Published in final edited form as:

Acta Physiol (Oxf). 2019 February ; 225(2): e13190. doi:10.1111/apha.13190.

Klotho protein supplementation reduces blood pressure and renal hypertrophy in db/db mice, a model of type 2 diabetes

Tsuneo Takenaka¹, Hiroyuki Kobori¹, Takashi Miyazaki², Hiromichi Suzuki², Akira Nishiyama³, Naohito Ishii⁴, Maho Yamashita⁵, and Matsuhiko Hayashi⁵

¹International University of Health and Welfare, Minato, Japan

²Saitama Medical University, Moroyama, Japan

³Kagawa University, Takamatsu, Japan

⁴Kitasato University, Sagamihara, Japan

⁵Keio University, Shinjuku, Japan

Abstract

Aims: Klotho interacts with various membrane proteins, such as receptors for transforming growth factor (TGF)- β and insulin-like growth factor (IGF), to alter their function. Renal expression of klotho is diminished in diabetes. The present study examined whether exogenous klotho protein supplementation ameliorates kidney injury and renin-angiotensin system (RAS) in db/db mice.

Methods: We investigated the effects of klotho supplementation on diabetic kidney injury and RAS. Recombinant human klotho protein (10 μ g/kg/d) was administered to db/db mice daily.

Results: Klotho protein supplementation reduced kidney weight, systolic blood pressure, albuminuria, glomerular filtration rate, and 8-epi-prostaglandin F2 α excretion without affecting body weight. Although klotho supplementation did not alter glycated albumin, it reduced renal angiotensin II levels associated with reduced renal expression of angiotensinogen. Klotho supplementation improved renal expression of superoxide dismutase (SOD), and endogenous renal expression of klotho. Klotho supplementation reduced the levels of hypoxia-inducible factor, phosphorylated Akt, and phosphorylated mTOR and decreased the renal expression of TGF- β , tumour necrosis factor (TNF), and fibronectin.

Conclusions: These data indicate that klotho supplementation reduces blood pressure and albuminuria along with ameliorating renal RAS activation in db/db mice. Furthermore, these results suggest that klotho inhibits IGF signalling, induces SOD expression to reduce oxidative stress, and suppresses Akt-mTOR signalling to inhibit abnormal kidney growth. Collectively, the results suggest that klotho inhibits TGF- β and TNF signalling, resulting in a decline in renal fibrosis.

Correspondence: Tsuneo Takenaka, Department of Nephrology, Graduate School of Medicine, International University of Health and Welfare, Minato, Tokyo, Japan. takenaka@iuhw.ac.jp.

CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

Keywords

epithelial–mesenchymal transition; insulin-like growth factor; mTOR; superoxide dismutase; transforming growth factor; tumour necrosis factor

1 | INTRODUCTION

Diabetic nephropathy, a significant socio-economical problem, is the most prominent renal disease leading to renal replacement therapy. Thus, currently there is a lack of therapeutic methods for the treatment of patients with diabetic nephropathy. The targets of new medication are focused on albuminuria and/or renal fibrosis.^{1,2} Of note, kidney size as well as albuminuria and renal fibrosis are prognostic indicators for diabetic kidney diseases.^{3,4} Type 2 diabetic mice show significant renal hypertrophy and renal accumulation of insulin-like growth factor (IGF)-binding protein 1, similar to the findings in models of type 1 diabetes mellitus.⁵ In addition, renal cortical activity of phosphatidylinositol 3-kinase and Akt is elevated in type 2 diabetic mice.⁶ However, the effects of IGF receptor inhibition on type 2 diabetic nephropathy have not been fully elucidated.

Diabetes and insulin resistance are linked to inflammation. Tumour necrosis factor- α (TNF), which activates NF- κ B to promote renal fibrosis,⁷ can be induced by hyperglycaemia and advanced glycated end-products.⁸ Of interest, insulin resistance was improved in TNF-deficient obese animals.⁹ The activation of renin–angiotensin system (RAS) is one of the characteristics of diabetic nephropathy.¹⁰ Although the RAS induces renal fibrosis partially through the hypoxia-inducible factor (HIF) and transforming growth factor- β (TGF- β) pathways,^{11,12} the activation of RAS may also contribute to TNF induction in diabetic kidneys.¹³

Klotho is expressed mainly in the kidney, and functions as a coreceptor for fibroblast growth factor 23 to control phosphate–calcium metabolism.^{14,15} Klotho possesses anti-ageing properties; its sequence is similar to that of β -glycosidase.¹⁶ Distal nephrons express the membrane protein klotho, which is enzymatically cleaved. Its extracellular domain is released into the renal interstitium and subsequently into systemic circulation.^{14,15} Klotho is also excreted into the urine by transcytosis in the proximal tubules.¹⁷ Klotho interacts with various membrane proteins, such as receptors for IGF and TGF- β .^{16,18} Free klotho inhibits IGF signalling and subsequent Akt phosphorylation, thus inducing superoxide dismutase (SOD) that at least partly accounts for the longevity effect. Free klotho also binds to TGF- β receptors to suppress their signalling.

