Renal Production, Uptake, and Handling of Circulating aKlotho

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ABSTRACT

aKlotho is a multifunctional protein highly expressed in the kidney. Soluble aKlotho is released through cleavage of the extracellular domain from membrane aKlotho by secretases to function as an endocrine/paracrine substance. The role of the kidney in circulating aKlotho production and handling is incompletely understood, however. Here, we found higher aKlotho concentration in suprarenal compared with infrarenal inferior vena cava in both rats and humans. In rats, serum aKlotho concentration dropped precipitously after bilateral nephrectomy or upon treatment with inhibitors of aKlotho extracellular domain shedding. Furthermore, the serum half-life of exogenous aKlotho in anephric rats was four- to five-fold longer than that in normal rats, and exogenously injected labeled recombinant aKlotho was detected in the kidney and in urine of rats. Both in vivo (micropuncture) and in vitro (proximal tubule cell line) studies showed that aKlotho traffics from the basal to the apical side of the proximal tubule via transcytosis. Thus, we conclude that the kidney has dual roles in aKlotho homeostasis, producing and releasing aKlotho into the circulation and clearing aKlotho from the blood into the urinary lumen.


aKlotho was originally identified as an anti-aging gene as its disruption confers a premature aging-like syndrome.1 Overexpression of aKlotho reverses defects resulting from aKlotho deficiency and extends lifespan in mice.1,2 aKlotho has extremely pleiotropic effects on multiple organs and is involved in many physiologic processes.3,4 Two other paralogs that share homology to aKlotho, are βKlotho,5 and Klotho/lactase-phlorizin hydrolase-related protein (Klph); also called γKlotho.6 Both α- and β-Klotho function as co-receptors of different fibroblast growth factor (FGF) isoforms and serve distinct biologic actions.4,7–9

aKlotho is a type-I single-pass transmembrane protein with a long extracellular region harboring β-glucosidase-like motifs called K1 domains.1,5,6,10 aKlotho expression is restricted to a few organs including the kidney,1 parathyroid glands,11 and sinoatrial node12 where it forms tetrameric complexes with FGF1c, 3c, or 4 (2Klotho:2FGFR), and serves as the high-affinity receptor for circulating FGF23 to regulate mineral metabolism.4,13–15 βKlotho is expressed in the liver and fat and forms complexes with FGF1c or 4 to support FGF15/19 and FGF21

Received October 23, 2014. Accepted March 14, 2015.
Published online ahead of print. Publication date available at www.jasn.org.

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signaling. βKlotho is involved in regulation of caloric homeostasis and bile secretion. γKlotho is expressed in the skin and kidney, complexes with FGF1b, 1c, 2c, or 4 in vitro but its physiologic function is not known.

Of the three Klotho paralogs, αKlotho is the only one known to date to function both as a membrane-bound receptor (co-receptor for FGF23) and a circulating endocrine substance. The extracellular portion of αKlotho is released from the cell by proteolytic cleavage and is present in the blood, urine, and cerebrospinal fluid. Theoretically, soluble αKlotho can form a complex with FGF and FGF receptor (FGFR) thus fulfilling its role as a co-receptor. However, as a soluble protein, it has much weaker action than transmembrane αKlotho thus seriously questioning whether soluble αKlotho can serve as a “deliverable co-receptor” for FGF23. It is more likely that soluble αKlotho circulates to exert its effects independent of FGF23 and FGFR.

In the kidney, soluble αKlotho acts from the urinary luminal side as an autocrine or paracrine enzyme to regulate transporters and ion channels. αKlotho modifies the Na⁺-phosphate cotransporter NaPi-2a as a glucuronidase and the renal outer medullary K⁺ and TRPV5 calcium channel as a sialidase. In addition, αKlotho functions as a glucuronidase on the basolateral membrane modifying organic cation transport. These enzymatic effects are clearly not due to αKlotho’s function as a co-receptor for FGF23 and are FGF23-independent. The pharmacologic effect of injected soluble αKlotho is fully intact in the FGF23–/– mouse and in cultured cells in the complete absence of FGF23. Further support of the endocrine action of circulating αKlotho is the fact that the systemic administration of recombinant Klotho protein or viral delivery of αKlotho can rescue a multitude of the phenotypic features of genetic αKlotho deficiency in different organ systems.

