Klotho provides a protective role against podocyte injury via cell cycle-dependent manner under diabetic conditions

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I want to dedicate this paper to all of you with all of my heart. Thanks everyone.

Hyung Jung Oh

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ABSTRACT

Klotho provides a protective role against podocyte injury via cell cycle-dependent manner under diabetic conditions

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Background: Most studies on the protective effect of klotho against renal injury have been focused on tubulopathy instead of glomerular cells. The aim of this study was to test whether klotho can exert a protective effect against podocyte injury in an experimental model of diabetic nephropathy.

Methods: *In vitro*, mouse podocytes were cultured in a medium containing 5.6 mM glucose (NG) or a high (30 mM) glucose concentration (HG) with or without 200 pM recombinant klotho (rKL). Additionally, a knockdown of klotho was performed using a small interfering RNA to examine the effect of endogenous klotho. *In vivo*, 32 mice were injected with either a diluent (n = 16, control) or with streptozotocin intraperitoneally (n = 16, diabetes; DM) to induce diabetes. Eight mice from each

control and DM group were treated daily with rKL at 10 μg/kg/day. All the mice were euthanized 4 weeks after the development of diabetes.

Results: Protein klotho was expressed in podocytes depending on PPAR-γ. HG treatment increased the expression of cell cycle-related proteins and apoptotic markers, but these changes were significantly attenuated by rKL. Nonetheless, the small interfering RNA against the klotho gene in HG-treated podocytes failed to enhance cell cycle arrest and apoptosis. In line with *in vitro* findings, protein expression of cell cycle-related proteins and apoptotic markers was increased in DM mice compared to control mice, and these increased expression levels were significantly decreased by rKL. Moreover, hypertrophy of glomeruli in DM mice was significantly alleviated after rKL administration.

Conclusion: This study showed that klotho is expressed in glomerular podocytes, and its expression is regulated by PPAR-γ. Moreover, administration of klotho can attenuate glomerular hypertrophy via a cell cycle-dependent mechanism and thus decreases apoptosis. These findings provide new insights into the mechanisms of protective effects of klotho in diabetic nephropathy.

Key words: klotho, podocytes, high glucose, cell-cycle dependent mechanism, apoptosis, diabetic nephropathy

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I. INTRODUCTION

Klotho is known as an antiaging gene and is predominantly expressed in the distal convoluted tubules of the kidney, although it is also expressed in the brain choroid plexus, pituitary gland, pancreas, and reproductive organs.[1] It is downregulated after kidney injury such as angiotensin II-induced hypertensive renal damage,[2] ischemia-reperfusion acute kidney injury,[3] and diabetic nephropathy.[4-6] In agreement with the empirical studies on animals, studies on human subjects showed that mRNA and protein expression of klotho in kidneys are lower in patients with chronic kidney

disease (CKD) and those with end-stage renal disease than in healthy controls.[7-9] In addition, previous study revealed that circulating klotho levels decrease as CKD reaches the advanced stage and inversely correlate with estimated glomerular filtration rate among patients with CKD.[7]

Klotho exerts its effects as two distinct forms.[10] The membrane form is a cofactor in fibroblast growth factor 23 signaling in kidneys. This complex modulates phosphate homeostasis and vitamin D metabolism.[11,12] In contrast, the soluble form of klotho acts as a hormone with anti-oxidative-stress[13] and anti-inflammatory properties.[14] Although recent studies have shown a protective effect of klotho on renal injury in experimental nephropathy,[3,15-18] local effects of klotho on individual cells are still largely unknown. Given that distal tubules are the primary site of klotho production, most studies have been mainly focused on acute tubulointerstitial injury. Nonetheless, there is a lack of evidence that klotho has a therapeutic effect in glomerular injury or CKD models. Haruna et al.[16] reported that not only tubulointerstitial damage but also glomerulosclerosis are attenuated by klotho overexpression in mice with immune-complex mediated glomerulonephritis, suggesting that klotho may also be effective in alleviating glomerular injury. Nevertheless, the authors did not clarify whether such improvement is attributed to a direct effect of klotho on glomerular cells or a secondary phenomenon after klotho-induced amelioration of tubulointerstitial injury.[16]

Diabetic nephropathy is characterized by hypertrophy of both glomerular and tubular elements, progressive accumulation of extracellular-matrix components in the glomerular mesangium, and thickening of the glomerular and tubular basement membranes.[19] There are some pieces of evidence suggesting that klotho plays a protective role in experimental models of diabetic nephropathy. Some studies have consistently shown that the klotho level decreases in the kidneys of rodents with type I or II diabetes.[4,6,20] In addition, *in vitro* studies revealed that restoring klotho expression relieves tubular epithelial cell injury (caused by excessive glucose) by attenuating oxidative stress or inflammation.[4,20] On the other hand, researchers in these studies used tubular epithelial cells only and mainly evaluated tubulopathy under diabetic conditions.

Thus, glomerulopathy, the primary pathological change in diabetic nephropathy, has not yet been explored in depth, and no conclusion can be drawn regarding the effect of klotho on diabetic glomerular injury. Of note, klotho is reported to attenuate cellular senescence by suppressing the p53/21 pathway.[21] The upregulation of p21 can induce cell cycle arrest and result in cellular hypertrophy.[22-24] Meanwhile, one of the prominent features of the early stage of diabetic nephropathy is an increase in kidney size mainly because of glomerular and tubular hypertrophy.[19] Given the inhibitory effect of klotho on cellular senescence, it can be hypothesized that klotho can be effective in mitigating early changes of diabetic kidney injury by limiting renal hypertrophy. Therefore, I aimed to determine whether klotho has a protective effect against glomerular hypertrophy via a cell cycle-dependent mechanism in an experimental model of diabetic nephropathy.

II. MATERIALS AND METHODS

1. Podocyte culture and reagents

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (University of Miami, Miami, FL, USA) and were cultured as previously described.[25] Briefly, frozen podocytes were first grown under permissive conditions at 33°C in collagen-coated flasks in the RPMI 1640 medium (Gibco) containing 10% of fetal bovine serum, 50 U/ml γ-interferon, and 100 U/ml penicillin/streptomycin. The interferon γ was added to the medium (nonpermissive conditions), and the cells were allowed to differentiate at 37°C, with the medium changed on alternate days. The differentiation of podocytes grown for 14 days at 37°C was confirmed by the detection of synaptopodin, a podocyte differentiation marker, by reverse transcription-time polymerase chain reaction (RT-PCR) and western blotting (data not shown).

For in vitro studies, depending on the purpose of the experiments, cells were incubated with 50 μM peroxisome proliferator-activated receptor (PPAR)-γ agonist (pioglitazone, Tocris Bioscience, Bristol, UK), 20 μM PPAR-γ antagonist (GW 9662, Tocris Bioscience, Bristol, UK), and/or 200 pM recombinant klotho (rKL) (R & D Systems, Minneapolis, MN, USA).

2. RNA interference

In this study, small interfering RNA (siRNA) against mouse *klotho* was used to inhibit klotho expression. The sequence of anti-*klotho* siRNA was 5′-CCCAGAGAGTATGAAGAACAA-3′. The *klotho* siRNA was transiently transfected into differentiated podocytes with Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) for 24 hrs.

