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## Klotho Ameliorates Medullary Fibrosis and Pressure Natriuresis in Hypertensive Rat Kidneys

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### Abstract

Renal expression of klotho is reduced in hypertension. Experiments were performed to examine whether exogenous klotho protein supplementation ameliorates pressure natriuresis in early phase of hypertension, using stroke-prone spontaneously hypertensive rats (sp-SHR). The interactions between klotho protein and renal renin-Ang (angiotensin) system were examined with immunoprecipitation and cell culture methods. Uninephrectomy was performed in sp-SHRs to induce nephrosclerosis, and they were treated with exogenous klotho protein or vehicle.

Exogenous klotho protein supplementation to sp-SHR decreased blood pressure, renal Ang II levels, AGT (angiotensinogen) expression, HIF (hypoxia-inducible factor)-1 $\alpha$  abundance, and medullary fibronectin levels, with increased renal klotho expression and serum and urine klotho levels. Klotho supplementation also reduced kidney weight, renal phosphorylated Akt, and mTOR (mammalian target of rapamycin) abundance. Furthermore, klotho supplementation restored renal autoregulation of glomerular filtration rate and enhanced pressure-induced natriuresis in sp-SHR.

Klotho protein bound to AT1R (Ang II type-1 receptor) and decreased the presence of AT1R on

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Disclosures

None.

HK-2 (human proximal tubular) cells, attenuating inositol triphosphate generation. Klotho protein suppressed Ang II-induced increments of AGT expression in HK-2 cells. Collectively, the present data demonstrate that klotho binds with the AT1R to suppress Ang signal transduction, participating in inactivating renal renin-Ang system. Our results also suggest that exogenous klotho supplementation represses Akt-mTOR signaling to reduce renal hypertrophy and restore the autoregulatory ability of glomerular filtration rate in uninephrectomized sp- SHR. Finally, the present findings implicate that klotho supplementation inhibits HIF-1 $\alpha$  pathway and medullary fibrosis, contributing to enhancements of pressure natriuresis and reduction in blood pressure.

## Keywords

angiotensinogen; fibronectin; klotho protein; natriuresis; rats

Klotho is a type-1 membrane protein expressed mainly in the kidneys.<sup>1</sup> Peptidases of a disintegrin and metalloproteinase family cleave m-klotho (membrane-bound klotho) to release its extracellular domain, which then diffuses into the renal interstitium. Secreted klotho reaches various regions within the kidneys, such as the glomeruli and medulla, and then enters systemic circulation.<sup>2,3</sup> Thus, renal interstitial concentration of klotho could exceed serum klotho level. The kidneys are the main source of circulating klotho, which functions as an antiaging protein.<sup>4</sup> Secreted klotho binds to Wnt, thus inhibiting Wnt signaling, which promotes aging and activates the renin-Ang (angiotensin) system (RAS).<sup>5,6</sup> Both renal and circulating klotho levels are decreased in hypertensive nephrosclerosis.<sup>7</sup> Klotho interacts with many membrane proteins, such as transient receptor potential channel 1, transient receptor potential vanilloid 5, type 2 sodium-phosphate co-transporter, and receptors of fibroblast growth factor 23, IGF (insulin-like growth factor), parathyroid hormone, and TGF $\beta$  (transforming growth factor  $\beta$ ).<sup>1,4,8,9</sup>

Renal medulla plays an important role in the pathogenesis of hypertension. Pharmacological papillary necrosis prevents pressure-induced natriuresis,<sup>10</sup> thus supporting that medullary circulation determines blood pressure levels.<sup>11</sup> Hyperfiltration occurs in juxtamedullary nephrons but not in cortical nephrons of uninephrectomized spontaneously hypertensive rats (SHRs), suggesting that deep nephrons are exposed to excessive albumin excretion during hypertension.<sup>12,13</sup> However, glomerular blood flow is almost perfectly autoregulated in juxtamedullary nephrons of normotensive rats.<sup>14</sup> In SHRs, pathological lesions, such as fibrinoid necrosis, sclerosis, and tubulointerstitial changes are initially confined to the juxtamedullary zones, sparing the outer cortex. Normal kidneys possess resident fibroblasts in the medulla but almost no fibroblasts in the cortex. Ang II induces profibrotic changes in medullary fibroblasts by activating HIF (hypoxia-inducible factor)-1 $\alpha$ .<sup>15</sup> These findings indicate that the renal medulla is selectively damaged in the initial phase of hypertension.

The RAS contributes to the development and maintenance of high blood pressure. Ang II induces glomerular arteriolar constriction and enhances both proximal tubular reabsorption and tubuloglomerular feedback.<sup>16</sup> We previously showed that the renal RAS is involved in the pathogenesis of hypertension in SHRs.<sup>17</sup> Although Ang II maintains the autoregulation of glomerular filtration rate (GFR), it attenuates pressure natriuresis.<sup>16</sup> There is general

agreement that the renal RAS is regulated independently of the systemic RAS.<sup>18</sup> Activation of the renal RAS during Ang II-infused hypertension involves increased renal AGT (angiotensinogen) expression, which is triggered, at least partly, by AT1R (Ang II type-1 receptor) internalization.<sup>19</sup> In the present study, we examined the potential interaction between klotho and the AT1R and determined whether exogenous supplementation of rh-klotho (recombinant human klotho protein, 516 amino acids) ameliorated renal RAS, medullary fibrosis, and pressure natriuresis in early phase of hypertension.<sup>20</sup>

## Methods

The authors declare that all supporting data are available within the article and in the online-only Data Supplement.