The kidney has the highest level of expression of αKlotho, but it is important to know whether αKlotho circulating in serum is derived from the kidney. Serum αKlotho levels in patients with CKD are extremely variable probably due to assay-related variance. In contrast, renal αKlotho levels are uniformly reduced in CKD and the strongest evidence to date comes from renal tubule-specific deletion of αKlotho, which caused reduced serum αKlotho levels and systemic features closely resembling the phenotype in global αKlotho deletion mice; indicating that the kidney may be the principal source of endocrine αKlotho.

Even less studied than its source, the route of elimination of circulating αKlotho is completely unknown. There is no information on how this 130 kD protein is cleared from the circulation. We previously demonstrated the presence of intravenously injected exogenous extracellular domain of recombinant αKlotho protein in animal urine but how αKlotho gets into the urine is not known.

The present study addresses several fundamental questions of αKlotho biology. Understanding the source and clearance of Klotho is critical because restoration of αKlotho in deficient states reverses the phenotype which has great therapeutic potential. We show that the kidney is the principal contributor to circulating αKlotho and simultaneously, is also the major organ where circulating αKlotho is taken up from the circulation. Thus kidney disease is expected to affect both production and clearance of αKlotho in a complex fashion. Finally, circulating αKlotho is transported into the urine through transepithelial transcytosis, which provides an avenue for circulating αKlotho to function as luminal enzyme to modulate target proteins. These fundamental concepts are important for understanding the pathophysiologic mechanisms of αKlotho deficiency in kidney disease and any contemplation in replacement therapy.

RESULTS

The Kidney as a Source of Circulating αKlotho

While αKlotho mRNA (RT-PCR, in situ hybridization, and RNA blot) is expressed in multiple tissues including heart, aorta, colon, pituitary gland, thyroid gland, pancreas, and gonads, the strongest expression by far is in the kidney. αKlotho protein expression is detected in renal tubules, choroid plexus, islet cells in pancreas, and the parathyroid gland. However, the high expression in the kidney does not necessarily indicate that circulating αKlotho is of renal origin. To test whether the kidney is a major source of endocrine αKlotho in mammals, we measured serum αKlotho protein in supraprenal and infrarenal vena cava of normal rats by direct puncture and human subjects who underwent right heart catheterization. All patients had eGFR ≥60 ml/min/1.73 m². The medical conditions of the patients are shown in Supplementary Table 1. Similar infrarenal-to-supraprenal increment in caval αKlotho level was observed in both rat and human serum samples (Figure 1, A and B). We plotted serum αKlotho against serum erythropoietin, a well-known renal-derived hormone, and found that as serum erythropoietin rose (Figure 1C), and serum creatinine (S_{Cr}) decreased from infrarenal-to-supraprenal inferior vena cava (Supplementary Figure 1), αKlotho increased indicating that the kidney secretes αKlotho into the circulation.

We next performed the classic organ ablation experiment to test the nephrogenic origin of αKlotho. When we removed both kidneys, serum αKlotho level dropped significantly to about half the normal level in one day. The fall in serum αKlotho parallels the rise in S_{Cr} (Figure 1D) and BUN (Supplemental Figure 2). The anephric state did not permit studies to continue for longer than 40–50 hours.

Type-I transmembrane proteins can be cleaved and released by A disintegrin and metalloproteases (ADAM) which is a metalloprotease that functions as “sheddase” for membrane-anchored growth factors, cytokines, and receptors. ADAM plays an important role in modulation of cell signaling in physiology and pathophysiology. Studies on cultured...
cell and kidney slices indicated that α-secretase (ADAM 10/17) and β-secretase modulate αKlotho ectodomain shedding and becoming soluble αKlotho protein,18,19 by acting on two cleavage sites: one close to the juxtamembrane region (aa 950 approximately 981) and another between the KL1 and KL2 domains.53 To examine if secretases modulate circulating αKlotho in vivo, we injected α-secretase inhibitor doxycycline hyclate and/or β-secretase inhibitor III into the intraperitoneal cavity of normal mice for 2 days and determined serum αKlotho in 48 hours. Serum αKlotho levels were decreased with either one or both α- and β-secretase inhibitors (Figure 1E) supporting the notion that αKlotho release depended on secretases.

