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Postprint: The Role and Molecular Mechanism of Klotho in Renal Injury in Salt-Sensitive Hypertension

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Abstract

Background: Klotho is closely associated with the occurrence and development of kidney diseases, and salt-sensitive hypertension (SSH) often accompanies kidney diseases. Currently, studies on the role and molecular mechanism of klotho in SSH-induced renal injury are rarely reported.

Objective: To investigate the role and molecular mechanism of klotho in SSH-induced renal injury.

Methods: On June 15, 2021, the rat glomerular mesangial cell line HBZY1 was selected as the experimental cell line. The experimental cells were divided into a control group and a model group. A HBZY1 cell injury model induced by NaCl 137 mmol/L and angiotensin II (Ang II) 10^{-6} mmol/L was used to simulate SSH-induced renal injury, and cells were collected. Real-time quantitative PCR (qRT-PCR) and Western blotting were used to detect differences in klotho mRNA and protein expression. Klotho interference and overexpression vectors and angiotensin II type 1 receptor (AT1R) overexpression vector were constructed. The klotho interference experiment was divided into five groups, including a control group, an empty vector group, klotho-siRNA1, klotho-siRNA2, and klotho-siRNA3; the klotho overexpression experiment was divided into three groups, including a control group, an empty vector group, and a klotho overexpression group; the AT1R overexpression experiment was divided into three groups, including a control group, an empty vector group, and an AT1R overexpression group. The constructed vectors were transfected into cells and transfection efficiency was verified. After successful transfection, the experiment was conducted in two parts. The first part verified the renal protective effect of klotho, with experimental groups divided into four groups, including a control group, a model group, a klotho overexpression group, and a klotho interference group. The second part explored whether the renal protective effect of klotho

was related to AT1R, with the experiment divided into three groups, including a model group, a klotho overexpression group, and a klotho+AT1R overexpression group. After successful transfection, the following detections were performed: cell viability was detected by cell counting kit-8 (CCK-8) assay, cellular reactive oxygen species (ROS) content was detected by flow cytometry, malondialdehyde (MDA) and superoxide dismutase (SOD) contents in cell supernatant were detected by enzyme-linked immunosorbent assay (ELISA), and the interaction between klotho and AT1R was detected by co-immunoprecipitation (Co-IP).

Results: Compared with the control group, klotho mRNA level and protein expression in the model group were both decreased (t=7.102, 7.506, P=0.002, 0.002). Compared with the control group, the interference effect of klotho-siRNA2 was significant ($P<0.001$); klotho protein expression in the klotho overexpression group was significantly increased ($P<0.001$); AT1R protein expression in the AT1R overexpression group was significantly increased ($P<0.001$). Effects of klotho on cell viability and oxidative stress injury: Compared with the control group, cell viability in the model group decreased ($P<0.001$), intracellular ROS and MDA contents increased ($P<0.001$, P=0.004), and intracellular SOD content decreased (P=0.041); compared with the model group, cell viability in the klotho overexpression group increased ($P<0.001$), intracellular ROS and MDA contents decreased ($P<0.001$, P=0.003), and intracellular SOD content increased (P=0.018); compared with the model group, cell viability in the klotho interference group decreased ($P<0.001$), intracellular ROS and MDA contents increased ($P<0.001$, P=0.002), and intracellular SOD content decreased (P=0.001). Effects of klotho on cell viability and oxidative stress injury via AT1R: Compared with the model group, cell viability in the klotho overexpression group increased ($P<0.001$), intracellular ROS and MDA contents decreased ($P<0.001$, P=0.024), and intracellular SOD content increased (P=0.007); compared with the klotho overexpression group, cell viability in the klotho+AT1R overexpression group decreased ($P<0.001$), intracellular ROS and MDA contents in the klotho+AT1R overexpression group increased ($P<0.001$, P=0.001), and intracellular SOD content decreased (P=0.002). Co-IP confirmed that there is an interaction between klotho and AT1R.

Conclusion: Klotho exerts a protective effect in SSH-induced renal injury by interacting with AT1R and inhibiting oxidative stress injury.

