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The mechanism of attenuation of epithelial-mesenchymal transition by a phosphodiesterase 5 inhibitor via renal klotho expression

Seung T Han | Jae S Kim | Jun Y Lee | Min K Kim | Jin S Yoo | Byoung G Han | Seung O Choi | Jae W Yang

Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju, Gangwon-do, Korea

Correspondence
Jae Won Yang, Nephrology, Internal Medicine, Yonsei University Wonju College of Medicine, Wonju-si, Gangwon-do, Korea.
Email: kidney74@yonsei.ac.kr

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Summary

Phosphodiesterase-5 (PDE-5) inhibitors induces vasodilation in several organs by blocking cyclic GMP (guanosine monophosphate) degradation. However, the existence of alternative mechanism of action in case of an impaired nitric oxide (NO) system remains controversial. Previous studies suggested that decreased NO bioavailability may result in the downregulation of klotho expression, but the relationship between klotho and NO remains obscure. Therefore, we investigated whether a PDE-5 inhibitor could preserve epithelial–mesenchymal transition (EMT) and relationship exists between the NO and renal klotho expression. Ten-week-old SD rats (N = 24, 200 g, male) were divided (N = 6) into four groups, which received: A LSD, L-NAME 1 mg/mL in drinking water, Udenafil 5 mg/kg subcutaneously and both for 4 weeks. Urine nitrate/nitrite, NGAL (Neutrophil gelatinase-associated lipocalin), and cGMP were measured using ELISA. Kidney was subjected to evaluate PCNA (proliferative cell nuclear antigen), α-SMA (smooth muscle cell antigen), E-cadherin, and klotho expression. Urine cGMP decreased after treatment of PDE-5 inhibitor compared with control due to blocking degradation of cGMP ($P < .05$, control vs Udenafil and L-NAME with Udenafil groups). Urine NGAL increased after treating of L-NAME and attenuated after using PDE-5 inhibitor ($P < .05$, control vs L-NAME and L-NAME with Udenafil). PCNA, α-SMA, and E-cadherin (EMT markers) increased after L-NAME treatment and normalized after using PDE-5 inhibitor. Klotho expression showed trend to increase in the L-NAME with PDE-5 inhibitor group compared with the L-NAME group, however, eNOS expression did not change after treatment of L-NAME or PDE-5 inhibitor compared with control. PDE-5 inhibitor alleviates EMT in the kidney via klotho modulation independent of the NO system.

KEYWORDS
epithelial–mesenchymal transition, klotho, nitric oxide system, phosphodiesterase-5

1 | INTRODUCTION

Inhibition of cGMP (cyclic guanosine monophosphate) degradation by phosphodiesterase type 5 (PDE5)-targeted compounds has proven most successful in the treatment of pulmonary arterial hypertension (PAH) to date, although PDE5 inhibitors are usually used to treat erectile dysfunction.[1] However, In kidney diseases, PDE5 inhibition can reduce albuminuria in subjects with diabetes and ameliorate angiotensin II-induced

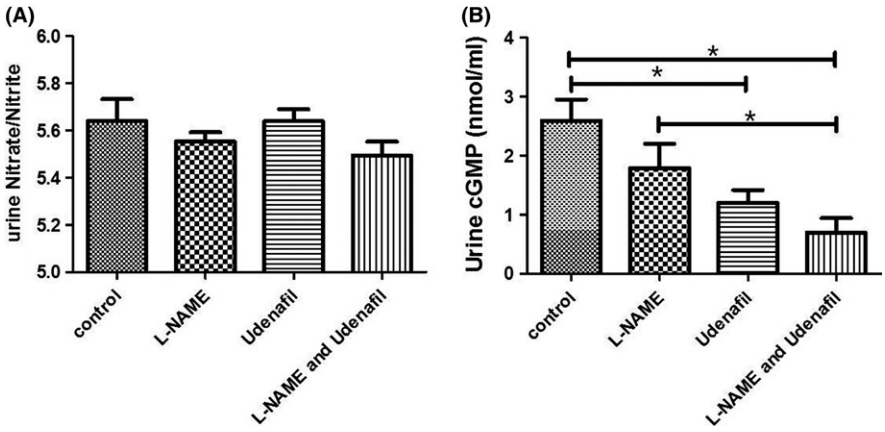

FIGURE 1 Comparison among the different groups of the effects on the nitric oxide system. (A) Urine nitrate/nitrite metabolite concentration. (B) Urine cGMP concentration detected using an ELISA kit. *$P < .05$

podocyte injury.[2,3] In an animal model of kidney ischaemic-reperfusion injury, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) showed toxic effect on glomerulus and tubule in several animal models, such as preeclampsia, spontaneous hypertensive rat.[4-7] These effects mediated via the modulation of the nitric oxide (NO) system by PDE5 inhibitors suggest that an increase in end-product in target organ can ameliorate tissue damage in several diseases.[7-9] We have previously reported that PDE-5 inhibitors can ameliorate cyclosporine A-induced renal injury.[10]