### In Vitro Cell-Free Analysis of Klotho Binding

As detailed previously,<sup>8</sup> antibodies against the human AT1R (10 µg; Abcam) were incubated with protein G-Sepharose beads (25% v/v; GE Health Care Life Sciences) in 0.1 mL binding buffer (Krebs-Ringer-Hepes buffer with 1% BSA) for 2 hours at room temperature. After washing the beads with the binding buffer, full-length human AT1R (1 µM; Novus Biologicals) was added to the beads suspended in 0.1 mL binding buffer, and the beads were incubated further at 4°C for 15 hours. Next, the beads were washed, treated with rh-klotho ( $10^{-10}$ – $10^{-8}$  M in 0.1 mL binding buffer), and incubated further at 4°C for 15 hours. The solution of rh-klotho was made freshly with HEPES buffer on the day of each in vitro experiment. The samples were centrifuged, and the beads were washed 3× with the binding buffer and 3× with the Krebs-Ringer-Hepes buffer lacking BSA. Proteins bound to the beads were detected by Western blotting with a monoclonal anti-klotho antibody (KM2076; provided by Hyowa-Hakko Kirin, Co, Ltd.). Similar experiments were performed using β-glucuronidase (Abcam) or kidney homogenates containing endogenous AT1R and m-klotho. Klotho shows β-glucuronidase activity and similar size to β-glucuronidase.<sup>1</sup>

In additional experiments, Sepharose beads coated with the AT1R were incubated in 0.2 mL binding buffer containing fixed amount of <sup>125</sup>I-Ang II ( $10^{-9}$  M) and increasing doses of rh-klotho ( $10^{-10}$ – $10^{-8}$  M) for 1 hour at room temperature.<sup>8</sup> Next, the beads were washed 3× with the binding buffer and 3× with the Krebs-Ringer-Hepes buffer lacking BSA and were subjected to a scintillation counter. Nonspecific binding was determined by replacing rh-klotho with unlabeled Ang II (Sigma-Aldrich) in an excess of 100-fold <sup>125</sup>I-Ang II.

### Cell Culture

Human proximal tubular (HK-2) cells were purchased from American Type Culture Collection (CRL-2190) and were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor until confluency.<sup>21</sup> The cells were then cultured in keratinocyte serum-free medium lacking both bovine pituitary extract and epidermal growth factor for 1 day before performing acute experiments. Ang II (1–10 nmol/L) was added to the culture medium in the presence or absence of rh-klotho ( $10^{-9}$  M). This dose of rh-klotho showed considerable binding to AT1Rs. Inositol triphosphate (IP3) levels in the culture medium were measured at 5, 10, and

20 minutes after adding Ang II by using an ELISA kit (Antibodies-Online). Because variations in intracellular IP3 levels are relatively large among cultured sheets, the medium was used as a sample.<sup>8</sup>

Separate experiments (PeproTech, Inc, Rocky Hill, NJ) were performed to examine the potential effects of klotho on AT1R internalization. As previously described,<sup>22</sup> HK-2 cells were stimulated with Ang II (100 nmol/L) in the presence or absence of rh-klotho ( $10^{-10}$ – $10^{-8}$  M) for 5 to 20 minutes at 37°C. Cells were treated with rh-klotho or vehicle for 30 minutes before Ang II challenge. Surface-bound ligands were removed by treatment with a gentle acid wash (50 mmol/L sodium citrate, 0.2 mmol/L sodium phosphate, 90 mmol/L NaCl, and 0.1% BSA [pH 5.0]) for 10 minutes at 4°C. Radioligand binding assay was performed at 4°C for 5 hours to quantify receptors remaining on cell surface. Internalized receptors were expressed as the percentage loss of cell surface binding compared with that in cells exposed to neither Ang II nor rh-klotho.

In additional studies, we assessed the effects of klotho protein on the induction of AGT and AT1R expression by Ang II in HK-2 cells.<sup>21</sup> First, rh-klotho ( $10^{-9}$  M) or vehicle was added to the media. After 30 minutes, Ang II (3 nM) was added. Cells were collected from the sheets under basal condition, 1 and 24 hours later from Ang II exposure to evaluate AGT and AT1R expressions.

### In Vivo Animal Study

Six-week-old male stroke-prone SHR were purchased from Charles River Labs (Yokohama, Japan). All experimental protocols were approved by the ethical committee for animal research of Keio University (permit No. 14 001-[0]).

At 7 weeks of age, the rats were anesthetized by intraperitoneally administering pentobarbital sodium (50 mg/kg), and left uninephrectomy was performed to induce nephrosclerosis for relatively short period.<sup>20,23</sup> As described in Table S1 in the online-only Data Supplement, renal expression of klotho was reduced by hypertension and uninephrectomy. At 8 weeks of age, the rats were divided into 2 groups (n=12 per group)<sup>1</sup>: rats treated with a vehicle (control group)<sup>2</sup> and rats treated with rh-klotho (PeproTech, Inc, Rocky Hill, NJ; klotho group). Rh-klotho ameliorates calcium and phosphate abnormalities in klotho-deficient animals, indicating that it is physiologically active.<sup>8</sup> Rh-klotho was dissolved in 1 mL saline with 0.1% BSA and administered daily through a subcutaneous injection (10 µg/kg per day). A vehicle control group received the same amount of saline containing 0.1% BSA. Systolic blood pressure was measured every 2 weeks by using tail-cuff method, and urine was collected every 2 weeks to measure albumin, creatinine, klotho, and PGF2α (prostaglandin F2a) levels. At 12 weeks of age, 12 rats (6 rats from each group) were anesthetized, and their femoral artery was cannulated to obtain blood samples for various analyses, including measurement of Ang II, FGF (fibroblast growth factor) 23, and klotho levels. In addition, the kidneys were removed, weighed, and cut in half. One half of the kidneys was frozen quickly in liquid nitrogen for the analyses, and the other half was fixed in 4% formalin solution for pathology.<sup>6</sup> The rats were then euthanized by administering an overdose of the anesthetic. Blood samples were centrifuged at 4°C for 10 minutes. Serum, plasma, urine, and tissues were deep-frozen until their use.<sup>3</sup>