Full Text

The Role and Molecular Mechanism of Klotho in Renal Injury Induced by Salt-Sensitive Hypertension

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Abstract Background: Klotho is closely associated with the occurrence and development of kidney disease, and salt-sensitive hypertension (SSH) is often

accompanied by renal disease. Currently, few studies have reported on the role and molecular mechanism of klotho in SSH-induced renal injury. **Objective:** To investigate the role and molecular mechanism of klotho in renal injury induced by SSH.

Methods: On June 15, 2021, the rat glomerular mesangial cell line HBZY1 was selected as the experimental cell model. Cells were divided into control and model groups. A cell injury model was established using 137 mmol/L NaCl and 10^{-6} mmol/L angiotensin II (Ang II) to simulate renal injury in SSH, after which cells were collected. Differences in klotho mRNA and protein expression were detected by real-time fluorescent quantitative PCR (qRT-PCR) and Western Blot. Interference and overexpression vectors for klotho and an overexpression vector for angiotensin II type 1 receptor (AT1R) were constructed. Klotho interference experiments were divided into five groups: control, empty vector, klotho-siRNA1, klotho-siRNA2, and klotho-siRNA3. Klotho overexpression experiments were divided into three groups: control, empty vector, and klotho overexpression. AT1R overexpression experiments were divided into three groups: control, empty vector, and AT1R overexpression. The constructed vectors were transfected into cells and transfection efficiency was verified.

After successful transfection, the experiment was conducted in two parts. The first part verified the renal protective effect of klotho, with subjects divided into four groups: control, model, klotho overexpression, and klotho interference. The second part explored whether the renal protective effect of klotho was related to AT1R, with subjects divided into three groups: model, klotho overexpression, and klotho+AT1R overexpression. After successful transfection, cell viability was detected by cell counting kit-8 (CCK-8) assay, reactive oxygen species (ROS) content was measured by flow cytometry, malondialdehyde (MDA) and superoxide dismutase (SOD) levels in cell supernatant were determined by enzyme-linked immunosorbent assay (ELISA), and the interaction between klotho and AT1R was detected by co-immunoprecipitation (Co-IP).

Results: Compared with the control group, klotho mRNA and protein levels in the model group decreased significantly ($t=7.102$, 7.506; $P=0.002$, 0.002). The klotho-siRNA2 interference effect was most significant ($P<0.001$). Klotho protein expression increased significantly in the klotho overexpression group ($P<0.001$), and AT1R protein expression increased significantly in the AT1R overexpression group ($P<0.001$). Effects of klotho on cell viability and oxidative stress injury: Compared with the control group, cell viability in the model group decreased ($P<0.001$), intracellular ROS and MDA content increased ($P<0.001$, $P=0.004$), and SOD content decreased ($P=0.041$). Compared with the model group, cell viability in the klotho overexpression group increased ($P<0.001$), intracellular ROS and MDA content decreased, and SOD content increased ($P<0.001$, $P=0.003$, $P=0.018$). Compared with the model group, cell viability in the klotho interference group decreased ($P<0.001$), while intracellular ROS and MDA content increased and SOD content decreased ($P<0.001$, $P=0.002$, $P=0.001$). Effects of klotho on cell viability and oxidative stress injury through

AT1R: Compared with the model group, cell viability increased (P<0.001), intracellular ROS and MDA content decreased, and SOD content increased (P<0.001, P=0.024, P=0.007) in the klotho overexpression group. Compared with the klotho overexpression group, cell viability decreased (P<0.001), ROS and MDA content increased, and SOD content decreased (P<0.001, P=0.001, P=0.002) in the klotho+AT1R overexpression group. Co-IP confirmed an interaction between klotho and AT1R.

Conclusion: Klotho plays a protective role in renal injury in SSH by inhibiting oxidative stress injury through interaction with AT1R.