The number of studies examining the role of fibroblast growth factor-23 (FGF23) and klotho in chronic kidney disease (CKD) has risen exponentially over the past decade. Tissue levels of the FGF23 co-receptor klotho declines in early CKD and this deficiency is linking to accelerated ageing, cellular senescence, vascular calcification, oxidative stress, and renal fibrosis. At present, methodological difficulties limit the utility of soluble klotho measurements, but animal studies have demonstrated the beneficial effects of klotho delivery in CKD.[11] The in vivo reduction in endogenous NO production in klotho-deficient mice is reflected by the reduced urinary excretion of NO metabolites and cGMP (an indicator of NO synthesis).[12,13] Further, in-vitro experimental findings lend support for klotho-induced enhancement of NO production in human endothelial cells via modulation of the activity of endothelial nitric oxide synthase (eNOS), the enzyme responsible for generation of NO.[14,15]

Epithelial–mesenchymal transition (EMT) has been implicated in cancer progression and metastasis, wound healing, and the development of fibrotic disorders, including pulmonary, hepatic, and renal fibrosis.[16-18] EMT in the kidney can be induced by CKD-associated renal hypoxia, which is thought to result from a combination of structural and functional changes that include decrease in the number of capillaries, compromised peritubular blood flow resulting from glomerular injury, vasoconstriction due to changes in the levels of vasoactive factors and signaling molecules such as angiotensin II, endothelin, and NO.[19]

Therefore, in this study, we investigated whether PDE5 inhibition could ameliorate EMT via klotho expression in the kidney.

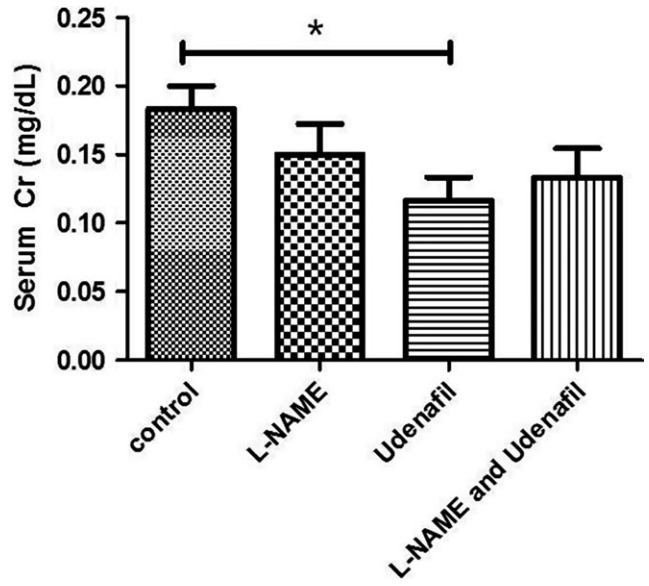

FIGURE 2 The comparison of serum creatinine levels among the groups. *$P < .05$

2 | RESULTS

2.1 | Comparison of the effects of a phosphodiesterase 5 inhibitor on the NO system among the groups

Urine nitrate/nitrite and cGMP levels were measured and normalized to creatinine levels to determine the success of the experimental design. The nitrate/nitrite metabolite levels showed a decreasing trend in the L-NAME and L-NAME with Udenafil groups compared to that in the control and Udenafil groups. The urine cGMP levels were 2.59 ± 0.88, 1.79 ± 0.99, 1.20 ± 0.22, and 0.69 ± 0.59 nmol/μL for the control, L-NAME, Udenafil and L-NAME with Udenafil groups, respectively ($P < .05$, control vs Udenafil and L-NAME with Udenafil groups) (Figure 1A, B).

2.2 | The comparison of renal function and degree of injury among the groups

In the Udenafil group, kidney function showed a slight increase as indicated by the decrease in serum creatinine. However, other groups did not show a statistically significant change (Figure 2). PCNA expression in terms of PCNA-positive tubular cells/unit area was 0.11 ± 0.06, 0.31 ± 0.14, 0.17 ± 0.02, and 0.19 ± 0.08 mL for the control, L-NAME, Udenafil, and L-NAME with Udenafil groups, respectively