3. The chromatin immunoprecipitation (ChIP) assay

This assay was performed as previously described.[26] Briefly, 4 to 6 × 10^6 cells were cross-linked with 1% formaldehyde at a high glucose concentration (30 mmol) in

phosphate-buffered saline (PBS) for 20 min at room temperature. The cross-linked cells were washed twice with cold PBS containing protease inhibitors, and the lysates were sonicated, and immunoprecipitation was performed with antibodies to PPAR-γ (Abcam, PLC, Cambridge, UK) or control IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoprecipitates were captured using protein A-conjugated magnetic beads (Invitrogen), and the bound proteins were eluted. I obtained ChIP-enriched DNA using phenol/chloroform extraction followed by ethanol precipitation. Input DNA samples as well as antibody-enriched ChIP DNA samples were analyzed by quantitative real-time PCR using the primers (shown below) within the *klotho* enhancer promoter. The primer sequences for amplifying the *klotho* promoter were sense 5′-AGTGCATCTCCTGCTCCATT-3′ and anti-sense 5′-CTTGCTGTGCTTTGAACAGG-3′. Data were analyzed using the 2 methods and normalized to input samples as previously described.[26]

4. Flow cytometry

Flow cytometry was performed to analyze cell cycle distribution. Cells were trypsinized and pelleted by centrifugation at 1000 × *g* for 5 min at room temperature. After 2 washes with ice-cold PBS, the cell pellets were fixed in ice-cold 70% ethanol and stored at –20°C. Before analysis, the cells were treated with 500 U/ml RNase A, and DNA was stained with 50 μg/ml propidium iodide (Sigma-Aldrich, St Louis, MO, USA) for 1 hr at room temperature. Cell cycle distribution was determined using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA); at least 10,000 cells per sample were analyzed. The data were processed in the FlowJo software.

5. Western blot analysis

Podocytes harvested from the culture plates and kidney cortex were homogenized in lysis buffer consisting of sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. Aliquots corresponding to 50 μg of total

protein were mixed with Laemmli sample buffer, heated at 100°C for 5 min, and subjected to electrophoresis at 50 μg/lane in an 8%–12% polyacrylamide denaturing (SDS-containing) gel. Proteins were then transferred to a Hybond-ECL membrane in a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A [1×PBS, 0.1% of Tween 20, and 8% of nonfat milk] for 1 hr at room temperature, followed by an overnight incubation at 4°C with an appropriate dilution of primary antibodies specific for PPAR-γ, klotho (Abcam), cell cycle-related proteins such as p21, p27, and p53 (Santa Cruz Biotechnology), apoptosis-associated proteins such as Bcl-2, Bax (Santa Cruz Biotechnology), and cleaved caspase 3 (Cell Signaling Technology), and β-actin (Sigma-Aldrich). The membrane was then washed once for 15 min and twice for 5 min in 1× PBS with 0.1% of Tween 20. Next, the membrane was incubated in buffer A containing a 1:1000 or 1:2000 dilution of horseradish peroxidase-conjugated donkey anti-goat IgG antibody (Amersham Life Science) or anti-rabbit IgG antibody (Cell Signaling Technology) depending on the primary antibodies. Several washes were done, and the membrane was developed with a chemiluminescent reagent (ECL; Amersham Life Science). The density of bands was measured in the ImageJ software, version 1.49 (NIH, Bethesda, Maryland, USA; online at http://rsbweb.nih.gov/ij).

6. Quantitative real-time PCR

Total RNA from mouse podocytes was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). I analyzed the quality of RNA on agarose gels, and measured the quantity using a NanoDrop. By means of the cDNA Archival Kit (Applied Biosystems, Foster City, CA, USA), 1 μg of total RNA was reverse-transcribed. Quantitative real-time PCR was performed using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with a total volume of 20 μl in each well, containing 10 μl of the SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 μl of cDNA, and 5 pM sense and antisense primers. The PCR conditions were as follows: 35 cycles of denaturation at 94.5°C for 30 sec, annealing at

60°C for 30 sec, and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed in all PCRs. The sequences of primers used for *klotho* were as follows: sense 5′-TGGGTCACTGGGTCAATCTCT-3′ and anti-sense 5′-CCGGCACGATAGGTCATGTT-3′. I examined the melting curve to verify that a single product was amplified. For quantitative analysis, all the samples were normalized to 18S gene expression using the $^{\Delta\Delta}$CT method.

7. Histological and immunofluorescent assays

I used formalin-fixed, paraffin-embedded kidney slices stained with periodic acid-Schiff (PAS) reagent for routine histological examination. The slides were examined, and photographs of glomeruli were taken at 40x magnification with a camera-attached Olympus DP73 microscope.

For immunofluorescent staining for podocytes and mesangial cells, those glomerular cells were grown in 6-well plates. The cells were fixed in acetone for 10 min at 4°C and air-dried for 10 min at room temperature, and the cells were blocked with 10% donkey serum for 20 min at room temperature and incubated with antibodies specific for mouse klotho (Abcam), p21, and p53 (Santa Cruz Biotechnology) overnight at 4°C. After washing, a Cy2 (green)-conjugated anti-goat IgG antibody (Research Diagnostics, Flanders, NJ, USA) was added, with incubation for 60 min and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

8. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Apoptosis was also evaluated by the TUNEL assay using a commercially available kit (Chemicon International, Temecula, CA, USA). TUNEL-positive glomerular cells in formalin-fixed renal tissue were identified by examining at least 30 glomeruli at 400× magnification.

9. Morphometric measurement of glomerular volume

Volumes of the glomeruli were determined as described previously.[27] Briefly, paraffin-embedded tissue slices were subjected to PAS staining. The surface areas of 50 glomeruli cut at the vascular pole traced along the outline of the capillary loops were measured using a computer-assisted color image analyzer (Image-Pro Ver. 2.0, Media Cybernetics, Silver Spring, MD, USA). Glomerular volume was calculated using the equation: $VG = \beta / k\ 9\ (Area)^{3/2}$, where $\beta = 1.38$ (the shape coefficient for spheres), and $k = 1.1$ (the size distribution coefficient).

10. **Animals and the surgical protocol**

All animal experiments were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Thirty-two C57BL/6 mice were injected with either diluent [n = 16, Control (C)] or 65 mg/kg streptozotocin intraperitoneally [n = 16, Diabetes (DM)]. Diabetes was confirmed if tail vein blood glucose levels were above 300 mg/dl on the third day post-injection. After confirmation of diabetes, osmotic minipump (Alzet, DURECT Corp, Cupertino, CA, USA) was implanted intraperitoneally into 8 mice from each group to ensure continuous delivery of rKL at 10 μg/kg per day. In order to induce greater glomerular injury, diabetic mice were subjected to unilateral nephrectomy (UNx), while a sham operation was performed on control mice. The mice were given free access to water and standard laboratory chow during the 4-week study period. Body weights were measured weekly, and the kidney weights were measured at the time of euthanasia. Serum glucose and 24-hr urinary albumin were also quantified at the time of euthanasia. Blood glucose was quantified by means of a glucometer, and 24-hr urinary albumin excretion was analyzed by an ELISA kit (Nephrat II, Exocell, Philadelphia, PA, USA).

11. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows, ver. 13.0 (SPSS, Chicago, IL, USA). The results were analyzed using the Kruskal-Wallis

nonparametric test for multiple comparisons. Significant differences according to the Kruskal-Wallis test were confirmed by the Mann-Whitney *U* test. Differences with P values less than 0.05 were considered statistically significant.

III. RESULTS

1. Cell culture studies

A. Klotho is expressed in glomerular cells

To determine whether klotho is expressed in glomerular cells, PCR and immunofluorescence analyses were performed. RT-PCR analysis showed that mRNA expression of klotho was well pronounced in mouse podocytes and mesangial cells (Figure 1A). The immunofluorescent assay also identified the cytoplasmic localization of klotho in these cells (Figure 1B).

