Involvement of α-klotho in growth hormone (GH) signaling

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Growth hormone (GH) exerts multiple effects on different organs directly or via its main mediator, insulin-like growth factor1 (IGF1). In this study, we focused on the novel relationship between GH action and the antiaging hormone α-klotho. Immunofluorescent staining of α-klotho was observed in the renal distal tubules and pituitary glands of somatostatin- and GH-positive cells in wild-type (WT) mice. Treatment of 4-week-old WT mice with GH increased IGF1 mRNA expression in the pituitary gland, liver, heart, kidney, and bone but increased α-klotho mRNA expression only in the pituitary gland, kidney, and bone. Increased α-klotho protein levels were observed in the kidney but not in the pituitary gland. No induction of α-klotho RNA expression by GH was observed in juvenile mice with kidney disease, indicating GH resistance. Furthermore, GH and α-klotho supplementation in HEK293 cells transfected with GHR increased Janus kinase 2 mRNA (a GH downstream signal) expression compared to supplementation with GH alone. In conclusion, we suggest that 1) the kidney is the main source of secreted α-klotho, which is detected in blood by the downstream action of GH, 2) α-klotho induction by GH is resistant in kidney disease, and 3) α-klotho might be an enhanced regulator of GH signaling.

Key Words: growth hormone, α-klotho, kidney, CKD, GH-resistant

α-Klotho, identified in α-klotho mutant mice (kl/kl mice) exhibiting symptoms of early senescence, is highly expressed in the kidneys and is known to be a factor in the homeostasis of calcium (Ca) and inorganic phosphate (Pi).[1,2] It is also expressed in various extrarenal tissues, including the choroid plexus, parathyroid gland, and pituitary gland.[1] The α-klotho protein exists in membrane and secreted forms. α-klotho proteins are single-pass transmembrane proteins with a molecular weight of 130 kDa that are enzymatically cleaved at two sites in the extracellular domain by a disintegrin and metalloproteinase (ADAM)10 and ADAM17. This cleavage releases the entire extracellular domain or two internal domains, KL1 and KL2, which circulate throughout the body in a secreted form.[3,4] RNA splicing also results in the synthesis of a secreted klotho protein containing the KL1 subunit.[5] Membrane-type α-klotho, a coreceptor for fibroblast growth factor (FGF)23 in the kidney, suppresses the functional expression of the Pi transporter and inhibits active vitamin D synthesis.[2,6,7] Conversely, secreted α-klotho is found in blood, urine, and cerebral fluid, acting as a circulating hormone with systemic effects, regulating ion channels, transporters, insulin/insulin-growth factor1 (IGF1) signaling, and exhibiting antioxidant effects.[2] The kl/kl or α-klotho knockout (KO) mice exhibit significant hyperphos-

phatemia and elevated blood vitamin D levels.[8,9] Although both types of mice show normal development until approximately 3 weeks of age, they subsequently display growth retardation and severe aging phenotypes, leading to an average lifespan of approximately 60 days.[1] The kl/kl mice have shown reduced vesicle numbers in growth hormone (GH)-producing cells, resulting in GH resistance and a lack of weight gain even with GH administration.[1,10]

GH, a peptide secreted by the anterior pituitary gland, is regulated by the hypothalamic hormones [growth hormone-releasing factor and growth hormone-inhibiting factor (somatostatin)]. GH secreted from the pituitary gland binds to GH binding protein and is transported to target organs where it exerts various metabolic functions (including the proper development and growth of bone, skeletal muscle, and adipose tissue) either directly or indirectly via IGF1, which is mainly produced in the liver.[11,12] Intracellular signal transduction after GH binding to its receptor requires the activation of Janus kinase 2 (JAK2), which stimulates the phosphorylation of signal transducer and activator of transcription (STAT) proteins known as MAPK/ERK and PI3K/AKT.[11,12]

GH deficiency (GHD) is a common complication associated with several diseases of the pituitary gland and hypothalamus.[13] It has been reported that children with GHD show decreased blood levels of the secreted form of α-klotho and that their blood levels increase with GH treatment.[14] Furthermore, this secretion into the blood was reported to be mediated by IGF1.[14] Patients with GH-producing tumors (which lead to various systemic abnormalities due to GH overproduction) show a significant increase in circulating secreted α-klotho levels.[15–18] However, following the removal of GH-producing tumors, the rapid decrease in α-klotho suggests that the cause of the elevation in secreted α-klotho levels are unclear. Moreover, α-klotho plays a positive regulatory role in GH secretion.[19] Thus, a close association between α-klotho and the GH/IGF1 axis has been reported; however, not everything about this association is clear.

In addition, chronic kidney disease (CKD) and its complications in children are associated with changes in the GH/IGF1 axis, including impaired renal postreceptor GH signaling and growth retardation associated with a GH-resistant state due to chronic inflammation.[4,12,20,21] The kidney is the organ with the highest klotho expression, and renal α-klotho expression and blood α-klotho levels decrease with the progression of CKD.[22] Thus, we speculate that the decrease in α-klotho may be involved in GH resistance in patients with CKD. In this study, we focused on the relationship between GH action and α-klotho to clarify

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doi: 10.3164/jcbn.23-127
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J. Clin. Biochem. Nutr. | **May 2024** | vol. 74 | no. 3 | 221–229

that GH resistance during CKD may be associated with α-klotho deficiency.