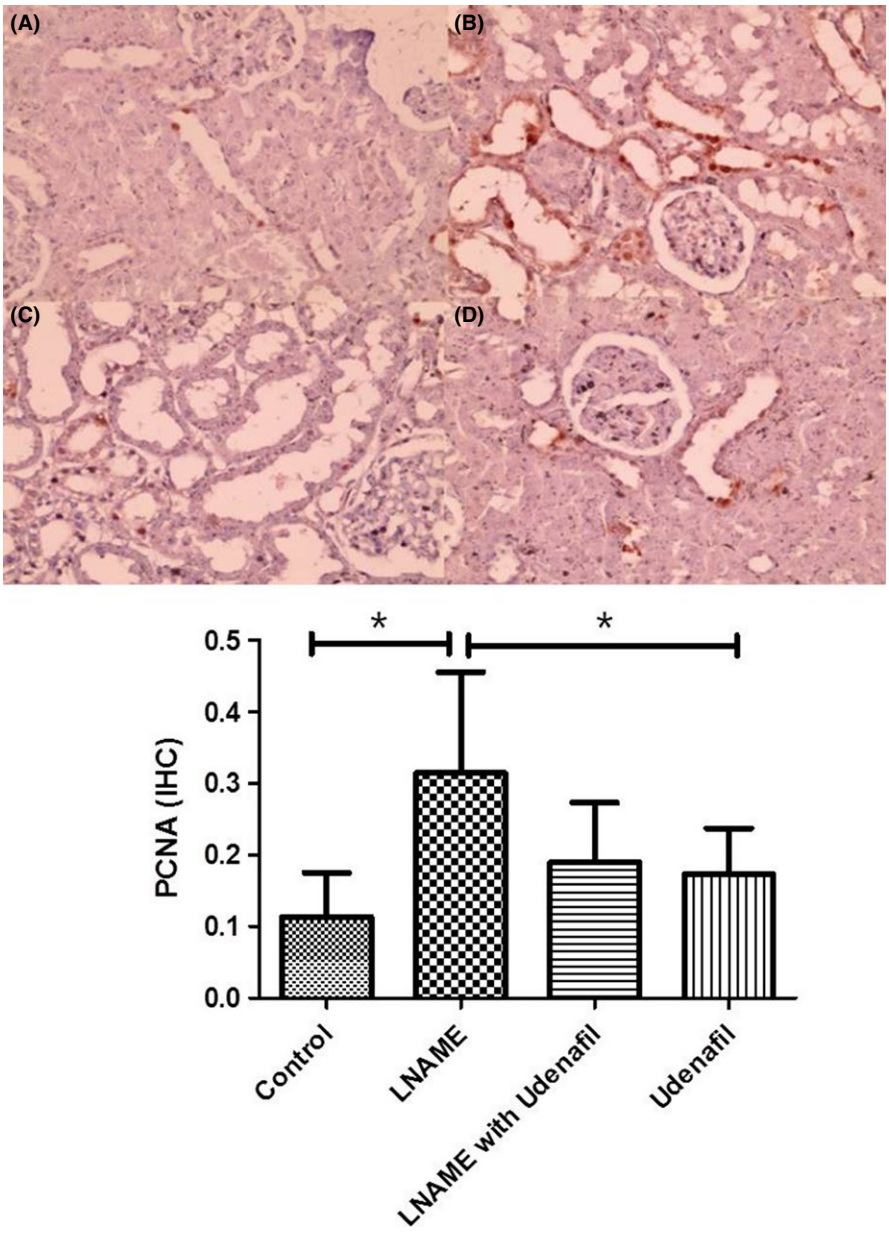

FIGURE 3 Comparison of proliferative cell nuclear antigen (PCNA) expression in immunohistochemical stain among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) L-NAME with Udenafil. *$P < .05$

($P < .05$, control vs L-NAME, and L-NAME vs L-NAME with Udenafil) (Figure 3). The urine NGAL levels were 279.8 ± 126.8, 651.0 ± 195.3, 473.7 ± 114.9, and 326.5 ± 279.4 ng/mL for the control, L-NAME, Udenafil, and L-NAME with Udenafil groups, respectively ($P < .05$ control vs L-NAME and L-NAME with Udenafil) (Figure 4). There were no functional changes in the kidney among the groups, but acute injury to tubular cells was well established in the L-NAME group, and this damage was ameliorated by Udenafil treatment.

2.3 | The changes of epithelial–mesenchymal transition markers after treatment

α-SMA showed increased expression in the L-NAME group compared to that the L-NAME with Udenafil group (control 0.45 ± 0.02, L-NAME 0.95 ± 0.05, Udenafil 0.50 ± 0.02, and L-NAME with Udenafil 0.31 ± 0.11). E-cadherin protein expression decreased in the L-NAME group compared to that in the other groups (control 0.911 ± 0.01, L-NAME 0.36 ± 0.09, Udenafil 0.99 ± 0.01, and L-NAME with Udenafil 0.62 ± 0.03) (Figure 5). Considered together with the changes in PCNA expression, these results suggest that L-NAME treatment induced EMT in the kidney and that Udenafil has a protective effect in the kidney.

2.4 | The mechanism of attenuation of epithelial–mesenchymal transition after treatment

Klotho mRNA expression increased in the L-NAME with Udenafil group compared with that in the L-NAME group determined by RT-PCR (control 0.98 ± 0.01, L-NAME 0.30 ± 0.05, Udenafil 0.68 ± 0.06, and L-NAME with Udenafil 0.54 ± 0.13) (Figure 6). Klotho protein density, as determined by immunohistochemical stain, showed the same trend among the groups. After treatment with Udenafil, klotho

