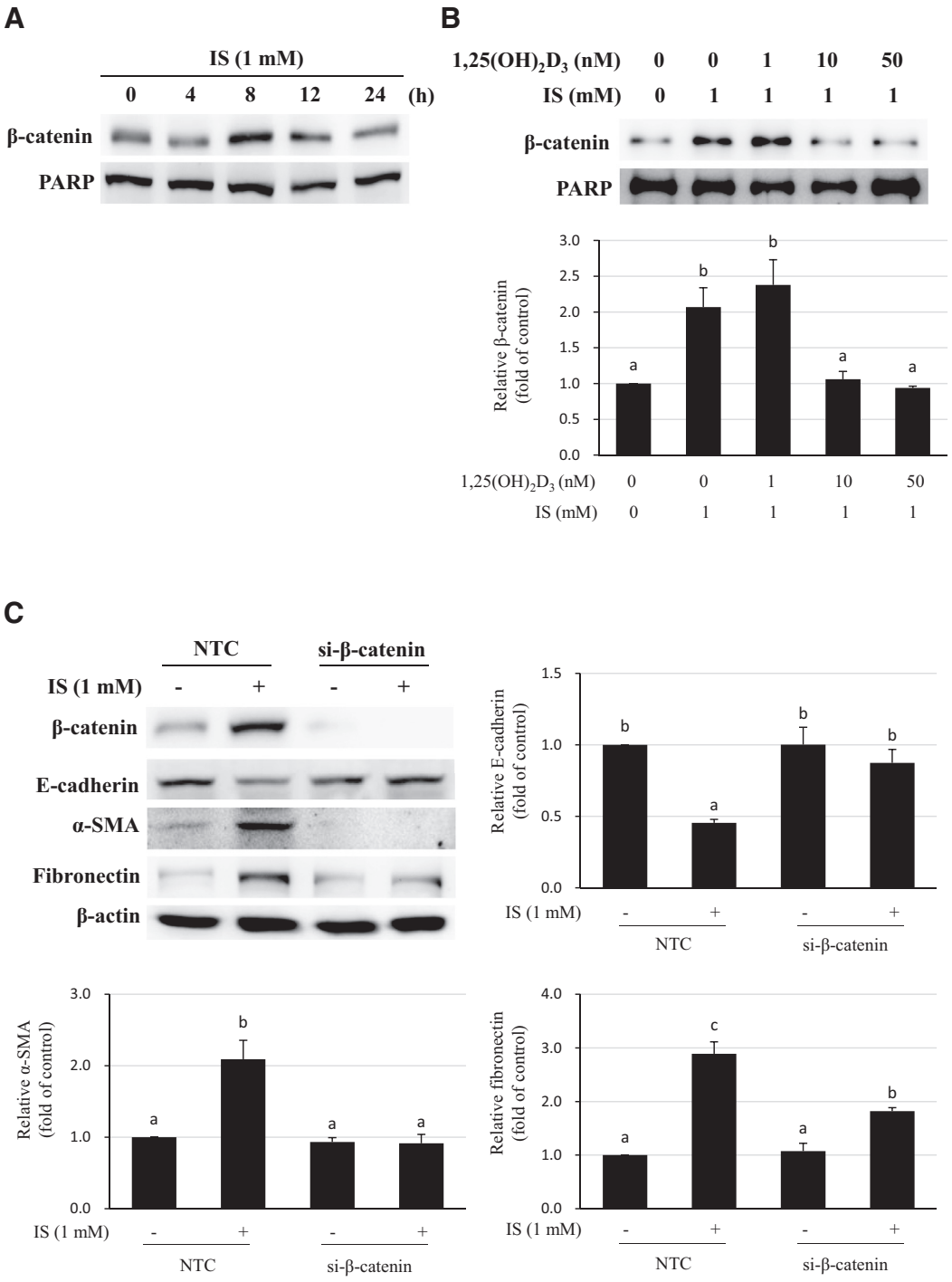

Fig. 3. Effect of 1,25(OH)$_2$ D$_3$ on IS-induced β-catenin nuclear accumulation and β-catenin–mediated expression of E-cadherin, α-SMA, and fibronectin in HK-2 cells. (**A**) Cells were treated with 1 mM IS for the indicated time periods. (**B**) Cells were pretreated with or without 1, 10, or 50 nM 1,25(OH)$_2$ D$_3$ for 1 h before the addition of IS. Eight hours after IS addition, cells were harvested and aliquots of nuclear protein (10 μg) were used for Western blot analysis. (**C**) Cells were transfected with si-β-catenin and challenged with 1 mM IS for 24 h. Aliquots of total protein (10 μg) were used for Western blotting. One representative experiment out of three independent experiments is shown. PARP and β-actin were used as internal loading control for nuclear and whole cell protein extracts, respectively. Bands were quantified and normalized to loading control bands on the same blot. Values are means ± SD, n = 3. Bars with different letters are significantly different ($P < 0.05$). 1,25(OH)$_2$ D$_3$, 1,25-dihydroxyvitamin D$_3$; α-SMA, alpha-smooth muscle actin; IS, indoxyl sulfate; NTC, non-targeting control; PARP, poly (ADP-ribose) polymerase.

Discussion