Materials and Methods

Experimental animals. Mice were maintained under pathogen-free conditions and handled per the Guidelines for Animal Experimentation of Tokushima University School of Medicine. Male C57B6/J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were weaned at 3 weeks of age and given free access to water and standard mouse chow containing 0.8% phosphorus (Oriental MF; ORIENTAL YEAST CO., LTD, Osaka, Japan). Klotho KO mice were purchased from Clare Japan Corporation (Tokyo, Japan) and Jackson Laboratories (Bar Harbor, ME), respectively, and heterozygous pairs were crossed to obtain the corresponding knockout mice.[9]

Recombinant human GH (Wako, Osaka, Japan) was dissolved in saline. Mice were injected subcutaneously with recombinant human GH (4 mg/kg) at 0:00 and 8:00. Several organs (heart, liver, kidney, pituitary gland, and bone) were removed. Control mice were administered an equivalent volume of a vehicle.

Adenine-induced nephritis is produced in mice by oral gavage of adenine dissolved in a vehicle (carboxymethyl cellulose).[23,24] Three-week-old male mice were administered adenine (50–100 mg/kg body weight) thrice weekly for 2 weeks. Control mice were orally administered an equivalent volume of the vehicle.

This article does not contain any human studies performed by any authors. The mice were handled per the Guidelines for Animal Experimentation of Tokushima University School of Medicine (T2019-126, 2023-3).

Biochemical measurements. Plasma blood urea nitrogen (BUN) and plasma Pi were determined using commercial kits (Wako).

RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction. Total RNA was extracted from several tissues of mice and cells using ISOGEN (Wako) per the manufacturer's instructions. After treatment with DNase (Invitrogen, Carlsbad, CA), cDNA was synthesized with or without the Moloney murine leukemia virus, reverse transcriptase (Invitrogen), and oligo(dT)12–18 primers. Quantitative polymerase chain reaction (PCR) was performed using StepOnePlus (Applied Biosystems, Foster City, CA). The reaction mixture with specific primers was 10 µl of SYBR Premix Ex Taq, ROX Reference Dye II (Perfect Real Time, Takara, Osaka, Japan). The PCR primer sequences are shown in Table 1.

Protein sample purification and immunoblotting. The whole homogenate was obtained from mouse pituitary glands and kidneys and used for immunoblotting analyses as previously described.[9,25] Protein samples were added to the sample buffer in the presence of 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred by electrophoresis to Immobilon-P polyvinylidene difluoride (Millipore, Billerica, MA) and treated with diluted antibodies. Signals were detected using Immobilon Western (Millipore).

Immunofluorescence staining. Mouse kidneys and pituitary glands were fixed with 4% paraformaldehyde solution (pH 7.2) overnight at 4°C, washed with PBS, cryoprotected with 10% and 20% sucrose at 4°C, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), and frozen in hexane at −80°C. Frozen sections (5 µm thick) were collected on MAS-coated slides (Matsunami Glass IND, Ltd., Osaka, Japan) and air-dried. For immunofluorescence microscopy, serial frozen cells were incubated with primary antibodies overnight at 4°C. Alexa Fluor 488 anti-rat (Molecular Probes, Eugene, OR) antibodies and Alexa Fluor 568 anti-mouse and rabbit (Molecular Probes) antibodies were used as secondary antibodies for 60 min at room temperature. The sections were then mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, UK). Images were taken using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Antibodies. Rat anti-α-klotho (Transgenic Inc., Fukuoka, Japan), rabbit anti-prolactin (Abcam Inc., Cambridge, MA), rabbit anti-GH (FabGennix International, inc., Frisco, TX), rabbit anti-somatostatin (Bioss Antibodies, Woburn, MA), mouse anti-actin (Millipore), and mouse anti-villin (Millipore) antibodies were purchased. Rabbit anti-NaPi2a antibodies were generated as previously described. Actin was used as an internal control for immunoblotting. NaPi2a and villin were used as markers of the renal proximal tubule. Prolactin, somatostatin, and GH were used as markers of the secretory cell. Horseradish peroxidase-conjugated anti-rat or anti-mouse IgG was used as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The diluted antibodies for immunoblotting were as follows: anti-α-klotho (1:2,000) and anti-actin (1:10,000). The diluted antibodies used for immunofluorescence staining were as follows: anti-NaPi2a (1:500), anti-α-klotho (1:50), anti-prolactin (1:50), goat anti-GH (1:50), goat anti-somatostatin (1:50), and anti-villin (Millipore; 1:500).

Detection of serum α-klotho. Mouse serum (125 µl) was mixed with 12.5 µl of 10% SDS, boiled at 100°C for 4 min, and cooled to room temperature. The sample was mixed with 0.4 ml

Table 1. primer for quantitative PCR

Primer name	Sense	Antisense
Mouse α-klotho	CAATGGCTTTCCTCCTTTAC	TGCACATCCCACAGATAGAC
GAPDH	CATCCACAGTCTTCTGGGTG	CTGCACCACCAACTGCTTAGC
Mouse IGF-1	ACCTCAGACAGGCATTGAGG	GTTTGTCGATAGGGACGGGG
Mouse ADAM10	TTTGGATCCCCACATGATTCTG	GGTTGGCCAGATTCAACAAAA
Mouse ADAM17	GGGTTTTGCGACATGAATGG	GAAAACCAGAACAGACCCAAC
Mouse GHR	TCTGGAAAGCCTCGATTCAC	TCAGGGCATTCTTTCCATTC
JAK2	TTGTGGTATTACGCCTGTGTATC	ATGCCTGGTTGACTCGTCTAT
Human α-klotho	GGCCCAGAAACTCTGGAAAGA	AAGCTATGAAAGCCAGTAAAGACT
Human IGF-1	GACAGGGGCTTTTATTTCAAC	CTCCAGCCTCCTTAGATCAC
Human STAT5A	GCAGAGTCCGTGACAGAGG	CCACAGGTAGGGACAGAGTCT
Human STAT5B	CCGCTGCATCCGCCATATATTG	ATTCTCTGTGTCCTGCGTGAC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF1, Insulin-like Growth Factor I; ADAM, a disintegrin and metalloproteinase; GHR, growth hormone receptor; JAK2, janus kinase 2; STAT5, signal transducer and activator of transcription 5.

