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Overexpression of klotho protein modulates uninephrectomy-induced compensatory renal hypertrophy by suppressing IGF-I signals

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ABSTRACT

The *klotho* gene is highly expressed in the distal convoluted tubule of the kidney, while its encoded protein has many physiological and pathophysiological renal roles. We investigated the effect of klotho protein on physiological compensatory renal hypertrophy after nephrectomy in *klotho* transgenic (KLTG) mice. Renal hypertrophy was suppressed in KLTG mice compared with wild-type mice, and this was associated with suppression of insulin growth factor-1 (IGF-1) signaling by klotho protein. *In vitro*, IGF-1 signaling was suppressed in human proximal tubular cells transfected with the klotho plasmid. Our data suggest that klotho modulates compensatory renal hypertrophy after nephrectomy via suppression of the IGF-1 signaling pathway, indicating a novel physiological role for klotho protein in the kidney.

1. Introduction

Klotho is a newly identified anti-aging factor [1]. Several phenotypes of *klotho* mutant mice developed a syndrome resembling human aging that was characterized by shortened life span, growth retardation, arteriosclerosis, skin and muscle atrophy, and osteoporosis. *Klotho* gene expression is high in the distal convoluted tubule of the kidney and is associated with elevated serum levels of 1,25-dihydroxyvitamin D3, phosphate, and calcium levels through fibroblast growth factor 23 (FGF23) in aging-like mouse phenotypes [2–4].

We have previously reported the reno-protective effects of klotho in ICR-derived glomerulonephritis mice, a progressive renal injury mouse model [5]. Renal protection by klotho was shown not to be dependent on the effects of FGF23 or phosphate metabolism but rather on its anti-oxidative effects. Additional effects of klotho include the suppression of Wnt signaling [6] and the induction of IGF-I and insulin pathway resistance [7,8]. Furthermore, *klotho* is also a target gene of peroxisome proliferator-activated receptor γ [9,10].

Renal mass reduction by unilateral nephrectomy-induced compensatory glomerular and renal hypertrophy is carried out to maintain residual renal function. Several signaling pathways are involved in this process, including the IGF-1 pathway, which is one of the most important pathways for compensatory renal hypertrophy after uninephrectomy [11,12]. Renal hypertrophy is preceded by increased renal levels of IGF-1, which is presumably involved in tubular cell proliferation and hypertrophy after loss of renal mass [12]. However, the precise molecular mechanism underlying renal hypertrophy under these conditions remains unclear. Furthermore, the role of klotho in compensatory renal hypertrophy has not yet been elucidated.

Here, we hypothesized that klotho regulates compensatory renal hypertrophy through suppression of the IGF-1 pathway. To verify this hypothesis, we examined whether transgenic overexpression of the *klotho* gene can inhibit renal hypertrophy after renal mass reduction.

2. Methods

2.1. In vivo experimental protocol

The experimental protocol (No. 10-014) was approved by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School (Kurashiki, Japan). Transgenic mice overexpressing the *klotho* gene (EFmKL46) were kindly provided by Yo-Ichi

Abbreviations: IGF, insulin like growth factor; mTOR, mammalian target of rapamycin; FGF23, fibroblast growth factor 23; WT, wild type; KLTG, *klotho* transgenic; hPTECs, human proximal tubular cells; gp91TAT, gp91ds-*tat*; gp91SCR, scrambled gp91; NAD(P)H oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; IRS, insulin receptor substrate; rpS6, ribosomal protein S6; 4EBP, 4E binding protein; ROS, reactive oxidative species.

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Nabeshima (Kyoto University, Kyoto, Japan) [7]. These mice expressed exogenous *klotho* gene under the control of the human elongation factor 1α promoter in addition to their endogenous gene expression. As a result, there was a 2-fold higher klotho concentration in the blood of *klotho* transgenic mice when compared to wild type (WT) [7].

Klotho transgenic mice were backcrossed with C57/BL6J background mice more than 10 times. Male klotho transgenic (KLTG) mice weighing 27–33 g were used for the study and littermate WT mice were used as controls. The WT mice ($n = 20$) and KLTG mice ($n = 20$) were divided randomly into two groups ($n = 10$ per group). The mice in one group were unilaterally nephrectomized (WT Nx and KLTG Nx, respectively) and those in the other group underwent sham surgery (WT sham and KLTG sham, respectively). Two weeks after right nephrectomy or sham surgery, mice were sacrificed for collection of left kidney tissue and blood samples. The 24-h urine samples were collected on the day before sacrifice. Kidney weight was measured and corrected for body weight. Part of the kidney was used for histological examination. Renal cell hypertrophy was determined by total protein/DNA ratio using the remaining kidney [13].