Klotho expression is reduced in the kidney of type 2 diabetes rats.¹⁹ Our data indicated that endogenous klotho expression was reduced in db/db mice kidney. Decreased levels of endogenous klotho could derepress TGF- β and IGF signalling. In addition to RAS activation, signalling through the TGF- β and NF- κ B pathways decline renal expression of klotho.^{20,21} Conversely, exogenous klotho protein supplementation suppresses the RAS and hypertension in various kidney disease models.^{11,12} However, the effects of klotho protein supplementation on diabetic kidney have not been fully examined. The present studies have

investigated whether exogenous klotho protein supplementation ameliorates kidney injury and RAS in db/db mice.

2 | RESULTS

Table 1 summarized the effects of exogenous klotho protein supplementation on renal function. Although body weight, plasma glucose and glycated albumin levels in the 20-week-old db/db mice were greater than in the control ($P < 0.01$), klotho supplementation did not alter them. In the present study, kidney weight, heart weight glomerular filtration rate (GFR), systolic blood pressure (SBP), albuminuria, and 8-epi-prostaglandin F2 α (PGF2 β) excretion were larger in db/db mice than in the control ($P < 0.05$), and klotho supplementation attenuated these values in db/db mice ($P < 0.05$). Conversely, lithium clearance was lower in db/db mice than in the control ($P < 0.05$), and klotho supplementation reversed the decrements in db/db mice ($P < 0.05$).

Table 2 described the influence of exogenous klotho protein supplementation on RAS and endogenous klotho expression. Kidney angiotensin II levels and renal expression of angiotensinogen (AGT) were greater in the 20-week-old db/db mice ($P < 0.05$) than in the control, and klotho supplementation ameliorated them in db/db mice ($P < 0.05$). Although plasma angiotensin II levels were similar among four groups, plasma aldosterone level was higher in db/db mice than in the control ($P < 0.01$); plasma aldosterone levels were not altered by klotho supplementation. Renal expression, serum, and urine levels of klotho were lower in db/db mice than in the control ($P < 0.05$), and exogenous klotho protein supplementation abolished these decrements in db/db mice. While klotho administration increased serum klotho levels in the control mice ($P < 0.05$), renal function and RAS were not affected in the control mice. Thus, the following analyses were focused on db/db mice.

Klotho supplementation decreased renal expressions of TGF- β , fibronectin, and collagen I in 20-week-old db/db mice ($P < 0.05$ for all, Figure 1A-C). In contrast, klotho preserved renal expression of E-cadherin in db/db mice ($P < 0.05$, Figure 1D). Accordingly, exogenous klotho protein supplementation considerably reduced fibrosis index (1.4 ± 0.4 vs 0.6 ± 0.2 , $P < 0.05$) and nuclear staining of Smad3 ($20 \pm 5/\text{field}$ vs $8 \pm 2/\text{field}$, $P < 0.01$) in the kidney (Figure 1E,F).

However, klotho supplementation suppressed the phosphorylation of Akt, mTOR, and p70S6K in the kidney (Figure 2A-C). In accordance, klotho reduced renal tubular staining of phosphorylated mTOR ($45\% \pm 10\%$ vs $15\% \pm 4\%$, $P < 0.01$) in db/db mice (Figure 2D).

Exogenous klotho protein supplementation enhanced SOD expression in the kidney and aorta (Figure 3A,B). Accordingly, klotho declined renal abundance of hypoxia-inducible factor (HIF)-1 α in db/db mice (Figure 3C). Klotho suppressed renal TNF expression as well as circulating TNF levels in 20-week-old db/db mice (Figure 3D, E). Consistently, klotho supplementation reduced the phosphorylation of inhibitory kappa β (Ik β) in the kidney (Figure 3F).

As shown in Figure 4A, a two-way analysis of variance (ANOVA) indicated that the exposure of human proximal tubular (HK-2) cells to hydrogen peroxide elicited time-

dependent increases in angiotensinogen (AGT) expression, and AGT induction was significantly attenuated in the presence of klotho (n = 6 for each). Similar trends were observed for TNF expression (Figure 4B). In addition, IGF diminished SOD expression in HK-2 cells, and klotho considerably mitigated this response (Figure 4C).

3 | DISCUSSION

Renal hypertrophy is characterized by proximal tubular growth and elongation, which facilitate proximal tubular uptake, reducing the delivery to the macula densa and tubuloglomerular feedback (TGF).²² The present data indicate that exogenous supplementation of klotho protein caused a decline in kidney weight, in conjunction with physiological effects of increasing lithium clearance, supporting the notion that klotho suppressed renal hypertrophy of db/db mice. Klotho inhibits IGF signalling.¹⁶ Accordingly, our findings demonstrate that klotho supplementation inhibited the phosphorylation of Akt, mTor, and p70S6K, participating in the suppression of renal hypertrophy in db/db mice. Furthermore, klotho diminished renal tubular staining of phosphorylated mTOR in db/db mice. Collectively, our data are consistent with those of Sataranatarajan et al²³ that rapamycin treatment reduced kidney weight in db/db mice, suggesting that klotho supplementation curtailed the Akt-mTOR pathway, contributing to the repression of kidney hypertrophy in db/db mice.

