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ORIGINAL ARTICLE

Klotho preservation by Rhein promotes toll-like receptor 4 proteolysis and attenuates lipopolysaccharide-induced acute kidney injury

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Abstract

Renal anti-aging protein Klotho exhibits impressive properties of anti-inflammation and renal protection, however is suppressed early after renal injury, making Klotho restoration an attractive strategy of treating renal inflammatory disorders. Here, we reported that Klotho is enriched in macrophages and Klotho preservation by Rhein, an anthraquinone derived from medicinal plant *rhubarb*, attenuates lipopolysaccharide (LPS)-induced acute inflammation essentially via promoting toll-like receptor 4 (TLR4) degradation. LPS-induced pro-inflammatory NF-κB signaling and cytokine expressions coincided with Klotho repression and toll-like receptor 4 (TLR4) elevation in macrophages, renal epithelial cells, and acutely- inflamed kidney. Intriguingly, Rhein treatment effectively corrected the inverted alterations of Klotho and TLR4 and mitigated the TLR4 downstream inflammatory response in a Klotho restoration and TLR4 repression-dependent manner. Klotho inducibly associated with TLR4 after LPS stimulation and suppressed TLR4 protein abundance mainly via a proteolytic process sensitive to the inhibition of Klotho's putative β-glucuronidase activity. Consistently, Klotho knockdown by RNA interferences largely diminished the anti-inflammatory and renal protective effects of Rhein in a mouse model of acute kidney injury incurred by LPS. Thus, Klotho suppression of TLR4 via deglycosylation negatively controls TLR-associated inflammatory signaling and the endogenous Klotho preservation by Rhein or possibly other natural or synthetic compounds possesses promising potentials in the clinical treatment of renal inflammatory disorders.

Key messages

- Klotho is highly expressed in macrophages and repressed by LPS in vitro and in vivo.
- Klotho inhibits LPS-induced TLR4 accumulation and the downstream signaling.
- Klotho decreases TLR4 via a deglycosylation-associated proteolytic process.
- Rhein effectively prevents acute inflammation-incurred Klotho suppression.
- Rhein reversal of Klotho attenuates LPS-induced acute inflammation and kidney injury.

Keywords Klotho · Rhein · TLR4 · Kidney inflammation · Acute kidney injury

Introduction

Deregulated inflammation remains the central pathogenesis involved in various acute or chronic kidney diseases. Strategies aiming at maintaining endogenous anti-inflammatory mediators are promising for effective anti-inflammation therapy and renal protection. Klotho, a kidney tubule cell-enriched anti-aging protein essential for kidney homeostasis, is a sensitive biomarker and a therapeutic target of renal diseases owing to its sensitivity to renal injury and remarkable renoprotective properties [1, 2]. Klotho presents as both a membrane-bound and a soluble protein. The membrane Klotho mainly functions as a co-receptor for fibroblast growth factor − 23 (FGF-23) [3, 4], through which it regulates renal mineral and hormone metabolisms [5, 6], while soluble Klotho, generated from alternative splicing or from ectodomain shedding of membrane Klotho and released into blood and cerebral-spinal fluid, beneficially affects

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renal local and systemic pathological processes including oxidative stress, fibrosis, inflammation, and cell senescence by interrupting the key cellular signaling controlling these processes [7–10]. In addition, soluble Klotho possesses β-glucuronidase (a member of glycosidase family) property capable of deglycosylating many ion and peptide transporters and affecting their stability and the downstream signaling [11, 12]. Klotho reportedly inhibits inflammation at various stages of toll-like receptor (TLR) signaling cascade including IKK (inhibitor of IκB kinase) activation, IκB (inhibitor of kappaB) phosphorylation and degradation, or NF-κB (nuclear factor-kappaB) nucleus translocation [13–16], but with unclarified cellular targets.

Klotho level is quickly reduced in renal patients and the experimental animals of acute or chronic kidney diseases [1] and mice lacking Klotho display enhanced inflammatory damage in animal models of renal diseases [17, 18]. On the other hand, Klotho transgenic expression or exogenous supplementation effectively improved the inflammation-associated renal pathological processes [9, 19], suggesting that Klotho is a prominent anti-inflammation protein with therapeutic potentials. We have recently found that Rhein, a lipophilic anthraquinone compound isolated from medicinal plant *rhubarb*, up-regulates Klotho via increasing Klotho transcription both in vitro and in vivo, which confers its renoprotective activities [20, 21]. Rhein displays multiple pharmacological bioactivities against imbalanced oxidative stress, fibrogenesis, deregulated glucose/lipid metabolism, and excessive inflammation [22, 23] and is broadly used as an alternative medication in the clinical treatment of various inflammatory renal disorders in eastern Asia countries [24]. Like Klotho, Rhein's anti-inflammation functions are mainly attributed to its inhibition of TLR-associated inflammatory signaling [25, 26], raising the possibility that the anti-inflammation actions of Rhein and Klotho are mechanistically connected.

TLR family consists of at least 13 members (TLR1–13) in mammalian and forms the first line of host defense against invading microorganisms and endogenous inflammatory derivatives. TLR4, the best characterized and the first member of the family, recognizes pathogen-associated molecular patterns (PAMPs) derived from gram-negative bacteria such as lipopolysaccharide (LPS) or danger-associated molecular patterns (DAMPs) generated from endogenous inflammatory processes [27]. TLR4 once activated by ligand binding transduces its downstream signaling mainly through IKK/IκB/NF-κB signaling pathway, in which IKK phosphorylates IκB and induces its degradation, consequently leading to NF-κB nuclear translocation and the transcriptional inductions of pro-inflammatory cytokines/mediators. The basal TLR4 level is low in various inflammatory or non-inflammatory cells, but quickly accumulated after inflammatory stimulation through its own activation in a positive feedforward fashion, therefore facilitating an efficient inflammatory response to eliminate invading pathogens and prevent damage to nearby tissues. However, inflammatory responses must be tightly restricted both spatially and temporally. TLR4 is negatively regulated by endocytosis and lysosomal degradation process to avoid prolonged inflammation and tissue damage [28]. TLR4 is heavily glycosylated during biosynthesis similar to many membrane glycoproteins, and its glycosylation modifications ensure its membrane trafficking, stability, and the downstream inflammatory signaling transduction [29, 30].