The Kidney as a Portal of Clearance of Circulating αKlotho from Blood
It is not known how αKlotho is cleared from the circulation. To examine this, we injected fluorescent exogenous recombinant αKlotho protein into the intraperitoneal cavity of normal and anephric rats. The levels of circulating exogenous αKlotho...
protein in anephric rats were similar to those in normal rats immediately after injection (Figure 2A), but the half-life of exogenous αKlotho protein in normal rats was much shorter than that in anephric rats (Figure 2B) and the half-life of endogenous αKlotho upon nephrectomy closely approximates that of exogenous αKlotho in the anephric rats (Figure 2B, gray and black closed circles).

**Distribution of Exogenous αKlotho Protein in Rodent Organs**
The lengthening of the half-life of exogenous αKlotho with nephrectomy implies that the kidney may be clearing αKlotho. Alternatively, the anephric state can alter αKlotho clearance by other organs. To distinguish these possibilities, we examined the anatomic fate of exogenous labeled αKlotho protein in the kidney. After an intravenous injection of 125I-labeled αKlotho protein (125I-αKlotho) into normal rats, we found that the kidney is a major organ for exogenous αKlotho protein uptake, with much lower signals in other organs (Figure 3A). The signal of the 125I-albumin control is unquestionably different from that of 125I-labeled αKlotho (Figure 3A), indicating that the signal of αKlotho in the kidney is not just due to trapping of αKlotho in renal vascular beds. Quantitative scintillation counting in tissue homogenates showed high signal of 125I-labeled αKlotho in the kidney and in urine compared with that in the blood (Figure 3B), supporting that the kidney may be a major organ of αKlotho uptake as well as its excretion.

We found a high signal in the renal medulla and papilla immediately after injection of 125I-αKlotho, which diminished by 30 min and disappeared by 120 min (Figure 3C). This signal likely came from free 125I released from 125I-αKlotho which we detected (data not shown) and is largely unavoidable. To independently confirm the high signal in the kidney, we covalently labeled recombinant αKlotho with infrared dye and examined αKlotho distribution in several organs in normal rats. Similar to the radioactive labeling, exogenous infrared labeled αKlotho protein was prominently distributed in the kidney and spleen, sparsely in the heart (Figure 3D), and not detectable in aorta, brain, and muscle (data not shown).

To characterize the clearance profile of αKlotho from the circulation into urine, we intravenously injected 125I-αKlotho into normal rats and simultaneously measured the radioactivity in the urine and serum over 2 hours. The serum αKlotho signal quickly decreased by half over approximately 4 min (due to redistribution) followed by a slower steady decline (Supplemental Figure 3A). The 125I-αKlotho clearance pattern was clearly different from that of 125I-albumin, which was mainly retained in the circulation (Supplemental Figure 3B). As serum 125I-αKlotho radioactivity decreased, urinary radioactivity rose simultaneously (Supplemental Figure 3C). In comparison, radioactivity of albumin in the urine remained low (Supplemental Figure 3D). These findings support the notion that αKlotho protein is cleared from blood through the kidney into the urine.

**Distribution of Exogenous αKlotho Protein in the Kidney**
As discussed previously, upon injection of 125I-αKlotho to rats, autoradiography showed that the signal in the renal cortex appeared first and gradually increased followed by a signal in the inner medulla later. The signal in the cortical rim persisted up to 120 minutes (Figure 3C). The infrared (IR) labeled αKlotho imaging clearly demonstrates that exogenous αKlotho was not in glomeruli but exclusively present in renal tubules in the cortex and the outer strip of the medulla (Figure 4A).