[**Key words**] Hypertension; Salt-sensitive hypertension; Kidney injury; Oxidative stress; Klotho; Angiotensin II; Receptor, angiotensin, type 1

Salt is an important predisposing factor for hypertension. Salt sensitivity of blood pressure refers to an elevated blood pressure response to high salt intake, and the associated hypertension type is called salt-sensitive hypertension (SSH). Epidemiological surveys show that 51% of hypertensive populations have salt sensitivity, and SSH accounts for 50%-60% of hypertensive patients in China. Moreover, compared with primary hypertension, SSH patients have higher incidence and more severe damage in target organs such as blood vessels, heart, and kidneys. SSH-induced renal injury is the result of long-term high salt load and can progress to chronic kidney disease (CKD) and even end-stage renal disease.

Klotho, identified as an anti-aging gene, is primarily expressed in the kidneys. Its encoded secreted protein (klotho protein) can enter the circulation in a hormone-like manner to exert beneficial effects. Studies have found that any form of renal injury leads to decreased klotho expression, and klotho deficiency occurs at any stage of CKD while also accelerating CKD progression. Currently, klotho has emerged as a novel biomarker for evaluating disease status and prognosis in CKD patients and plays an important role in CKD treatment. However, to date, few studies have reported on the role and molecular mechanism of klotho in SSH-induced renal injury. Therefore, this study used an HBZY1 cell injury model induced by 137 mmol/L NaCl and 10^{-6} mmol/L angiotensin II (Ang II) to simulate SSH renal injury. By observing the effects of klotho on HBZY1 cell viability and related indicators, we aimed to elucidate the role and molecular mechanism of klotho in SSH renal injury, which is of great significance for hypertension management and cardiovascular disease prevention.

1.1 Experimental Materials

1.1.1 Experimental Cells Rat glomerular mesangial cell line HBZY1 (Procell, catalog number: CL-0092).

1.1.2 Experimental Reagents Major reagents included: Opti-MEM®, TRIzol Reagent (CWBio, catalog number: CW0580S); Ultrapure RNA extraction kit (CWBio, catalog number: CW0581M); HiScript II Q RT SuperMix for

qPCR (+gDNA wiper) (Vazyme, catalog number: R223-01); 2×SYBR Green PCR Master Mix (Lifeint, catalog number: A4004M); Reactive Oxygen Species Assay Kit (KeyGen Bio, catalog number: KGT010-1100 assays); Superoxide Dismutase (SOD) ELISA kit (Enzyme-linked Biotechnology, catalog number: MM-0386R2); Malondialdehyde (MDA) assay kit (Enzyme-linked Biotechnology, catalog number: MM-0385R2); Ultra-sensitive luminescent solution (Thermo Fisher, catalog number: RJ239676); PVDF membrane (Millipore, catalog number: IPVH00010); Primary internal reference antibody: Mouse Monoclonal Anti-GAPDH (ZSGB-BIO, catalog number: TA-08, 1/2000); Secondary antibody: HRP-labeled goat anti-mouse IgG (H+L) (ZSGB-BIO, catalog number: ZB-2305); Target primary antibody: Rabbit Anti-klotho (Santa Cruz, catalog number: sc-515942, 1/1000); Target primary antibody: Rabbit Anti-AT1R (Proteintech, catalog number: 25343-1-AP, 1/1000); BCA protein concentration assay kit (MDL, catalog number: MD913053); SDS-PAGE precast gel kit (MDL, catalog number: MD911919); RNase Free ddH_2O (Hunan Ategene Biotechnology, catalog number: RO1012); β-actin (Affinity, catalog number: AF7018); Horseradish peroxidase (HRP) (Beijing Solarbio Science & Technology, catalog number: P8020); Phosphate buffered saline (PBS) (BioVision, catalog number: 2128-1000); Lipofectamine 3000 (Thermo Fisher, catalog number: L3000015); P3000 (Qiagen, catalog number: 9013150); RIPA lysis buffer (Beijing Pulilai Gene Technology, catalog number: C1055).