In this study, we found that Klotho, in addition to being renal epithelium-enriched, is highly expressed in macrophages. We show that Klotho prevents LPS-induced TLR4 accumulation likely via promoting its proteolysis. More importantly, Rhein is capable of preventing the inflammatory Klotho loss and Klotho restoration dependently attenuated the excessive inflammation and acute kidney injury induced by LPS. Thus, our study reveals a novel anti-inflammation mechanism of Klotho and the therapeutic potentials of Klotho preservation by Rhein or other natural or synthetic substances in the clinical treatment of various inflammatory kidney diseases.

Materials and methods

Animal studies of acute kidney injury

Use of animal and the experimental procedures were in accordance with the animal use guidelines and approved by the Institutional Animal Care and Use Committee of Nanjing University School of Medicine (Nanjing, China). C57BL/6 male mice of 6–8 weeks of age were purchased from the model animal research center of Nanjing University and housed in the animal facility on site under standard temperature (22 ± 2 °C), humidity (50–60%), and light conditions (12 h light/dark cycles).

Mouse model of LPS-induced AKI was adopted from a previous study [31]. Mice were assigned to one of four groups ($n = 6$ in each group): (1) control: PBS injection; (2) LPS: one LPS intraperitoneal injection (10 mg per kilogram body weight); (3) Rhein: mice receiving Rhein (120 mg/kg daily by oral gavage) [21]; and (4) LPS mice pretreated with Rhein 1 day before, and experiment went for 24 h. For siRNA in vivo study, small interfering RNA (siRNA) of mouse Klotho was employed, which targeted GCGACTAC CCAGAGAGTAT [32]. The control siRNA contained a scrambled RNA sequence. Mice were first injected with either siRNA-control or siRNA-Klotho 1 day before, and then subjected to AKI model as above ($n = 6$ in each group). After experiment completion, mice were sacrificed by CO_2

inhalation and mouse kidneys, and serum samples were collected and stored at − 80 °C for further analysis.

Histology and serum biochemistry study

Kidney sections were prepared, and the sections (5 μm) were stained by hematoxylin and eosin (H&E) method following a previous protocol [33]. The histological samples were scored blindly by a lab observer. The magnitude of tubular injury including tubular dilatation, tubular cell flattening, and vacuolization was scored on the basis of the percentage of affected tubules over total observable tubules under a high-power field light microscope. Ten randomly selected fields were examined for each sample. Mouse serum creatinine (Cre) and blood urine nitrogen (BUN) were measured with a Hitachi 7180 automatic analyzer (Hitachi Ltd., Tokyo, Japan).

Cell culture

Mouse macrophage RAW264.7 (RAW), human monocytic THP-1, renal tubule epithelial HK2, and human embryo kidney HEK293 cells (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, USA) and maintained at 37 °C in a humidified incubator of 5% CO_2. Rhein (R7269), lipopolysaccharide (LPS), cycloheximide (CHX), MG132, 3-methyladenine (3-MA), Chloroquine (CQ) (Sigma-Aldrich, USA), β-glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, Selleck, USA), or recombinant mouse Klotho containing amino-acid 35-982 (extracellular domain) (1819-KL, R&D systems, USA) were added to cells as indicated.

Western blot

Cell lysates from mouse kidney tissue or cultured cells were prepared and subjected to Western blot assay following the procedures described before [33]. Glycosylated TLR4 was precipitated with agarose-bound *Sambucus nigra* lectin and eluted following the manufacturer's protocol (AL-1303, Vector Laboratories, USA). The primary antibodies to Klotho (TransGenic, Japan and ABclonal Biotech. China), β-actin (Invitrogen, USA), TLR4, HA and Myc tag, E-cadherin, IκBα, or phosphorylated-IκBα (Santa Cruz Biotech, USA), were incubated with the blots at predetermined concentrations, then with HRP-conjugated secondary antibodies (Yifeixue Biotech, Nanjing, China). Western blots were developed using an ECL plus Western blotting detection system (Vazyme, Nanjing, China). The protein quantities were analyzed with Image J software (NIH, USA).

Reverse-transcription PCR and quantitative real-time PCR

Kidney mRNA isolation, cDNA conversion, RT-PCT, and qRT-PCR were performed essentially as before [33]. The primer sequences are as following: TNFα-F: CATCTTCT CAAAATTCGAGTGAC, TNFα-R: TGGGAGTA GACAAGGTACAAC CC; IL-1β-F:TGGAAAAG CGGTTTGTCTTC, IL-1βR: TACCAGTTGGGGAA CTCTGC; NOS2-F: CAGCTGGGCTGTACAAACCTT, NOS2-R: CATTGGAAGTGAAGCGT TTCG; TLR4-F: ACCTGGCTGGTTTACACGTC, TLR4-R: CTGCCAGA GACA TTGCAGAA; Klotho-F: GGCTTTCCTCCTTT ACCTGAAAA, Klotho-R: CACA TCCCACAGATAGAC ATTCG; GAPDH-F: GGCCCGGTGCTGAGTATGTC, GAPDH-R: TGCCTGCTTCACCACCTTCT.