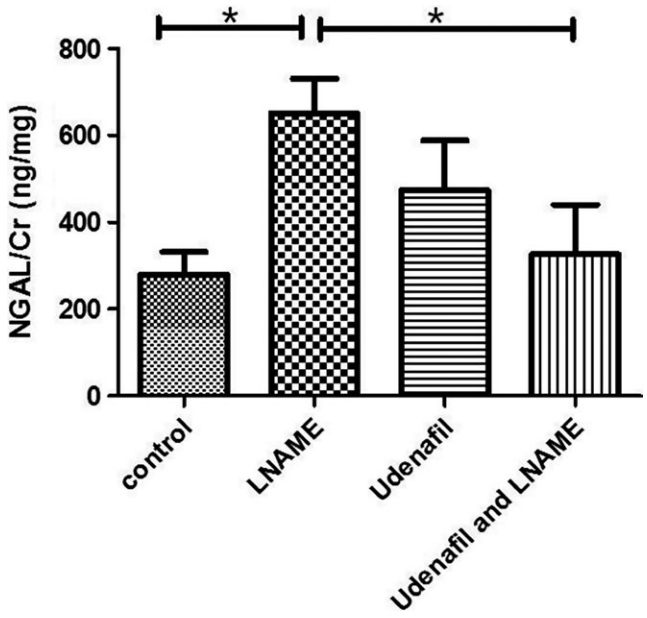

FIGURE 4 Comparison of the urine NGAL/Cr ratio among the groups. *$P < .05$

mRNA and protein expression in tubular cells increased compared with that in the L-NAME group. Renal eNOS protein expression, measured by immunohistochemical staining, decreased in the Udenafil group; however, eNOS protein expression in the L-NAME and L-NAME with Udenafil groups was not significantly different from that in the control (Figure 7).

3 | DISCUSSION

Epithelial–mesenchymal transition was induced by several factors in cancer cell and nitric oxide system had epithelial–mesenchymal transition in this study using L-NAME was not established well, however, Seccia etc. proved that endothelin-1 drives epithelial–mesenchymal transition in hypertensive nephroangiosclerosis.[20] L-NAME also can make hypertension, podocyte injury, and tubular damage in hypertensive rat model. There is no study measuring EMT markers in the kidney using L-NAME toxicity until now, we can confirm the change of E-cadherin, alpha SMA, PCNA in the tubular cell after treating L-NAME. Therefore, we can use L-NAME induced tubular injury model for EMT study.

PDE5 inhibitors represent a class of drugs traditionally used to treat erectile dysfunction and pulmonary hypertension. Recent evidence suggests that PDE5 inhibitors may have additional therapeutic effects such as cardioprotection and cerebrovascular protection.[21] However, the direct effect and mechanism of PDE5 inhibitors in renal fibrosis are not fully understood. Recently the long-acting PDE5 inhibitor PF-00489791 was assessed in a multinational, multicenter, randomized, double-blind, placebo-controlled, parallel group trial of subjects with type 2 diabetes mellitus and overt nephropathy receiving angiotensin converting enzyme inhibitor or angiotensin receptor blocker background therapy. Compared to placebo, 12-week treatment with PF-00489791 resulted in a significant reduction of 15.7% in the urinary albumin-to-creatinine ratio).[22] Results of several animal studies suggested that PDE5 inhibitors such as sildenafil, vardenafil, and tadalafil have a protective effect on renal ischaemic-reperfusion injury.[23-26] To demonstrate the protective effect of a PDE5 inhibitor on an impaired NO system in the kidney, we postulated that the central molecule between EMT and cGMP would be eNOS. However, eNOS expression in the kidney tissue did not show significant difference among groups in our pilot study. Richter et al. suggested that klotho is the key molecule involved in the modulation of the NO system. In the presence of klotho, FGF23 induces NO release in human coronary artery endothelial cells, and its stimulating effects on ROS production are counterbalanced by increased ROS degradation. In states of klotho deficiency, FGF23-mediated NO synthesis is blunted and the rate of ROS formation exceeds that of ROS degradation.[27] We focused on klotho expression in our animal model to prove the mechanism of PDE5 inhibition under conditions of NO system deterioration.

Our animal model was designed to demonstrate the effect of cGMP increase under impaired NO donation conditions in kidney tissue. One study showed the effects of peroral sildenafil administration in the macaque monkey (*Macaca fascicularis*) by performing chemical analysis

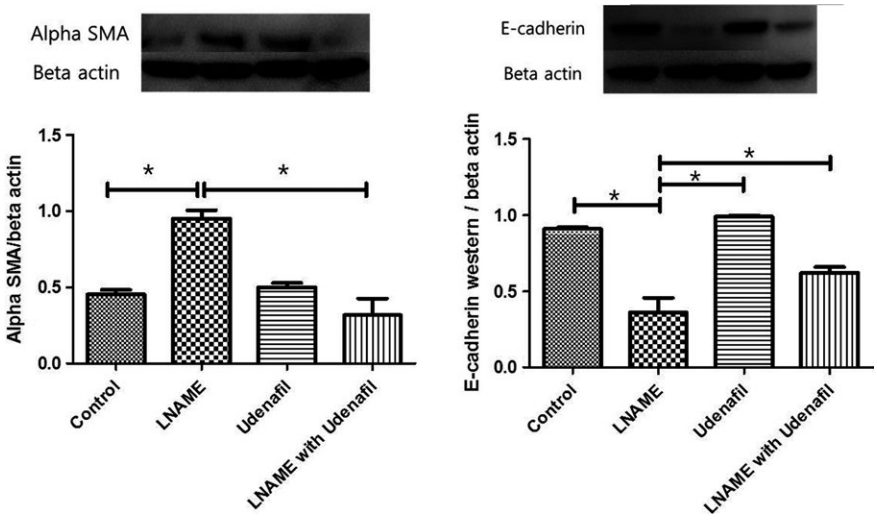

FIGURE 5 Comparisons of kidney alpha SMA and E-cadherin expression among the groups as measured by western blot. *$P < .05$