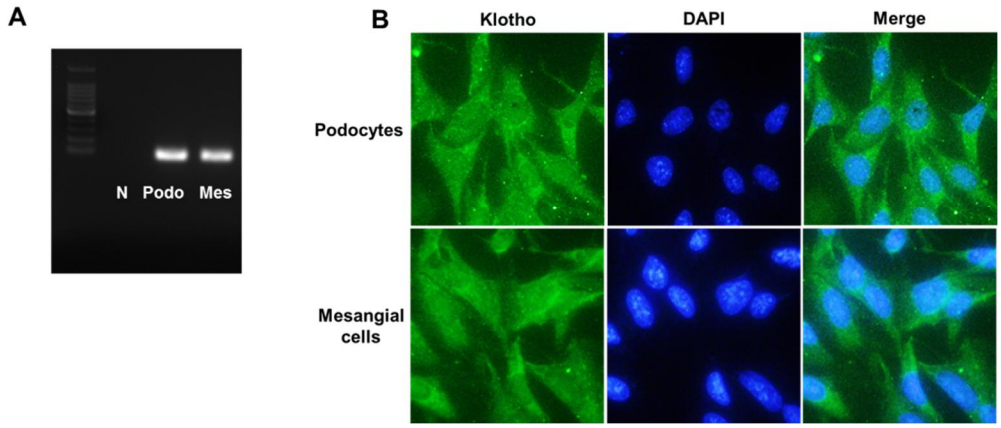

Figure 1. Expression of klotho in podocytes and mesangial cells. (A) Reverse transcription PCR revealed mRNA of klotho was expressed in podocytes and mesangial cells. (B) Immunofluorescence study also showed cytoplasmic distribution of klotho in these cells. Abbreviation; N, negative control; podo, podocyte; mes, mesangial cell.

B. The klotho gene is a target of PPAR-γ

Some studies showed that klotho is a downstream effector of PPAR-γ in kidney tubular epithelial cells.[28] To identify the relation between PPAR-γ and klotho in glomerular podocytes, the evaluation of the expression of klotho and PPAR-γ in response to incubation with a high glucose concentration (HG) was performed. Podocytes were incubated with a normal concentration of glucose (LG, 5.6 mM), mannitol, or HG (30 mM) and harvested at 6, 12, 24, or 48 hrs after the treatment.

Western blot analysis showed that protein expression of PPAR-γ was significantly increased up to 24 hrs in HG-treated podocytes compared to LG-treated cells but decreased at 48 hrs (Figure 2A). In accordance with PPAR-γ expression, protein expression of klotho was also initially increased at 12- and 24-hr time points but decreased at 48 hrs in these cells in response to HG. The bimodal expression of PPAR-γ and klotho was not observed in podocytes treated with mannitol. To further clarify this relation, the expression of klotho in these cells was examined after treatment with a PPAR-γ agonist (pioglitazone) or antagonist (GW9662). Protein expression of klotho was significantly increased by pioglitazone but decreased by GW9662 (Figure 2B). Furthermore, ChIP assay was performed to reveal the interaction between PPAR-γ and klotho. There is no typical binding site for PPARs in the 5′-flanking region of the klotho gene, but two noncanonical PPAR-responsive element (PPRE) motifs exist at positions −3698 and −2760 upstream of the ATG start codon in the promoter region of the klotho gene as previously reported.[28] Therefore, a primer spanning the putative PPRE within the 5′-flanking region of the klotho gene was designed and the binding of this region to PPAR-γ was examined. The ChIP assay clearly revealed immunoprecipitation of this PPRE region by an anti-PPAR-γ antibody but not by a control (IgG) antibody (Figure 2C). Taken together, all these findings suggest that the klotho gene is a target of PPAR-γ and that expression of klotho is dependent on PPAR-γ.

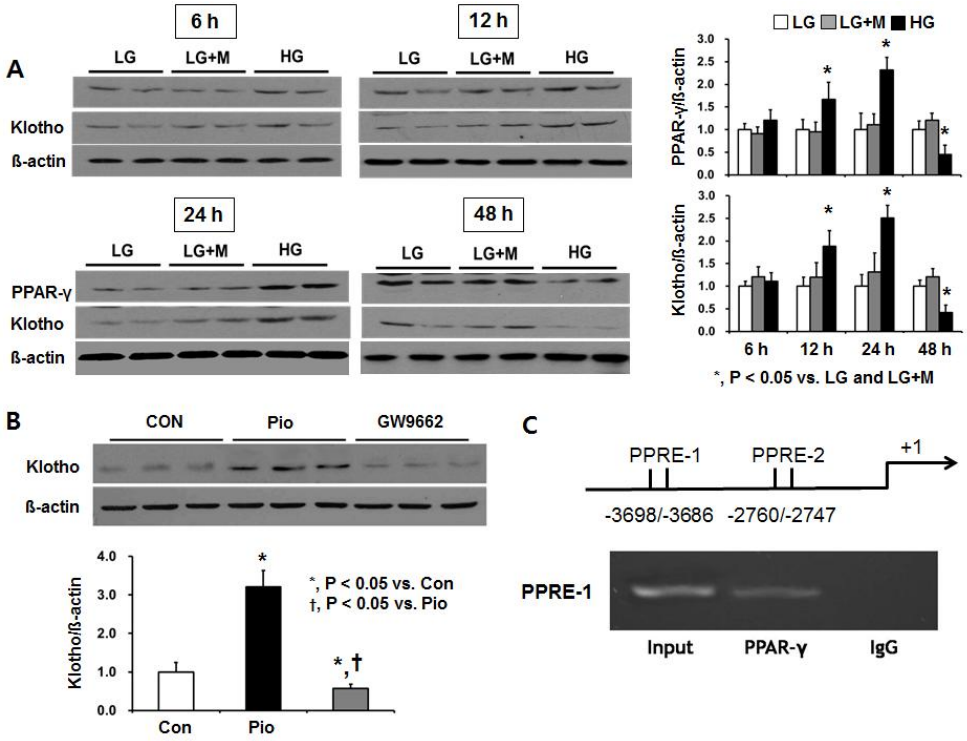

Figure 2. Klotho is a downstream target of PPAR-γ. (A) The protein expression of klotho is concordant to that of PPAR-γ. (B) The protein expression of klotho is increased by PPAR-γ agonist (pioglitazone) and decreased by PPAR-γ antagonist (GW9662). (C) ChIP assay identified a clear binding activity of the promoter region of klotho gene to PPAR-γ antibody. Abbreviation; LG, low glucose; HG, high glucose; con, control; pio, pioglitazone; GW, GW9662.

C. Klotho inhibits apoptosis in HG-treated podocytes via a cell cycle-dependent mechanism

Next, the effect of klotho was examined on cell apoptosis and cycle-related proteins. As shown in Figure 3, the protein expression levels of p21, p27, and p-p53, which are known as cell cycle-related proteins, were significantly increased in the HG group compared to the control group, whereas the increased expression levels of these proteins in the HG group were significantly attenuated by rKL treatment. In addition,

the ratio Bax/Bcl2 and protein expression of cleaved caspase 3 were significantly increased in the HG group compared to the control group, whereas rKL significantly attenuated the increased expression of apoptosis-related proteins in the HG group (Figure 3).

Moreover, the effect of klotho on podocyte injury using a PPAR-γ agonist and antagonist was evaluated. Pioglitazone treatment significantly ameliorated HG-induced podocyte damage by reducing the increased expression of cell cycle-related proteins and apoptotic markers. Administration of the PPAR-γ antagonist negated this favorable effect of the PPAR-γ agonist on HG-induced injury. Addition of rKL reversed the podocyte damage caused by the PPAR-γ antagonist (Figure 4). These findings further delineated the relation between PPAR-γ and klotho.