Plasmid constructions, Klotho over-expression, and knockdown

HA-tagged mouse Klotho (pUSEHA-Klotho) and myc-tagged TLR4 (pCDNA-TLR4-6myc) expression plasmids were constructed from RAW cell mRNA by RT-PCR in pUSEHA (Upstate, USA) or pcDNA-6myc vector containing six copies of myc tag at carboxyl terminal, respectively. The primer set used was cKlotho-F: TAAGCGGCCGCCATG CTAGCCCGCGCCCCTC and cKlotho-R: CGCTCGAG TTAGCTGAAAACTCTGCTGTGG; cTLR4-F: CGGGAT CCATGATGCCTCCC TGGCTCCTG and cTLR4-R: GGGGTACCGGTCCAAGTTGCCGTTTCTT (the restriction enzyme sites were underlined). A human shRNA-Klotho plasmid for Klotho knockdown in cells was described before [20].

Co-immunoprecipitation assay

RAW cells or HEK293 cells transfected with an HA-tagged Klotho expression plasmid were treated with LPS and/or Rhein plus LPS for various time, and then, co-immunoprecipitation assays were performed reciprocally with antibodies specific for TLR4, Klotho, or HA as before [34].

Luciferase assay

HEK293 cells were co-transfected with luciferase reporter plasmid containing three copies of IκB (NF-κB binding site derived from IκB promoter) plus a renilla control plasmid with Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The transfected cells were treated with Rhein and/or LPS for 24 h, and then, the reporter luciferase activities were determined as the results of reporter activities divided by renilla activities and normalized. All the transfection experiments were performed in triplicate and repeated at least three times independently.

Immunofluorescent staining

For immunofluorescent staining, HK2 cells were transfected with either shRNA-control or shRNA-Klotho plasmid and cultured on cover-slips. Cells were then treated with Rhein and/or LPS for 30 min and incubated with an anti-NF-κB antibody (Santa Cruz, USA) 1 h at room temperature. Subsequently, the cells were washed and incubated with Alexa Fluor 488 fluorescein-conjugated secondary antibody and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, from Sigma-Aldrich, USA) for 10 min. Images were taken by confocal microscopy (Olympus, Japan).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). The statistical difference was analyzed by Student's *t* test for comparisons of two groups or one-way ANOVA for two factor experiments or one-way ANOVA followed by Tukey's test for comparisons of multiple groups. $P < 0.05$ or $P < 0.01$ was considered statistically significant or very significant.

Results

Rhein inhibition of LPS-induced acute inflammation and AKI parallels with TLR4 suppression and Klotho restoration in mouse kidney

To gain insight into the functional anti-inflammation connections of Klotho and Rhein in acute inflammation, we adopted a mouse model of LPS-induced AKI. Mice receiving LPS injection for 24 h expectedly displayed severe renal epithelial damage scored by renal tubule dilatation, flattening, or vacuolization (Fig. 1a, b) and the functional loss such as increased serum creatinine and blood urine nitrogen (BUN) (Fig. 1c). AKI kidneys also showed reduced expressions of the epithelial marker E-cadherin and NF-κB inhibitor IκBα (Fig. 1d, e), accompanied by excessive inflammatory cytokine induction of TNFα, NOS2, and IL-1β (Fig. 1f). However, Rhein pretreatment effectively mitigated the renal injury and the functional loss, as well as the abnormal expressions of inflammatory signaling molecules and mediators (Fig. 1a–f). Intriguingly, LPS also caused concomitant Klotho down-regulation and TLR4 elevation, while Rhein treatment significantly corrected the