For the other 12 rats, 2 days before acute experiments, the chow containing LiCl (5 mmol/Kg) was given to obtain measurable concentrations of lithium, and its clearance as an index of end-proximal tubular flow without acute diuresis.<sup>24</sup> On the day of acute experiment, rats were anesthetized with intraperitoneal Inactin (100 mg/kg; Byk Gulden, Konstanz, Germany). To directly monitor renal perfusion pressure (RPP), femoral and carotid arteries were cannulated.<sup>17,25</sup> Mean blood pressure was used as RPP. Jugular vein was also cannulated (PE50) to infuse saline containing 1% BSA, 7.5% Inutest (Laevosan-Gesellschaft, Linz/Donau Austria). The ureter was cannulated (PE10) with a midline abdominal incision to collect urine in a preweighed tube. Adjustable clamps were placed on the aorta above and below the renal artery to control RPP. Rats were allowed to breathe air enriched with 100% oxygen (aimed at FiO<sub>2</sub> of 40%), which markedly improves the stability of arterial blood pressure. After completion of surgery, 1 hour of equilibration was allowed. Before initiating pressure natriuresis study, basal blood pressure was checked. The upper clamp was tightened to decrease RPP approximately by 20 mm Hg. After 30 minutes of equilibration, 2 separate 20-minute clearance studies were performed. The upper clamp was loosened to return RPP to the baseline. After 30 minutes of equilibration, clearance studies were performed in 2 separate 20-minute periods. The lower clamp was tightened to increase RPP by about 20 mm Hg. After 30 minutes of equilibration, 2 20-minute clearance studies were performed. Inulin clearance was considered as GFR. Because GFR altered with the variations of RPP in control group, fractional excretion of sodium was used as the index for natriuresis. All clearance studies were performed in 12-week-old uninephrectomized stroke-prone SHR.

Please refer online-only Data Supplement for detailed *in vivo* study, reverse transcription polymerase chain reaction, Western blotting, and pathology.

Data are expressed as mean±SEM. Statistical analysis was performed using Student *t* test, linear regression analysis, or ANOVA followed by Newman-Keuls test. *P* value of <0.05 was considered statistically significant.

## Results

The present study was, to our knowledge, the first to show that klotho interacted with the AT1R (Figure 1A). Our results exhibited that 10<sup>-9</sup> M rh-klotho was bound to beads coated with the human AT1R. Moreover, m-klotho was detected when these beads were treated with kidney homogenates, which should contain both the AT1R and m-klotho. In contrast, β-glucuronidase did not show substantial binding to AT1R (Figure S1). Multiple-way ANOVA for repeated measurements revealed that Ang II induced dose- and time-dependent elevations of IP3 in HK-2 cells<sup>19</sup> and that klotho protein suppressed Ang II-induced IP3 elevations (*P*<0.005 for each). At 5 and 10 minutes, Ang II (1 and 3 nM) elicited smaller increments of IP3 in the presence of klotho protein (Figure 1B). However, our results showed that increasing doses of rh-klotho did not inhibit the amounts of Ang II bound on AT1Rs (Figure 1C). Ang II elicited time-dependent increases in AT1R internalization in HK-2 cells, and klotho protein altered Ang II-induced AT1R internalization (*P*<0.001 for each). At 10<sup>-9</sup> M and higher, rh-klotho also enhanced the amounts of AT1Rs lost following to Ang II stimulation for 5 minutes (Figure 1D). In addition, klotho protein lowered AT1Rs on HK-2 cells by itself. Thus, Ang II could internalize smaller amounts of tubular AT1Rs in

the presence of klotho protein. As depicted in Figure 1E, ANOVA showed that Ang II caused time-dependent increments of AGT and AT1R expressions in HK-2 cells, and klotho protein attenuated them ( $P<0.005$  for each). Although rh-klotho ( $10^{-9}$ M) failed to alter AGT and AT1R expression by an hour of Ang II exposure, rh-klotho attenuated the expression of AT1R and AGT induced by the treatment with Ang II for a day in HK-2 cells.

Although systolic blood pressure was elevated with time, daily supplementation of klotho protein significantly attenuated systolic blood pressure at 4 experimental weeks (Figure 2A). In addition, daily klotho protein supplementation declined time-dependent increase in albumin excretion (Figure 2B). Similarly, exogenous klotho protein supplementation decreased time-dependent increases in 8-epi-PGF2 $\alpha$  excretion by experimental week 4 (Figure 2C). Although exogenous klotho protein supplementation did not affect the temporal increase in body weight without considerable changes in appetite, drinking, and activity (Figure 2D), it decreased kidney weight by experimental week 4 (Figure 2E). Our data constitute new demonstration that klotho protein supplementation restored autoregulatory ability (Figure 3A) in association with considerable increments of lithium clearance at experimental week 4 (Figure 2F). Compared with control group ( $0.42\pm 0.09$ ), GFR autoregulatory index was attenuated in klotho group ( $0.02\pm 0.02$ ,  $P<0.01$ ). Of note, at the baseline mean blood pressure levels, whole kidney GFR in klotho-treated animals ( $1.35\pm 0.05$  mL/min at  $128\pm 2$  mm Hg) was significantly less than the controls ( $1.76\pm 0.06$  mL/min at  $147\pm 3$  mm Hg,  $P<0.01$ ). Furthermore, exogenous klotho protein supplementation markedly sharpened pressure natri- uresis responses in uninephrectomized stroke-prone SHR (Figure 3B). Compared with control group ( $0.024\pm 0.009$  %/ mm Hg), the slope for fractional excretion of sodium against RPP was accentuated by klotho supplementation ( $0.074\pm 0.014$  %/mm Hg,  $P<0.05$ ).