2.2. Serum IGF-1 concentration

Serum was separated from blood and used for IGF-1 concentration measurements using an enzyme-linked immuno sorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

2.3. Histological examination

Paraffin sections were cut into 4-μm slices and subjected to periodic acid-Schiff staining. Glomerular volumes were evaluated by light microscopy. Volumes of the glomerular tuft were calculated from midsection areas using the maximal planar area method [14]. Glomerular volumes and total glomerular cell numbers were measured for at least 25 randomly selected glomeruli from the renal cortex of each animal (total of 300–400 glomeruli from 8 to 10 mice per group).

2.4. In vitro experimental protocol

Human proximal tubular cells (hPTECs) (Cell Systems Corporation, Kirkland, WA) at passage 6–8 were cultured in 10-cm dishes and propagated in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated newborn bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin [15]. Mouse *klotho* cDNA was cloned into the pCAGGS vector (pCAGGS-klotho), an expression vector containing the CMV/chicken β-actin enhancer/promoter [16]. pCAGGS vector-cloned *lacZ* cDNA was used as a control. LipofectAMINE 2000 (Invitrogen, Camarillo, CA) was used to transfect these plasmids [17].

hPTECs were stimulated by recombinant human IGF-1 (R&D Systems) for 30 min and cells were also co-incubated with NAD(P)H oxidase-specific inhibitor gp91TAT (50 μM; Sigma–Aldrich Japan K.K., Tokyo, Japan) or gp91SCR (50 μM; Sigma–Aldrich Japan K.K.) [18]. After stimulation, cell lysates were collected to detect NAD(P)H oxidase activity using a lucigenin chemiluminescence assay [19].

2.5. Western blot analysis

Renal protein was extracted using Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Equal protein quantities of cell lysate (30 μg) were separated on a 10–15% SDS–polyacrylamide gel, electrotransferred onto a polyvinylidene difluoride membrane, and probed with indicated primary antibody and the appropriate secondary antibody conjugated with horseradish peroxidase goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies against phospho IRS1, phospho Akt, phospho rpS6, phospho 4EBP, and antibodies for each total protein were obtained from Cell Signaling Technology (Beverly, MA). Antibody was visualized using an enhanced chemiluminescence method (ECL plus; GE Healthcare Japan, Tokyo, Japan). The integrated band density ($n = 5$ per group) was quantified using NIH Image software v.1.61.

2.6. Statistical analysis

Values are expressed as mean ± SEM. Parameters were evaluated with a two-tailed unpaired Student's *t*-test or one-way analysis of variance for comparison of multiple means. A p value <0.05 was considered significant.

3. Results

3.1. Compensatory renal hypertrophy was suppressed in klotho transgenic mice

Physiological data for each group are shown in Table 1. There were no significant changes in body weight or mean blood pressure between groups (blood pressure, data not shown). There was also no significant difference in kidney function, as assessed by serum creatinine levels, between WT sham and WT Nx, whereas serum creatinine was significantly higher in KLTG Nx than in KLTG sham. No pathological changes such as inflammatory cell infiltration or fibrotic changes (data not shown) were observed in the glomerular or interstitial areas.

Fig. 1A shows representative macro kidney images for each group. Under basal conditions, kidney size was originally larger in KLTG sham than in WT sham (Fig. 1B; KLTG sham/WT sham, 1.24 ± 0.07). In WT Nx, the remaining kidney was increased remarkably after uninephrectomy (Fig. 1B; WT Nx/WT sham, 1.54 ± 0.11), while compensatory renal hypertrophy was markedly suppressed in KLTG Nx (Fig. 1B; KLTG Nx/WT sham, 1.26 ± 0.04). The total protein/DNA ratio was increased in WT Nx compared with WT sham, but there were no differences between KLTG Nx and KLTG sham (Fig. 1C; WT Nx/WT sham, 1.65 ± 0.01; KLTG sham/WT sham: 0.9 ± 0.02; KLTG Nx/WT sham: 1.20 ± 0.02).