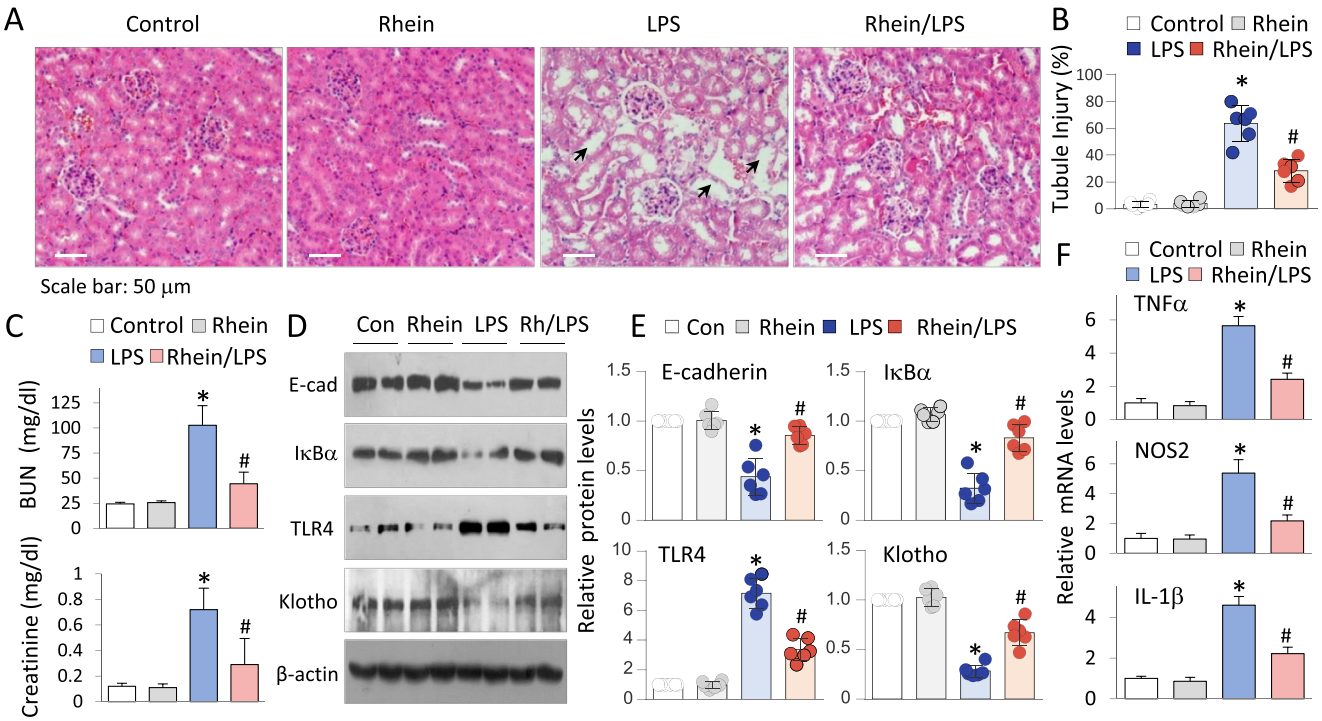

Fig. 1 Rhein inhibition of LPS-induced acute inflammation and AKI parallels with TLR4 suppression and Klotho restoration in mouse kidney. Mice were treated with Rhein in presence or absence of LPS for 24 h. **a** Representative photomicrographs of H&E-stained kidney sections. Tubular damage, including tubular dilatation, flattening, or vacuolization in tubular cells (indicated by dark arrows), was observed in mice receiving LPS injection. **b** Kidney tubule injury scores of Fig. 1a. Data are presented as the ratio of damaged renal tubule including tubular dilatation, flattening, or vacuolization in tubular cells over the total visible tubules in the cortex area ($n = 6$ in each group). **c** Average concentrations of serum creatinine (Cre) and blood urine nitrogen BUN) from control, Rhein, LPS, and Rhein-treated LPS mice. **d** Representative renal protein levels of Klotho, TLR4, E-cadherin (E-cad), and IκBα assayed by Western blotting (two randomly selected samples from each group were shown). **e** Quantifications of Fig. 1c. **f** Average renal mRNA levels of TNFα, NOS2, and IL-1β from mice in Fig. 1a were assayed by qRT-PCR and quantified as fold changes ($n = 6$ in each group). Data were presented as mean ± SD (standard deviation). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. LPS treatment

inverted alterations, suggesting that Rhein attenuation of renal inflammation closely correlates with its beneficial regulation of aberrant TLR4 and Klotho expressions in kidney.

Rhein inhibition of LPS-induced acute inflammation parallels with TLR4 suppression and Klotho restoration in mouse macrophages

Klotho is reportedly expressed in renal distal tubes and human renal tubule HK2 cells, but silenced in most other cell types [35]. A recent study detected both cytoplasmic and membrane-bound Klotho by immunofluorescence staining from human peripheral blood and peritoneal monocytes [36], suggesting the full length Klotho expression. Another study reported that mouse macrophage RAW cells expressed only the secret form of Klotho (65kd) by Western blot [37]. However, we routinely detected substantial Klotho protein of full length (130 kd) in human embryo kidney cell HEK293, RAW, and human monocytic THP-1 cells (Fig. 2a, left panel). Further examination revealed that RAW and THP-1 cells expressed less but noticeable secret Klotho (about 65 kd) (Fig. 2a, right panel), supporting that both full length and secret isoforms of Klotho participate in cellular processes of inflammatory cells. RAW cells expressed basal toll-like receptor 4 (TLR4), whereas LPS treatment dose-dependently increased its abundance accompanied by a marked Klotho protein reduction (Fig. 2b). Interestingly, LPS-induced Klotho suppression and TLR4 induction in HK2 were prohibited by Rhein treatment (Fig. 2c, d), which also effectively corrected the Klotho protein/mRNA reductions and TLR4 accumulation induced by LPS in RAW cells (Fig. 2e, f). Further, Rhein dose-dependently inhibited the LPS-induced expression of tumor necrosis factor-alpha (TNFα), interleukin 1-beta (IL-1β), and inducible nitric oxide synthase (iNOS/NOS2) (Fig. 2g, h), indicating that Rhein up-regulation of Klotho likely relates to its inhibition of TLR4 and the inflammatory cytokine expressions.

Rhein preservation of Klotho decreases TLR4 and TLR4 dependently attenuates LPS-induced inflammatory signaling

To explore the potential role of Klotho restoration by Rhein in TLR4 suppression, we assessed whether Klotho gain or loss affects the TLR4 down-regulation. The results showed that LPS induced TLR4 accumulation in control HK2 cells, but over-expression of an HA-tagged Klotho blunted the TLR4