Clinical evidence strongly supports that fibrosis is a characteristic feature of CKD. The EMT has been shown to play a crucial role in progression of renal fibrosis in CKD, although the major contribution of EMT to the trigger of kidney fibrosis remains controversial [26,27]. More evidences supported initiation of an EMT program of TECs leading to a vicious cycle of damage and host response, accelerating progression of chronic renal fibrosis [28–30]. In the unilateral ureteral obstruction animal model,

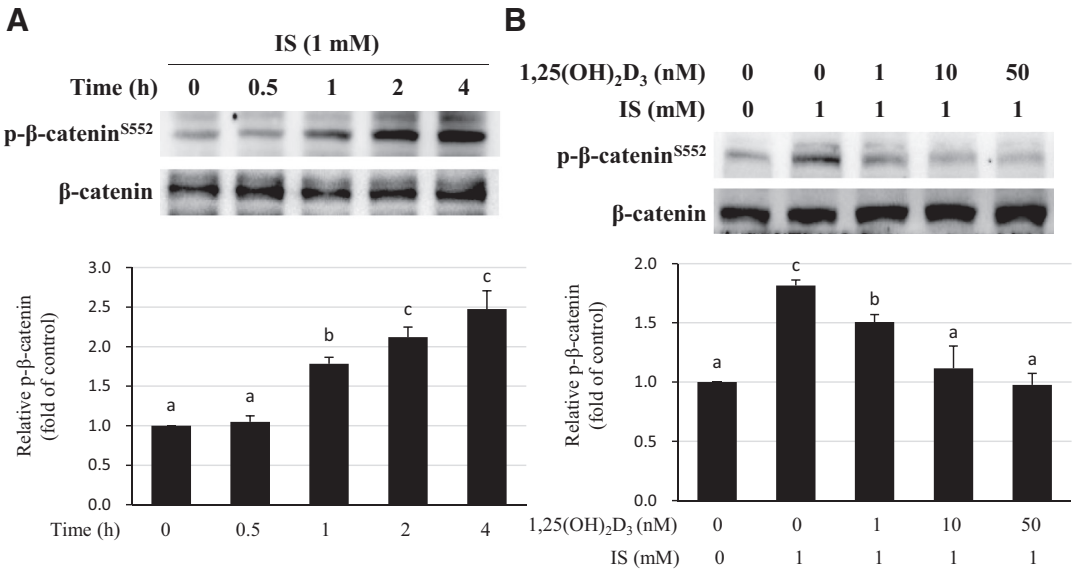

Fig. 4. Effect of 1,25(OH)$_2$ D$_3$ on IS-induced phosphorylation of β-catenin at Ser552 in HK-2 cells. (**A**) Cells were treated with 1 mM IS for the indicated time periods. (**B**) Cells were pretreated with or without 1, 10, or 50 nM 1,25(OH)$_2$ D$_3$ for 1 h and then incubated with IS for 1 h. Cells were harvested and aliquots of whole cell protein (10 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown. β-catenin was used as internal loading control for nuclear protein extracts. Bands were quantified and normalized to loading control bands on the same blot. Values are means ± SD, n = 3. Bars with different letters are significantly different ($P < 0.05$). 1,25(OH)$_2$ D$_3$, 1,25-dihydroxyvitamin D$_3$; IS, indoxyl sulfate.