Levine et al²⁴ have described that a single-nephron GFR is upregulated in association with diminished TGF in db/db mice. In the present study, klotho reduced GFR, suggesting that klotho supplementation ameliorated glomerular hyperfiltration in db/db mice. Although marked hyperglycaemia may affect lithium clearance,²⁵ klotho supplementation did not alter glycaemic states. Taken together, these results suggest that klotho induced an increase in lithium clearance, which subsequently enhanced the delivery to the macula densa and TGF, ameliorating glomerular hyperfiltration and albuminuria in db/db mice.

The present results show for the first time that exogenous klotho protein supplementation reduced renal angiotensin II level and renal expression of AGT in db/db mice, resulting in the decline of blood pressure and albuminuria.^{26,27} Renal expression of AGT is increased by the inhibition of GSK3 β ,¹¹ which is activated by Akt. RAS-induced elevation of blood pressure serves as the mechanism of kidney injury.^{27,28} Beneficial effects of klotho could relate to its blood pressure effect. Indeed, klotho reduced heart weight in db/db mice. Klotho supplementation induces both renal and aortic expressions of SOD, decreasing PGF2 α excretion, an indicator of oxidative stress.¹⁶ In consistent with in vivo data, the in vitro data indicated that klotho reversed IGF-induced reductions in SOD expression in HK-2 cells. Oxidative stress stimulated AGT expression, which was attenuated by klotho. Together, these results suggest that klotho attenuates renal RAS including AGT in db/db mice, partly via SOD induction.

Starkey et al²⁹ have reported that NF- κ B is activated in db/db mice. Our data provide new evidence that oxidative stress stimulates TNF expression in HK-2 cells. The in vitro results are consistent with the in vivo observations that exogenous klotho protein supplementation reduced renal expression of TNF and circulating TNF levels, as klotho augments SOD.¹⁶

Gao et al³⁰ have reported that plasma TNF is elevated in db/db mice. The inactivation of renal RAS by klotho may be involved in reducing renal expression of TNF in db/db mice.¹³ Accordingly, klotho supplementation decreased the phosphorylation of renal $\text{I}\kappa\text{B}$ in the present study. Furthermore, klotho specifically inhibited RelA Ser 36 phosphorylation without affecting IKK-mediated $\text{I}\kappa\text{B}$ degradation.⁸ Consequently, these findings suggest that klotho suppresses the NF- κB pathway directly by inhibiting its translocation and indirectly by impeding TNF at least partially through suppressing oxidative stress in db/db mice.

In the present study, exogenous klotho protein supplementation suppressed fibrosis index and renal expressions of fibronectin and collagen I in db/db mice kidney. Our data suggest that klotho-induced suppression of renal RAS participated in reducing renal expression of TGF- β and renal abundance of HIF-1 α . Klotho inhibits TGF- β signalling.¹⁸ Consistently, nuclear staining of Smad3 declined in the klotho-treated group. Klotho supplementation preserved renal expression of E-cadherin, supporting that klotho inhibited epithelial mesenchymal transition (EMT).^{11,31} Using an $\text{I}\kappa\text{B}$ dominant-negative transgene, we previously demonstrated that NF- κB activation plays a profibrotic role in renal fibrosis.⁷ Collectively, these observations suggest that klotho inhibits renal fibrosis in db/db mice via multiple pathways, including TGF- β , HIF-1 α , and NF- κB .

Exogenous klotho protein supplementation increased serum klotho levels towards those of the control mice,¹⁶ justifying the dose of klotho used in the present study. Our data indicate that exogenous klotho protein supplementation preserved endogenous renal expression of klotho. Klotho supplementation suppressed oxidative stress and renal RAS, both of which diminish klotho expression.^{20,32} TGF- β epigenetically inhibits renal expression of klotho.²¹ These findings suggest that exogenous klotho protein supplementation halts the feedback loop which reduces endogenous expression of klotho in db/db mice.

Our study has several limitations. First, there have been reports on streptozotocin-induced diabetic rats with klotho gene transfer and Ins2Akita diabetic mice crossed with klotho transgenic mice.^{33,34} The present study focused on db/db mice, using klotho protein supplementation which may be more ethically acceptable for diabetic patients than gene transfer. Second, klotho decreased PGF2 α excretion. Thus, it is possible that klotho may hamper NAD(P)H oxidases.³⁵ Third, plasma aldosterone was higher in obese db/db mice than in the lean controls. Adipocyte-derived aldosterone may be involved.³⁶ Fourth, klotho supplementation did not alter the glycaemic state in db/db mice. Klotho blunts insulin sensitivity,¹⁶ whereas klotho gene delivery reduced blood glucose by increasing insulin secretion in 10-week-old db/db mice.³⁷ Finally, klotho administration did not alter kidney weight in control mice. Thus, disease-related decrements of endogenous klotho may underlie deleterious renal hypertrophy.³⁸

In summary, the results of this study indicate that klotho supplementation reduced blood pressure and albuminuria and ameliorated RAS and fibrosis in db/db mice. Furthermore, the results are consistent with the notion that klotho inhibits IGF signalling, inducing SOD expression to reduce oxidative stress and suppressing Akt-mTOR signalling to decrease abnormal kidney growth. Finally, the present findings suggest that klotho inhibits TGF- β

expression and TGF- β signalling, leading to the decline of EMT and renal fibrosis associated with diabetes.