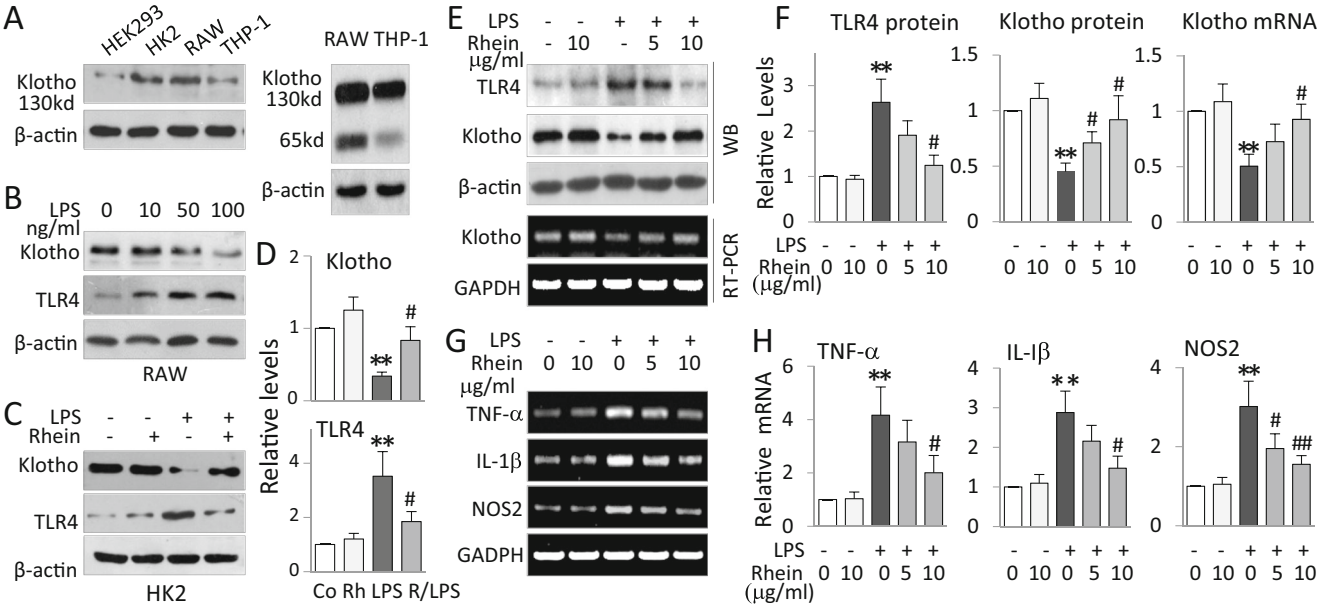

Fig. 2 Rhein inhibition of LPS-induced acute inflammation is accompanied by TLR4 suppression and Klotho restoration in mouse macrophages. **a** Klotho expression in renal cells and macrophages. Klotho protein expression was tested from human embryo kidney cell HEK293, human renal tubule epithelium HK2 cells, mouse macrophage RAW264.7 (RAW), and human monocytic THP-1 cells by Western blotting (left panel). Klotho expression in RAW and THP-1cells after longer exposure (right panel). **b** RAW cells were treated with increasing amounts of LPS (10, 50, 100 ng/ml) for 12 h (upper panel), or **c** HK2 cells were treated with LPS (300 ng/ml) for 12 h in presence or absence of Rhein (10 μg/ml), and then Klotho and TLR4 protein levels were assayed by Western blotting. **d** quantification of Fig. 2c. **e** RAW cells were treated with LPS (100 ng/ml) in absence of presence of Rhein (5 and 10 μg/ml) for 12 h; then TLR4, Klotho protein, and mRNA were assayed by Western blotting (upper panel) or RT-PCR (lower panel), respectively. **f** Quantifications of Klotho protein, Klotho mRNA, and TLR4 protein in Fig. 2e. **g** Rhein inhibition of LPS-induced pro-inflammatory cytokine expressions. RAW cells were treated with Rhein (5 or 10 μg/ml) in absence or presence of LPS (100 ng/ml) for 6 h, and then TNF-α, IL-1β, and NOS2 mRNAs were assayed by RT-PCR. GAPDH served as an internal control. **h** Quantifications of TNF-α, IL-1β, and NOS2 mRNAs in Fig. 2g. The results were presented as mean ± SD of three independently performed experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS treatment

induction in both RAW and HEK 293 cells (Fig. 3a). On the contrary, the HK2 cells transfected with a small hairpin RNA (shRNA) specific for Klotho potentiated the basal and LPS-induced TLR4, but abolished the Rhein inhibition of TLR4 enhanced by LPS (Fig. 3b). To investigate the functional significance of LTR4 down-regulation by Rhein in TLR-associated inflammatory cellular signaling, we found that Rhein reduced the LPS-induced IκBα phosphorylation and degradation, but over-expression of a myc-tagged TLR4 diminished the inhibitory effects (Fig. 3c). Further, TLR4 overexpression attenuated Rhein inhibition of LPS-induced NF-κB transcription of a plasmid reporter containing 3 NF-κB binding sites derived from IκB promoter (Fig. 3d), revealing that Klotho preservation and TLR4 down-regulation invertedly mediate Rhein inhibition of inflammatory signaling.

Rhein down-regulates TLR4 through TLR4 degradation

To gain further insight into the nature of Rhein down-regulation of TLR4 and its impacts on LPS-induced inflammatory signaling, we performed a time course study examining the effects of Rhein on the kinetics of LPS-induced TLR4 accumulation. LPS time-dependently up-regulated TLR4 and down-regulated Klotho over a 24-h period of time, but Rhein inhibited the inverted alterations (Fig. 4a). We also examined TLR4 mRNA levels and found that LPS increased TLR4 mRNA and Rhein almost blunted the increment (Fig. 4b). These results suggest that TLR4 activation enhances its own transcription, which in turn increases its protein abundance, but do not differentiate whether Rhein acts on TLR4 or on its downstream signaling molecules. We then directly assayed the effect of Rhein on TLR4 protein stability. We first stimulated cells with LPS overnight to induce a substantial TLR4 protein accumulation, and then treated cells with a protein synthesis blocker cycloheximide (CHX) and measured the TLR4 protein decay overtime. The results showed that Rhein significantly accelerated TLR4 decay by 4 and 12 h (Fig. 4c) comparing to non-Rhein-treated and time-matched cells. Since proteasome-associated ubiquitination pathway and lysosomal autophagic process are two major mechanisms mediating protein degradation, we used the pharmacological strategies to investigate which pathway is responsible for the TLR4 reduction. A previous study reported that TLR4 can