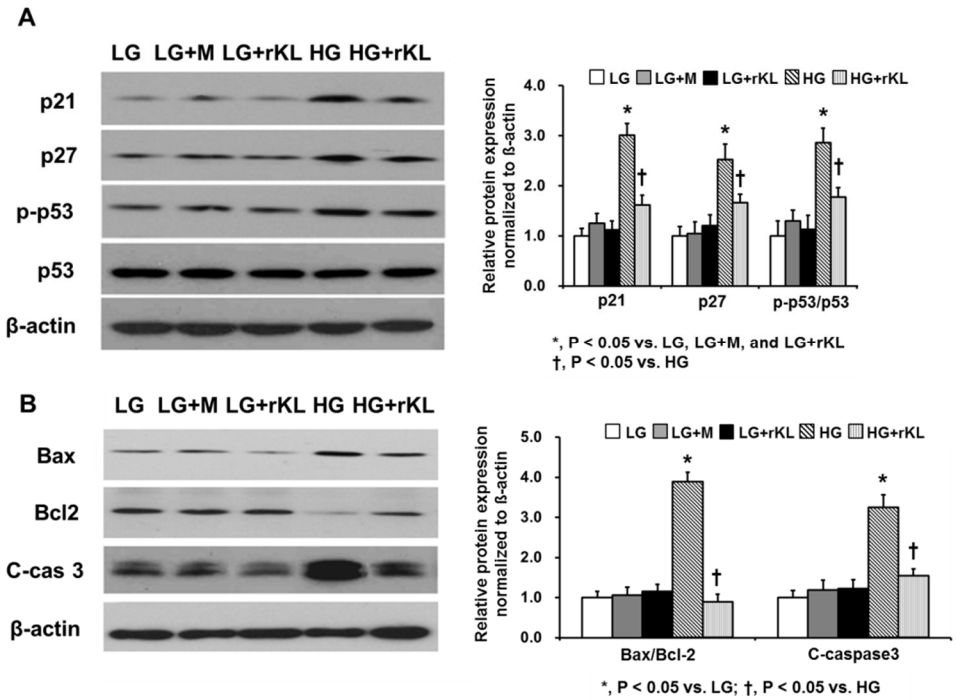

Figure 3. Changes in expression of cell cycle-related proteins and apoptotic markers by recombinant klotho in HG-treated podocytes. (A) The expression of cell cycle-related proteins such as p21, p27, and p-p53 were significantly increased in HG-treated podocytes. rKL significantly attenuated the increased expression of these

proteins. (B) The expression of Bax/Bcl2 ratio and cleaved caspase-3 were significantly increased in HG-treated podocytes. The increased expressions of these apoptosis-related proteins were significantly mitigated by rKL treatment. Abbreviation; LG, low glucose; M, mannitol; rKL, recombinant klotho; HG, high glucose; c-cas 3, cleaved caspase-3.

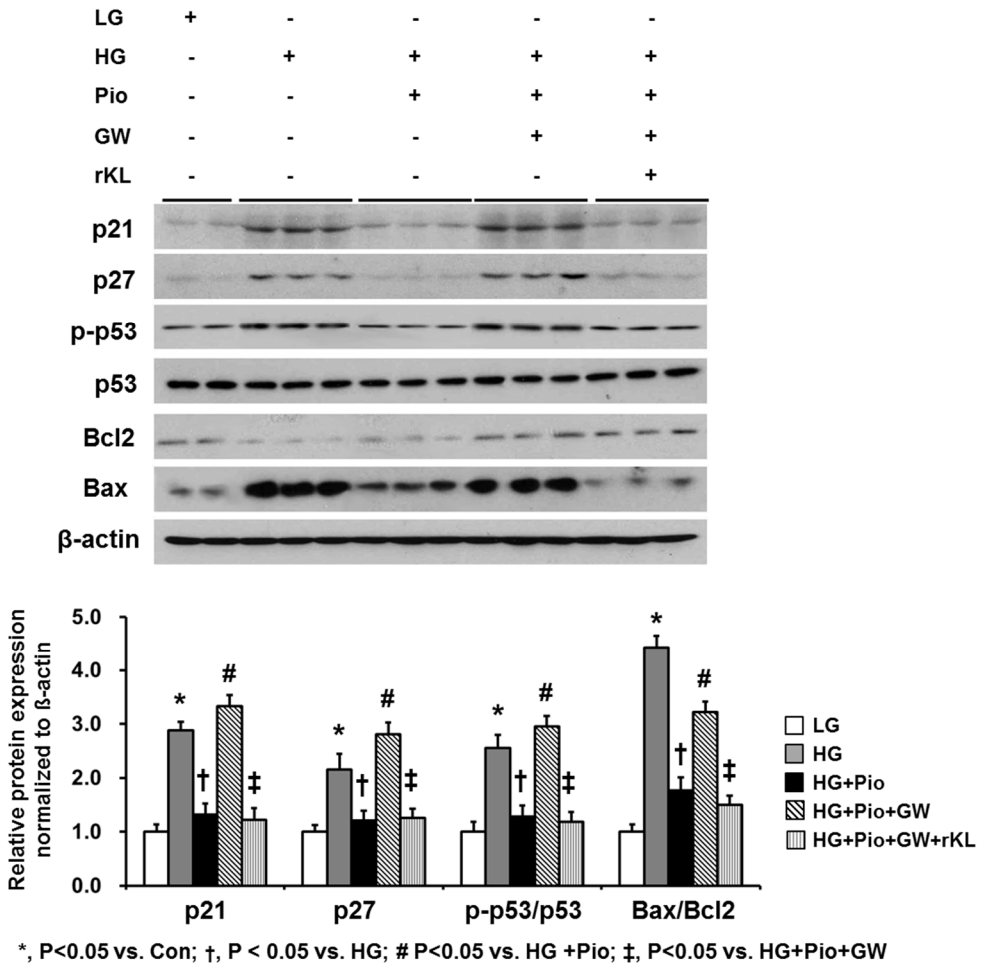

Figure 4. Effect of klotho on HG-treated podocyte injury in conjunction with PPAR-γ agonist and antagonist. Pioglitazone treatment significantly reduced the increased expression of cell-cycle related proteins and apoptotic markers in HG-treated podocytes. This favorable effect of PPAR-γ agonist on HG-induced damage was negated by PPAR-γ antagonist administration. Adding rKL restored podocyte damage

caused by PPAR-γ antagonist. Abbreviation; LG, low glucose; HG, high glucose; Pio, pioglitazone; GW, GW9662; rKL, recombinant klotho.

D. Klotho inhibits the ERK1/2 and p38 signaling pathways in HG-induced podocyte injury

To further explore the mechanism behind effectiveness of klotho against HG-induced podocyte injury, ERK1/2 and p38 signaling pathways were examined. Western blot analysis revealed that p-ERK1/2 and p-p38 expression levels were significantly increased in HG-treated podocytes, but rKL attenuated activation of ERK1/2 and p38 signaling pathways (Figure 5).