inhibition of fibrotic injury–induced EMT program in TECs, led to protection of TEC integrity, restored proliferation and dedifferentiation-associated repair and regeneration, attenuated myofibroblast accumulation, fibrosis, and immune infiltration [28]. Inadequate accumulation of IS causes free radical production, reduces superoxide scavenging activity, leading to tubular cell injury in the kidneys [31]. Injury to TECs results in loss of functional parenchyma and further promotes EMT program of TECs to exacerbate chronic fibrosis [28]. These results support the notion that targeting the EMT program is a potential therapeutic approach for protecting functional parenchyma in kidney fibrosis. Previous studies indicated that IS can accelerate EMT and subsequently cause renal fibrosis [32]. However, the molecular mechanisms underlying IS-induced EMT in renal tubular cells remain unclear. In the present study, we examined the protection by 1,25(OH)$_2$ D$_3$ against IS-induced EMT in HK-2 cells. The results indicated that inhibition of IS-induced EMT-associated protein expression by 1,25(OH)$_2$ D$_3$ was, at least in part, via down-regulation of the PI3K/Akt/β-catenin signaling pathway.

N-cadherin, α-SMA, and fibronectin have been examined as markers of renal EMT, which, when overexpressed, contribute to renal fibrosis [33]. Previous studies showed that the mean concentration of IS in the plasma of healthy individuals is ~5 μmol/L, whereas the concentration is up to 285 μmol/L (0.285 mM) in patients with renal failure [34]. In some uremic patients, the concentration of IS in predialysis uremic plasma is ~80 μg/mL (0.318 mM) [35]. Treatment with IS at a concentration of 25 μg/mL for 48 h significantly downregulates the expression of E-cadherin and ZO-1, whereas it upregulates α-SMA expression in NRK-52 E rat renal TECs [32]. In the present study, a high dose of IS (1 mM) was used to examine the effect on EMT in HK-2 cells; however, 0.25 mM IS also significantly increased the protein level of α-SMA and fibronectin (Fig. 1). The present results provided evidence that accumulation of >0.25 mM IS led to a loss of epithelial adhesion properties and subsequently caused EMT in human renal proximal TECs.

β-catenin has been recognized as a marker of fibrosis, and the β-catenin/T-cell factor (TCF)/lymphoid enhancer factor (LEF) complex directly regulates gene expression associated with EMT, including the expression of α-SMA [23] and fibronectin [36]. Knockdown of β-catenin abolishes the induction of α-SMA in TGF-β1–treated rat lung epithelial-6-T-antigen alveolar type II epithelial cells [23]. Induction of β-catenin–mediated EMT signaling contributes to the pathogenesis of fibrotic diseases, such as lung fibrosis, liver fibrosis, skin fibrosis, and renal fibrosis [6]. IS promotes renal fibrosis through the induction of DNA hypermethylation of sFRP5, leading to the activation of Wnt/β-catenin signaling [4]. Induction of β-catenin nuclear translocation and α-SMA expression are involved in the endoplasmic reticulum (ER) stress-induced EMT [7], and IS has been known to induce ER stress in cultured human proximal tubular cells [37]. In the present study, IS significantly increased the nuclear accumulation of β-catenin as well as the protein expression of β-catenin, α-SMA, and fibronectin (Fig. 3A and C), which is consistent with findings from our previous study that elevation of β-catenin is positively correlated with expression of α-SMA and fibronectin [38]. Upon knockdown of β-catenin by β-catenin siRNA, the induction of fibronectin and α-SMA by IS was abolished and the inhibitory effect of IS on E-cadherin was reversed (Fig. 3C). These results suggest that IS modulates EMT-associated protein expression through induction of the β-catenin signaling pathway.