Although exogenous supplementation of klotho protein did not significantly reduce plasma Ang II concentrations (Figure 4A), it considerably decreased renal Ang II levels (Figure 4B) and renal AGT expression (Figure 4C) in uninephrectomized stroke-prone SHR. Klotho protein supplementation substantially increased renal klotho expression (Figure 4D) and elevated both serum klotho levels and urinary klotho excretion (Figure 4E and 4F) to the levels similar to normotensive control rats.<sup>6</sup> Of note, klotho supplementation failed to alter serum calcium, phosphate, and FGF23 levels in this model (Table S2). Early phase of kidney disease model may relate to small effects of klotho supplementation on mineral metabolism. In contrast, klotho protein supplementation significantly decreased phosphorylated Akt (Figure 5A) and mTOR (mammalian target of rapamycin; Figure 5B) abundance.

Because hypertension initially damages the juxtamedullary nephrons<sup>13</sup> and because HIF-1 $\alpha$  induces profibrotic changes in medullary fibroblasts,<sup>15</sup> we assessed medullary fibrosis by performing immunohistochemical analysis. Klotho protein supplementation decreased TGF $\beta$  expression (Figure 6A). Moreover, klotho protein supplementation marginally ameliorated  $\alpha$ -SMA ( $\alpha$  smooth muscle actin) (Figure 6B) but not Snail abundance (Figure 6C). Exogenous klotho supplementation considerably ameliorated renal abundance of HIF-1 $\alpha$  (Figure 6D). Furthermore, klotho protein supplementation decreased fibronectin staining of renal medulla but not cortex (Figure 6E). Similarly, fibronectin expression was reduced by klotho protein supplementation in renal medulla (Figure 6F) but not cortex (Figure 6F).

Moreover, the results of fibrosis indexes using Masson trichrome staining manifested similar trends to those of immunohistochemical analyses on fibronectin (Figure S2).

## Discussion

Hypertensive nephrosclerosis is a common chronic kidney disease that requires renal replacement therapy. Although antihypertensive therapy is the main strategy for delaying the progression of nephrosclerosis, it may not completely prevent the progression to end-stage renal failure. Established hypertensive nephrosclerosis is characterized by tubulointerstitial fibrosis, arteriosclerosis, arteriolosclerosis, and glomerular sclerosis. Hypertension initially damages the juxtamedullary nephrons,<sup>12,13</sup> and resultant medullary pathology further increases blood pressure.<sup>10,11</sup> Thus, high blood pressure and renal medullary lesions form a vicious cycle, which could be disrupted by employing therapeutic strategies focused on medullary fibrosis.

The present data constitute new demonstrations that klotho interacts with AT1Rs to diminish their localization on the cell surface. Two domains of the carboxyl terminus and 1 domain of the third intracellular loop are important for the internalization of activated AT1R.<sup>22</sup> AT1R stimulation by Ang II results in the translocation of  $\beta$ -arrestin to the AT1R and subsequently elicits the internalization of the receptor.<sup>26–28</sup> Binding of klotho to the AT1R may elicit conformational changes in these regions, thus enhancing the internalization of the receptor to reduce surface density. In addition to AT1R stimulation, the internalized Ang II induces AGT expression.<sup>19</sup> These *in vitro* data are consistent with the *in vivo* results that klotho protein supplementation attenuated renal AGT expression and elevations of blood pressure. The present findings also showed that exogenous klotho supplementation inhibited Akt phosphorylation. We recently showed that GSK3 $\beta$  (glycogen synthase kinase 3 $\beta$ ) inhibition increased AGT expression in the kidneys.<sup>6</sup> GSK3 $\beta$  is activated by the Akt pathway to control glucose metabolism. Collectively, the present findings suggested that klotho-induced modulations of renal RAS involve multiple pathways, AT1R internalization, as well as Akt phosphorylation and oxidative stress in hypertensive kidneys.

The present study indicated that klotho protein supplementation decreased TGF $\beta$  expression and  $\alpha$ -SMA abundance in hypertensive kidneys. However, exogenous klotho supplementation did not alter Snail abundance. Hypertensive kidneys show weak TGF $\beta$  and Snail levels compared with kidneys with adriamycin nephropathy.<sup>6</sup> Albuminuria strongly induces TGF $\beta$  expression in tubular cells of the nephron.<sup>29</sup> However, albumin excretion in uninephrectomized SHR was much lower than that in rats with adriamycin nephropathy. Ang II induces TGF $\beta$  expression in renal tubular cells, thus inducing epithelial-mesenchymal transition in an autocrine manner.<sup>30</sup> Klotho inhibits TGF $\beta$  signaling.<sup>9</sup> These may account for the discrepant results. We previously showed that the contribution of epithelial-mesenchymal transition in renal fibrosis differs among various underlying kidney diseases.<sup>31</sup> In addition, cortical fibrosis was not apparent in the present study. Together, these results extended the above-mentioned findings<sup>31</sup> and suggest that the contribution of epithelial-mesenchymal transition to renal fibrosis is relatively small in the early phase of hypertension.

Renal fibrosis is the common final pathway that leads to end-stage renal failure.<sup>31</sup> To our knowledge, the present study showed for the first time that klotho protein supplementation ameliorated PGF2 $\alpha$  excretion and HIF-1 $\alpha$  abundance in hypertensive kidneys. Ang II stabilizes HIF-1 $\alpha$ , which promotes both fibrosis and cell growth.<sup>32</sup> The excretion of PGF2 $\alpha$  represents an index of oxidative stress.<sup>6,7</sup> Thus, these findings suggested that klotho-induced decrease in renal Ang II levels and oxidative stress contributed to the decrements of HIF-1 $\alpha$  abundance. Wang et al<sup>15</sup> showed that Ang II induced profibrotic changes by activating HIF-1 $\alpha$  in medullary fibroblasts. In the present study, we found that exogenous klotho supplementation inhibited medullary fibronectin expression and sharpened pressure-induced natriuresis responses. Our data extended those of Bank et al<sup>13</sup> who showed that tubulointerstitial changes were first confined to the juxtamedullary zones except the outer cortex in SHRs and suggest that klotho-induced alterations of medullary pathology underlie the augmentation of pressure natriuresis. Taken together, the present study indicated that klotho protein supplementation attenuated renal medullary fibrosis in hypertensive rats and suggested that klotho could crosstalk with the renal RAS to suppress medullary fibrosis, contributing to enhancing pressure-induced natriuresis in hypertensive kidneys.