4 | METHODS

The db/db mouse strain was used as a model of type 2 diabetes with nephropathy.³⁹ The db/db mice have an inactivating mutation in the leptin receptor gene, resulting in a shorter domain of the receptor which is unable to transduce signals. Their phenotypes include of obesity, insulin resistance, and diabetes, similar to type 2 diabetes in humans. In the early phase of diabetes, the db/db mice show elevated albumin excretion in association with hyperglycaemia and renal hypertrophy. The db/db mice develop progressive renal derangements including the loss of renal function. However, db/db mice fail to exhibit significant glomerular basement thickening.³⁹ Indeed, there are no mouse models of diabetes that show nodular glomerular lesions, which are typical for human diabetic nephropathy. Therefore, instead of elucidating detailed glomerular pathology, we focused on renal function, fibrosis, and hypertrophy in the present study.

4.1. | Whole animal study

Seven-week-old male db/db and control (db/m) mice were purchased from Sankyo Lab Service Corporation (Edogawa, Tokyo). The mice were given free access to water and standard chow. All experimental protocols were approved of by the ethical committee for animal research of Keio University (permit number: 14091-[1]). This study was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were taken to minimize the suffering of the animals. For acclimation, each mouse was housed separately for 1 week in a metabolic cage kept in a temperature-controlled room with a 12/12-hour light/dark cycle.⁴⁰

Exogenous supplementation of recombinant human klotho protein (rh-klotho, 10 μ g/kg/d; PeproTech, Rocky Hill, NJ, USA) or vehicle was started on 8-week-old mice (n = 10 for each group) with daily subcutaneous injections. This dose of rh-klotho ameliorated calcium and phosphate abnormalities in klotho-deficient animals, indicating that it is physiologically active.^{15,41} SBP was measured every 4 weeks by the tail cuff method and urine was collected every 4 weeks to measure albumin and PGF2 α levels. From 19 weeks of age, mice were fed chow containing LiCl (5 mmol/kg) to obtain measurable concentrations of lithium as an index of end-proximal tubular flow without acute diuresis.²⁵ At 20 weeks, GFR was measured by plasma decay kinetics of FITC-inulin.^{42,43} A bolus of around 4 μ L/g body wt FITC-inulin was injected into the retro-orbital plexus of mice. From saphenous vein, approx. 20 μ L of blood was collected 1, 3, 5, 10, 15, 35, 55, and 75 minutes later. Plasma fluorescence was measured using a microplate reader (SH-9000; Corona, Ibaraki, Japan). Inulin concentration was measured using an FITC-inulin standard, with the clearance calculated using a two-phase decay curve.

Under the anaesthesia with pentobarbital (50 mg/kg), the abdomen was opened by midline section. Bilateral renal arteries and veins were ligated and both kidneys were removed. Then, the blood was taken from the heart (about 1 mL). The mice were killed by administering an overdose of the anaesthetic before the removal of heart and aorta. Glycated

albumin, glucose, aldosterone, and angiotensin II were measured. One kidney and aorta from each mouse was frozen quickly in liquid nitrogen. Blood samples were centrifuged at 4°C for 10 minutes. Serum, plasma, urine, and tissues were deep-frozen until the use.¹¹ Because physiological parameters were not altered by klotho administration in the control mice, no further experiments were performed for the control mice with and without klotho treatment. As detailed previously,²⁵ kidney angiotensin II was measured using radioimmunoassay. Glycated albumin (LifeSpan BioScience, Seattle, WA, USA), klotho (Antibody Online, Aachen, Germany), and TNF (Antibody Online) were measured by ELISA. The other kidney was fixed in 4% formalin solution.⁴⁴

4.2. | Reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from the kidney and aorta by using a commercial kit (Isogen; Wako Pure Chemical, Osaka, Japan), as described previously.⁴⁵ Approx. 50 mg of the tissue was homogenized in 1 mL Isogen reagent in a microcentrifuge tube. Next, 200 µL chloroform was added to the tube, and the samples were vortexed for 30 seconds. After centrifugation, the aqueous phase was transferred to a new microcentrifuge tube containing 0.7 volumes of isopropanol, and RNA was recovered by centrifuging at 15 000 rpm at 4°C for 15 minutes. The pellet was washed in 70% cold ethanol, centrifuged, dried in a vacuum centrifugal evaporator (Tomy MV-100; Tomy Digital Biology, Tokyo, Japan) for 10 minutes, and dissolved in 50 µL diethyl pyrocarbonate-treated water. Concentration of the dissolved RNA was determined by measuring its absorbance at 260 nm. Next, 1 µg of the total RNA was used for performing first-strand cDNA synthesis in a 20-µL reaction mixture using a commercial kit (iScript cDNA Synthesis kit; Bio-Rad Laboratories, Tokyo, Japan). Next, PCR was performed in a 50-µL reaction mixture containing 2 µL synthesized cDNA, real-time PCR reagent (iQ SYBR Green Supermix; Bio-Rad), and specific primers for target genes. The PCR was performed in a thermal cycler (iCycler iQ System; Bio-Rad) using 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. Relative levels of target mRNAs were determined using the comparative threshold (CT) method. Relative quantification ratio was determined according to a calibrator which allowed statistical comparison of gene expression among samples. In addition, CT values of a reference gene (*Gapdh*) and the target genes in each sample were determined using the 2^{-CT} method. Changes in mRNA expression levels of the target genes were calculated after normalization to the mRNA expression levels of *Gapdh*.