Phosphorylation of β-catenin at carboxylic acid-terminal residues on Ser552 by PI3K/Akt can increase its stabilization, leading to its accumulation and translocation into the nucleus as well as enhance β-catenin/TCF reporter activation [25,39]. Inappropriate accumulation of IS in uremic serum induces the production of TGF-β1, and the secreted TGF-β1 stimulates the production of TIMP-1 and collagen, accelerates tubular cell injury, and subsequently induces interstitial fibrosis [3]. Previous evidence has shown that TGF-β1 induces EMT through activation of the PI3K/Akt pathway in many epithelial cell lines [40]. We further clarify whether TGF-β1 involved in IS-induced the phosphorylation of Akt at Ser473, and the activity of 1,25(OH)$_2$ D$_3$ is exerted on TGF-β1–mediated PI3K/Akt signaling in our culture system. Previous studies showed that treatment with 5 mg/L IS for 72 h caused a significant increase in TGF-β1 mRNA expression in mouse

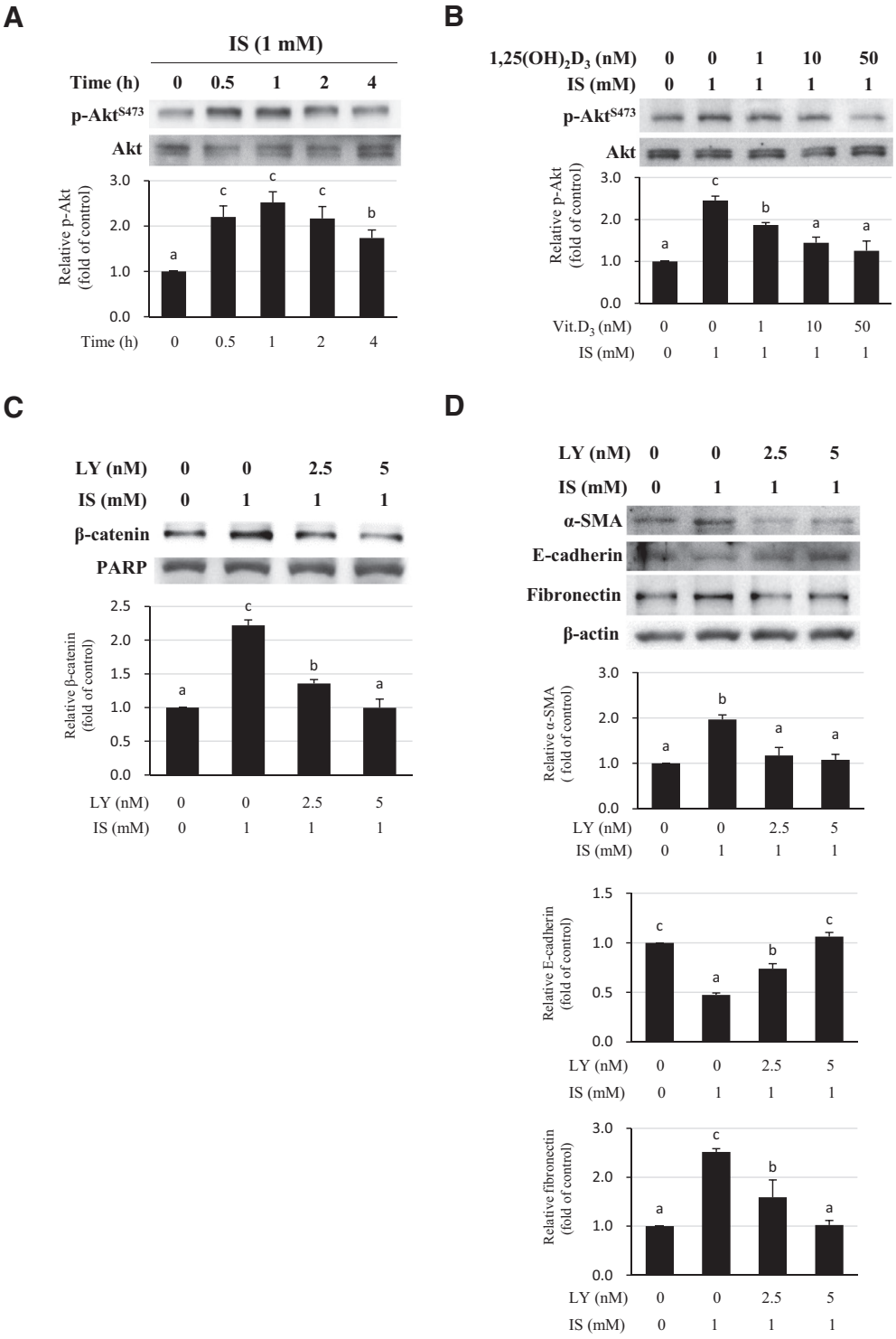

Fig. 5. Effect of 1,25(OH)$_2$ D$_3$ and PI3K inhibitor on IS-mediated phosphorylation of Akt at Ser473, β-catenin nuclear accumulation, and expression of E-cadherin, α-SMA, and fibronectin in HK-2 cells. (**A**) Cells were treated with 1 mM IS for the indicated time periods. (**B**) Cells