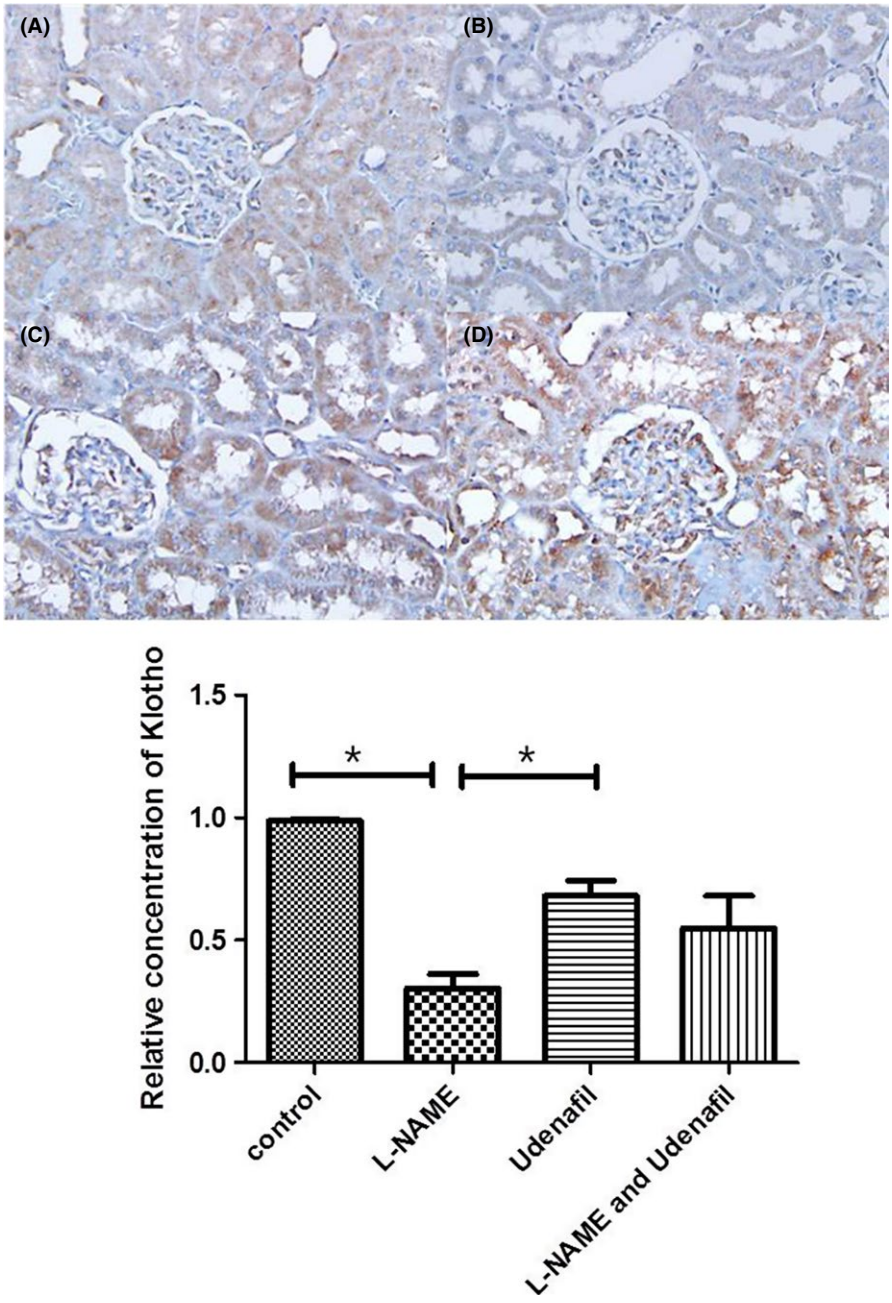

FIGURE 6 The comparison of protein density done by immunohistochemical stain and mRNA Klotho expression determined by RT-PCR in the kidney among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) LNAME with Udenafil. *$P < .05$

of plasma and cerebrospinal fluid (CSF) using liquid chromatography coupled with tandem mass spectrometry. The results showed that drug levels in the CSF were high enough to inhibit PDE5 activity, which was also demonstrated by the significant increases in CSF cGMP levels.[28] In our animal model, urine cGMP levels decreased in the L-NAME with Udenafil group compared with that in the other groups; although the cGMP level in the kidney tissue was not quantified, the effect of PDE5 inhibitor was enough to enable assessment of group differences.