To further localize the segment of renal tubules in the cortex where exogenous αKlotho is taken up, we intravenously injected doubly-labeled fluorescent αKlotho into normal mice.
There was no detectable signal of labeled IgG in the kidney (negative control, data not shown), whereas a clear aKlotho signal is present in the cortex starting 2 minutes after injection and a stronger signal was observed in 30 minutes. Labeled-aKlotho was present in both proximal and distal tubules (Figure 4B) but not in the glomeruli (not shown).

To delineate the locale of exogenous aKlotho further, we injected recombinant extracellular domain of mouse aKlotho into homozygous aKlotho hypomorphic (kl/kl) mice.1 As expected, immunoelectron microscopy (EM) did not detect endogenous aKlotho protein in the kidney of kl/kl mice (data not shown). Exogenous aKlotho was clearly found inside the glomerular capillary, the glomerular basement membrane (GBM), but not in Bowman’s space despite extensive search over many images from many glomeruli (Figure 4C). However, exogenous aKlotho was present in the lumen, apical and basolateral membranes, and cytoplasm in both distal and proximal tubules, and in peri-tubular capillary lumen (Figure 4, D–F, Supplemental Figure 4).

High radioactive counts were present both in the serum and urine (Supplemental Figure 3, A and C). To exclude the possibility that radioactivity in urine and serum was merely free125I released from 125I-aKlotho, we resolved the urine proteins by SDS-PAGE followed by autoradiography. There was indeed some free125I that ran off the gel (data not shown) but full-length aKlotho was clearly detectable in urine (Figure 5A). Small amounts of albumin were also detected in urine but very limited (Figure 5B).

The fact that aKlotho is not filtered may be intuitively expected for a 130 kDa protein. Since aKlotho functions as an enzyme,13,21,54 it can theoretically modify the glycans and hence reduce the negative charge barrier of the GBM with resultant leak of aKlotho into urine. To exclude that, we treated fresh normal kidney slices with aKlotho, glucuronidase, or sialidase, and examined their effects on negative charge. aKlotho did not affect glomerular negative charge rendering glomerular leak unlikely (Supplemental Figure 5).

To further explore how aKlotho gets into the urine, we measured radioactivity in the proximal lumen urine using free-flow micropuncture.21 Radioactivity was highest in the urine but undetectable in Bowman’s space (Figure 5C), which is consistent with immunoelectron micrographs (Figure 4C) showing no visible aKlotho antigen in Bowman’s space. 125I-aKlotho was detected in high quantity in the lumen which is remarkably different from that of the control protein125I-albumin (Figures 3B and 5C).

In light of the fact that injected IR-labeled full length aKlotho is present in urine,42 we conclude that exogenous aKlotho protein is cleared from blood by the kidney and excreted into urine at the level of the renal tubule. We do not exclude other possibilities such as hepatic and splenic clearance as exogenous aKlotho was also found in the liver and spleen (Figure 3A).

**Transcytosis of aKlotho by Renal Proximal Tubules**

The absence and presence of aKlotho in Bowman’s space and proximal lumen, respectively, suggests that aKlotho traffics across renal tubules from basolateral membrane to luminal membrane. We next used OK cells, an opossum kidney cell line with renal proximal tubular features55 as an *in vitro* model...
to examine αKlotho transcytosis. αKlotho was taken up by OK cells from both apical and basal media, but only αKlotho internalized from the basal side was secreted into apical media (Figure 6A); internalized αKlotho from apical media was not released into basal media (Figure 6B) suggesting unidirectional trafficking. There was no transcytosis of albumin or IgG across OK cells either from basal to apical or from apical to basal side (data not shown). Taken together with the dot blot data shown in Figure 6C, one can say that αKlotho is taken up by OK cells from the basal side, then crosses the cell to the apical side which occurs as early as 30 minutes and plateaus by 8 hours.