3.2. Physiological glomerular response was inhibited in klotho transgenic mice

Fig. 2A shows representative glomerular images for each group. There were no significant pathological changes in glomeruli, although glomerular volume increased remarkably in WT Nx compared with WT sham (Fig. 3B; WT Nx/WT sham, 1.40 ± 0.04). Glomerular size in KLTG sham was slightly, but not significantly, larger than in WT sham (Fig. 3B; KLTG sham/WT sham, 1.27 ± 0.06). Glomerular size was not increased in KLTG Nx (Fig. 3B; KLTG Nx/WT

Table 1
Changes in body weight, renal function and urinary data after uninephrectomy in wild type or *klotho* transgenic mice.

	WT		KLTG	
	Sham	Nx	Sham	Nx
BW (g)	23.4 ± 0.3	22.3 ± 0.3	22.5 ± 0.2	22.0 ± 0.5
S-CRN (mg/dL)	0.13 ± 0.03	0.15 ± 0.01	0.11 ± 0.01	0.14 ± 0.01*
U-Alb (g/g CRN)	0.11 ± 0.02	0.10 ± 0.01	0.10 ± 0.02	0.09 ± 0.01
U-Na (mEq/mg CRN)	3.1 ± 0.4	2.6 ± 0.1	2.2 ± 0.2	2.6 ± 0.5

WT, wild-type mice; KLTG, *klotho* transgenic mice; sham, sham operated group; Nx, nephrectomised group; BW, body weight; S-CRN, serum-creatinine; U-Alb, urinary albumin; U-Na, urinary Na.
* $p < 0.05$ versus KLTG sham. $n = 10$ per group.

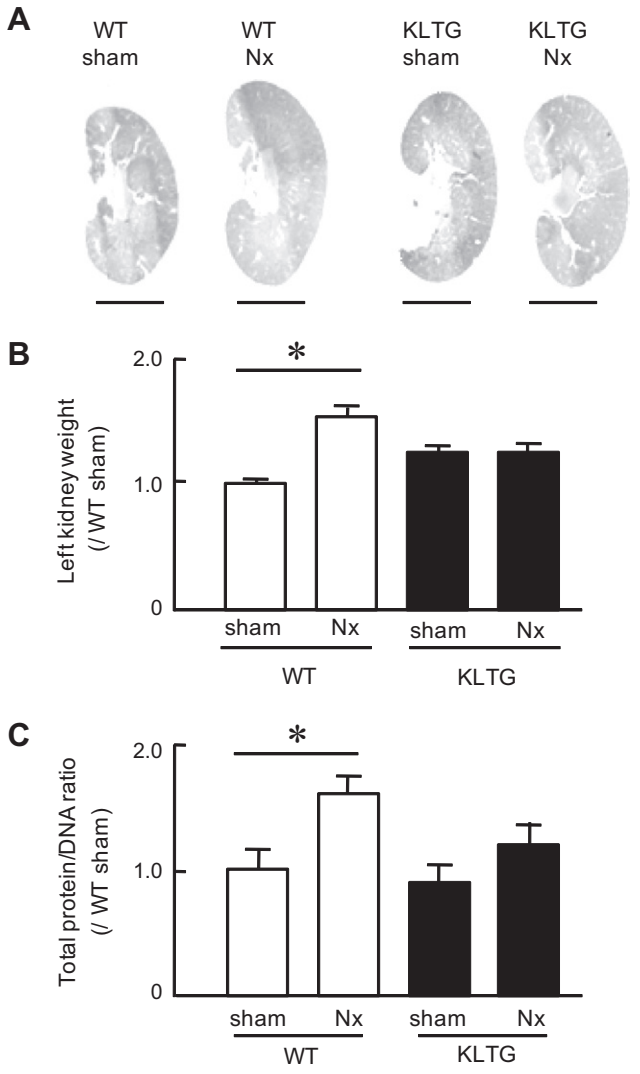

Fig. 1. (A) Representative whole kidney sections. Bar, 5 mm. WT, wild type mice; KLTG, *klotho* transgenic mice; sham, sham-operated group; Nx, nephrectomized group. (B) Relative left kidney weight 2 weeks after right kidney nephrectomy or sham operation. $^{*}p < 0.05$. (C) Relative cell hypertrophy determined by total protein/DNA ratio. $^{*}p < 0.05$.