CKD is a prevalent disease affecting 13% of adults in the United States, with 8% having an estimated glomerular filtration rate (eGFR) of <60 mL/min per 1.73 m^2 according to the National Health and Nutrition Examination Survey (1999-2004).[29] In recent years, considerable evidence has accumulated to suggest that CKD is a state of relative renal and systemic NO deficiency caused by a combination of decreased renal and vascular NO production and increased NO bioinactivation.[30] Because of the important protective role played by NO in the health of both renal and cardiovascular systems, it is likely that a deficit in NO will accelerate CKD progression and increase cardiovascular risk, making the NO pathway a promising therapeutic target.[31]

The relationship between the NO system and klotho was not clearly elucidated in this study, but we showed that chronic NOS inhibition markedly reduced renal klotho protein expression. Moreover, a previous study showed that treatment with atorvastatin or pitavastatin completely prevented the reduction of klotho expression induced by NOS inhibition.[32] Klotho protein exists in both a secreted and a membrane form. Its extracellular domain can be shed from the cell surface

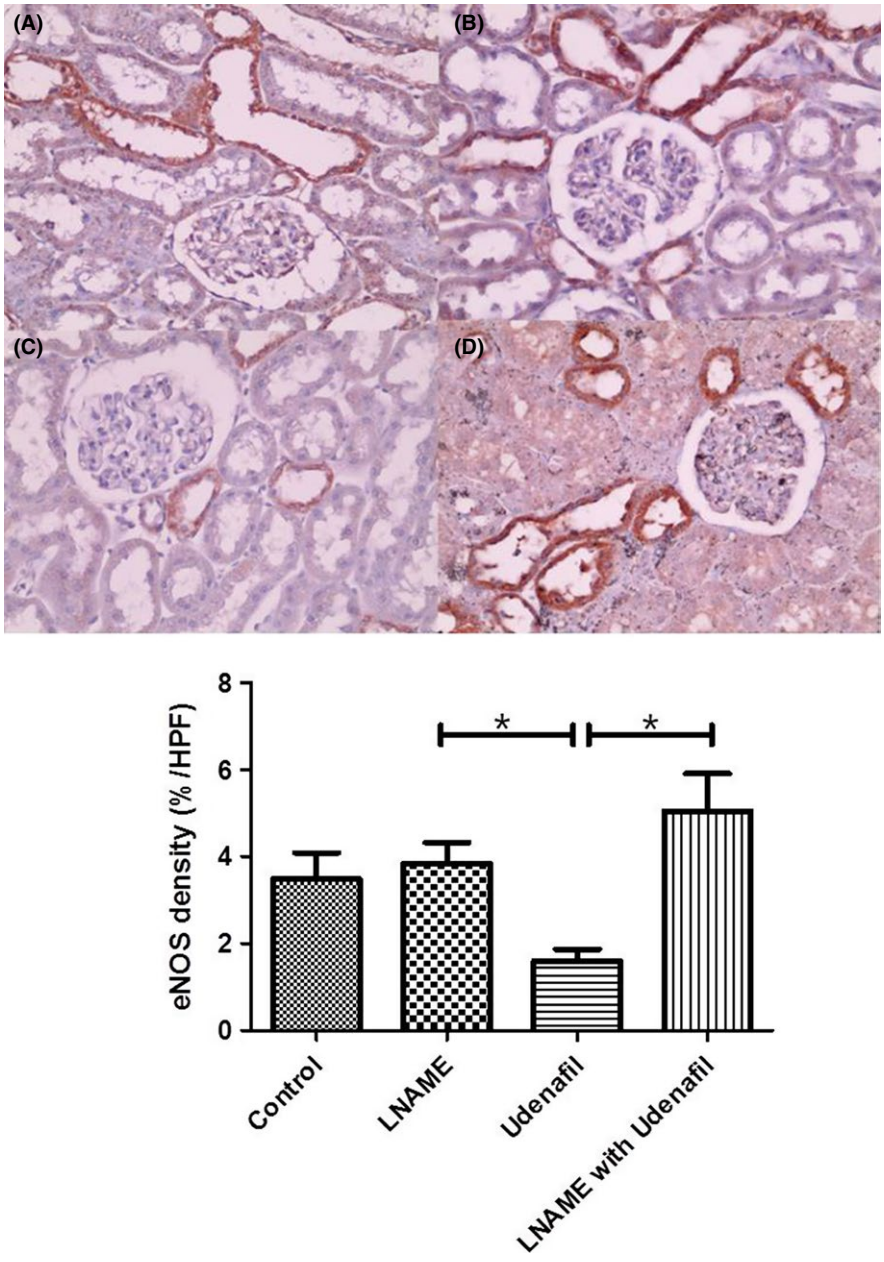

FIGURE 7 Comparison of eNOS protein expression in the kidney among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) L-NAME with Udenafil. *$P < .05$

after cleavage by secretases and released into the circulation to act as an endocrine factor. Soluble klotho is a multifunctional protein present in biological fluids including blood, urine, and CSF. It plays important roles in antiaging, energy metabolism, inhibition of Wnt signaling, anti-oxidation, modulation of ion transport, control of parathyroid hormone and active vitamin D production, and antagonism of the renin-angiotensin-aldosterone system.[33] Soluble klotho and active vitamin D levels decrease and FGF23 levels increase at early CKD stages, whereas parathyroid hormone levels increase at more advanced CKD stages.[34]

In the ischaemia-reperfusion injury rodent model, klotho in the kidneys, urine, and blood decreased; klotho levels in all these organs were restored upon recovery. Reduction in kidney and plasma klotho levels occurred earlier than reduction of NGAL, a known biomarker of kidney injury.[35] Our study also showed that klotho mRNA and protein levels in the kidney were inversely correlated with NGAL expression, although the time sequence of this phenomenon was not clear. Klotho might have some beneficial effect on L-NAME-induced kidney injury.