**DISCUSSION**

The main findings in this study are: (1) the kidney produces and releases soluble αKlotho into the systemic circulation by secretases-mediated shedding of the ectodomain of αKlotho, (2) the kidney is an important organ to clear soluble αKlotho from the circulation, (3) αKlotho traffics across renal tubules from basolateral to intracellular location and is then secreted across the apical membrane into the urinary lumen. αKlotho is found in urine and serum of rodents and humans, and the kidney has the highest abundance of αKlotho compared with other organs. Clinical and experimental animal studies showed low renal tubular αKlotho expression in both acute kidney injury and CKD and low circulating αKlotho levels in kidney diseases of a variety of etiologies in animals and humans. These data suggest that the kidney contributes to circulating αKlotho. Now we provide direct experimental evidence that the kidney is the source of circulating αKlotho by demonstrating step-up of αKlotho concentration from infrarenal to suprarenal vena cava in both mice and humans. Further evidence for a renal source of circulating αKlotho is that serum αKlotho falls rapidly
upon bilateral nephrectomy although one cannot rule out the possibility that the acute anephric state has suppressive effect on extrarenal source of αKlotho. We cannot extend our bilateral nephrectomy experiment beyond 2–3 days to test whether circulating αKlotho levels disappear entirely. Both proximal convoluted tubules and distal convoluted tubules express αKlotho protein and transcripts, but we do not know whether both segments release αKlotho into the circulation. It is unclear whether other organs contribute to circulating soluble αKlotho in physiologic or pathologic states. Lindberg and colleagues generated a conditional partial αKlotho deletion in renal tubules with a similar phenotype as global αKlotho deletion. In addition, serum αKlotho level was reduced by approximately 80% compared with wild-type and αKlotho-/- mice with chronic higher levels of αKlotho. Free-flow micropuncture from Bowman’s space showed low radioactivity after injection with 125I-αKlotho (Figure 5C). Thus, the exogenous αKlotho that ends up in the urine is tubular in origin probably through transcytosis across proximal renal tubules. This was further tested by adding αKlotho to the basolateral media of OK cells and confirmed by the presence of αKlotho in the apical media (Figure 6). In contrast, there is no traffic of αKlotho from apical media to basolateral media. Transcytosis may serve as a way to clear circulating αKlotho, or as a mean to channel αKlotho to the lumen to modulate transporters or channels on the apical membrane of renal tubules. Two points are noteworthy. Firstly, both proximal and distal tubules take up circulating αKlotho (Figure 4B) but we cannot discern at present the relative contribution of each of these segments to trans-tubular αKlotho transport. Secondly, αKlotho in the urine can be hematogenously derived via transcytosis but it can also originate from the renal tubules as well. Currently, we do not know the relative contribution from circulation versus renal tubules; and from proximal tubules versus distal tubules to urinary αKlotho.

In conclusion, the kidney is the principal organ in the maintenance of αKlotho homeostasis by contributing to circulating αKlotho. The clearance of circulating αKlotho is also partially mediated by the kidney. Hematogenously derived αKlotho is present in renal tubular lumen which is a key site of physiologic action of αKlotho. Therefore, the kidney assumes several functions in αKlotho homeostasis: contribution to systemic endocrine αKlotho, clearance of circulating αKlotho, and delivery of αKlotho to the tubular lumen. With the renal source of circulating αKlotho established, the next step would be to delineate why and how αKlotho is drastically reduced with both acute and CKD which is of immense clinical significance. In addition, establishing extrarenal sources of αKlotho and testing whether there is upregulation of αKlotho production from extrarenal sources when renal production fails will also be of paramount importance.
Klotho protein with C-terminal 6His tag was added to the basal side, and subjected to immunoblot for quantitation of Klotho protein with anti-His antibody (upper panel). Klotho at each time point in each compartment. Data are expressed as means ± SD from three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student–Newman–Keuls test, and significant differences were accepted when *P < 0.05 versus 0 min. (B) αKlotho with C-terminal 6His tag was added to the apical medium. At given time points after incubation, media were collected from apical and basal side and subjected to immunoblot for quantitation of αKlotho protein with anti-His antibody (left panel). Right panel is a summary of total amount of αKlotho at each time point in each compartment. Data are expressed as means ± SD from three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student–Newman–Keuls test. None of the changes were statistically significant. (C). Time course of transcytosis. Media were collected from the apical side when αKlotho with C-terminal 6His tag was added to the basal side, and subjected to dot blot αKlotho protein with anti-His antibody (upper panel). Bottom panel shows arbitrary densitometric units of αKlotho protein. Three independent experiments showed identical results.