1.1.3 Experimental Instruments Fluorescent PCR instrument (Bio-Rad, catalog number: CFX Connect™ Real-Time); Ultra-high sensitivity chemiluminescence imaging system (Bio-Rad, catalog number: Chemi Doc™ XRS+); Automatic chemiluminescence image analysis system (Shanghai Tanon Science & Technology, catalog number: Tanon-5200); NovoCyte™ flow cytometer (ACEA Biosciences, catalog number: NovoCyte2060R); Automatic microplate reader (Liuyi, catalog number: WD-2102B).

1.2 Experimental Methods

1.2.1 Cell Modeling and Grouping The experiment began on June 15, 2021. First, experimental cells were divided into control and model groups. The control group was cultured normally, while the model group was stimulated with 137 mmol/L NaCl and 10^{-6} mmol/L Ang II for 24 hours to establish an SSH renal injury cell model. Cells were then collected, and differences in klotho mRNA and protein expression were detected by qRT-PCR and Western Blot. To verify the renal protective effect of klotho and its underlying mechanisms, klotho interference and overexpression vectors and an AT1R overexpression vector were constructed. Klotho interference experiments were divided into five groups: control, empty vector, klotho-siRNA1, klotho-siRNA2, and klotho-siRNA3. The empty vector group was transfected with blank plasmid, while klotho-siRNA1, klotho-siRNA2, and klotho-siRNA3 groups were transfected with siRNA plasmid vectors containing corresponding sequences. Klotho overexpression experiments were divided into three groups: control, empty vector, and klotho over-

expression. The empty vector group was transfected with blank plasmid, while the klotho overexpression group was transfected with recombinant plasmid containing the klotho gene. AT1R overexpression experiments were divided into three groups: control, empty vector, and AT1R overexpression. The empty vector group was transfected with blank plasmid, while the AT1R overexpression group was transfected with recombinant plasmid containing the AT1R gene. The constructed vectors were transfected into cells and transfection efficiency was verified.

After successful transfection, the experiment was conducted in two parts. The first part verified the renal protective effect of klotho, with subjects divided into four groups: control, model, klotho overexpression, and klotho interference. The second part explored whether the renal protective effect of klotho was related to AT1R, with subjects divided into three groups: model, klotho overexpression, and klotho+AT1R overexpression. After successful transfection, cell viability was detected by CCK-8 assay, ROS content was measured by flow cytometry, MDA and SOD levels in cell supernatant were determined by ELISA, and the interaction between klotho and AT1R was detected by Co-IP.

1.2.2 Cell Transfection When cell density reached 70%, the culture medium was replaced. Two sterile EP tubes were prepared, each containing 125 l Opti-MEM®. One tube received 5 l Lipofectamine 3000, while the other received 2.5 g plasmid and 5 l P3000. After mixing, they were incubated at room temperature for 5 minutes. The contents of the two tubes were then mixed together and incubated at room temperature for 15 minutes. The mixture was added dropwise to the corresponding wells of a six-well plate, and cells were returned to the incubator. After 4 hours of transfection, 1 ml of complete medium containing 20% serum was added to each well of the six-well plate. Subsequent experiments were performed 48 hours later.

1.2.3 CCK-8 Assay Transfected cells were digested, resuspended, and counted, then plated at a density of 7,000 cells per well. After cell adhesion, the medium in the 96-well plate was replaced with fresh medium (100 l per well) after 24 hours. CCK-8 reagent (10 l) was added to each well and incubated in the culture incubator for 2 hours. Absorbance (OD) values at 450 nm were measured using a microplate reader.

1.2.4 Flow Cytometry The reactive oxygen species fluorescent probe DCFH-DA was diluted 1:1,000 with serum-free medium to a final concentration of 10 mol/L. Cell culture medium was removed, and diluted DCFH-DA was added to cells and incubated at 37°C for 20 minutes. Cells were washed three times with serum-free medium to remove excess DCFH-DA that had not entered cells. After adding 1 ml PBS, cells were centrifuged at 1,500 rpm for 5 minutes, the supernatant was discarded, and cells were resuspended in 300 l PBS for flow cytometry analysis.