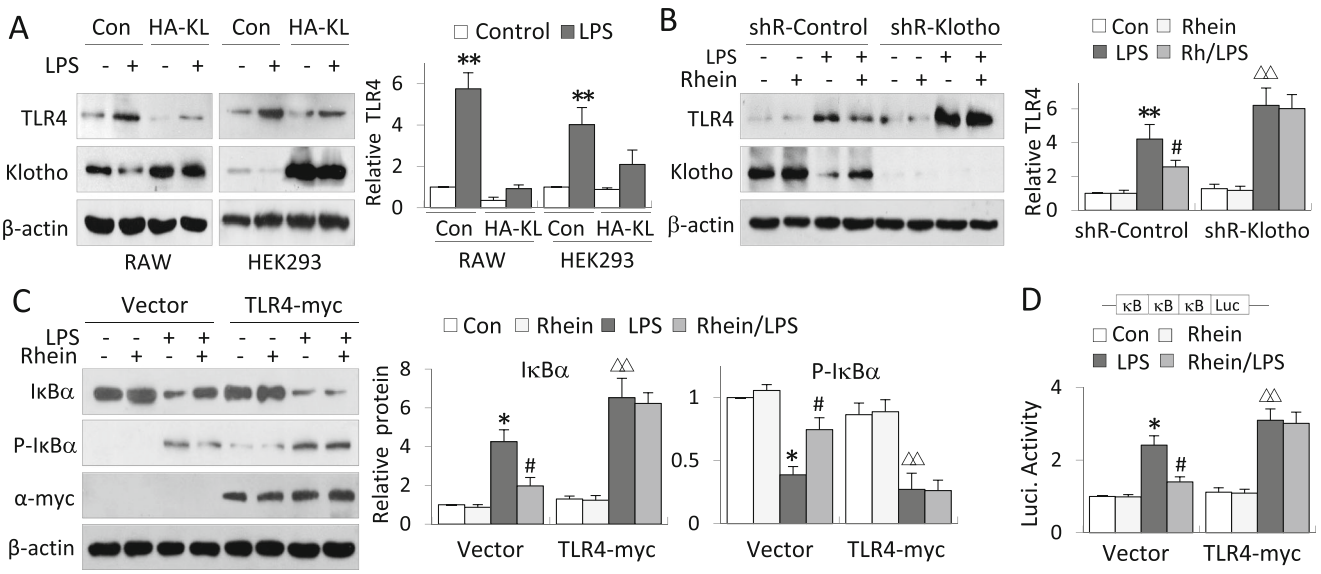

Fig. 3 Rhein preservation of Klotho decreases TLR4 and TLR4 dependently attenuates LPS-induced inflammatory signaling. **a** Klotho over-expression abolishes LPS-induced TLR4 accumulation. RAW and HEK293 cells were transfected with a control or a plasmid over-expressing HA-tagged Klotho (HA-KL), and then treated without or with LPS (100 ng/ml for RAW or 300 ng/ml for HEK293) for 12 h. The cell lysates were examined for TLR4 and Klotho expression by Western blotting. The bar graph on right: quantifications of Fig. 3a. **b** Klotho knockdown diminishes Rhein inhibition of TLR4 accumulation induced by LPS. HK2 cells were transfected with a control or a shRNA-Klotho plasmid, and then treated with Rhein (10 μg/ml) in presence or absence of LPS (300 ng/ml) for 12 h. Cell lysates were tested for TLR4 and Klotho expressions by Western blotting. The bar graph on right: quantification of Fig. 3b. **c** HEK293 cells were transfected with control or a plasmid expressing myc-tagged TLR4, and then treated with LPS (300 ng/ml) in presence or absence of Rhein (10 μg/ml) for 30 min. IκBα and phosphorylated IκBα (P-IκBα) were assayed by Western blotting. The over-expression of myc-tagged TLR4 was verified with an anti-myc antibody. The bar graph on right: quantifications of Fig. 3c. **d** Luciferase reporter assay. HEK293 cells were transfected with a NF-κB luciferase reporter plasmid containing three copies of NF-κB binding site derived from IκB promoter (depicted on top) plus a renilla luciferase reporter as internal control. Twenty-four hours later, cells were untreated or treated with LPS (300 ng/ml) in presence or absence of Rhein (10 μg/ml) for 24 h, followed by luciferase assay. The luciferase activities were normalized with that of renilla's and presented as fold changes. Data were presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\triangle\triangle}P < 0.01$ vs. control in shRNA-Klotho (Fig. 3b) or TLR4-myc (Fig. 3c, d)-transfected cells