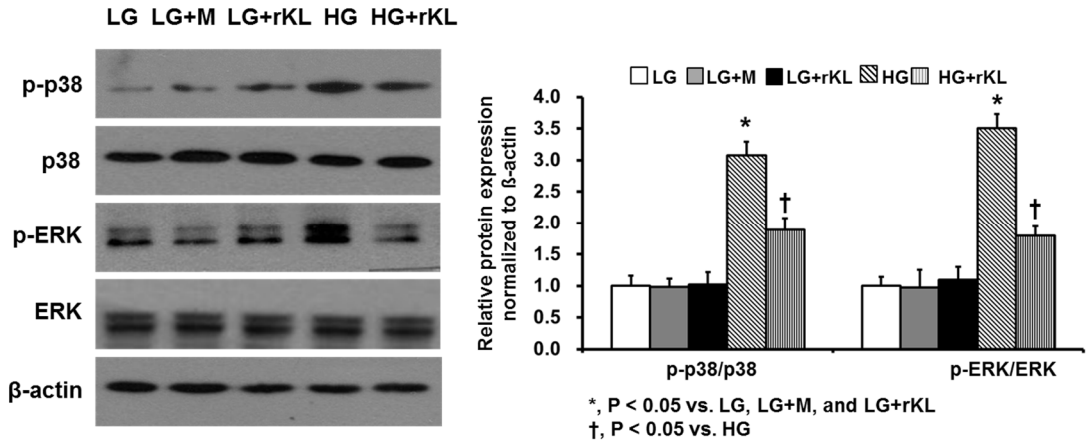

Figure 5. Effect of klotho on ERK1/2 and p38 signaling pathway in HG-induced podocyte injury. (A) Protein expression levels p-ERK1/2 and p-p38 were significantly increased in HG-treated podocytes compared to LG-treated podocytes. (B) Klotho administration significantly attenuated the increased expression of p-ERK1/2 and p-p38 in HG-induced podocytes. Abbreviation; LG, low glucose; M, mannitol; rKL, recombinant klotho; HG, high glucose.

E. A knockdown of klotho does not aggravate cell cycle arrest and apoptosis in HG-treated podocytes

To verify the effect of endogenous klotho on podocytes, the klotho gene in podocytes

was knocked down using a siRNA. Two siRNAs markedly decreased klotho expression (Figure 6A). The second siRNA was chosen for further experiments because klotho expression was almost abrogated by this reagent. Unexpectedly, the increased protein expression levels of p21, p27, and p-p53 in the HG group were not increased further by the siRNA knockdown of klotho (Figure 6B). Furthermore, the downregulation of klotho did not further aggravate the HG-induced apoptosis in the podocytes. Protein expression of apoptosis-related proteins was obviously increased in the HG group compared to the LG group, but the knockdown of klotho did not further increase cleaved caspase 3 expression. Only the Bax/Bcl2 ratio was slightly increased by the klotho knockdown (Figure 6C)

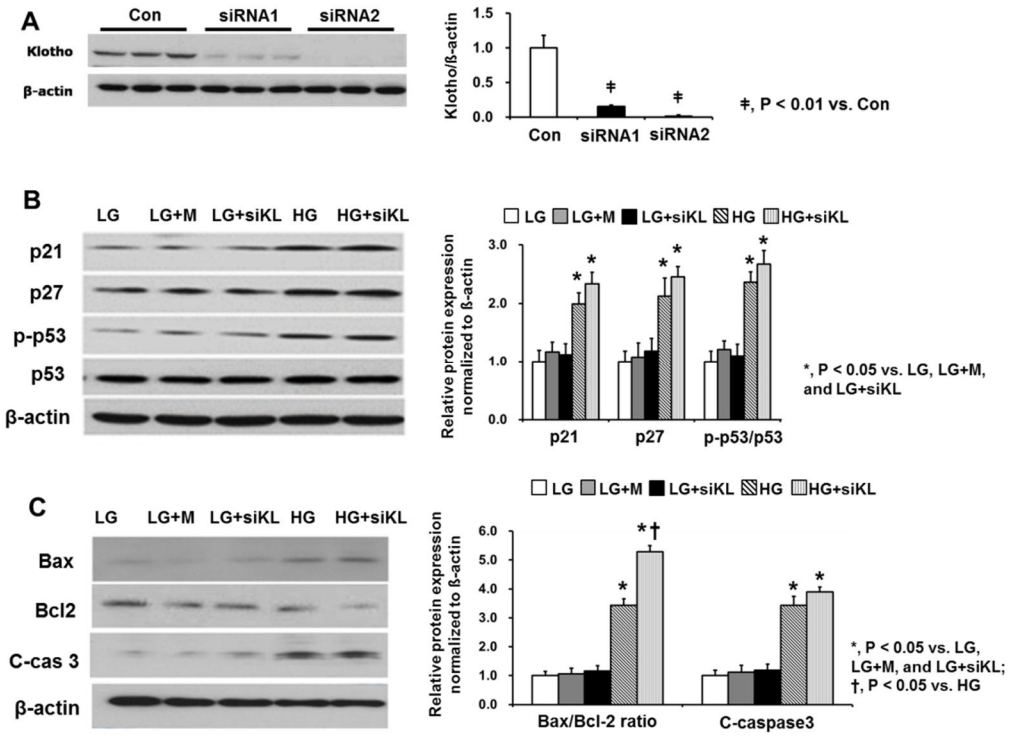

Figure 6. Effect of siRNA on cell cycle-related proteins and apoptosis-related proteins in podocytes. (A) Klotho siRNAs markedly decreased expression of klotho. (B) siRNA knockdown of klotho did not further elevate the HG-induced increases in

expression of p21, p27, and p-p53. (C) The increased expression of cleaved-caspase 3 in HG group was not further upregulated by siRNA knockdown of klotho. However, absence of klotho slightly increased Bax/Bcl2 ratio in HG-treated podocytes. Abbreviation; con, control; L, low glucose; M, mannitol; siKL, knockdown of klotho; H, high glucose; c-cas, cleaved caspase-3.

F. Flow cytometric analysis of the cell cycle arrest in podocytes

By means of flow cytometry, the cell cycle distribution of podocytes was examined to confirm the results of the western blot analyses. HG treatment significantly increased the proportion of cells in the G0 or G1 phase compared to LG treatment (80.5% vs. 65.5%, $P < 0.05$), whereas the number of cells in the S phase decreased more strongly in the HG group than in the LG group (4.6% vs. 2.1%, $P < 0.05$). rKL treatment reversed this pattern, which was 72.9% in the G0 or G1 phase and 3.7% in the S phase, respectively (Figure 7A to C).

Nevertheless, the siRNA knockdown of the klotho gene failed to enhance the cell cycle arrest in the podocytes. Although HG treatment significantly increased the G0/G1 population and decreased the S population as compared to the LG treatment, the downregulation of klotho did not significantly alter the HG-induced increase in the G0/G1 population and the HG-induced decrease in the S population (Figure 7A, D, and E).