of the TNE lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, pH 7.5) and centrifuged at 17,700 × g for 15 min at 4°C. The supernatant was collected and rotated at 4°C overnight with 1 μg anti-α-klotho antibody (R&D system, Minneapolis, MN). The next day, a 15-μl aliquot of protein A agarose beads (Santa Cruz Biotechnology, Inc., Dallas, TX) was added to the sample and rotated at 4°C for 1 h. Protein A agarose beads were centrifuged at 3,000 × g for 1 min at 4°C and washed four times with the TNE lysis buffer. The agarose beads were removed from the supernatant, eluted in the SDS sample buffer, and heated at 95°C for 5 min. The sample was analyzed by immunoblotting as described above.

Cell culture and plasmid transfection. HEK293 cells were purchased from the American Type Culture Collection (Rockville, MD). HEK293 cells were maintained in an appropriate medium, DMEM/F-12 HAM (D8062; Sigma-Aldrich, St. Louis, MO), containing 10% fetal bovine serum (Gibco, Carlsbad, CA) and penicillin-streptomycin (Gibco).[26] For the analysis of GH signaling with soluble klotho, HEK293 cells were seeded in a six-well plate (Nunc, Roskilde, Denmark) with a poly-L-lysine coating (R&D system) at 6.5 × 10^5 cells/well. Overall, 24 h after plating, HEK293 cells were transfected with pCMV-Tag2A (Takara Bio) or N-terminus FLAG fused mouse GH receptor (mGHR/pCMV-Tag2A) by using Polyethyleneimine "Max" (Mw40,000) (PEI-MAX reagent; Polyscience). Following preincubation of HEK293 cells in media containing 1% FBS for 16 h before treatment, cells were treated with/without 100 ng/ml recombinant-soluble klotho (R&D system) and/or 100 ng/ml recombinant human GH (Wako) in media containing 1% FBS and incubated. Eighteen hours after treatment, HEK293 cells were lysed in the TNE lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, pH 7.5) with a protease inhibitor and protein phosphatase inhibitor for protein analyses or ISOGEN (Wako) for reverse transcriptase PCR.

Statistical analysis. Data are expressed as the mean ± SE. The significance of differences between the two experimental groups was established by one-way or two-way ANOVA followed by Student's t test. Statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, ver. 4.2.1). More precisely, it is a modified version of the R commander (ver. 2.8-0) designed to add statistical functions frequently used in biostatistics.[27] A p value of <0.05 was considered statistically significant.

Results

Tissue distribution and immunofluorescence staining of α-klotho. α-klotho mRNA expression in wild-type (WT) mice was examined via real-time PCR analyses using mouse tissues. α-klotho mRNA expression was observed in the kidney, testis, femur, submandibular glands, brain, and pituitary gland (Fig. 1A). In WT mice, immunofluorescent staining of the α-klotho protein was strongly detected in the renal distal tubules (Fig. 1B). Furthermore, in the mouse pituitary gland, although α-klotho-positive staining was not observed in prolactin-positive cells, it was detected in somatostatin-positive and growth hormone-positive cells (Fig. 1C). In α-klotho KO mice, we observed no positive staining in the kidneys or pituitary glands (Fig. 1B and C).

GH administration induces α-klotho expression in the kidneys of mice. GH was administered to 4-week-old mice per the schedule shown in Fig. 2A. IGF1 mainly mediates the action of GH. Therefore, we first examined the expression of IGF1 mRNA in several mouse organs (Fig. 2B). GH significantly increased mice IGF1 mRNA levels in the pituitary, heart, liver, kidney, femur, and calvaria compared to the vehicle control. In contrast, GH significantly increased α-klotho mRNA levels in the pituitary, kidney, femur, and calvaria (but not in the heart and liver) compared to the vehicle control (Fig. 2C). Immunoblot analyses showed no increase in α-klotho protein expression in the pituitary glands of mice in the GH-treated group compared to those of mice in the vehicle control (Fig. 2D). In contrast, renal α-klotho protein levels were significantly higher in the GH-treated group than in the vehicle control group, with sizes of ~70 and 130 kDa (Fig. 2E). α-klotho protein expression was confirmed by immunostaining of kidney sections (Fig. 2F). Next, the expression of ADAM10 and ADAM17 mRNA was examined in the pituitary gland and kidney of mice treated with GH (Fig. 2G). In the pituitary glands of mice in the GH-treated group, there was a significant increase in ADAM10 mRNA expression compared to the vehicle controls but no difference in ADAM17 mRNA expression. In contrast, in the kidney, GH significantly increased ADAM10 and ADAM17 mRNA expression compared to vehicle controls (Fig. 2G). Furthermore, we strongly detected α-klotho proteins with a size of ~70 kDa in the serum of GH-treated mice compared to the vehicle control (Fig. 2H).