4.3. | Western blotting

Each kidney sample (50 mg) was homogenized in Tris-HCl buffer (pH 7.5) containing 150 mmol/L NaCl, 0.1% Triton X-100, and 1.0 mmol/L phenylmethylsulphonyl fluoride on an ice bath.⁴⁴ The homogenates were centrifuged and the obtained supernatants were electrophoresed. Protein concentration of the samples was measured using BSA as the standard. Proteins in the homogenates were treated with 2-mercaptoethanol at 95°C for 10 minutes. Next, samples containing 30 µg proteins were mixed with a sample buffer, boiled for 10 minutes, loaded, and resolved on a 7.5% polyacrylamide gel. Separated proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% BSA/TBS-T solution (50 mmol/L Tris-HCl [pH 8.0], 150

mmol/L NaCl, and 0.1% Tween 20) for 3 hours at room temperature. Each blocked membrane was incubated overnight at 4°C with a primary antibody. After incubation, the membranes were washed three times with TBS-T solution and were incubated with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2000; Binding Site, Birmingham, UK) for 1 hour. Target proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, UK). Protein expression levels were analysed using a densitometry system (AE-6920-MF; ATTO, Tokyo, Japan).

4.4. | Pathology

Paraffin-embedded renal tissue samples were cut into 2–3-µm-thick sections and were stained using the Masson trichrome technique.¹¹ Ten consecutive microscopic fields of the renal cortex (at a final magnification of 100×) were randomly selected for each kidney section. Interstitial fibrosis (IF) was evaluated using the following scale: 0, no fibrosis; 1, less than ¼ IF in the visual field; 2, ¼ to ½; 3, ½ to ¾; and 4, more than ¾. Immunohistochemistry was performed with peroxidase staining. Phosphorylated mTOR staining was semiquantified by HS score. For Smad staining, the number of Smad-positive nuclei was counted in 10 microscopic fields, and its index was expressed as the mean number per field.⁷ Two independent pathologists examined the tissue for scoring in an observer-blinded fashion.

4.5. | Cell culture

The effects of klotho protein on the induction of AGT and TNF expression by oxidative stress were assessed in HK-2 cells.⁴⁶ HK 2 cells were purchased from the American Type Culture Collection (CRL-2190) and were cultured in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) until reaching confluency.¹² The cells were then cultured in K-SFM lacking both BPE and EGF for 1 day before performing acute experiments. First, rh-klotho (10^{-9} mol/L) or vehicle was added to the media.¹² After 1 hour, hydrogen peroxide (0.4 mmol/L) was added.⁴⁶ Cells were collected from the sheets under basal conditions 48 hours later from oxidant exposure to evaluate AGT and TNF expressions. In a separate study, HK-2 cells were treated with IGF-1 (50 ng/mL) in the presence or absence of rh-klotho. Cells were collected 48 hours later to assess SOD expression.

Data are expressed as the mean $\pm$ SEM, unless otherwise stated. Statistical analysis was performed using a Student's *t* test, linear regression analysis, or ANOVA followed by a Newman–Keuls test. *P* values <0.05 were considered to be statistically significant.

ACKNOWLEDGMENTS

This study was supported by grants from the Japanese Ministry of Education, Science, Sports, and Technology (JSPS 16K3457190). The authors thank Ms. Hiroko Sano, International University of Health and Welfare, for her excellent technical help during preparing the manuscript. Some of data in this manuscript were presented at the annual meeting of the European Society of Hypertension, in June 2017 in Milan, Italy, and was published there as an abstract.