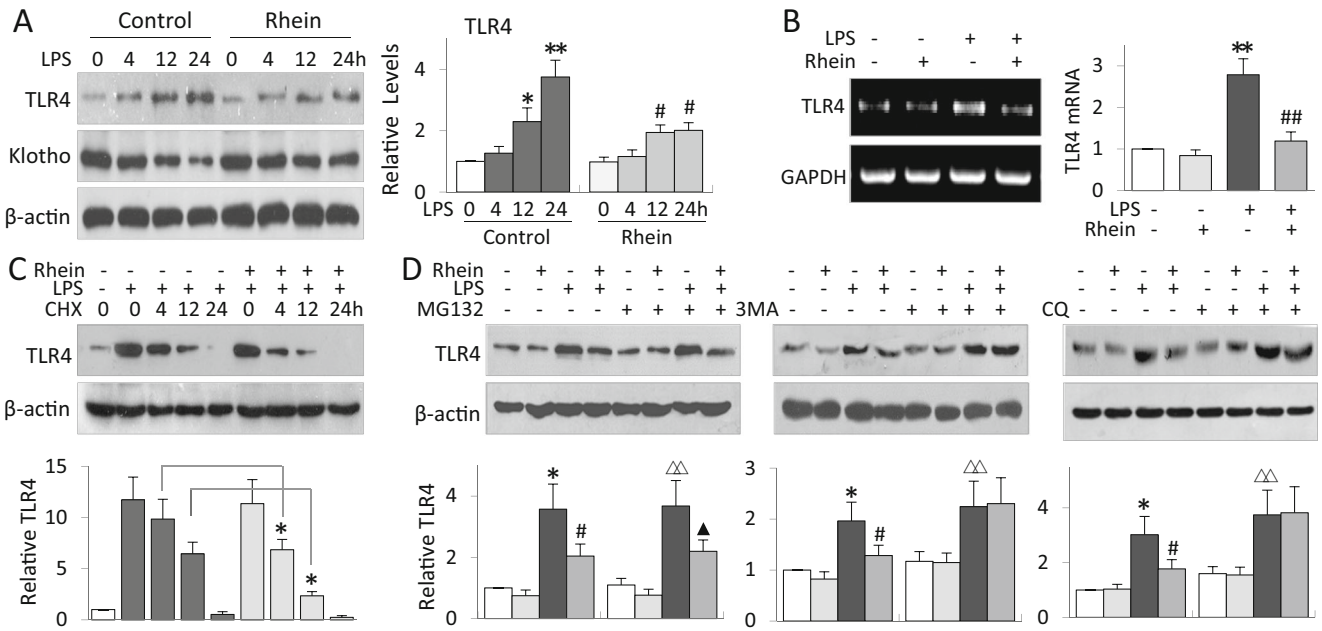

Fig. 4 Rhein down-regulates TLR4 through promoting its proteolysis. **a** Time course study of Rhein inhibition of TLR4 and Klotho suppression incurred by LPS. RAW cells were treated with LPS (100 ng/ml) for 4, 12, or 24 h in absence or presence of Rhein (10 μg/ml), then TLR4 and Klotho proteins were assayed by Western blotting. The bar graph on right: quantification of TLR4 protein levels in Fig. 4a. **b** Rhein inhibition of LPS-induced TLR4 mRNA. RAW cells were treated with LPS (100 ng/ml) and/or Rhein (10 μg/ml) for 6 h; then, TLR4 mRNA was assayed by RT-PCR. The bar graph on right: quantification of TLR4 mRNA levels in Fig. 4b. **c** Rhein accelerates TLR4 degradation. RAW cells were treated with LPS (100 ng/ml) overnight to induce TLR4, and then with cycloheximide (CHX, 1 μg/ml) in presence or absence of Rhein (10 μg/ml). Cell lysates were collected at 4, 12, and 24 h and TRL4 protein levels were assayed by Western blotting. The bar graph under the figures: quantification of TLR4 protein levels in Fig. 4c. **d** Protein degradation blockade assay. RAW cells were treated with Rhein (10 μg/ml) and/or LPS (100 ng/ml) in presence or absence of a proteasome inhibitor MG132 (1 μM, left), a general lysosome inhibitor 3MA (1 mM, middle) or a specific lysosome inhibitor chloroquine (CQ, 25 μM, right) for 24 h, and then TLR4 protein levels were examined by Western blotting. Quantifications of each assay were underneath the cognate figures. Data were presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control or time-matched non-CHX treatment (Fig. 4a); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS; $^{\triangle}P < 0.05$ vs. control and $^{\blacktriangle}P < 0.05$ vs. LPS within MG132-treated group

degrade via lysosome-associated autophagy pathway [28]. Indeed, proteasome inhibitor MG132 did not affect the Rhein reduction of TLR4, but a broad autophagy and phosphoinositide 3-kinase (PI3K) inhibitor 3-methyladenine (3-MA) and a more specific lysosome inhibitor chloroquine (CQ) prohibited the reduction (Fig. 4d), suggesting that Rhein decreases TLR4 probably through a lysosome-associated proteolytic process.

Klotho targets and degrades TLR4 via a deglycosylation process

Soluble Klotho contains intrinsic glycosidase activity and reportedly deglycosylates several membrane ion channels and transporters [6, 12, 38]. We decided to test if Klotho down-regulation of TLR4 involves its β-glucuronidase activity by using a β-glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL) that reportedly inhibited the Klotho deglycosylation of excitatory amino acid transporters (EAATs) and peptide transporters (PEPTs) [39, 40]. DSAL treatment increased the basal and LPS-induced TLR4 levels (Fig. 5a, comparing lanes 1 to 5 and 3 to 7) and eliminated the Rhein inhibition of TLR4 induced by LPS (Fig. 5a, comparing lanes 3 to 4 and lanes 7 to 8). Further, while Klotho over-expression inhibited LPS-induced TLR4 (Fig. 5b, comparing lanes 2 to 5), the effect is diminished in presence of DSAL (Fig. 5b, comparing lanes 2 to 5 and 5 to 6), suggesting that Klotho down-regulation of TLR4 involves Klotho's putative β-glucuronidase activity that likely deglycosylating TLR4. To clarify whether TLR4 is a substrate of Klotho glucuronidase activity, we employed a reciprocal co-immunoprecipitation assay to test whether Klotho physically associates with TLR4. Initially, we did not detect the interaction between endogenous Klotho and TLR4 under either resting or LPS-stimulated condition (12-h treatment), probably due to the facts that TLR4 is low in resting cells and Klotho is repressed by LPS. We then treated the cells with LPS for 4 h and found that LPS induced a weak, but noticeable, association between endogenous Klotho and TLR4 (Fig. 5c left panel). Further, ectopic over-expression of HA-tagged Klotho in HEK293 cells displayed stronger interaction with TLR4 under LPS-stimulated condition, which was inhibited by Rhein treatment (Fig. 5c, right panel). To further confirm this, we examined Klotho deglycosylation of TLR4. Klotho has been shown to