The disappearance of GH responsiveness in an adenine-induced kidney disease model. GH resistance, which is commonly observed in kidney disease, contributes to growth retardation and muscle wasting, along with resistance to IGF1.[4] In particular, pediatric CKD has been reported to be associated with dramatic changes in the GH and IGF1 axes, leading to growth retardation.[11,21] Next, we examined the response of GH, IGF1, and α-klotho in a pediatric kidney disease model (Fig. 3). Three-week-old WT mice were divided into untreated (control) and treated (adenine) groups. To generate a kidney disease model, adenine was administered as described in the methods section, and plasma BUN, a marker of renal function, was measured on day nine after its administration to confirm renal failure (Fig. 3A). There were no significant differences in plasma Pi levels between the control and adenine groups (Fig. 3B). In the kidney, renal insufficiency suppressed α-klotho mRNA expression, which is consistent with previous reports (Fig. 3C). In the pituitary gland, α-klotho mRNA was not suppressed in the adenine group compared to the controls. In bone, α-klotho mRNA expression decreased in the adenine group compared to the controls (data not shown). In the pituitary gland, α-klotho mRNA was not suppressed in the adenine group compared to the controls; however, in bone tissue, as in the kidney, α-klotho mRNA expression was reduced in the adenine group compared to the controls (data not shown).

Next, the control and adenine groups were separated into two further subgroups (vehicle and GH groups). We examined the response to GH in the control and adenine-treated groups (Fig. 3D–G). GH significantly increased JAK2 mRNA, a downstream signal, GHR mRNA, and IGF1 mRNA expression in the kidneys of the control group but not in those of the adenine group (Fig. 3D–F). With normal renal function, GH significantly increased α-klotho mRNA in the kidney compared to the vehicle. However, with renal impairment, GH did not increase renal α-klotho mRNA expression (Fig. 3G).