Eryptosis, the suicidal death of erythrocytes, is characterized by erythrocyte shrinkage, blebbing, and phospholipid scrambling of the cell membrane. Eryptosis is enhanced in mouse models of sickle cell anemia and thalassemia, as well as in mice lacking functional annexin 7, cGMP-dependent protein kinase type I, AMP-activated protein kinase, Janus kinase 3, anion exchanger 1, adenomatous polyposis coli, or klotho.[36,37] Therefore, we postulated this mechanism was that deficiencies of cGMP and klotho can induce cell death and have interrelationship. Our study results showed that induction of cGMP independent of NO can restore klotho expression and reverse renal injury induced by L-NAME.

The origin and precise molecular and functional differences between mesenchymal cells and fibroblasts during EMT are not

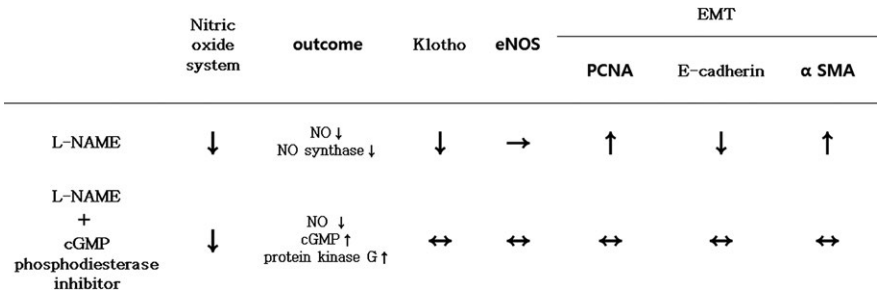

FIGURE 8 Summary of the relationship between the nitric oxide (NO) system and epithelial–mesenchymal transition

completely understood. Renal fibrosis is a pathological condition characterized by excessive accumulation of extracellular matrix and it is a common pathway for the progression of different renal diseases. Transforming growth factor-β1 (TGF-β1) is implicated in the pathogenesis of fibrosis in both glomerular and interstitial compartments of the kidney. Klotho acts as an endogenous inhibitor of multiple growth factors including TGF-β1.[38,39] In one study, renal fibrosis was induced by unilateral ureteral obstruction (UUO) in mice with reduced klotho expression (kl/+ mice) and they were then compared with wild-type mice. The UUO kidneys from kl/+ mice had significantly higher levels of fibrosis markers such as α-SMA, fibronectin, and TGF-β1 than those from wild-type mice.[40] E-cadherin, a key component of cell-cell adhesion junctions, is essential for the formation of epithelia during embryonic development and for the maintenance of adult epithelial homeostasis; its loss is associated with increased tumor cell invasiveness.[41] L-NAME-induced renal injury increased the levels of EMT markers such as PCNA and α-SMA, and decreased the levels of anti-EMT markers such as E-cadherin.[42] Thus, EMT can be improved by a PDE5 inhibitor treatment via klotho attenuation.

Kidney fibrosis induced by EMT was aggravated by reduced klotho levels, which can suppress TNF-α expression and attenuate NF-κB activation.[43,44] TGF-β1 mediated upregulation of PDE4 activity promotes EMT in alveolar epithelial cells in lung tissue.[45] One study showed that the klotho-induced reversal of EMT in SiHa cells is associated with downregulation of transcriptional factor Slug/Twist and resultant upregulation of E-cadherin.[46] We also could demonstrate the same change of TWIST 1,2 mRNA (EMT signaling markers) in the kidney after treatment (data not shown). Until now, there is no study has demonstrated a direct connection between renal EMT and PDEs. We postulate that the core molecule of attenuating EMT after tubular injury involved might be klotho, because eNOS expression (NO system) was not changed after treatment (Figure 8).

In conclusion, we suggest that, in a poor NO environment, PDE5 inhibitors can have protective effect on EMT via the klotho pathway independent of NO system.

4 | MATERIALS AND METHODS

4.1 | Animals

Ten-week-old male Sprague-Dawley rats (body weight 200-250 g) were housed with standard chow and tap water available ad libitum. All animal procedures and care protocols were approved by Yonsei University at Wonju Campus IACUC (Institutional Animal Care and Use Committee).

4.2 | Animal groups and experimental design

The 10-week-old male Sprague-Dawley rats (N = 24; weight 200 g) were divided into four groups, which received the following treatments for 4 weeks: a low salt diet (control group; N = 6); L-NAME 1 mg/mL in drinking water (L-NAME group; N = 6);[47-49] Udenafil 5 mg subcutaneously (SQ) (Udenafil group; N = 6); and both L-NAME and udenafil (L-NAME and udenafil group; N = 6). The experimental drugs used were L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, Sigma-Aldrich Co., St. Louis, MO, USA) and Udenafil (Zydena, Dong-A Pharmaceutical Co., Seoul, Korea), a PDE-5 inhibitor. Low salt diet can increase sensitivity of renal injury, because its activation of renin-angiotensin system can augment L-NAME induced tubular cell damage. We decided the dosage and duration of L-NAME and udenafil following several other study protocols.[7,47-49] On day 28, blood samples were collected from the jugular vein, and both kidneys were extracted. A portion of each kidney was fixed in 10% neutral formalin and prepared as tissue blocks embedded in paraffin. An additional portion of the kidney was cooled rapidly in liquid nitrogen and stored at −70°C.