1.2.5 ELISA Assay Required strips were removed from aluminum foil bags after equilibrating at room temperature for 60 minutes. Remaining strips were

sealed in self-sealing bags and stored at 4°C. Standard and sample wells were prepared. Standard wells received 50 l of different concentrations of standards, sample wells received 50 l of samples, and blank wells received nothing. HRP-labeled detection antibody (100 l) was added to each well, wells were sealed with sealing film, and incubated at 37°C in a water bath or incubator for 60 minutes. Liquid was discarded, wells were dried on absorbent paper, and washing buffer (350 l) was added to each well. After standing for 1 minute, washing buffer was discarded and wells were dried on absorbent paper. This washing process was repeated five times. Substrate A and B (50 l each) were added to each well and incubated at 37°C for 15 minutes protected from light. Stop solution (50 l) was added to each well, and OD values at 450 nm were measured within 15 minutes.

1.2.6 qRT-PCR Samples were fully lysed with TRIzol to extract RNA. RNA concentration and purity were measured, and RNA was reverse transcribed into cDNA using HiFiScript cDNA First Strand Synthesis Kit. Using cDNA as template, detection was performed on a fluorescent quantitative PCR instrument with β-actin as internal reference to calculate relative mRNA expression levels in each group. The reaction system included: 2×SYBR Green PCR Master Mix (10 l), cDNA (1 l), forward primer (0.4 l), reverse primer (0.4 l), and RNase Free ddH$_2$O (8.2 l). Klotho primers: forward 5' -ACTTTCTTCTGCCCTATTTCACG-3' , reverse 5' -CCAGGTAATCGTTGTATTTTATCGG-3' ; β-actin primers: forward 5' -GCCATGTACGTAGCCATCCA-3' , reverse 5' -GAACCGCTCATTGCCGATAG-3' .

1.2.7 Western Blot Cells were lysed with lysis buffer, placed on ice for 15 minutes, and centrifuged at 12,000 r/min for 10 minutes. The supernatant was collected, buffer solution was added, and samples were boiled for 5 minutes and stored at -20°C. Protein concentration in cell supernatant was detected using BCA protein assay kit, and a standard curve was prepared. SDS-PAGE gels were prepared, samples were loaded, and electrophoresis was performed at 60 V for protein compression and 80 V for protein separation (120 minutes). Gels containing internal reference or target bands were cut. Sponge, filter paper, gel, membrane, filter paper, and sponge were assembled in sequence. The target tape complex was immersed in pre-cooled 1× transfer buffer, and transfer was performed at 300 mA constant current. Blocking solution containing 3% skim milk prepared with 1×TBST was used to block for 1 hour. PVDF membrane was incubated with primary antibody overnight. Membrane was washed by soaking in 1×TBST for 10 minutes, discarded, and repeated three times. PVDF membrane was incubated with secondary antibody for 2 hours. Membrane was washed by soaking in 1×TBST for 10 minutes, discarded, and repeated three times. PVDF membrane was soaked in luminescent solution and placed in the sample area of the ultra-high sensitivity chemiluminescence imaging system for imaging.

1.2.8 Co-IP After cell lysis with RIPA lysis buffer, protein concentration was

measured by BCA assay. Equal amounts of protein were first pre-treated with IgG and A/G protein cross-linked agarose beads, then incubated with Klotho antibody and agarose beads. Finally, equal amounts of protein were subjected to gel electrophoresis and membrane transfer, followed by detection with corresponding antibodies.

1.3 Statistical Analysis GraphPad Prism 8.0 software was used for statistical analysis. All experiments were performed at least three times independently, and results were expressed as ($\bar{x}\pm s$). Comparisons between two groups were performed using independent sample t-test, comparisons among multiple groups were performed using one-way ANOVA, and multiple comparisons among groups were performed using Tukey' s test. $P<0.05$ was considered statistically significant.

2.1 Effect of NaCl and Ang II Treatment on klotho mRNA and Protein in HBZY1 Cells

Compared with the control group, klotho mRNA and protein levels in the model group decreased significantly ($t=7.102$, 7.506; $P=0.002$, 0.002) [Figure 1: see original paper].