The presence of α-klotho enhanced GH action in HEK293 cells. Finally, we investigated the involvement of GH and α-klotho using HEK293 cells. HEK293 cells do not express GH action-related molecules, including α-klotho, GHR, and IGF-1 (Fig. 4A). Therefore, we transfected GHR and examined the response of the downstream signaling molecule, JAK2, in the presence and absence of α-klotho supplementation (Fig. 4B and C). HEK293 cells transfected with GHR, recombinant hGH, and recombinant α-klotho supplementation significantly increased JAK2 mRNA levels compared to GH or α-klotho alone (Fig. 4C).
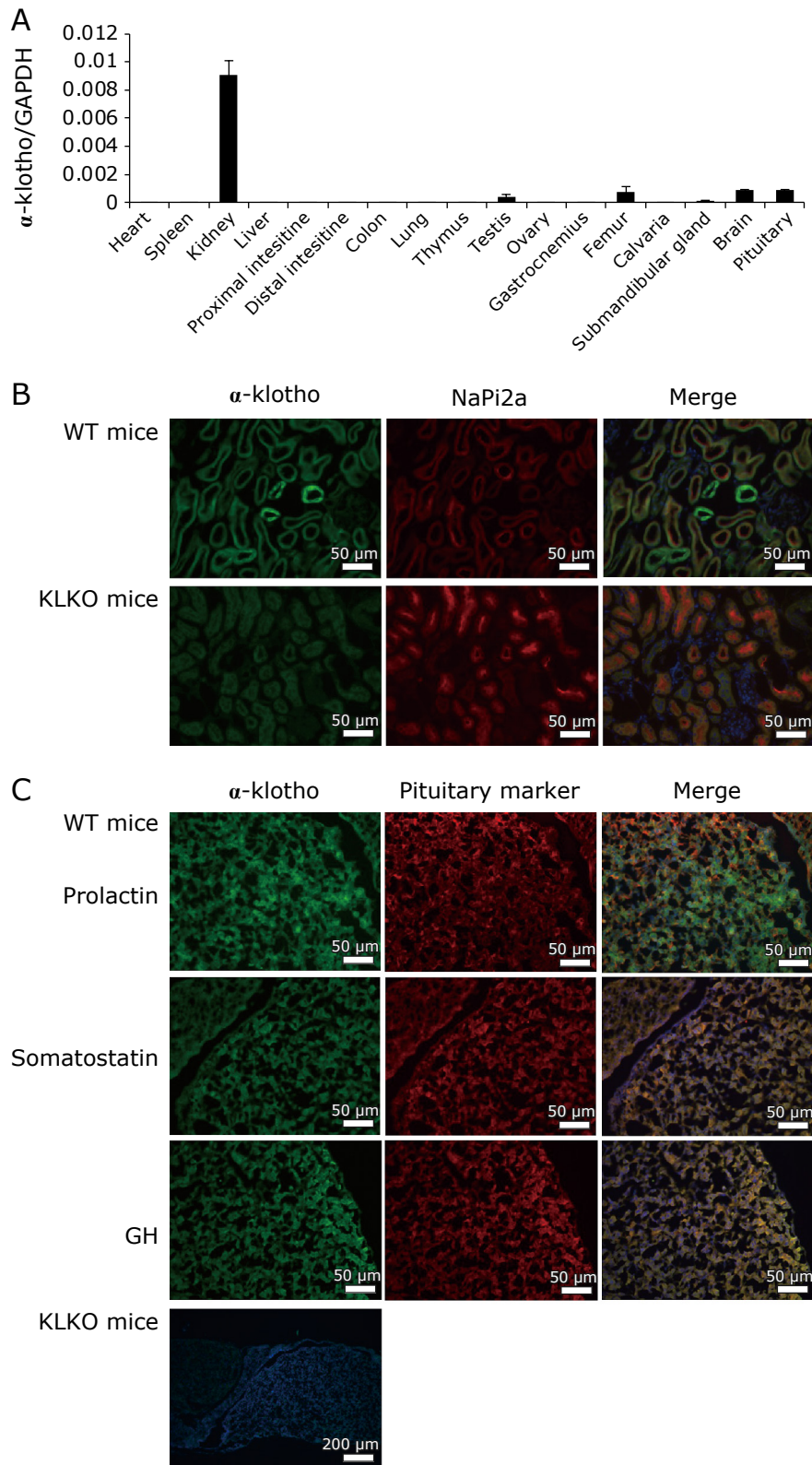
Fig. 1. α-Klotho expression and tissue localization in WT mice. (A) α-klotho mRNA expression of whole organs by real-time PCR. Four-week-old WT male mice (*n* = 3–5) were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Values are presented as the mean ± SE. (B, C) Immunofluorescence staining α-klotho (green) in frozen sections of the (B) kidney and (C) pituitary of 4-week-old WT mice. Blue: nucleus. Red: NaPi2a, prolactin, somatostatin, and GH. See color figure in the on-line version.
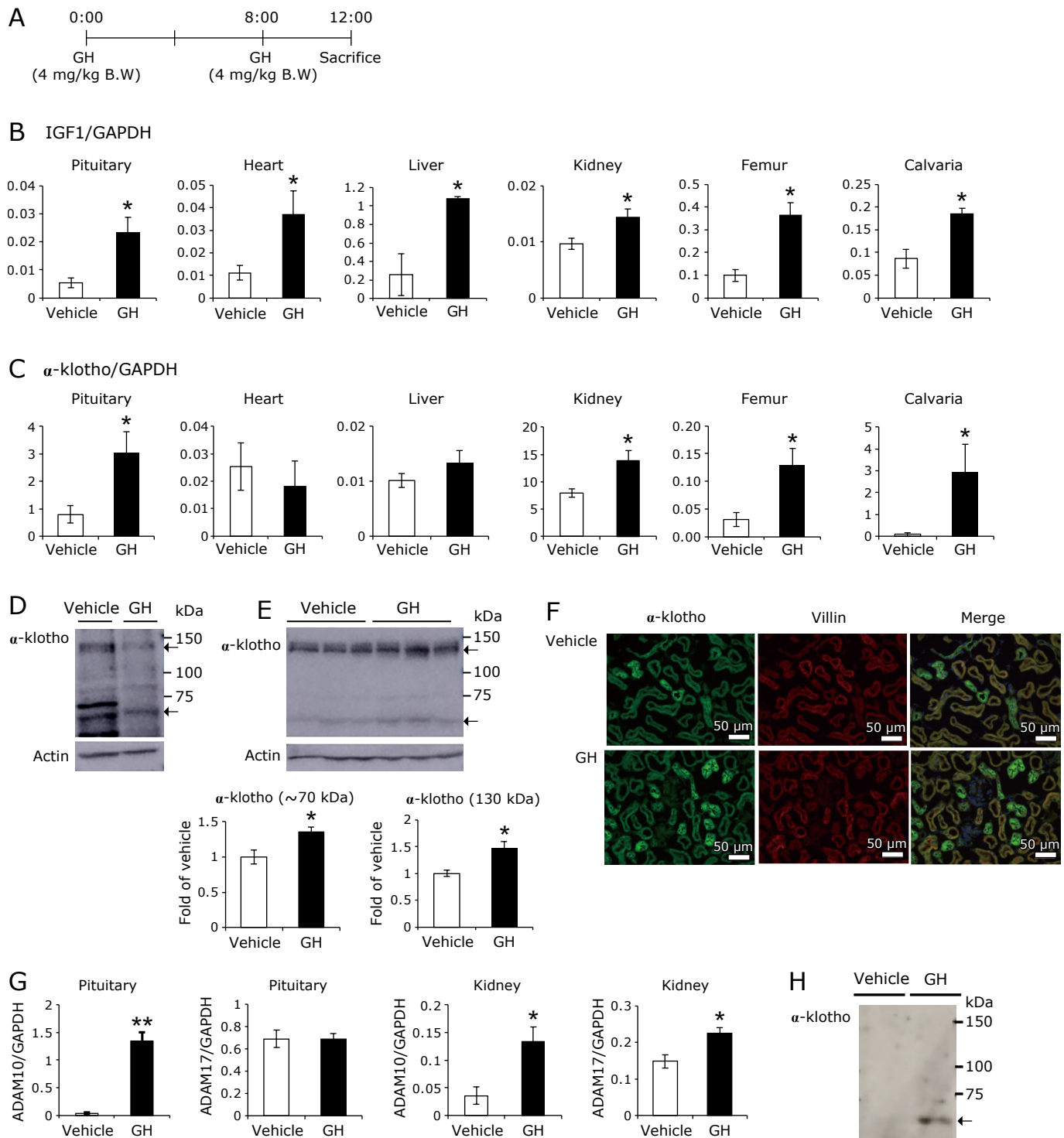
Fig. 2. GH administration induces α-klotho expression in the kidneys of WT mice. WT mice (*n* = 3–6) were administered GH (4 mg/kg) as shown in the experiment's schedule (A). (B) IGF1 mRNA expression and (C) α-klotho mRNA expression of pituitary gland, heart, liver, kidney, femur, and calvaria by real-time PCR. GAPDH