Funding information

REFERENCES

1. Alicic RZ, Tuttle KR. Novel therapies for diabetic kidney disease. *Adv Chronic Kidney Dis*. 2014;21(2):121–133. [PubMed: 24602462]
2. Pergola PE, Raskin P, Toto RD, et al. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N Engl J Med*. 2011;365(4):327–336. [PubMed: 21699484]
3. Inomata S Renal hypertrophy as a prognostic index for the progression of diabetic renal disease in non-insulin-dependent diabetes mellitus. *J Diab Comp*. 1993;7:28–33.
4. Kleinman KS, Fine LG. Prognostic implications of renal hypertrophy in diabetes mellitus. *Diabetes Metab Rev*. 1988;4:179–189. [PubMed: 3281809]
5. Segev Y, Eshet R, Yakir O, Haim N, Phillip M, Landau D. Systemic and renal growth hormone-IGF1 axis involvement in a mouse model of type 2 diabetes. *Diabetologia*. 2007;50(6):1327–1334. [PubMed: 17443310]
6. Feliars D, Duraisamy S, Faulkner JL, et al. Activation of renal signaling pathways in db/db mice with type 2 diabetes. *Kidney Int*. 2001;60(2):495–504. [PubMed: 11473632]
7. Inoue T, Takenaka T, Hayashi M, et al. Fibroblast expression of an I κ B dominant-negative transgene attenuates renal fibrosis. *J Am Soc Nephrol*. 2010;21(12):2047–2052. [PubMed: 20847140]
8. Zhao Y, Banerjee S, Dey N, et al. Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation. *Diabetes*. 2011;60 (7): 1907–1916. [PubMed: 21593200]
9. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature*. 1997;389(6651):610–614. [PubMed: 9335502]
10. Wang J, Shibayama Y, Kobori H, et al. High glucose augments angiotensinogen in human renal proximal tubular cells through hepatocyte nuclear factor-5. *PLoS One*. 2017;12(10):e0185600. [PubMed: 29053707]
11. Takenaka T, Inoue T, Miyazaki T, et al. Klotho suppresses the renin-angiotensin system in adriamycin nephropathy. *Nephrol Dial Transplant*. 2017;32(5):791–800. [PubMed: 27798196]
12. Takenaka T, Inoue T, Miyazaki T, et al. Klotho ameliorate medullary fibrosis and pressure natriuresis in hypertensive rat kidneys. *Hypertension* 2018; 10.1161/HYPERTENSIONAHA.118.11176
13. Siragy HM, Awad A, Abadir P, Webb R. The angiotensin II type 1 receptor mediates renal interstitial content of tumor necrosis factor- α in diabetic rats. *Endocrinology*. 2003;144(6): 2229–2233. [PubMed: 12746279]
14. Takenaka T, Watanabe Y, Inoue T, Miyazaki T, Suzuki H. Fibroblast growth factor 23 enhances renal klotho abundance. *Pflugers Arch*. 2013;465(7):935–943. [PubMed: 23467972]
15. Takenaka T, Inoue T, Miyazaki T, Hayashi M, Suzuki H. Xeno-Klotho inhibits parathyroid hormone signaling. *J Bone Miner Res*. 2016;31(2):455–462. [PubMed: 26287968]
16. Kurosu H, Yamamoto M, Clark JD, et al. Suppression of aging in mice by the hormone Klotho. *Science*. 2005;309(5742):1829–1833. [PubMed: 16123266]
17. Hu MC, Shi M, Zhang J, et al. Renal production, uptake, and handling of circulating α Klotho. *J Am Soc Nephrol*. 2016;27 (1):79–90. [PubMed: 25977312]
18. Doi S, Zou Y, Togao O, et al. Klotho inhibits transforming growth factor- β 1 (TGF- β 1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem*. 2011;286(10):8655–65. [PubMed: 21209102]
19. Aizawa H, Saito Y, Nakamura T, et al. Downregulation of the Klotho gene in the kidney under sustained circulatory stress in rats. *Biochem Biophys Res Commun*. 1998;249(3):865–871. [PubMed: 9731228]
20. Mitani H, Ishizaka N, Aizawa T, et al. In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. *Hypertension*. 2002;39(4):838–843. [PubMed: 11967236]