2.2 Verification Results of klotho Interference and Overexpression and AT1R Overexpression

Protein expression differed significantly among the control, empty vector, klotho-siRNA1, klotho-siRNA2, and klotho-siRNA3 groups ($F=23.13$, $P<0.001$). All three klotho interference sequences showed interference effects compared with the control group, with klotho-siRNA2 showing the best interference effect ($P<0.001$) [Figure 2: see original paper]. Protein expression differed significantly among the control, empty vector, and klotho overexpression groups ($F=414.80$, $P<0.001$). Klotho protein expression increased significantly in the klotho overexpression group compared with the control group ($P<0.001$) [Figure 3: see original paper]. Protein expression differed significantly among the control, empty vector, and AT1R overexpression groups ($F=53.97$, $P<0.001$). AT1R protein expression increased significantly in the AT1R overexpression group compared with the control group ($P<0.001$) [Figure 4: see original paper].

2.3 Effect of klotho on Cell Viability

Cell viability differed significantly among the control, model, klotho overexpression, and klotho interference groups ($F=537.60$, $P<0.001$). Compared with the control group, cell viability in the model group decreased significantly ($P<0.001$). Compared with the model group, cell viability increased in the klotho overexpression group and decreased in the klotho interference group ($P<0.001$) [Figure 5: see original paper].

2.4 Effect of klotho on Oxidative Stress Injury of Cells

ROS, MDA, and SOD levels differed significantly among the control, model, klotho overexpression, and klotho interference groups (F=1,677.00, 54.33, 47.15; P<0.001). Compared with the control group, intracellular ROS and MDA content increased (P<0.001, P=0.004) and SOD content decreased (P=0.041) in the model group. Compared with the model group, ROS and MDA content decreased and SOD content increased in the klotho overexpression group (P<0.001, P=0.003, P=0.018), while ROS and MDA content further increased and SOD content further decreased in the klotho interference group (P<0.001, P=0.002, P=0.001) [Figure 6: see original paper].

2.5 Effect of Klotho on Cell Viability via AT1R

Cell viability differed significantly among the model, klotho overexpression, and klotho+AT1R overexpression groups (F=335.50, P<0.001). Compared with the model group, cell viability increased in the klotho overexpression group (P<0.001). Compared with the klotho overexpression group, cell viability decreased in the klotho+AT1R overexpression group (P<0.001) [Figure 7: see original paper].

2.6 Effect of Klotho on Oxidative Stress Injury of Cells via AT1R

ROS, MDA, and SOD levels differed significantly among the model, klotho overexpression, and klotho+AT1R overexpression groups (F=7,815.00, 26.77, 22.54; P<0.001, P=0.001, P=0.002). Compared with the model group, ROS and MDA content decreased and SOD content increased in the klotho overexpression group (P<0.001, P=0.024, P=0.007). Compared with the klotho overexpression group, ROS and MDA content increased and SOD content decreased in the klotho+AT1R overexpression group (P<0.001, P=0.001, P=0.002) [Figure 8: see original paper].

2.7 Interaction between klotho and AT1R

Co-immunoprecipitation experiments demonstrated that AT1R expression could be detected after pull-down with klotho antibody, indicating that an interaction indeed exists between klotho and AT1R [Figure 9: see original paper].

In 1997, KURO-O et al. identified klotho as an anti-aging gene through studies of genetically defective mice. The study found that deficiency of the klotho gene triggered a series of aging-related degenerative manifestations including arterial calcification, osteoporosis, skin atrophy, growth retardation, gait disorders, and shortened lifespan. Initial animal experiments revealed that klotho is mainly expressed in the kidneys, and elimination of renal Klotho expression reduced circulating klotho levels by 80%, consistent with findings in human studies. In recent years, various renal injury models including adriamycin nephropathy, cyclosporine A nephropathy, doxorubicin nephropathy, and diabetic nephropathy

models have all shown decreased klotho expression. Our study found that klotho mRNA and protein expression decreased significantly in the model group compared with the control group, suggesting that klotho expression declines in SSH renal injury, consistent with previous research.