was used as an internal control. Values are presented as the mean ± SE. *p<0.05. Western blotting analysis of α-klotho protein in the pituitary gland (D) and kidney (E) whole homogenate. Each lane was loaded with 30 μg of protein. Actin was used as an internal control. Values are presented as the mean ± SE. *p<0.05. (F) Immunofluorescence staining of α-klotho (green) in the kidney frozen section. Blue: nucleus, red: villin. Scale bar: 50 μm. (G) ADAM10 and ADAM17 mRNA expression of the pituitary gland and kidney by real-time PCR (*n* = 5). GAPDH was used as an internal control. Values are presented as the mean ± SE. *p<0.05. (H) Immunoprecipitation of α-klotho protein using a vehicle or GH-administered mouse serum. See color figure in the on-line version.
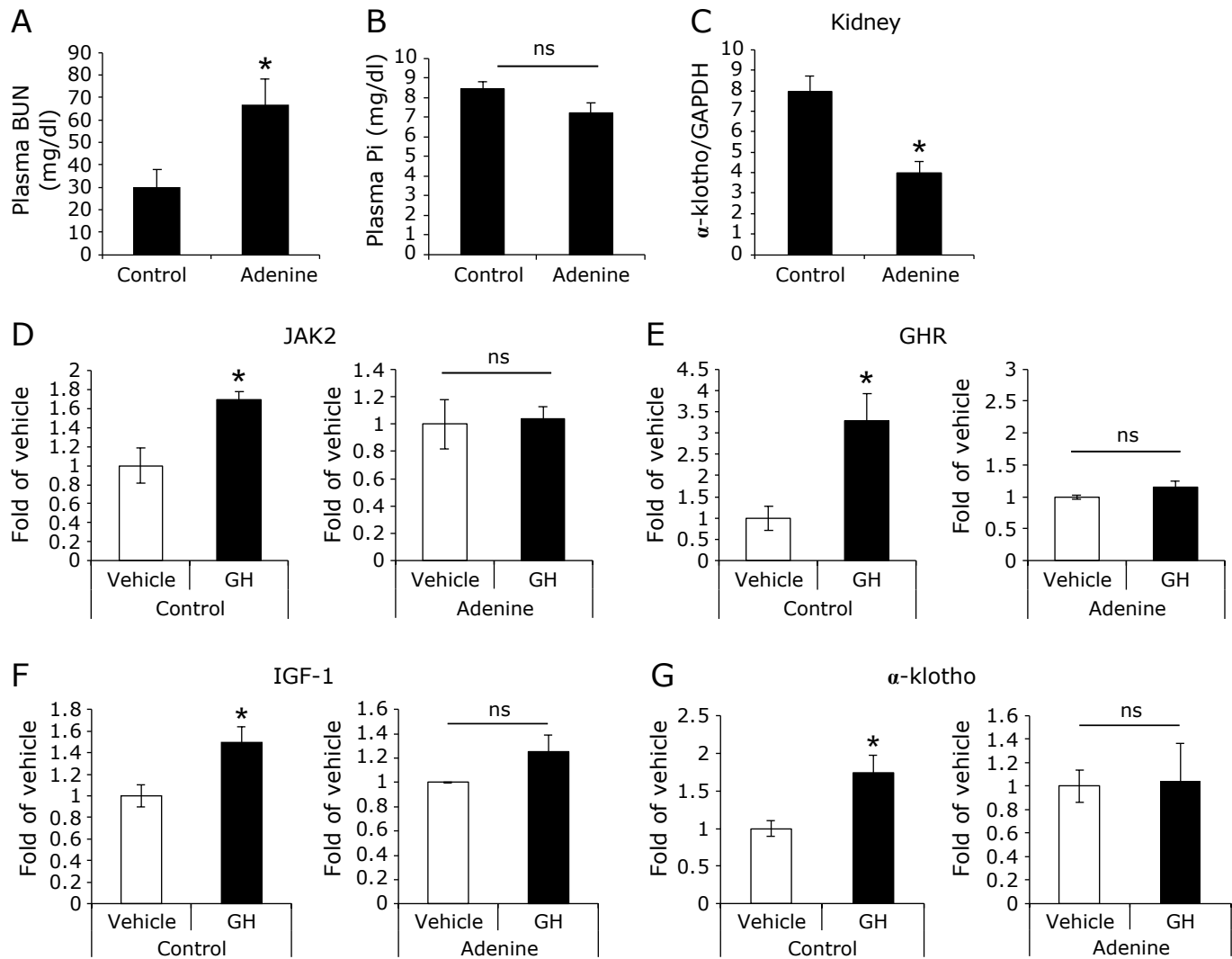
Fig. 3. The disappearance of GH responsiveness in the adenine-induced kidney disease model. WT mice ($n = 10$) were induced renal failure by administering adenine (50–100 mg/kg) dissolved in carboxymethyl cellulose.[23,24] (A) plasma BUN, (B) plasma Pi concentrations, (C) real-time PCR renal α-klotho mRNA. GAPDH was used as an internal control. Data are presented as the mean ± SE, $n = 5$. *$p<0.05$; ns, not significant. The GH administration schedule is shown in Fig. 2A. Real-time PCR renal JAK2 (D), GHR (E), IGF1 (F), and α-klotho (G) mRNA expression ($n = 3–6$). GAPDH was used as an internal control. Values are presented as the mean ± SE. *$p<0.05$; ns, not significant.