Compensatory renal hypertrophy is characterized by proximal tubular growth and elongation, which diminish the delivery to macula densa and then tubuloglomerular feedback.<sup>33,34</sup> The present results that exogenous klotho supplementation decreased phosphorylated Akt levels in hypertensive kidneys are consistent with the notion that klotho interacts with IGF receptor to inhibit its signaling.<sup>4</sup> In addition, our study is the first to show that klotho protein supplementation inhibited mTOR phosphorylation in the kidneys. Chen et al<sup>35</sup> showed that class III PI3K (phosphoinositide 3-kinase)-mTOR signaling mediates compensatory renal hypertrophy. Furthermore, kidney weights of rats receiving exogenous klotho protein were lower than those of the controls, supporting that klotho suppressed compensatory renal hypertrophy. This was supported by physiological significance that lithium clearance was increased by klotho protein supplementation. These observations indicate that klotho reduced proximal tubular reabsorption and suggest that klotho-induced increase in distal delivery is involved in controlling tubuloglomerular feedback and GFR autoregulation. Of course,<sup>14</sup> we would not exclude the possibility that klotho-induced reductions of blood pressure could ameliorate renal arteriolopathy (Figure S3). Consequently, the present findings are consistent with those of Kumar et al<sup>36</sup> and suggested that klotho-induced inhibition of the Akt-mTOR pathway decreased compensatory renal hypertrophy. However, mTOR signaling is important for normal growth, implicating that klotho supplementation may not suit for children with renal diseases, but for adult patients with diabetic nephropathy<sup>37</sup> and polycystic kidney disease.<sup>38</sup>

Our study has several limitations. First, although our data did not prove endogenous klotho loss as a mechanism of hypertension, klotho gene delivery to 12-week-old SHR prevented the progression of hypertension.<sup>39,40</sup> The present study focused on early phase of hypertension, using klotho protein supplementation that may ethically be more acceptable than gene transfer. It remained to be determined whether klotho improved the phenotypes or attenuated the development of disease-related phenotypes. Second, genetically modified ATIRs were not used. Thus, precise structural basis for klotho interactions remained to be elucidated. Third, klotho decreased IP3 levels after Ang II stimulation. However, we did not



examine the effects of klotho supplementation on AT1R desensitization.<sup>28</sup> Fourth, exogenous klotho supplementation enhanced endogenous klotho expression. Marginal reductions in FGF23 might have induced natriuresis,<sup>41</sup> possibly contributing to the present results. Finally, healthy control arm and experimental group under AT1R blockade were not included in the present study. Because the present study only assessed the relative benefit of exogenous klotho treatment, cautions should be exercised before generalizing the results.

## Perspectives

The present study was the first to show that klotho interacted with the AT1R to modulate its internalization and to inactivate the renal RAS. Furthermore, our results indicated that klotho suppressed medullary fibrosis through the HIF-1 $\alpha$  pathway, enhancing pressure natriuresis. Our results also suggested that klotho supplementation decreased the Akt-mTOR signaling to ameliorate renal hypertrophy and GFR autoregulation. These results provide translational basis to evaluate the utility of exogenous klotho protein supplementation for treating patients with hypertensive nephrosclerosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Novelty and Significance****What Is New?**

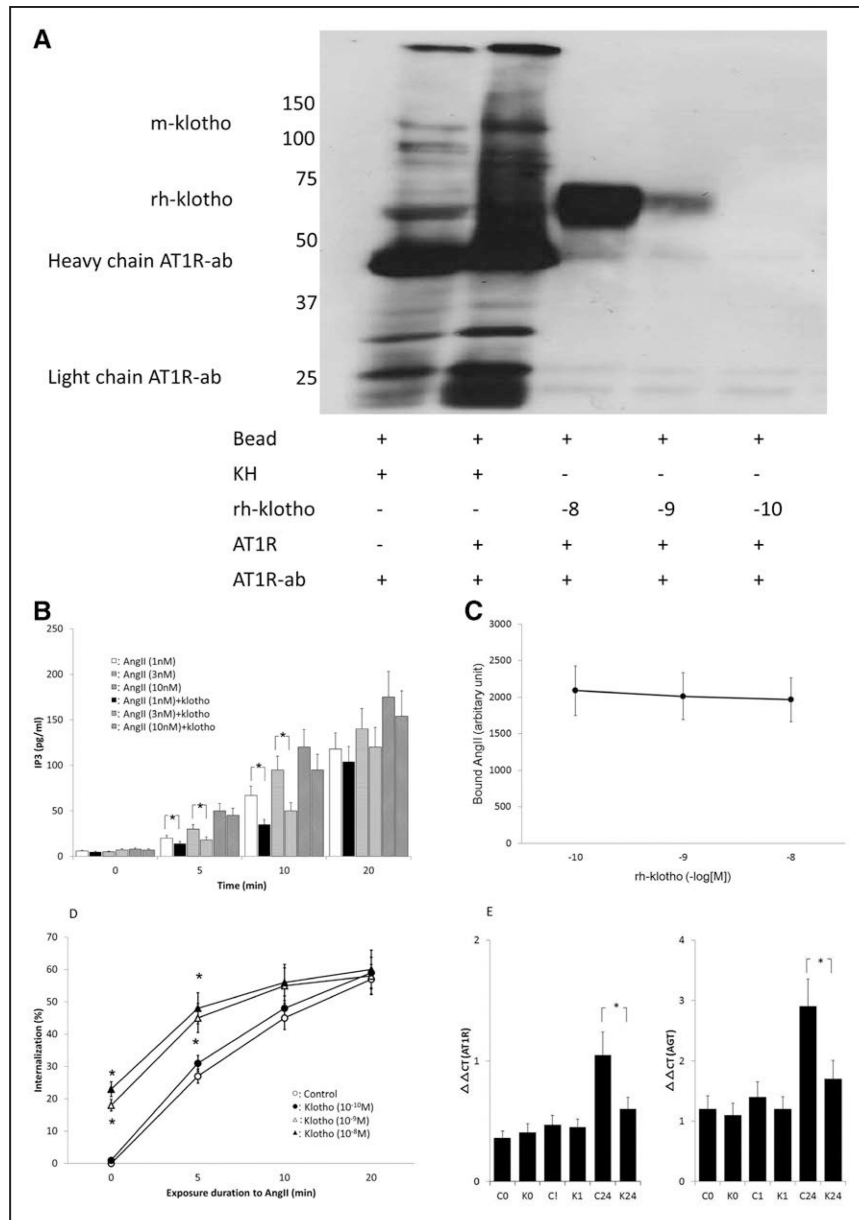
- Klotho interacts with the AT1R (Ang II [angiotensin II] type 1 receptor) to inactivate the renal renin-Ang system.
- Klotho suppresses medullary fibrosis through the HIF (hypoxia-inducible factor)-1 $\alpha$  pathway in early hypertension.
- Klotho supplementation decreases Akt-mTOR (mammalian target of rapamycin) signaling.