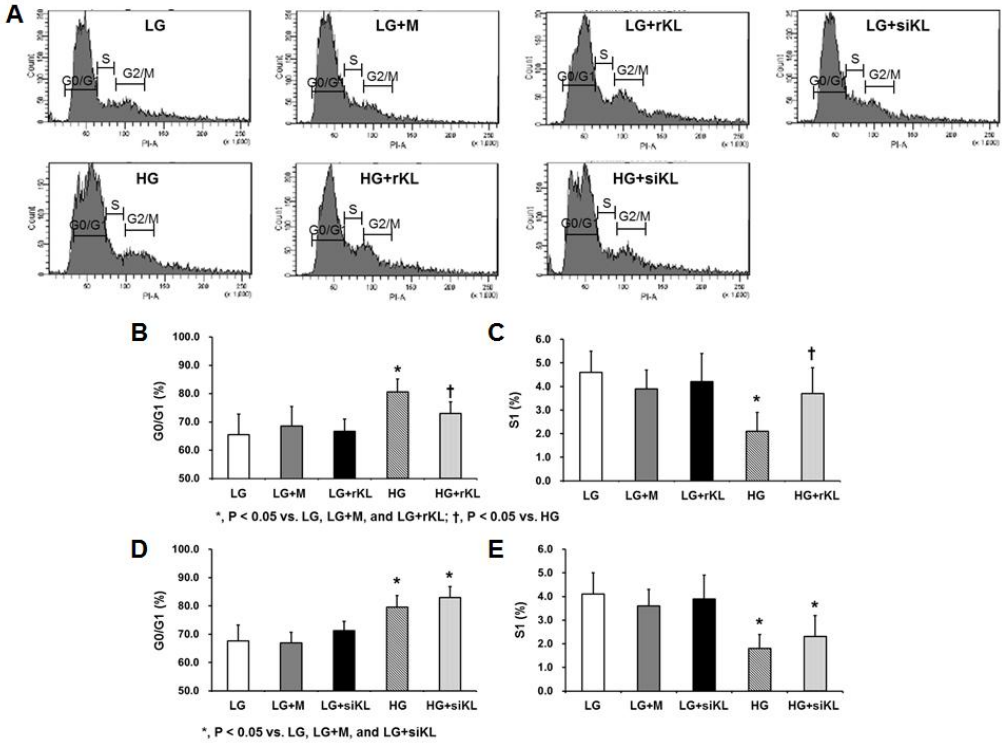

Figure 7. Flow cytometry analysis for cell cycle arrest of podocytes. (A) Flow cytometry curves (B) HG treatment significantly increased cells in the G0/G1 phase. This increase was significantly decreased by rKL treatment. (C) HG treatment significantly decreased cells in the S phase, which was restored by rKL treatment. (D) Cells in the G0/G1 phase were significantly increased by HG treatment compared to LG treatment, but klotho knockdown did not further increase the G0/G1 population. (E) Cells in the S phase were significantly decreased by HG treatment compared to LG treatment. RNA interference of klotho gene did not further lower the HG-induced decreases in the S population. Abbreviation; LG, low glucose; M, mannitol; HG, high glucose; rKL, recombinant klotho; siKL, knockdown of klotho.

2. Animal studies

A. Klotho attenuates kidney hypertrophy and decreases albuminuria in diabetic mice

The body and kidney weights were measured among all animals during the 4-week experimental period. The body weight loss was significantly greater, but the increase in kidney weight was significantly higher in the DM/UNx mice compared to C mice ($P < 0.05$). The ratio of kidney weight to body weight in the DM/UNx mice (1.4 ± 0.11) was significantly higher than that in C mice (0.74 ± 0.09; $P < 0.01$), and this increase in DM mice was significantly attenuated by the administration of rKL ($P < 0.05$). The mean blood glucose levels in DM/UNx and DM/UNx mice treated with rKL were significantly higher as compared to control mice and control mice treated with rKL ($P < 0.001$). Compared to the control mice (4.33 ± 0.04 μg/day) and control mice treated with rKL (4.17 ± 0.08 μg/day), 24-hr urinary albumin excretion was significantly higher in DM/UNx mice (12.27 ± 0.24 μg/day; $P < 0.01$), and rKL treatment significantly reduced albuminuria in DM/UNx mice ($P < 0.05$; Table 1).

Table 1. Animal data

	Control (N=8)	Control + rKL (N=8)	DM/UNx (N=8)	DM/UNx + rKL (N=8)
Body Wt (g)	25.0±0.3	24.0±0.4	21.0±0.2*	21.0±0.9*
Kidney Wt (g)	0.18±0.01	0.18±0.01	0.30±0.01*	0.23±0.01*§
Kidney Wt/Body Wt (g/g)	0.74±0.09	0.74±0.13	1.40±0.11*	1.01±0.09*,§
Blood glucose (mg/dl)	121.2±2.5	123.1±2.2	497.2±15.1#	488.7±17.2#
Urinary albumin excretion (ug/day)	4.33±0.04	4.17±0.08	12.27±0.24#	8.61±0.21#,§

*, $P < 0.05$ vs. control and control + rKL; #, $P < 0.01$ vs. control and control + rKL; §, $P < 0.05$ vs. DM/UNx group

Abbreviations: DM, diabetes mellitus; rKL, recombinant klotho; UAE, urinary albumin excretion; UNx, unilateral nephrectomy; Wt, weight

B. Klotho inhibits cell cycle arrest and attenuates apoptosis in diabetic kidneys

To validate the results of the *in vitro* experiment, the changes in protein expression of

cell cycle-related proteins and apoptotic markers in diabetic kidneys after rKL treatment were evaluated. Western blot analysis revealed increased protein expression of p21, p27, and p-p53 in the DM/UNx group compared to the control group, whereas the upregulation of these cell cycle-related proteins was significantly attenuated by rKL administration (Figure 8A). rKL treatment also significantly inhibited the activated ERK1/2 and p38 pathways in the DM/UNx group (Figure 8B). Next, apoptotic markers were examined whether klotho can reduce cell mortality caused by the diabetic injury. The ratio of protein expression levels Bax/Bcl2 and expression of cleaved caspase 3 were significantly increased in the DM/UNx group compared to control groups; the upregulation of these apoptotic markers was abrogated by rKL (Figure 8C). The TUNEL assay also detected greater numbers of TUNEL-positive cells in diabetic glomeruli than in the control, and rKL administration significantly decreased these numbers (Figure 9).

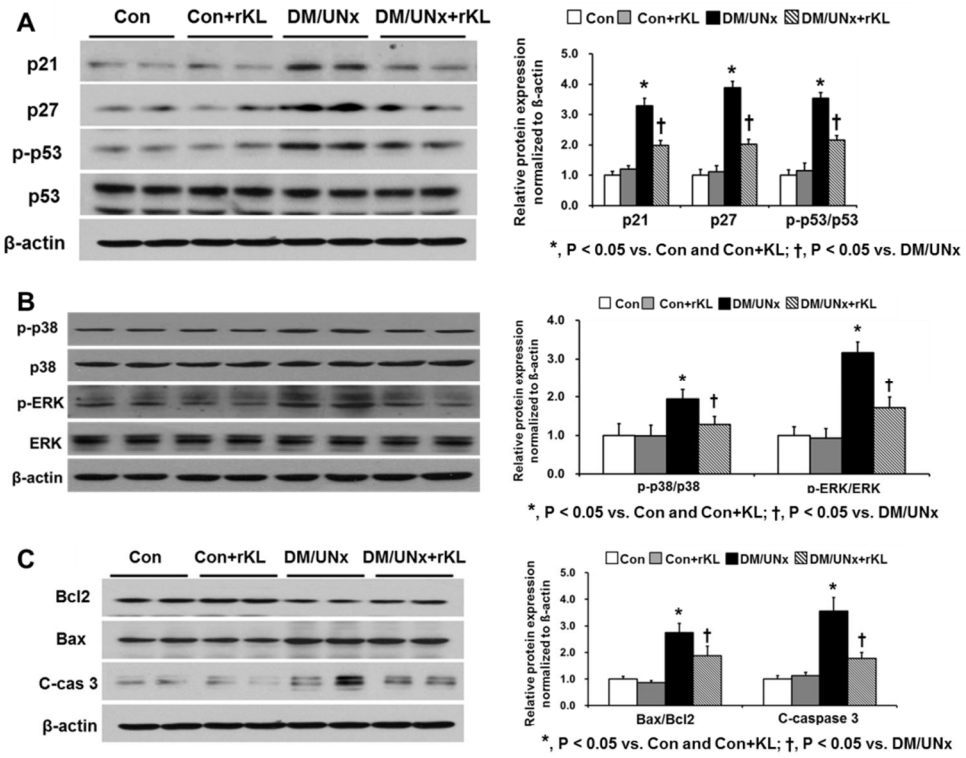

Figure 8. Effects of klotho on cell cycle arrest and apoptosis in diabetic kidney. (A) Compared to control group, protein expression levels of cell cycle-related proteins such as p21, p27, and p-p53 were significantly increased in DM/UNx group, which were attenuated by rKL treatment. (B) rKL significantly inhibited the activated ERK1/2 and p38 signaling in DM/UNx group. (C) Bax/Bcl2 ratio and cleaved caspase-3 expression were significantly increased in DM/UNx group and the increases in apoptosis markers were significantly mitigated by rKL treatment. Abbreviations: DM, diabetes mellitus; rKL, recombinant klotho; UNx, unilateral nephrectomy; c-cas 3, cleaved caspase-3.