Discussion

In this study, we investigated the role of α-klotho in GH function in mice. GH increased α-klotho mRNA expression in mouse kidney, pituitary, and bone tissue; however, increased a-klotho protein expression was observed only in the kidney. Although we did not perform a detailed signal analysis in this study, the GH/IGF1 pathway induced the expression of α-klotho, ADAM10, and ADAM17 in mouse kidneys, resulting in the release of secreted α-klotho from the kidney into the blood. We detected α-klotho protein in the blood with a size of ~70 kDa, corresponding to the secreted form containing KL1, considering antibody recognition. Thus, the kidney is the principal contributor of secreted α-klotho, including the KL1 site, from GH administration. Indeed, renal tubule-specific α-klotho KO mice manifested serum α-klotho levels that were reduced by approximately 80% compared to WT mice.[28]

However, the physiological roles of the induction of α-klotho expression by GH are unclear. GH exerts multiple effects on different organs (including the pituitary gland, bone, and kidneys) either directly or via IGF1.[11,12,29–31] GH and IGF1 act coordinately regarding growth and anabolic effects, whereas GH and IGF1 act antagonistically in many cases regarding metabolic effects.[29–31] Some of the properties of GH have been well characterized, such as its actions on the liver (increasing IGF1 production, synergistic with insulin), cartilage (growth), and adipose tissue (increasing lipolysis, antagonistic to insulin).[29–31] In addition, the GH/IGF1 system has a huge influence on the kidney, including glomerular and tubular function, normal kidney development, the glomerular filtration rate, and tubular water, sodium, Pi, and calcium handling.[11,12,21] Regarding α-klotho, it is involved in Pi and vitamin D metabolism as a coreceptor for FGF23 in the kidney.[1,2] Furthermore, soluble klotho has been reported to be secreted from the kidney into the blood and exerts a variety of effects (suppresses inflammatory responses and fibrosis, regulates autophagy, reduces oxidative stress, and protects mitochondrial function).[32–38] Furthermore, a short klotho-derived peptide, including the KL1 domain, has recently been reported to efficiently improve renal fibrosis and ameliorate renal function.[39] There may be a need for soluble klotho in such
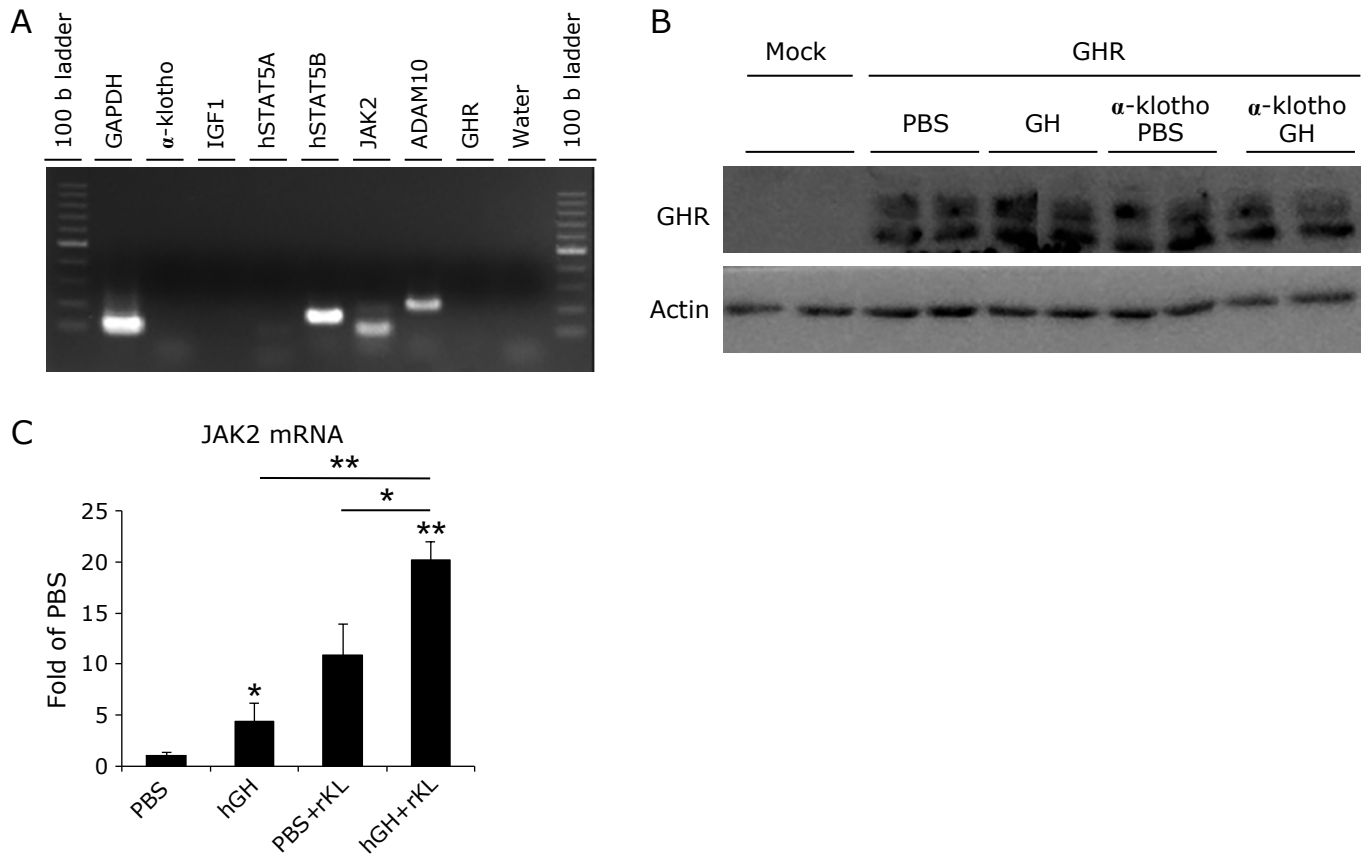
Fig. 4. The presence of α-klotho-enhanced GH action in HEK293 cells. GH, recombinant α-klotho protein, and GH/recombinant α-klotho protein were supplemented to HEK293 cells transfected with the GH receptor (n = 3). (A) PCR confirmed endogenous molecule mRNA expression in HEK293 cells. (B) Western blotting confirmed GHR protein expression transfected into HEK293 cells. (C) Real-time PCR was performed to determine JAK2 mRNA expression. GAPDH was used as an internal control. Values are presented as the mean ± SE. *p<0.05; **p<0.01. Experiments were performed in triplicates.