21. Ruiz-Andres O, Sanchez-Niño MD, Moreno JA, et al. Downregulation of kidney protective factors by inflammation: role of transcription factors and epigenetic mechanisms. *Am J Physiol Renal Physiol*. 2016;311(6):F1329–F1340. [PubMed: 27760772]
22. Vallon V, Thomson SC. Renal function in diabetic disease models : the tubular system in the pathophysiology of the diabetic kidney. *Annu Rev Physiol*. 2012;74:351–375. [PubMed: 22335797]
23. Sataranatarajan K, Mariappan MM, Lee MJ, et al. Regulation of elongation phase of mRNA translation in diabetic nephropathy: amelioration by rapamycin. *Am J Pathol*. 2007;171(6):1733–1742. [PubMed: 17991718]
24. Levine DZ, Iacovitti M, Robertson SJ, Mokhtar GA. Modulation of single-nephron GFR in the db/db mouse model of type 2 diabetes mellitus. *Am J Physiol Regul Integr Comp Physiol*. 2006;290(4):R975–R981. [PubMed: 16339386]
25. Takenaka T, Inoue T, Ohno Y, et al. Elucidating mechanisms underlying altered renal autoregulation in diabetes. *Am J Physiol Regul Integr Comp Physiol*. 2012;303(5):R495–R504. [PubMed: 22739351]
26. Crowley SD, Gurley SB, Herrera MJ, et al. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA*. 2006;103(47):17985–17990. [PubMed: 17090678]
27. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev*. 2007;59 (3): 251–287. [PubMed: 17878513]
28. Griffin KA. Hypertensive kidney injury and the progression of chronic kidney disease. *Hypertension*. 2017;70(4):687–694. [PubMed: 28760941]
29. Starkey JM, Haidacher SJ, LeJeune WS, et al. Diabetes-induced activation of canonical and noncanonical nuclear factor-kappaB pathways in renal cortex. *Diabetes*. 2006;55(5):1252–1259. [PubMed: 16644679]
30. Gao X, Belmadani S, Picchi A, et al. Tumor necrosis factor-alpha induces endothelial dysfunction in Lepr(db) mice. *Circulation*. 2007;115(2):245–254. [PubMed: 17200442]
31. Inoue T, Umezawa A, Takenaka T, Suzuki H, Okada H. The contribution of epithelial-mesenchymal transition to renal fibrosis differs among kidney disease models. *Kidney Int*. 2015;87(1):233–238. [PubMed: 25007169]
32. Mitobe M, Yoshida T, Sugiura H, Shirota S, Tsuchiya K, Nihei H. Oxidative stress decreases klotho expression in a mouse kidney cell line. *Nephron Exp Nephrol*. 2005;101(2):e67–e74. [PubMed: 15976510]
33. Deng M, Luo Y, Li Y, et al. Klotho gene delivery ameliorates renal hypertrophy and fibrosis in streptozotocin-induced diabetic rats by suppressing the Rho-associated coiled-coil kinase signaling pathway. *Mol Med Rep*. 2015;12(1):45–54. [PubMed: 25695625]
34. Kadoya H, Satoh M, Haruna Y, Sasaki T, Kashiwara N. Klotho attenuates renal hypertrophy and glomerular injury in Ins2Akita diabetic mice. *Clin Exp Nephrol*. 2016;20(5):671–678. [PubMed: 26586006]
35. Sharma K Obesity oxidative stress, and fibrosis in chronic kidney disease. *Kidney Int Suppl* (2011). 2014;4(1):113–117. [PubMed: 25401040]
36. Briones AM, Nguyen Dinh Cat A, Callera GE, et al. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction. *Hypertension*. 2012;59:1069–1078. [PubMed: 22493070]
37. Forsberg EA, Olauson H, Larsson T, Catrina SB. Effect of systemically increasing human full-length Klotho on glucose metabolism in db/db mice. *Diabetes Res Clin Pract*. 2016;113:208–210. [PubMed: 26806457]
38. Blantz RC, Steiner RW. Benign hyperfiltration after living kidney donation. *J Clin Invest*. 2015;125(3):972–974. [PubMed: 25689262]
39. Allen TJ, Cooper ME, Lan HY. Use of genetic mouse models in the study of diabetic nephropathy. *Curr Diab Rep*. 2004;4 (6):435–440. [PubMed: 15539008]

40. Takenaka T, Inoue T, Okada H, et al. Altered gap junctional communication and renal haemodynamics in Zucker fatty rat model of type 2 diabetes. *Diabetologia*. 2011;54(8):2192–2201. [PubMed: 21573906]
41. Chen TH, Kuro-O M, Chen CH, et al. The secreted Klotho protein restores phosphate retention and suppresses accelerated aging in Klotho mutant mice. *Eur J Pharmacol*. 2013;698(1-3):67–73. [PubMed: 23041151]
42. Qi Z, Whitt I, Mehta A, et al. Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *Am J Physiol Renal Physiol*. 2004;286:F590–F596. [PubMed: 14600035]
43. Nakano D, Doi K, Kitamura H, et al. Reduction of tubular flow rate as a mechanism of oliguria in the early phase of endotoxemia revealed by intravital imaging. *J Am Soc Nephrol*. 2015;26(12):3035–3044. [PubMed: 25855781]
44. Takenaka T, Inoue T, Ohno Y, et al. Calcitriol supplementation improves endothelium-dependent vasodilation in rat hypertensive renal injury. *Kidney Blood Press Res*. 2014;39(1):17–27. [PubMed: 24821359]
45. Takenaka T, Inoue T, Miyazaki T, et al. Antialbuminuric actions of calcilytics in the remnant kidney. *Am J Physiol Renal Physiol*. 2015;309(3):F216–F226. [PubMed: 26017971]
46. Kurosaki Y, Imoto A, Kawakami F, et al. Oxidative stress increases megalin expression in the renal proximal tubules during the normoalbuminuric stage of diabetes mellitus. *Am J Physiol Renal Physiol*. 2018;314(3):F462–F470. [PubMed: 29187367]

FIGURE 1.

Impact of exogenous klotho protein supplementation on renal expressions of TGF- β (A), collagen I (B), fibronectin (C), and E-cadherin (D), Smad3 distribution (E), and interstitial fibrosis (F) in db/db mice (db). The * indicates statistically significant differences between the two groups (n = 10 for each)

FIGURE 2.

Influences of exogenous klotho protein supplementation on phosphorylation of Akt (A, 56 kDa), mTOR (B, 289 kDa), and p70-S6K (C, 70 kDa), and phosphorylated mTOR staining (D) in db/db mice (db). The * indicates statistically significant differences between the two groups (n = 10 for each). db + k depicts db/db mice with klotho supplementation

FIGURE 3.