were pretreated with or without 1, 10, or 50 nM 1,25(OH)$_2$ D$_3$ for 1 h and then incubated with IS for 1 h. Cells were pretreated with or without 2.5 and 5 nM LY294002 for 1 h and then incubated with 1 mM IS. After IS treatment for 8 or 24 h, cells were harvested and aliquots of 10 μg of (**C**) nuclear protein or (**D**) whole cell protein were used for Western blot analysis. One representative experiment out of three independent experiments is shown. Akt, PARP, and β-actin were used as internal loading control. Bands were quantified and normalized to loading control bands on the same blot. Values are means ± SD, n = 3. Bars with different letters are significantly different ($P < 0.05$). 1,25(OH)$_2$ D$_3$, 1,25-dihydroxyvitamin D$_3$; α-SMA, alpha-smooth muscle actin; IS, indoxyl sulfate; PARP, poly (ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase.

proximal renal tubular cells (PKSV-PRs) [41]. In HK-2 cells, a dramatical induction in TGF-β1 mRNA and protein expression was performed after incubation with IS for 48 and 72 h, respectively [42]. These results indicated that long-term incubation of IS was necessary for new protein synthesis of TGF-β1 in HK-2 cells. Thus, the extracellular secretion and autocrine activity of TGF-β1 by IS