Figure 6. αKlotho transcytosis from basal to apical side in OK cells. OK cells were seeded on Transwell plates and grown to confluence to separate apical and basolateral side. (A) αKlotho with C-terminal 6His tag was added to the basal medium. At given time points after incubation, media were collected from both apical and basal side, and subjected to immunoblot to quantify exogenous αKlotho protein with anti-His antibody (left panel). Right panel is a summary of total amount of αKlotho at each time point in each compartment. Data are expressed as means ± SD from three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student–Newman–Keuls test, and significant differences were accepted when *P < 0.05 versus 0 min. (B) αKlotho with C-terminal 6His tag was added to the apical medium. At given time points after incubation, media were collected from apical and basal side and subjected to immunoblot for quantitation of αKlotho protein with anti-His antibody (left panel). Right panel is a summary of total amount of αKlotho at each time point in each compartment. Data are expressed as means ± SD from three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student–Newman–Keuls test. None of the changes were statistically significant. (C). Time course of transcytosis. Media were collected from the apical side when αKlotho with C-terminal 6His tag was added to the basal side, and subjected to dot blot αKlotho protein with anti-His antibody (upper panel). Bottom panel shows arbitrary densitometric units of αKlotho protein. Three independent experiments showed identical results.

CONCISE METHODS

Human Studies
Nine human subjects (49.0 ± 6.2 years) who underwent right heart catheterization were enrolled for this study (Supplementary Table 1), which was approved by the Institutional Review Board at the University of Texas Southwestern Medical Center, and all subjects gave informed consent. During right heart catheterization, suprarenal and infrarenal vena caval blood samples were obtained and sera were immediately separated after centrifugation at 4°C and stored at −80°C for future study.

Serum αKlotho was determined by immunoprecipitation-immunoblot assay described previously.21,42,63 Briefly, 0.1 ml serum was immunoprecipitated with a synthetic anti-αKlotho Fab (sb106)63 and immune complex was eluted with Laemmli sample buffer, and subject to immunoblot with KM2076 antibody.21,42,63 The specific signals on the autoradiograms based on 130 kD mobility were quantified with ImageJ Program (National Institutes of Health [NIH], Bethesda, Maryland).

Animal Studies
αKlotho hypomorphic (kl/kl) mice were described previously.1 kl/kl mice and their wild-type (WT) littermates were maintained at the Animal Research Center of the University of Texas Southwestern Medical Center. Currently all mice are 129S1/SV (129 SV) background age from 6 to 8 weeks. Normal Sprague-Dawley (SD) rats (220–250 g body weight) were purchased from Charles River Laboratories (Wilmington, MA). For αKlotho clearance study, rats underwent bilateral nephrectomy (aneurplenic rats) or laparotomy with manual manipulation of the kidneys (sham rats). Rats or mice were intravenously or intraperitoneally injected once with labeled full extracellular domain of recombinant mouse αKlotho protein (rMKl) (R&D Systems, Minneapolis, MN) at a dose of 0.1 mg/kg body weight.

To examine if secretases modulate blood αKlotho, doxycycline hyclate (Sigma-Aldrich, St. Louis, MO), an α-secretase inhibitor at 25 mg/kg/day, and/or β-secretase inhibitor III (Calbiochem, Billerica, MA) at 2.5 mg/kg/day were intraperitoneally injected into normal WT mice daily for 2 days, blood and kidneys were harvested at 48 hours to determine serum and renal αKlotho. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center, Dallas, TX.