systemic effects on GH and GH/IGF1. Indeed, previous reports have shown that the effects of GH administration disappear in kl/kl mice.[10]

Growth retardation is a common consequence of CKD in childhood.[20,21] Although growth retardation is correlated with the degree of renal impairment, even mild reductions in the glomerular filtration rate are associated with short stature.[20,21] The patients were shorter at the start of dialysis, and the younger the age, the more severe the growth retardation, with little improvement in the SD score for height. Particularly in infancy, growth is the fastest in childhood, with one-third of all growth occurring during the first 2 years of life, and it depends largely on nutrition.[20,21] However, this growth is significantly reduced in congenital diseases with an early onset of CKD. The etiology of growth retardation in pediatric CKD is thought to be multi-factorial, with a key component associated with changes in the GH/IGF1 axis.[20,21] CKD is a GH-resistant state and not a GH deficiency.[4,12,20,21] Indeed, GH levels are slightly elevated in CKD patients because of decreased renal clearance and half-life prolongation.[11,20,21,40] In addition, CKD appears to be a condition of the membrane and secreted α-klotho deficiency.[41–44] It is possible that α-klotho is one of the key regulators of GH/IGF signaling; therefore, α-klotho deficiency may be involved in GH resistance in CKD.

Defects in GH signaling include impaired postreceptor GH activation, the phosphorylation of downstream STAT proteins in the JAK2 signaling pathway, and the overexpression of suppressors of cytokine signaling (SOCS) (an inhibitor of JAK2/

STAT5 signaling) and GHR.[12,20] In addition, elevated levels of SOCS mRNA expression in skeletal muscle, liver, and epiphyseal growth plates have been reported in a rat model of CKD.[45] In the present study, we confirmed GH resistance to α-klotho expression in a pediatric adenine-induced kidney disease model. GH was not associated with a significant increase in renal GHR, JAK2, IGF1, and α-klotho mRNA in the kidney disease model. Similar findings were observed in pituitary and bone tissues (data not shown). In kl/kl mice with reduced klotho expression, the phosphorylation of JAK2 and STAT3 was signifi-cantly reduced in the hippocampus.[46] Although further detailed studies are needed for confirmatory purposes, our results suggest that α-klotho affects the GH signal JAK/STAT, which is the downstream signal of GH.

In this study, the role of α-klotho in GH action was examined using HEK293 cells. In HEK293 cells with the recombinant-soluble α-klotho protein, the response of JAK2 was enhanced. This suggests that α-klotho is involved in the enhancement of GH signals. The α-klotho possesses β-glucosidase activity, which hydrolyzes the sugar chains of target proteins and regulates their function and localization.[2] Conversely, it has also been reported to bind to Wnt and TGFβ receptors independently of enzyme activity.[47,48] Future studies are required to determine whether α-klotho is involved in signal enhancement via enzymatic activity or by direct binding to GHR. In addition, the α-klotho protein was detected in somatostatin-positive and GH-positive cells in the mouse pituitary gland. α-klotho has been recognized for its role in the promotion of GH secretion through a positive

feedback mechanism.[14,19] In addition, somatostatin has a role in inhibiting the secretion of other hormones, such as GH, insulin, and glucagon.[49] Although a more detailed analysis is needed, our findings suggest that α-klotho might play a negative role via somatostatin.

Finally, in the present study, we found that GH promoted renal α-klotho protein expression and α-klotho secretion, including the KL1 site, into the blood from the kidney. We also found that α-klotho may positively regulate GH signaling. These findings suggest that decreased α-klotho expression may constitute an etiology of GH resistance in kidney disease. Thus, this study suggests that α-klotho can overcome GH resistance. GH secretion is high during childhood and peaks in adolescence, after which it declines with age. However, it is still secreted in adulthood and plays an important role in regulating metabolism and maintaining health.[29] Treatment with recombinant α-klotho protein or increased endogenous α-klotho expression may overcome GH resistance and restore GH action in the body, as well as provide various biological defense actions of α-klotho.

Author Contributions

Study design and conception: MK, TS, and HS. Performing the experiments: MK, AK, MM, AH, AI, KT, MU, YS, and HS. Data analysis: MK and HS. Preparation of Figures: MK and HS. Writing–Original draft: MK, and HS. Review and approval of the manuscript: MK, TS, KM, and HS.

Acknowledgments

Conflict of Interest

No potential conflicts of interest were disclosed.

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