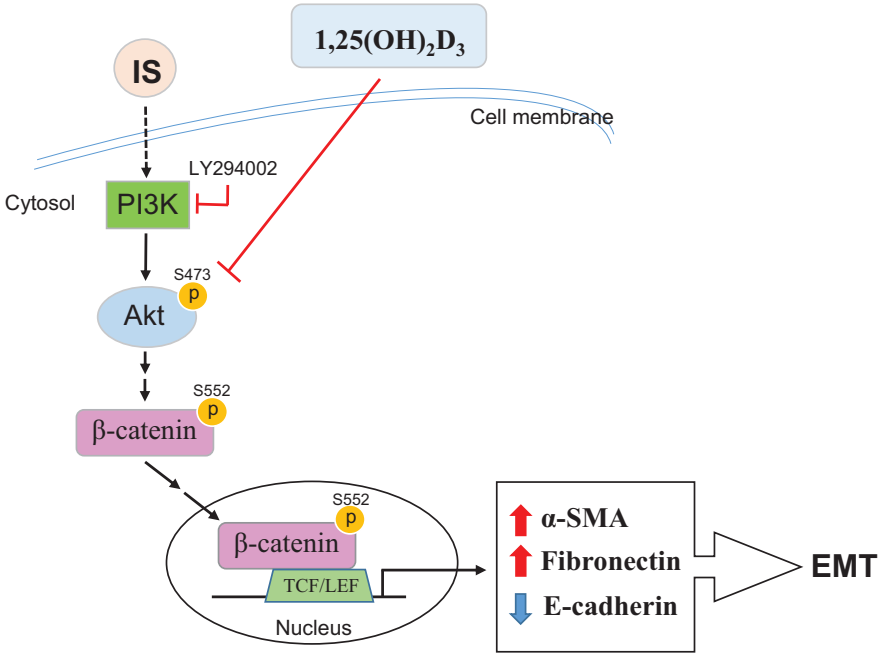

Fig. 6. 1,25(OH)$_2$ D$_3$ effectively modulates IS-mediated EMT-associated protein expression via inactivation of PI3K/Akt/β-catenin signaling pathway in HK-2 cells. 1,25(OH)$_2$ D$_3$, 1,25-dihydroxyvitamin D$_3$; α-SMA, alpha-smooth muscle actin; EMT, epithelial-to-mesenchymal transition; LEF, lymphoid enhancer factor; IS, indoxyl sulfate; PI3K, phosphoinositide 3-kinase; TCF, T-cell factor.

short-term treatment was examined. As noted in Figure 5A, the phosphorylation of Akt (Ser473) was rapidly and transiently elevated by IS treatment within 1 h, whereas the secretion of TGF-β1 has no significant elevation after IS treatment for 0.5 and 1 h (Supplementary Fig. 1). Actually, a significant elevation of TGF-β1 secretion was only performed after IS treatment at 6 and 12 h, whereas this IS induction of TGF-β1 was significantly decreased by 1,25(OH)$_2$ D$_3$ in HK-2 cells (Supplementary Fig. 1). Although treatment with 20 μM SB431542, a potent and specific inhibitor of TGF-β1 receptor, slightly decreased IS-induced the phosphorylation of Akt (S473) (Supplementary Fig. 2), the incubation with TGF-β1 antibody to indirect block the TGF-β1–mediated signaling pathway dose not inhibits the phosphorylation of Akt (S473) after IS treatment for 1 h (Supplementary Fig. 3). These results suggest that 1,25(OH)$_2$ D$_3$ is capable of inhibiting the phosphorylation of Akt within IS short-term incubation, belongs to an early event and a TGF-β1–independent manner. However, we cannot rule out that activation of TGF-β1–mediated Akt phosphorylation and its downstream signaling pathway presented in IS long-term treatment. The issue of the effect of 1,25 (OH)$_2$ D$_3$ on IS-induced TGF-β1 signaling activation will be considered in a future study.

In the present study, IS induced the phosphorylation of Akt at Ser473 (Fig. 5A and B), subsequently elevated p-Ser522-β-catenin expression (Fig. 4A), and induced accumulation of β-catenin in the nucleus (Fig. 5C). Treatment with LY294002 significantly inhibited IS-induced nuclear accumulation of β-catenin (Fig. 5C) as well as the protein expression of α-SMA and fibronectin in HK-2 cells. Animal study showed that treatment with IS significantly increased the phosphorylation of Akt (S473) and the nuclear localization of β-catenin in the tubular cells of the B6 mice [43]. These results suggest that IS induced EMT-associated protein expression at least in part through activation of Akt/β-catenin in HK-2 cells. A previous study revealed that treatment with ERK1/2 inhibitor (PD98059) or p38 MAPK inhibitor (SB203580) resulted in no significant effect on IS-induced EMT in NRK-52 E renal proximal tubular cells [32]. We also studied whether MAPKs were involved in IS-induced EMT in HK-2 cells. As noted (Supplementary Fig. 4), pretreatment with 10 μM ERK1/2 or p38 MAPK inhibitors did not affect the EMT-associated protein expression in the presence of IS, which agrees with the previous study [32]. Although the phosphorylation of JNK was clearly increased at 60 min after IS treatment (Supplementary Fig. 5), and treatment with JNK inhibitor dramatically decreased IS-induced α-SMA and fibronectin protein expression (Supplementary Fig. 4), 10 or 50 μM of SP600125 did not suppress IS-induced nuclear accumulation of β-catenin (Supplementary Fig. 6). These results indicate that JNK-dependent and β-catenin independent signaling pathways may play a role in IS-induced EMT in HK-2 cells.