Antibodies and Other Key Reagents
Rat monoclonal anti-human Klotho antibody, KM20761,2 was used for immunoblotting and immunoelectron microscopy; and the synthetic αKlotho antibody sb10663 was used for immunoprecipitation of serum Klotho. Anti-His antibody was purchased from Invitrogen (Carlsbad, CA), NaCl cotransporter (NCC) from Chemicon, (EMD Millipore, Billerica, MA), Lotus-Tetragonolobus lectins (LTA) from Vector laboratories (Burlingame, CA), and other chemicals were obtained from Sigma-Aldrich, except otherwise noted. Culture media were purchased from Invitrogen; penicillin and streptomycin from Cambrex (East Rutherford, NJ); infrared dye 800 CW from LI-COR Biosciences (Lincoln, NE), carboxy-tetramethylrhodamine succinimidyl ester from G-Biosciences (St. Louis, MO);
Transwell plates from Corning Inc. (Corning, NY); enhanced chemiluminescence detection kit from GE Healthcare (Piscataway, NJ); nitrocellulose and polyvinylidene difluoride (PVDF) membranes from EMD Millipore; soluble murine Klotho protein containing the extracellular domain (amino acid number 35–982) from R&D Systems; 125I material from GE Healthcare.

**αKlotho Labeling**

Different methods were used to label C-terminal 6-His tagged αKlotho protein (R&D Systems, Minneapolis, MN) or albumin (Sigma-Aldrich, St. Louis, MO) with 125I, infrared (IR)-dye or fluorescein. Iodine labeling of purified αKlotho protein was performed using IODO-GEN (Pierce, Rockford, IL) and 125I according to the labeled with two types of convoluted tubules was performed using our published methods.21 Iodine labeling of purified αKlotho protein was performed using IODO-GEN (Pierce, Rockford, IL) and 125I according to the protocols provided by the manufacturers. Specific activity of 125I-I-αKlotho was determined by trichloroacetic acid precipitation. For IR dye labeling, recombinant mouse αKlotho protein was reacted with the IR dye 800 CW according to the kit’s instructions (LI-COR Biosciences). Labeled protein was confirmed by observation of the correct size band of labeled αKlotho on 7.5% SDS-PAGE with Odyssey Infrared Imaging System (LI-COR Biosciences). αKlotho was labeled with two types of fluorescent dye: Alexa 555 C2 Maleimide (λex 555 nm, λem 565 nm) (Invitrogen) and TAMRA-SE (λex 546 nm, λem 579 nm) using Hook-Dye labeling kit (G Biosciences) at sulfhydryl, arginine, and lysine residues to enhance fluorescent signal. For in vitro cell culture experiments, 1 μg/ml of labeled αKlotho was added in apical or basal media of insert of 6-well Transwell plate (Corning Inc.). For up–take study, fluorescein-labeled αKlotho protein with C-terminal 6His tag was added into either apical or basal compartment and incubated for 4 hours. Apical or basal media was then collected for immunoblot for αKlotho with anti-His antibody. For determination of radioactivity, 10 μl of media from different compartments and cell lystate was subject to scintillation counting and also subjected to SDS-PAGE followed by autoradiography.

**Immunoblot**

Immunoblotting was performed as described.21,42 Briefly, total lysates were extracted from kidney tissues and OK cells in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) containing fresh phosphatase inhibitors and protease inhibitors. Protein content in samples was determined by the method of Bradford.21,42 Thirty μg of protein were fractionated by SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in nonfat milk, membranes were probed with anti-Klotho monoclonal antibody (KM2076)42 (overnight 4°C) and followed by secondary antibodies conjugated with horseradish peroxidase (GE Healthcare). Antibody against β-actin (Sigma-Aldrich) was used for loading control. Signal was visualized by enhanced chemiluminescence (GE Healthcare), and quantified densitometrically by the Scion/NIH Image software (Scion, Frederick, MD).

For assay of serum αKlotho, 100 μl of serum from rodents was subjected to immunoprecipitated enrichment by sb106 αKlotho64 and was immunoblotted by rat anti-human αKlotho (KM2076).