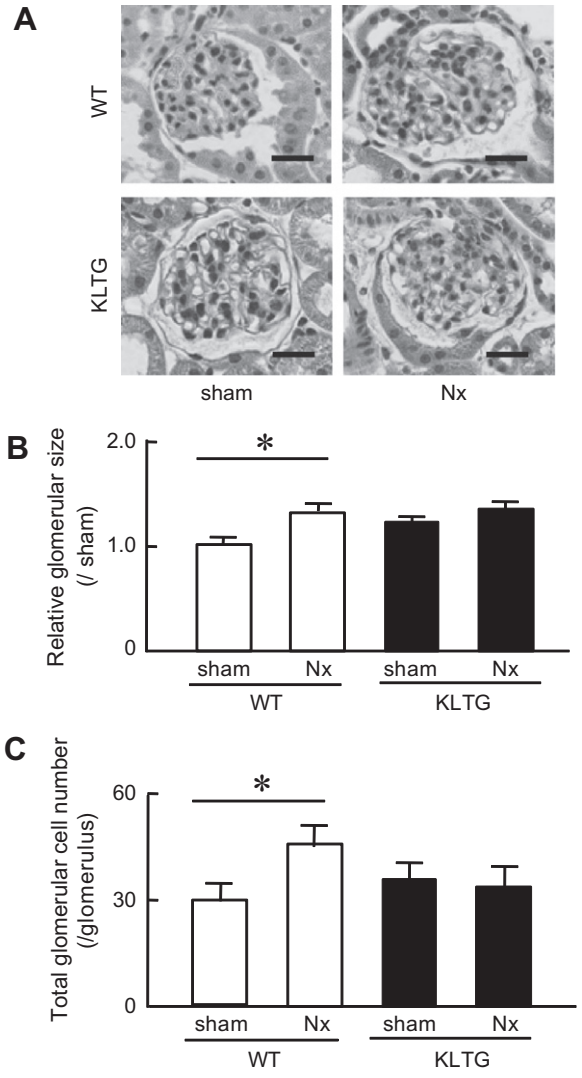

Fig. 2. (A) Histological findings in glomeruli. Bar, 25 μm. WT, wild type mice; KLTG, *klotho* transgenic mice; sham, sham-operated group; Nx, nephrectomized group. (B) Relative glomerular size in each group. $^{*}p < 0.05$. Glomeruli (300–400) were examined per group. (C) Total glomerular cell number per group. $^{*}p < 0.05$. Glomeruli (300–400) were examined per group.

sham, 1.32 ± 0.08) compared with KLTG sham (KLTG Nx/KLTG sham: 1.11 ± 0.12). Total glomerular cell number increased significantly after nephrectomy in WT mice (Fig. 2C; WT sham: 31 ± 5; WT Nx: 46 ± 6), but there was no change in KLTG mice (Fig. 2C; KLTG sham: 36 ± 5, KLTG Nx: 34 ± 8).

3.3. IGF-1 signaling was suppressed in the kidney of klotho transgenic mice

Serum IGF-1 concentrations were increased after uninephrectomy in WT mice (Fig. 3A; WT sham 252 ± 18 ng/ml, WT Nx 290 ± 16 ng/ml) and KLTG mice (KLTG sham 260 ± 11 ng/ml, KLTG Nx 288 ± 12 ng/ml). Renal IRS-1 was also activated in WT Nx, but IRS-1 phosphorylation was not enhanced by uninephrectomy in KLTG mice (Fig. 3B).

3.4. IGF-1-activated NAD(P)H oxidase activity in human proximal tubular cells in vitro

hPTECs transfected with the pCAGGS-klotho plasmid expressed twice as much klotho protein as those transfected with control pCAGGS-LacZ plasmids (data not shown). IGF-1 stimulated NAD(P)H oxidase activity in hPTECs transfected with the pCAGGS-LacZ plasmid but not hPTECs with the pCAGGS-klotho plasmid, although this effect was completely suppressed by gp91TAT pretreatment (Fig. 4A). gp91SCR pretreatment did not suppress NAD(P)H oxidase activity in hPTECs (data not shown).

3.5. Klotho protein modulated the IGF-1-mTOR pathway via suppression of NAD(P)H oxidase activity in vitro

IGF-1 stimulated phosphorylation of Akt in hPTECs transfected with the pCAGGS-LacZ plasmid. Furthermore, stimulation of IGF-1 induced phosphorylation of rpS6 and 4EBP (Fig. 4B–D). However, these effects were suppressed in hPTECs transfected with the pCAGGS-klotho plasmid (Fig. 4B–D). Phosphorylation of Akt, rpS6 and 4EBP were suppressed by the administration of gp91TAT, inhibitor of NAD(P)H oxidase.

4. Discussion

In the present study, we demonstrated that KLTG mice suppressed compensatory renal hypertrophy after uninephrectomy.