**What Is Relevant?**

- Exogenous klotho protein supplementation would be useful for hypertensive kidney diseases
- Renal renin-Ang system contributes at least partly to the pathogenesis of medullary fibrosis and high blood pressure.
- Klotho supplementation could ameliorate renal hypertrophy.

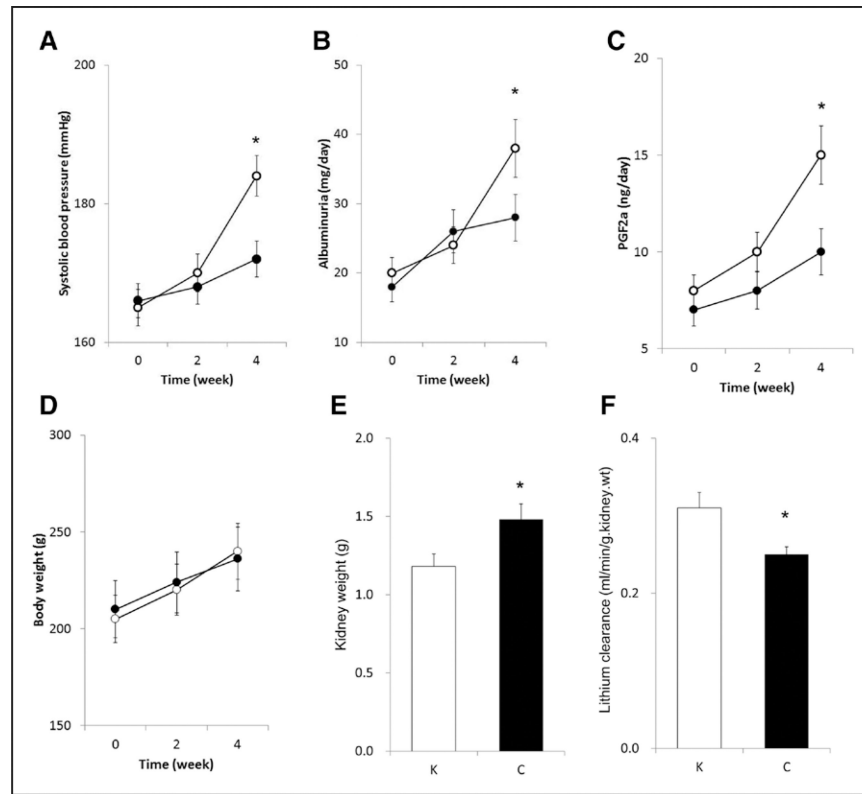
**Summary**

Klotho suppresses renal renin-Ang system at least partly by interacting with the AT1Rs. Klotho supplementation suppresses Akt-mTOR signaling, declining renal hypertrophy and improving glomerular filtration rate autoregulation. Medullary fibrosis in early hypertension involves HIF-1 $\alpha$  pathway, attenuating pressure natriuresis and elevating blood pressure.

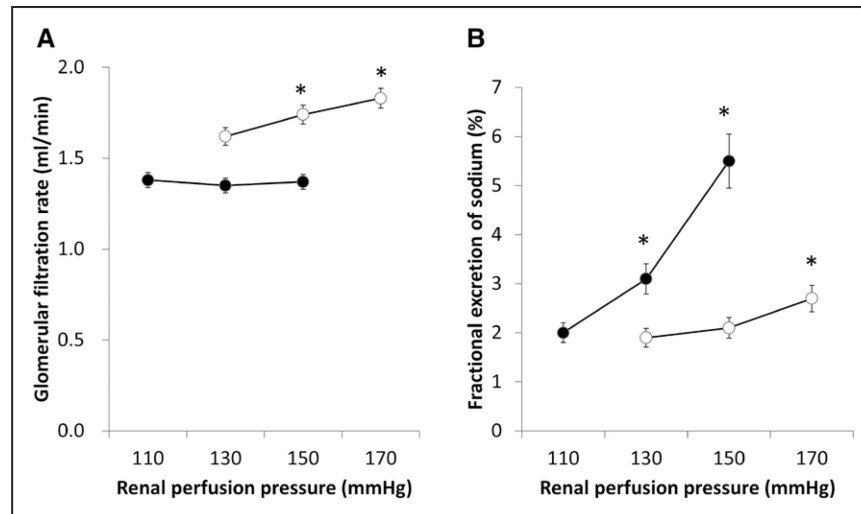


**Figure 1.** Summary of in vitro studies. **A**, Binding of the human AT1R (Ang II [angiotensin II] type-1 receptor) to rh-klotho (recombinant human klotho). This binding is evident at high concentrations of rh-klotho, which composed of *KL-1* gene (n=5). Kidney homogenate (KH), AT1R-ab (antibody against the AT1R), m-klotho (membrane-bound klotho), -8: 10<sup>-8</sup> M, -9: 10<sup>-9</sup> M, -10: 10<sup>-10</sup> M. **B**, Ang II stimulation increased inositol triphosphate (IP3) levels (n=6 for each). Vertical axis showed the amount of IP3. Ang II (1 and 3 nM) caused smaller increments of IP3 in the presence of klotho at 5 and 10 min. Open, horizontal line, and right oblique line bars indicate 1, 3, 10 nmol/L of Ang II exposure without klotho treatment. Closed, vertical line, and left oblique line bars depict 1, 3, 10 nmol/L of Ang II with klotho treatment, respectively. \*Significant difference between control and klotho-treated groups. **C**, Treatment with rh- klotho did not alter the binding of Ang II to the AT1R