Klotho participates in various pathophysiological processes and is considered an important regulator in the aging process. The three klotho proteins encoded by the Klotho gene—α-klotho, β-klotho, and γ-klotho—are all membrane-bound proteins with limited functions, primarily serving as receptors for fibroblast growth factors. Among them, α-klotho protein can be cleaved by proteases to release its extracellular domain, yielding soluble klotho protein. Studies have shown that soluble klotho protein possesses multiple biological functions including anti-inflammatory, antioxidant, anti-fibrotic, calcium-phosphate metabolism regulation, and glucose homeostasis modulation. Previous research has confirmed the protective role of klotho in renal injury. In an adenine-induced renal failure model, klotho-siRNA treatment exacerbated renal fibrosis progression and caused renal injury in sham-operated mice, while exogenous klotho supplementation reversed these effects. Furthermore, HU et al. found that klotho treatment after renal ischemia-reperfusion injury reduced α-smooth muscle actin (α-SMA) and type I collagen expression, inhibited renal fibrosis, and alleviated renal injury, suggesting therapeutic potential even after renal injury occurs.

Our study used klotho-siRNA interference and overexpression approaches to investigate the effects of klotho on HBZY1 cell viability. We found that cell viability decreased in the model group compared with the control group. Compared with the model group, cell viability increased in the klotho overexpression group but decreased further in the klotho interference group, suggesting that klotho has a protective effect on renal cells. KIMURA et al. found that ROS levels were low and antioxidant enzyme activities such as hydrogen peroxide and SOD were high in renal tissues of klotho-overexpressing mice, while klotho-deficient mice showed high ROS content and low antioxidant enzyme activity, indicating strong antioxidant activity of the klotho gene. Our study also found that intracellular ROS and MDA content increased while SOD content decreased in the model group compared with the control group. Compared with the model group, ROS and MDA content decreased and SOD content increased in the klotho overexpression group, while ROS and MDA content further increased and SOD activity further decreased in the klotho interference group. These findings suggest that klotho exerts its protective effects by inhibiting oxidative stress injury in HBZY1 cells.

Ang II is a potent mediator of oxidative stress and ROS-mediated signal transduction, inducing ROS production through activation of AT1R. In hypertensive mouse myocardial fibrosis, blocking AT1R significantly inhibited Ang II-induced ROS generation and collagen expression in cardiac fibroblasts. Additionally, in studies of Ang II-induced cardiomyocyte hypertrophy in neonatal rats, klotho significantly inhibited cardiomyocyte hypertrophy, and further research showed that klotho pretreatment significantly downregulated AT1R protein expression

without affecting angiotensin II type 2 receptor protein expression. In our study, ROS and MDA content decreased while SOD content and cell viability increased after klotho intervention compared with the model group, but these effects were reversed by AT1R overexpression. Moreover, Co-IP experiments confirmed an interaction between klotho and AT1R. These results suggest that klotho exerts its cytoprotective effects by interacting with the AT1R receptor to inhibit oxidative stress injury.

In summary, this study verified the protective role of klotho in SSH renal injury at the cellular level and demonstrated from a protein-protein interaction perspective that klotho exerts its protective effects by interacting with AT1R to inhibit oxidative stress injury. Future plans include further exploration of the specific interaction sites between klotho and AT1R in cellular experiments and investigation of the protective mechanism of klotho in SSH renal injury in animal models to provide a more comprehensive theoretical basis for clinical application and drug development.

Author Contributions: Wei Zhao contributed to conception and design, analysis and interpretation of results, and manuscript writing. Rongjie Tang and Shanshan Yang performed cell experiments. Fang Yang was responsible for quality control and revision of the manuscript. Feng Sun performed data collation. Qiufang Lian revised the manuscript, took overall responsibility, and provided supervision.

Conflicts of Interest: None declared.

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