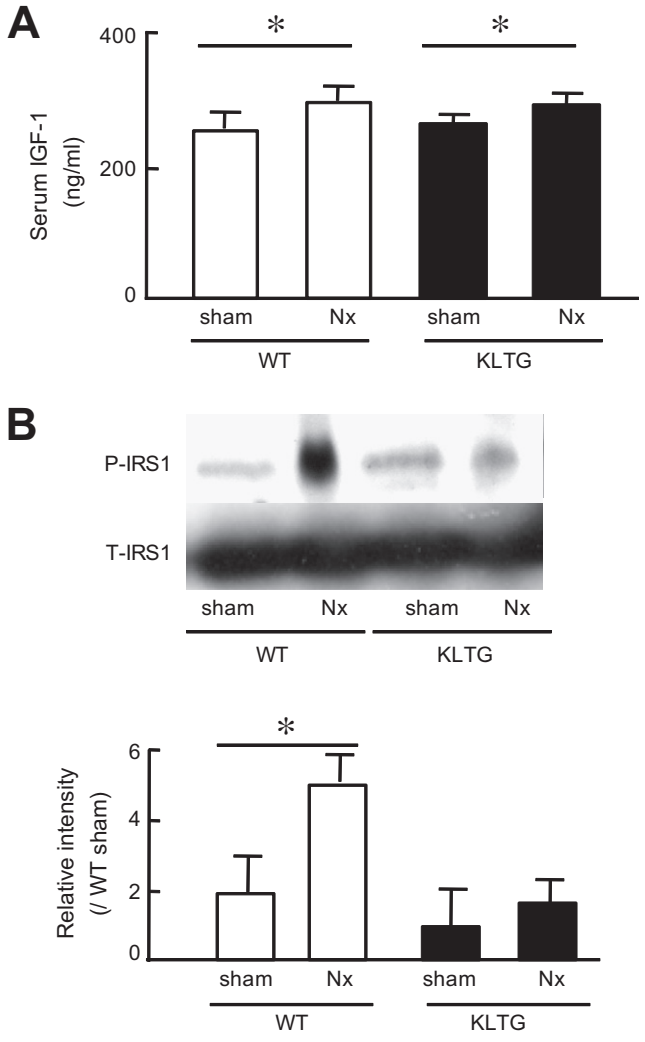

Fig. 3. (A) Serum IGF-1 concentrations. WT, wild type mice; KLTG, *klotho* transgenic mice; sham, sham-operated group; Nx, nephrectomized group. $^*p < 0.05$. (B) Phosphorylation of IRS1 and total IRS1 in the kidney. P-IRS1, phospho-IRS1; T-IRS1, total-IRS1. $^*p < 0.05$.

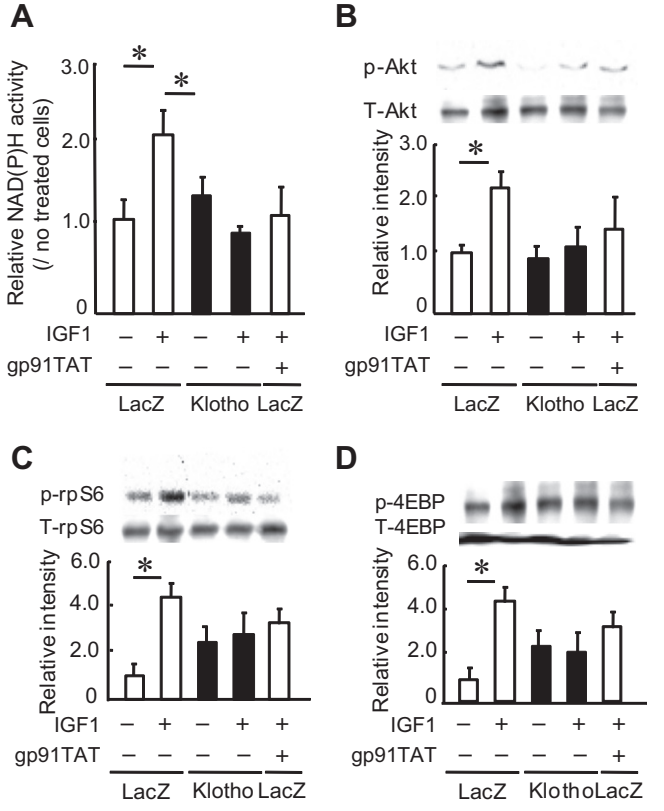

Fig. 4. (A) NAD(P)H oxidase activity assessed by lucigenin chemiluminescence assay. $^*p < 0.05$. (B–D) western blot analysis of Akt, rpS6, and 4EBP activation. (B) Representative western blot for phospho-Akt (p-Akt) and total Akt (T-Akt). $^*p < 0.05$. (C) Representative western blot for phospho-rpS6 (p-rpS6) and total rpS6 (T-rpS6). $^*p < 0.05$. (D) Representative western blot for phospho-4EBP (p-4EBP) and total 4EBP (T-4EBP). $^*p < 0.05$.