(n=4 for each). **D**, Ang II-induced time- dependent internalization of the AT1R (n=6 for each). Vertical axis showed the magnitude of AT1R internalization. Klotho enhanced the amounts of AT1Rs lost following to Ang II stimulation for 5 min. Open and closed circles indicate control and  $10^{-10}$  M rh-klotho-treated groups, respectively; open and closed triangles depict  $10^{-9}$  and  $10^{-8}$  M rh-klotho-treated groups, respectively. \*Significant difference compared with the control group. **E**, Ang II elicited time-dependent inductions of AT1R and AGT (angiotensinogen; n=6 for each). Target gene expression was compared by relative changes in comparative threshold (  $\Delta\Delta C_t$  ) using *Gapdh* as reference. \*Significant difference between control and klotho-treated groups. C0 indicates the values from control group at time 0; C1, the values from control group at 1 h; C24: the values from control group at 24 h; K0, the values from klotho-treated group at time 0; K1, the values from klotho-treated group at 1 h; and K24, the values from klotho-treated group at 24 h.

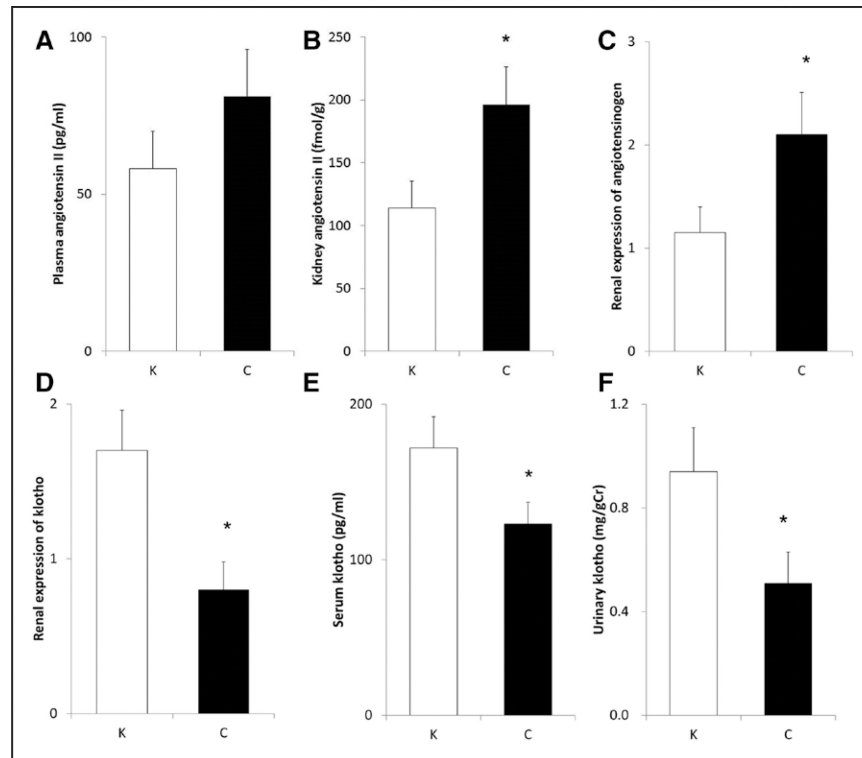


**Figure 2.** Influences of klotho protein supplementation on systolic blood pressure (A), albuminuria (B), 8-epi-PGF2α (prostaglandin F2a) excretion (C), body weight (D), kidney weight (E), and lithium clearance at basal blood pressure (F). Open and closed circles indicate control (C) and klotho (K)-treated groups (F: n=6 for each; the other parameters: n=12 for each), respectively. \*Significant difference between the 2 groups



**Figure 3.** Impacts of exogenous klotho protein supplementation on autoregulation of glomerular filtration rate (A), pressure-induced natriuresis (B). Open and closed circles indicate control and klotho-treated groups (n=6 for each). \*Significant difference from the values at the lowest renal perfusion pressure within the group.