4.3 | Biochemical markers

On day 28, serum creatinine was measured using a kinetic colorimetric assay with the Modular equipment (Hitachi High-Technologies Corporation, Tokyo, Japan). Urine nitrate/nitrite and cGMP levels were measured using an ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Urine neutrophil gelatinase-associated lipocalin (NGAL) levels were also measured using an ELISA kit (Abcam, Cambridge, UK).

4.4 | Immunohistochemistry

Kidney tissue blocks embedded in paraffin were prepared as 5-μm thick sections, and attached to the silane-coated glass slides. To recover antigenicity, the PT module (Lab Vision, Freemont, CA, USA) was used and heated at 99°C for 15 minutes. For the inactivation of endogenous peroxidase activity, the samples were pretreated with Hydrogen Peroxide Block (Thermo Fisher Scientific, Fremont, CA, USA) for 10 minutes, and washed with Tris-buffered saline plus

Tween 20 (TBST buffer; ScyTek Laboratories, Logan, UT, USA). To suppress nonspecific antigens, the samples were treated with Ultra V Block (Thermo Fisher Scientific, CA, USA) for 5 minutes followed by incubation with primary antibody for 2 hours. The primary antibodies used were as follows: klotho (anti-klotho antibody, Thermo Scientific), eNOS (anti-eNOS antibody ab66127, Abcam), proliferative cell nuclear antigen (PCNA; anti-PCNA antibody ab9252, Abcam) diluted 1:200, 1:25, and 1:200 respectively. The samples were treated with the horseradish peroxidase (HRP) polymer secondary antibody (Thermo Fisher Scientific) for 30 minutes and then incubated with TBST buffer. Background staining was performed with Mayer's hematoxylin, and then the slices were immersed in distilled water, sealed with Immu-Mount (Thermo Fisher Scientific), and examined under a microscope.

4.5 | RNA isolation and real-time PCR

The tissues were cut to 0.5-cm pieces, washed twice with phosphate-buffered saline (PBS), and then the RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). The concentration of RNA was measured at 260 and 280 nm with a spectrophotometer (Bio-Rad, Hercules, CA, USA). cDNA was synthesized from the isolated RNA by using the Quantitect Reverse Transcription kit (Qiagen). Less than 1 mg of RNA was mixed with 2 mL 7× gDNA Wipeout buffer and incubated at 42°C for 2 minutes. Next, 1 mL Quantiscript Reverse Transcriptase, 4 mL 5× Quantiscript Reverse Transcription buffer, and 1 mL RT primer mix were added, and incubated at 42°C for 30 minutes and at 95°C for 3 minutes. The cDNA synthesized in this manner was used as a template for PCR. For the PCR reaction mixture, 400 ng cDNA, 5 mL 2× QuantiTect Probe PCR Master Mix (Qiagen), 10 pmol primer, and 30 pmol probe were mixed. The QuantiTect Primer Assay (klotho: QT01570618, Qiagen) was used as the source of the primers. PCR was performed using the RotorGene Real-Time Q-PCR system (Corbett Research, Sydney, Australia). The PCR conditions included the HotStarTaq polymerase activation step; the samples were reacted at 95°C for 15 minutes, denaturation was performed at 94°C for 15 seconds, annealing was performed at 60°C for 1 minute, extension was performed at 72°C for 30 seconds, for a total of 50 cycles. Beta-actin was used as the control. The amount of mRNA obtained was calculated as the relative concentration using the value $2^{-\Delta\Delta Ct}$, and the results were compared.

4.6 | Protein preparation and western blotting

Kidney proteins were extracted with 1× cell lysis buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich). After centrifugation at 12500 g at 4°C for 30 min, supernatant was collected and 20 μL of lysate from each sample was run on a 10% dodecyl sulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were rinsed in TBST (10 mmol/L Tris-HCL, pH 7.4, 0.9% NaCl, 0.05% Tween 20, and 1 mmol/L EDTA) and blocked in blocking buffer (TBST containing 5% bovine serum albumin) for 1 hour at room temperature. PVDF membranes were then incubated with primary antibodies against β-actin (sc-47778, Santa Cruz Biotechnology, CA, USa), E-cadherin (anti-E-cadherin antibody, Thermo Scientific), and α-smooth muscle actin (α-SMA; anti-alpha SMA antibody ab5694, Abcam) overnight at 4°C. Subsequently, the membranes were washed, and then incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz) for 1 hour at room temperature. The membrane was developed with enhanced chemiluminescent (ECL) substrate (Li-cor, Lincoln, NE, USA) and exposed to UVP chemiluminescence (Biolite LLC, Upland, CA, USA).

4.7 | Statistical analyses

The SPSS version 12.0 and Graphpad Prism 5 program was used for statistical comparisons and making graph. The non-parametric Kruskal-Wallis and Mann-Whitney test were used. A *P* value less than .05 was considered significant.

ORCID

Jae W Yang http://orcid.org/0000-0003-3689-5865

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