A protective effect of 1,25-(OH)$_2$ D$_3$ on renal fibrosis has been reported; however, the mechanism underlying this effect has not been fully clarified. Serum 25(OH)D$_3$ levels <30 ng/mL (75 nM), which are recognized as vitamin D deficiency, are associated with vascular calcification in patients with CKD [44]. The normal 25(OH) D$_3$ level in the blood is 30 to 80 ng/mL (75–200 nM). In the present study, the dose of 50 nM of 1,25(OH)$_2$ D$_3$ we used showed dramatic protection in suppression of EMT in the presence of IS, suggesting that 1,25(OH)$_2$ D$_3$ supplements at levels >50 nM may exert a protective role in uremic vascular calcification and renal fibrosis. An animal study indicated that 1,25(OH)$_2$ D$_3$ suppressed renal fibrosis by inhibiting TGF-β/SMAD signaling in mice with unilateral ureteral obstruction [20]. Treatment with 1,25(OH)$_2$ D$_3$ was shown to repress the EMT induced by high glucose via inactivation of the RhoA/ROCK pathway in HK-2 cells [9]. The present study revealed that β-catenin is one of the important targets in the inhibition of EMT by 1,25(OH)$_2$ D$_3$. 1,25(OH)$_2$ D$_3$ has been shown to inhibit the transcriptional activity of β-catenin by two mechanisms. First, 1,25(OH)$_2$ D$_3$ rapidly increases the amount of vitamin D receptor (VDR) bound to β-catenin, thus reducing β-catenin/TCF-mediated transcriptional activity [45]. Second, 1,25(OH)$_2$ D$_3$ induces the expression of *CDH1* gene coding for E-cadherin, which sequesters β-catenin at the plasma membrane adherens junctions, accompanied by the nuclear export of β-catenin [46].

Activation of PI3K/Akt pathway is capable of phosphorylating glycogen synthase kinase-3 (GSK3) β at Ser9 and subsequently inhibiting its kinase activity [47]. Induction of PI3K/Akt2-mediated phosphorylation and inactivation of GSK3 β was involved in TGF-β1 induced EMT in renal TECs [48]. HK-2 cells were treated with 5 mM IS for 2 h in serum-free medium increased the phosphorylation of GSK-3 β at Ser9 [49]. However, treatment with 1 mM of IS for 2 h does not significantly increased the phosphorylation of GSK3 β in our experimental condition (data not shown), suggests that IS-induced GSK3 β phosphorylation in the concentration-dependent manners in HK-2 cells.

In the present study, $1,25(OH)_2$ D_3 abolished IS-mediated PI3K/Akt/β-catenin signaling and decreased the nuclear accumulation of β-catenin, which suggests that altering the localization and translocation of β-catenin from the cytosol into the nucleus was involved in inhibition of the EMT by $1,25(OH)_2$ D_3 in the presence of IS. In the present study, treatment with $1,25(OH)_2$ D_3 does not increase the protein level of VDR in the presence of IS. Actually, treatment with $1,25(OH)_2$ D_3 just reversed IS-decreased the protein level of VDR in HK-2 cells (Supplementary Fig. 7). Thus, the possibility that the VDR−β-catenin complex played a role in the inhibition of IS-induced EMT-associated protein expression by $1,25(OH)_2$ D_3 cannot be excluded. A previous study showed that IS can elevate ER stress and subsequently promote EMT in HK-2 cells [37]. Recent evidence indicates that treatment with $1,25(OH)_2$ D_3 protects against ER stress in endothelial cells [50]. Future work is warranted to determine whether inhibition of IS-induced EMT by $1,25(OH)_2$ D_3 occurs via suppression of ER stress in cultured human proximal tubular cells.

Findings from the present study are presented schematically in Figure 6. The findings strongly support a protective role of $1,25(OH)_2$ D_3 in IS-induced EMT in human renal tubular cells and suggest $1,25(OH)_2$ D_3 as a potential therapeutic target for treatment of renal fibrosis. $1,25(OH)_2$ D_3 effectively modulates the IS-mediated expression of E-cadherin, α-SMA, and fibronectin, at least in part, through inactivation of the PI3K/Akt/β-catenin signaling pathway.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2019.110554.

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