Hypertrophy of renal tubular cells, especially those in the proximal tubule, accounts for the majority of kidney size increase, and KLTG mice exhibited inhibition of both tubular cell hypertrophy and glomerular hypertrophy after uninephrectomy. Serum IGF-1 concentrations were also increased after nephrectomy in KLTG mice; however, renal phosphorylation of IRS-1 was suppressed. Klotho inhibits tubular hypertrophy by suppressing intracellular signal transduction downstream of IGF-1. *In vitro* experiments in this study indicated that IGF-1 stimulates NAD(P)H oxidase and revealed the phosphorylation of Akt, rpS6, and 4EBP in hPTECs, whereas these effects were all suppressed in klotho plasmid-transfected cells.

The *klotho* gene encodes a 130-kDa transmembrane protein that shares sequence homology with β-glucosidase [1]. Recently, several reports have shown that klotho is involved in not only calcium homeostasis but also many other activities. Previously, we reported that klotho serves as a potential renoprotective humoral factor by reducing mitochondrial oxidative stress [5]. In addition, klotho influences intracellular signaling pathways including those of cAMP, protein kinase C, and Wnt [6,20]. Moreover, klotho protein suppresses IGF-1 signaling [7].

Several studies suggested the importance of IGF-1 for compensatory hypertrophy. Renal IGF-1 levels were increased at 1–5 days after nephrectomy [21], while Shohat et al. reported that serum IGF-1 levels increased 10 days after uninephrectomy [22]. In this study, serum IGF-1 levels increased significantly after uninephrectomy in KLTG mice, but there was no significant increase in kidney IRS1 activation. Previously, the levels of IGF-1 in KLTG mice were reported to be normal or elevated, but their actions were inhibited [23]. Kurosu found that klotho circulates as a hormone and binds to cell surface receptors through which it represses the autophosphorylation of IGF-1 receptors [7]. This action of klotho protein would result in a reduction of IRS1 activation by inhibiting IGF-1 receptor signaling.

Reduction of renal mass by unilateral nephrectomy results in an immediate increase in glomerular flow to the remnant kidney, followed by compensatory glomerular hypertrophy by means of a complex mechanism. We previously reported that reactive oxygen species (ROS), via NAD(P)H oxidase activation, have a function as signal transducers of glomerular hypertrophy and cell proliferation in compensatory renal growth after uninephrectomy [24]. It has also been reported that IGF-1 stimulates ROS production in adipocytes [25] or vascular smooth muscle cells [26] via activation of NAD(P)H oxidase [27]. In this study, we demonstrated that IGF-1 stimulated ROS production through enhanced NAD(P)H oxidase activity in proximal tubular cells. This activity and subsequent ROS production were suppressed by the overexpression of klotho protein, suggesting that klotho modulates tubular hypertrophy by suppression of ROS production.

The mTOR pathway is the most important downstream signaling pathway of compensatory renal hypertrophy after nephrectomy. Rapamycin, an mTOR inhibitor, inhibited compensatory

tubular cell hypertrophy. Furthermore, compensatory renal hypertrophy was suppressed in rpS6 knock out mice that could not exhibit compensatory renal growth or tubular cell proliferation after nephrectomy [28]. This indicated that mTOR plays an essential role in mediating increased RNA and protein synthesis during compensatory renal hypertrophy. Here, we observed that IGF-1 activated the mTOR pathway and induced hPTEC hypertrophy via stimulation of NAD(P)H oxidase activity. Moreover, klotho protein was found to inhibit mTOR activity via suppression of the IGF-1 signaling pathway and modulation of protein synthesis in tubular cells.

Taken together, our data indicate that klotho modulates compensatory renal hypertrophy via suppression of the IGF-1 signaling pathway. As klotho was previously demonstrated to decrease in line with the progression of kidney injury, the resulting expression insufficiency could further aggravate the decline in renal function because of the absence of this compensatory mechanism.

Conflict of interest statement

None declared.

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