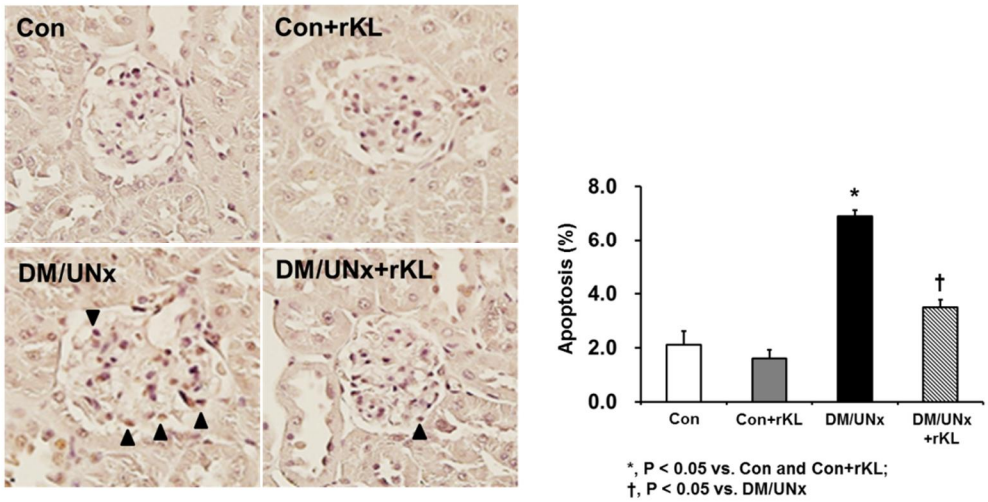

Figure 9. TUNEL assay on the effect of klotho on apoptosis. TUNEL positive cells were significantly higher in diabetic glomeruli compared to control. The positive cells were significantly diminished by rKL administration. Abbreviations: DM, diabetes mellitus; rKL, recombinant klotho; UNx, unilateral nephrectomy.

C. Klotho alleviates diabetic glomerular hypertrophy

To evaluate the effect of klotho on glomerular hypertrophy, glomerular volume was measured using images of PAS staining. Glomerular volume was significantly larger in the DM/UNx group compared to the control group. Klotho treatment significantly attenuated the increase in glomerular volume in the DM/UNx group (Figure 10).

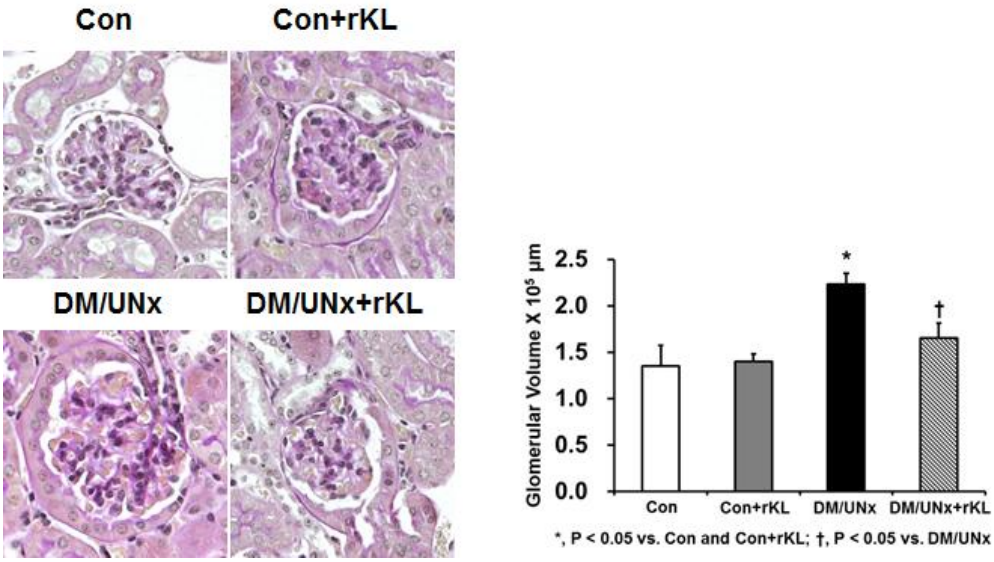

Figure 10. Morphologic analysis on glomerular volume. Glomerular volume was significantly larger in DM/UNx group than in control group. rKL treatment significantly diminished the hypertrophic glomeruli. Abbreviations: DM, diabetes mellitus; rKL, recombinant klotho; UNx, unilateral nephrectomy.

IV. DISCUSSION

This study showed that klotho is expressed in resident glomerular cells, and that the klotho gene is a target of PPAR-γ. In addition, it demonstrated that administration of rKL protects kidneys from injury under conditions of diabetes by inhibiting the cell cycle arrest and hypertrophy. However, blocking of endogenous klotho did not further worsen HG-induced podocyte damage.

Many properties of klotho have recently been highlighted in various studies. Klotho is originally known as a cofactor of FGF23 and mediates calcium-phosphate homeostasis.[11] There is evidence that klotho deficiency-induced disturbances of mineral metabolism result in vasculopathy.[20,29] Besides this function, klotho acts as a hormone that has anti-oxidative-stress, antisenescence, and antiapoptotic effects.[30,31] Because klotho is mainly produced in the distal convoluted tubules of the kidney, its expression levels are decreased in many experimental models of kidney disease, but clinical studies on humans are lacking. Several studies have shown that circulating klotho levels are decreased in patients with CKD,[7,9] and the decreased klotho levels are associated with oxidative stress in patients undergoing dialysis.[32]

The existing empirical evidence indicates that klotho is effective against kidney injury. Such studies involve various animal models such as hypertensive renal damage,[2,18] ischemia-reperfusion acute kidney injury,[3,17] unilateral ureteral obstruction,[33,34] and diabetic nephropathy.[6,35] On the other hand, these studies have been primarily focused on tubulointerstitial injury and have not evaluated glomerular injury.

A study by Haruna *et al.*[16] showed that overexpression of the klotho gene in mice with immune-complex mediated glomerulonephritis significantly relieves glomerulosclerosis and tubulointerstitial damage. In addition, Lin *et al.*[6] observed greater mesangial matrix expansion in $KL^{+/-}$ mutant mice with streptozotocin-induced diabetes than in control mice. Furthermore, a recent study by Deng *et al.*[35] revealed that the klotho transgene ameliorates glomerular hypertrophy in mice with streptozotocin-induced diabetes. Nevertheless, many uncertainties remain regarding whether klotho

acts directly on resident glomerular cells and via which mechanism klotho can exert the protective effect. With this background information in mind, here I studied the effect of klotho on podocytes and found that klotho attenuates cell injury via a cell cycle-dependent mechanism under conditions of diabetes.

Several studies have indicated that klotho is a downstream effector of PPAR-γ.[28,36] In fact, PPAR-γ binds to the 5′-flank promoter region of the klotho gene and regulates klotho expression.[28] In addition, pioglitazone, a PPAR-γ agonist, can increase klotho expression in aging kidneys.[36] In agreement with these findings, this study clearly showed that klotho expression is dependent on PPAR-γ and detected a significant interaction between PPAR-γ and the klotho gene in podocytes by a ChIP assay (Figure 2). PPAR-γ agonists are generally known as antidiabetic drugs, but they confer a variety of beneficial effects onto kidney cells independently of the lowering of glucose levels.[37] In this study, klotho expression in podocytes is upregulated by treatment with a PPAR-γ agonist and downregulated by a PPAR-γ antagonist. This finding points to a possible mechanism behind the effectiveness of the PPAR-γ agonist against diabetic glomerular injury.