**Immunoelectron Microscopy**

Mouse recombinant Klotho protein (0.1 mg/kg BW) was intraperitoneally injected once into kl/kl mice and mice were sacrificed 24 hours after injection. Kidneys were harvested and fixed with 2.5% paraformaldehyde via aortic perfusion, removed, and post-fixed in 4% paraformaldehyde (4°C for 4 hours). Immunogold labeling of ultrathin frozen tissue sections was performed as described.21 Kidney cortex was infiltrated with 2.3 M sucrose overnight, frozen in liquid nitrogen, and 70–80-nm-thick sections were made (Ultramicrotome Reichert Ultracut E; Leica Microsystems, Wetzlar, Germany) and mounted on Formvar-coated nickel grids. The sections were incubated scanned with Odyssey Infrared Imaging System (LI-COR Biosciences). Kidneys were snap-frozen in liquid N2 and stored in −80°C. Four μm cryosections were made and scanned directly without fixation using Odyssey Infrared Imaging System or StormTM 860 imager (GE Healthcare). For the experiment of injection of fluorescent labeled αKlotho, kidney sections were imaged by immunofluorescent microscopy, and immunohistologically stained with αKlotho, NaCl cotransporter (NCC), and Lotus tetragonolobus lectins (LTA).

**Opossum Kidney (OK) Cells**

OK cells were cultured and maintained in high-glucose (450 mg/dl) Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 g/ml) as described.21 For transcytosis study, OK cells were seeded on Transwell, and grown to complete confluence for 2 days (typical transepithelial resistance approximately 1–5 kΩ).22 For uptake study, fluorescein-labeled αKlotho protein with C-terminal 6His tag was added into either apical or basal compartment and incubated for 4 hours. Apical or basal media was then collected for immunoblot for αKlotho with anti-His antibody. For determination of radioactivity, 10 μl of media from different compartments and cell lystate was subject to scintillation counting and also subjected to SDS-PAGE followed by autoradiography.

**Distribution and Location of Labeled αKlotho in the Kidney**

For the experiment of injection of IR-labeled αKlotho, normal SD rats were used. Four μl of serum or fresh spot urine were solubilized in Laemmli sample buffer, fractionated by 7.5% SDS-PAGE, and
with KM2076 antibody and followed by incubation with gold-conjugated protein A (10-nm gold particles, Sigma-Aldrich) for 60 minutes. After staining with uranyl acetate, sections were visualized with Jeol 1200 EX transmission electron microscope (Jeol Ltd., Akishima, Japan).

Statistical Analyses
Data are expressed as the means±SD. Statistical analysis was performed using unpaired t test, or paired t test, or ANOVA followed by Student–Newman–Keuls test whenever appropriate. A P-value of≤0.05 was considered statistically significant. Unless otherwise stated, representative figures reflect the results in a minimum of three independent experiments.

ACKNOWLEDGMENTS

This work was in part supported by the National Institutes of Health (R01-DK092461, R01-091392, and R01-DK13686), O’Brien Kidney Research Center at the University of Texas Southwestern Medical Center (P30-DK07938), American Heart Foundation Western Affiliate Beginning-Grant-in-Aid (0865235F), the Simmons Family Foundation, the Charles and Jane Pak Foundation, the Pak Center Innovative Research Support, and the Canadian Institutes of Health Research (MOP-93725).

The authors would like to thank Dr. Philipp Scherer for assistance with infrared dye labeling and for providing infrared scan system, and Drs. Michel Baum, Jyothsna Gattineni and Xiao Yan for helpful discussions during the preparation of the manuscript.

Authors are grateful to Ms. Jean Paek for technical support, and to Ms. Carolyn Griffith for assistance in enrollment of research subjects.

A.B. was in part supported by Visiting Scholar Award from National Natural Science Foundation of China (81170660H0509, 81270408H0220), and Provincial Natural Science Foundation of Jiangsu, China (BK2011849).

DISCLOSURES
None.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014101030/-/DCSupplemental.