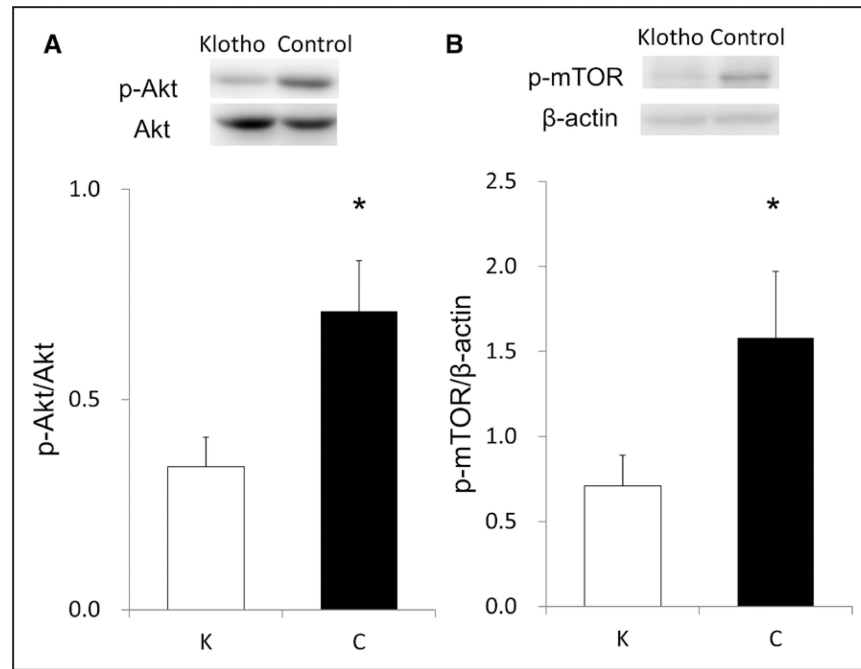




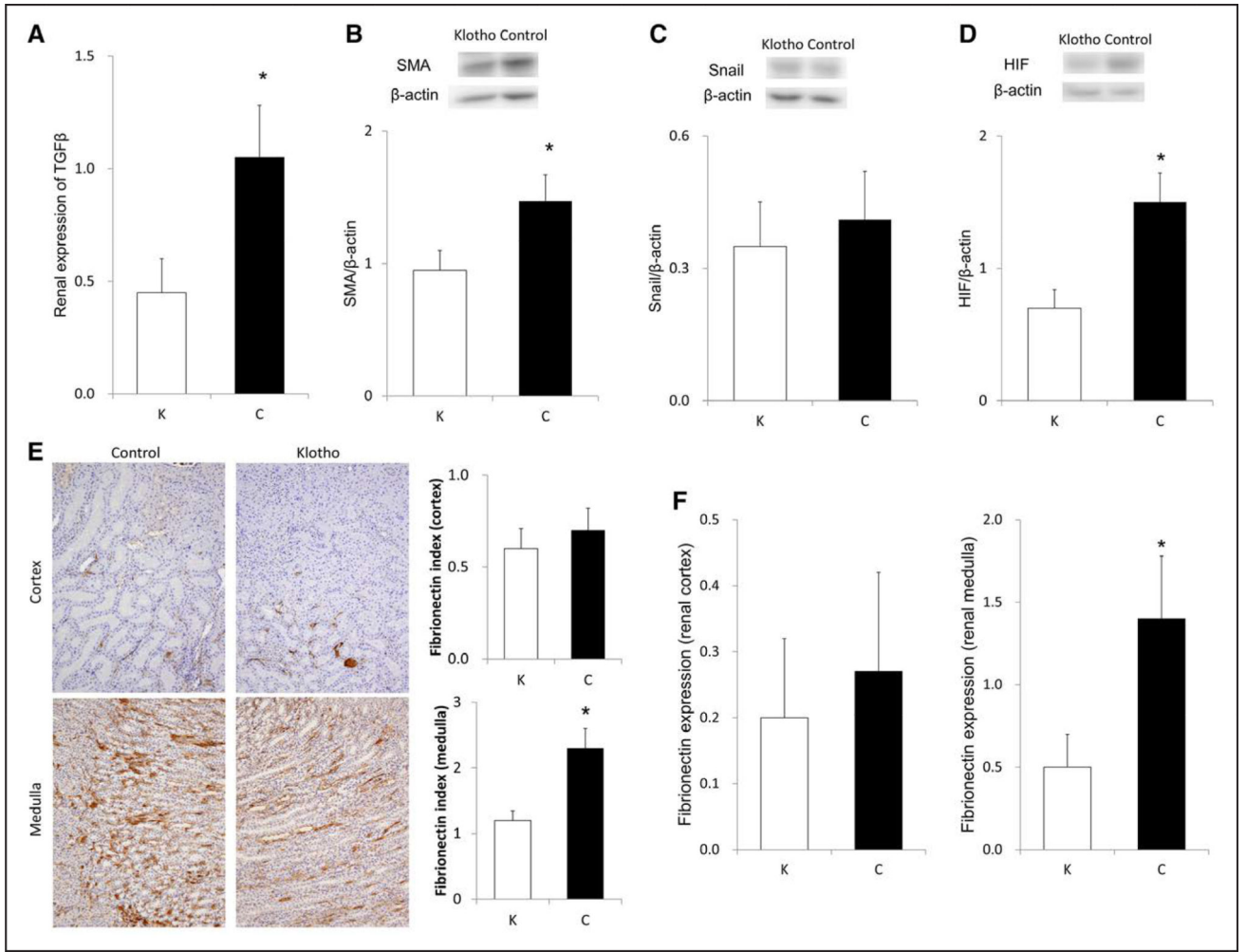
**Figure 4.**

Effects of exogenous klotho supplementation on plasma Ang II (angiotensin II) concentration (A), renal Ang II concentration (B), renal AGT (angiotensinogen) expression (C), renal klotho expression (D), serum klotho concentration (E), urine klotho levels (F).

\*Significant difference between the 2 groups. C indicates control group; and K, klotho group (n=6 for each).



**Figure 5.** Influences of klotho protein supplementation on renal p-Akt (phosphorylated Akt) abundance (**A**) and renal p-mTOR (phosphorylated mTOR, mammalian target of rapamycin) abundance (**B**). \*Significant difference between the 2 groups. C indicates control group; and K, klotho group (n=6 for each).



**Figure 6.** Impacts of exogenous klotho protein supplementation on renal TGFβ (transforming growth factor β) expression (A); renal α-SMA (α smooth muscle actin; B), Snail (C), and HIF (hypoxia-inducible factor)-1α abundance (D). Fibronectin staining in the renal cortex (E, upper) and medulla (E, lower). Klotho supplementation declined fibronectin index in the renal medulla (E, right). Fibronectin is stained in brown. Fibronectin index was calculated similarly to fibrosis index, by measuring the percentage of area stained in brown. Klotho also reduced fibronectin expression in the medulla (F, right). E: n=12 for each; the other parameters: n=6 for each. \*Significant difference between the 2 groups. C indicates control group; and K, klotho group.