Effects of exogenous klotho protein supplementation on aortic (A) and renal (B) expressions of superoxide dismutase (SOD), renal abundance of hypoxia-inducible factor-1α (C, 110 kDa, HIF-1α), renal expression of tumour necrosis factor-α (D, TNF-α), plasma concentration of TNF-α (E), and phosphorylation of Iκβ (F, 36 kDa) in db/db mice (db). β-actin was observed at 42 kDa. The * indicates statistically significant differences between the two groups (n = 10 for each). db + k depicts db/db mice with klotho supplementation

FIGURE 4.

Summary of in vitro studies in HK-2 cells. Hydrogen peroxide induced angiotensinogen expression (A) and klotho suppressed this response (For time: $F = 36$, $df = 1$, $P < 0.005$; for klotho treatment: $F = 14$, $df = 1$, $P < 0.001$; for interaction: $F = 5$, $df = 1$, $P < 0.05$; for error: $df = 20$). An interaction between time and klotho treatment may relate to transcytosis of klotho protein by proximal tubular cells.¹⁷ Similarly, hydrogen peroxide induced the expression of tumour necrosis factor- α (B, TNF- α), and klotho inhibited this (For time: $F = 85$, $df = 1$, $P < 0.001$; for klotho treatment: $F = 8$, $df = 1$, $P < 0.05$; for interaction: $F = 13$, $df = 1$, $P < 0.01$; for error: $df = 20$). Insulin-like growth factor repressed expression of superoxide dismutase (C, SOD), and klotho opposed this response (For time: $F = 96$, $df = 1$, $P < 0.001$; for klotho treatment: $F = 6$, $df = 1$, $P < 0.05$; for interaction: $F = 9$, $df = 1$, $P < 0.01$; for error: $df = 20$). Blue and grey bars depict control and klotho-treated groups respectively. The * indicates statistically significant differences between the two groups

TABLE 1

The effects of exogenous klotho protein supplementation on kidney function

	Control	Control + klotho	db/db	db/db + klotho
Body weight (g)	31.0 ± 1.2	30.2 ± 1.2	56.1 ± 2.6 ^{*,†}	57.2 ± 2.3 ^{*,†}
Kidney weight (g)	0.18 ± 0.02	0.18 ± 0.01	0.28 ± 0.02 ^{*,†}	0.23 ± 0.02 ^{*,†,‡}
Heart weight (g)	0.12 ± 0.01	0.11 ± 0.01	0.19 ± 0.02 ^{*,†}	0.15 ± 0.01 ^{*,†,‡}
Plasma glucose (mg/dL)	118 ± 12	120 ± 11	576 ± 42 ^{*,†}	571 ± 35 ^{*,†}
Glycated albumin $\frac{1}{4}$ g/mL	532 ± 65	535 ± 67	1212 ± 85 ^{*,†}	1231 ± 83 ^{*,†}
SBP (mm Hg)	103 ± 2	102 ± 2	113 ± 2 ^{*,†}	105 ± 2 [‡]
GFR (μ L/min)	290 ± 18	291 ± 18	419 ± 30 ^{*,†}	354 ± 27 ^{*,†,‡}
CLi (μ L/min)	98 ± 7	100 ± 8	65 ± 5 ^{*,†}	81 ± 6 ^{*,†,‡}
Albuminuria (μ g/d)	100 ± 13	98 ± 14	2489 ± 347 ^{*,†}	1501 ± 249 ^{*,†,‡}
PGF2a (ng/d)	45 ± 20	37 ± 18	409 ± 97 ^{*,†}	74 ± 32 [‡]

The data are expressed as means ± SD. SBP, GFR, CLi, and PGF2a indicate systolic blood pressure, glomerular filtration rate, lithium clearance, and 8-epi-prostaglandin F2a excretion. Newman-Keuls test was applied as post hoc.

Depict significant difference from

* control,

[†] control + klotho

[‡] db/db.

The influence of klotho supplementation on renin-angiotensin system and endogenous klotho expression

TABLE 2

	Control	Control + klotho	db/db	db/db + klotho
Plasma ANGII (fmol/mL)	98 ± 34	99 ± 34	117 ± 36	113 ± 27
Plasma aldosterone (pg/mL)	224 ± 22	222 ± 27	458 ± 37 ^{*,†}	405 ± 46 ^{*,†}
Kidney ANGII (fmol/g)	127 ± 16	125 ± 14	203 ± 33 ^{*,†}	131 ± 16 [‡]
Kidney AGT expression	0.76 ± 0.14	0.72 ± 0.15	1.96 ± 0.32 ^{*,†}	0.78 ± 0.23 [‡]
Serum klotho (pg/mL)	101 ± 13	129 ± 14 [*]	65 ± 10 ^{*,†}	97 ± 13 ^{‡,‡}
Urine klotho (pg/d)	551 ± 61	588 ± 76	244 ± 31 ^{*,†}	508 ± 71 [‡]
Kidney klotho expression	1.52 ± 0.25	1.64 ± 0.34	0.75 ± 0.15 ^{*,†}	1.50 ± 0.26 [‡]

The data are expressed as means ± SD. ANGII and AGT indicate angiotensin II, and angiotensinogen. Newman-Keuls test was applied as post hoc.

Depict significant difference from

* control,

‡ control + klotho

‡ db/db.