Renal hypertrophy is a prominent feature of early diabetic nephropathy. Glomerular and tubular hypertrophy mainly contributes to the increase in kidney size, but the latter is also partly due to inflammatory-cell infiltration, extracellular-matrix accumulation, and hemodynamic factors.[38,39] Our group previously demonstrated that podocytes and glomeruli under conditions of diabetes undergo hypertrophy that is characterized by increased expression of cell cycle-related proteins (such as p21 and p-p53), phospho-eukaryotic elongation factor 4E-binding protein 1, and phospho-p70 S6 ribosomal protein kinase.[40] In line with those findings, this study showed that expression levels of these cell cycle-related proteins are significantly increased in HG-treated podocytes and in diabetic kidneys. Of note, these changes were found to be significantly attenuated by rKL treatment. In addition, rKL significantly abrogated the HG-induced increase in the G0/G1 population of podocytes. Furthermore, the increased glomerular volume in diabetic kidneys was significantly diminished by the administration of klotho. These

findings indicate that klotho can limit podocyte and glomerular hypertrophy by inhibiting cell cycle arrest.

A number of research papers have indicated that p38 and extracellular signal-regulated protein kinase (ERK)-1/ERK-2 are also implicated in the hypertrophic response.[41] Podocytes undergo hypertrophy in response to mechanical stress, and this response requires activation of p21, ERK1/2, and Akt.[24,42] These findings were corroborated by a recent study by Huang et al.[43] In that study, the authors demonstrated that exogenous klotho decreases HG-activated p38 and ERK1/2 signaling and thereby attenuates cell hypertrophy. In agreement with those studies, rKL treatment significantly decreases the increased expression of p38 and ERK1/2 in HG-treated podocytes and in diabetic glomeruli. This finding seems to be additional evidence that klotho can modulate glomerular hypertrophy by suppressing p38 and ERK1/2 signaling.

It is not clear whether klotho has an autocrine effect in podocytes because the knockdown of the endogenous klotho gene by means of the siRNA did not further worsen cellular hypertrophy or cell death in HG-treated podocytes. HG-induced podocyte injury was alleviated only by rKL treatment. It is possible that HG-induced podocyte injury depleted klotho, thus blunted the effect of siRNA. Alternatively, klotho mediates the protective effects via paracrine action. It is well-known that klotho functions as a hormone.[13,31] It can be presumed that klotho production is much lower in podocytes than in the distal tubules, and thus the autocrine effect of klotho on podocytes is overridden by the paracrine action of klotho from the distal tubules. In fact, immunohistochemical study and ELISA in culture media showed significantly lower klotho levels in podocytes than in tubular epithelial cells (data not shown) although the presence of klotho was demonstrated by RT-PCR and Western blot analysis.

V. CONCLUSION

The present study demonstrated that klotho was present in glomerular podocytes and its expression was regulated by PPAR-γ. The administration of klotho attenuated podocyte and glomerular hypertrophy via cell cycle-dependent mechanism, and thus resulted in decreased apoptosis. Klotho is more likely to act in a paracrine manner rather than autocrine manner on podoctyes. These findings provide new insights to the mechanisms of protective effects of klotho in diabetic kidney disease.

1. Klotho is expressed in glomerular cells.
2. Klotho is a downstream target of PPAR-γ.
3. Klotho inhibits apoptosis in HG-treated podocytes via cell cycle-dependent mechanism.
4. Knockdown of klotho does not aggravate cell cycle arrest and apoptosis in HG-treated podocytes.
5. Klotho inhibits cell cycle arrest and attenuates apoptosis in diabetic kidney.
6. Klotho alleviates diabetic glomerular hypertrophy.

In conclusion, these findings suggest that klotho provides a protective role against glomerular hypertrophy through cell cycle-dependent manner in diabetic nephropathy.

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ABSTRACT (IN KOREAN)

당뇨로 인한 세포주기 변화로 발생하는 족세포 손상에 대한 klotho 의 보호 효과

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배경: 최근까지 여러 연구들은, 당뇨병성 신병증을 포함한 기타 실험적 신장병 모델을 통해 klotho의 신장병 진행에 대한 보호 효과를 보여주고 있지만, 대부분의 연구가 세뇨관에 국한되어 있다. 한편, 당뇨병성 신병증은, 사구체 병변이 주된 병리 변화이기 때문에, 당뇨병성 신병증 모델에서 klotho의 역할을 보고자 할 때, 사구체 및 사구체 세포를 이용한 실험은 의미가 있을 것이다.

방법: 생체 외 실험으로 족세포를 이용해 고포도당 (30 mM) 자극 후, klotho의 변화를 확인하고자 하였고, 재조합 klotho 투여 후 세포주기 관련 단백질 발현의 변화를 알아 보고자 하였다. 또한, 족세포로부터 발현된 klotho의 효과를 밝히고자, klotho knockdown을 시행하였다. 생체 내 실험으로 32 마리의 C57BL/6 생쥐를 이용하여, 16 마리에게 당뇨 유발을 위해 streptozotocin를 복강으로 투여하였으며, 이 중 8 마리에게는 osmotic minipump를 이용해 재조합 klotho (10 ug/kg per day)를 투여하였다. 반면, 대조군 16 마리 중에서 8 마리에게는 osmotic

minipump를 통해 생리식염수를 투여하였다. 모든 군에서 한쪽 신장 제거술을 시행하여 좀더 빠른 신장 악화를 유발하고자 하였으며, 당뇨 유발 4주 후 희생을 통해 신장을 적출하여 단백질, RNA의 변화를 확인하였고, 면역형광법 및 면역조직화학을 시행하였다.

결과: 족세포와 혈관사이세포에서 klotho mRNA 및 단백질이 발현됨을 확인하였고, 고포도당 자극 후 수치가 감소함을 확인하였다. 더 나아가, klotho가 PPAR-ɣ 변화에 따라 발현됨을 알았다. 족세포에서 고포도당 자극으로 증가되었던 세포주기 관련 단백질인 p21, p27, p-p53과 세포사멸 관련 단백질인 Bax/Bcl2 및 cleavage caspase-3가 재조합 klotho 투여 후 유의하게 감소됨을 확인 할 수 있었으나, klotho 발현을 억제하였음에도 고포도당 자극 후, 이들의 발현 정도는 klotho 발현을 억제하지 않은 세포와 비교 시, 더 악화되지 않았다. 생체 외 실험 결과와 유사하게, 당뇨 유발 동물 모델에서 세포 주기 및 세포 사멸과 관련된 상기 단백질 발현이 정상군에 비해 유의하게 증가된 반면, 재조합 klotho 투여군에서는 투여하지 않은 당뇨 유발 군에 비해 이 단백질 발현들이 유의하게 감소함을 확인할 수 있었다. 또한, 당뇨군에서 사구체 부피도 재조합 klotho 투여 후, 투여하지 않은 군에 비해 감소함을 확인하였다.

결론: 이상의 결과를 종합하여 볼 때, klotho는 사구체 족세포에서 발현되며, PPAR-ɣ에 의해 조절됨을 확인하였다. 실험으로 유도된 당뇨 모델에서 재조합 klotho의 투여로 세포 주기 관련 기전 및 사구체의 비대가 완화됨을 확인하였으며, 이는 세포 사멸과 관련되어 있음을 확인하였다. 이상으로 klotho는 당뇨병성 신장 질환 악화에 보호 역할을 할 수 있을 것으로 기대된다.

핵심되는 말: klotho, 족세포, 고혈당, 세포주기 방식, 세포사멸, 당뇨병성 신병증