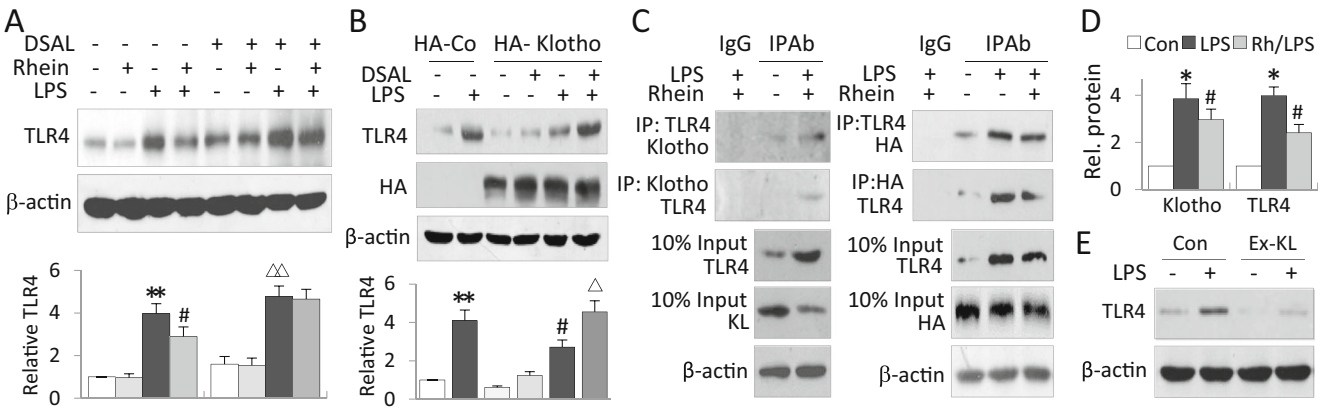

Fig. 5 Klotho targets and degrades TLR4 in a β-glucuronidase-sensitive manner. **a** Rhein β-glucuronidase-sensitive down-regulation of TLR4. RAW cells were treated with Rhein (10 μg/ml) and/or LPS (100 ng/ml) in presence or absence of β-glucuronidase inhibitor DSAL (1 μM) for 24 h, and then, TLR4 protein levels were assayed by Western blotting. Quantification is underneath the figure (**$p < 0.01$ vs. control; $^{\#}P < 0.05$ vs. LPS treatment; $^{\triangle\triangle}P < 0.01$ vs. control in DSAL-treated cells). **b** Klotho β-glucuronidase-sensitive down-regulation of TLR4. HEK293 cells were transfected with control or plasmid encoding HA-tagged Klotho, and then, cells were treated with LPS (100 ng/ml) in presence or absence of DSAL (1 μM) for 24 h. TLR4 and HA-tagged Klotho were assayed by Western blotting. Quantification of Fig. 5c is underneath the figure (**$p < 0.01$ vs. control, $^{\#}P < 0.05$ vs. LPS treatment in HA-vector cells; $^{\triangle}P < 0.05$ vs. LPS treatment in HA-Klotho cells. **c** Klotho targets TLR4. Left panel: RAW cells were treated with LPS (100 ng/ml) for 4 h. The cell lysates were subjected to reciprocal immunoprecipitations using Klotho or TLR4 antibody and then immune-blotting using TLR4 or Klotho antibodies, respectively. A 10% of initial cell lysates served as internal controls (Int). Right panel: The HEK293 cells were first transfected with an HA-Klotho expression plasmid, treated with Rhein (10 μg/ml) and/or LPS (100 ng/ml) for 12 h, and then, cell lysates were subjected to reciprocal co-immunoprecipitation assays using anti-HA or TLR4 antibody in IP and subsequent anti-TLR4 or HA antibody in Western blot as above. **d** Quantifications of immune-precipitated TLR4 and HA-Klotho of right panel in Fig. 5c (*$P < 0.05$ vs. non-LPS-treated control; $^{\#}P < 0.05$ vs. LPS treatment). Data were presented as mean ± SD of three independent experiments. **e** Soluble Klotho decreases TLR4. RAW cells were incubated with recombinant soluble Klotho (0.4 nM) with or without LPS (100 ng/ml) for 12 h; then, cell lysates were precipitated with agarose-bound *Sambucus nigra* lectin and the precipitants were examined by Western blotting with anti-TLR4 antibody

specifically remove α2,6-, but not α2,3-linked sialic acids from its substrate TRPV5 [41]. By using agarose-bound *Sambucus nigra* lectin that specifically recognizes α2,6-linked sialic acids, we showed that recombinant soluble Klotho containing only extracellular domain reduced the glycosylated TLR4 under both resting and LPS-stimulated conditions (Fig. 5e). Taken together, these results suggest that Klotho down-regulates TLR4 via reducing TLR4 glycosylation.

Rhein restoration of Klotho inhibits LPS-induced NF-κB signaling and inflammatory cytokine expression in macrophages

To confirm the critical role of Klotho restoration by Rhein in the inhibition of TLR4-associated inflammation, we examined the effect of Klotho knockdown on Rhein inhibition of TLR4-associated inflammatory signaling and inflammatory cytokine expressions. Klotho knockdown by shRNA in HK2 cells eliminated the Rhein inhibition of IκBα degradation (Fig. 6a), abrogated Rhein inhibition of NF-κB nuclear translocation (Fig. 6b), and the NF-κB reporter transcription incurred by LPS (Fig. 6c). Consistently, Rhein inhibition of LPS-induced pro-inflammatory cytokine expression of TNFα and NOS2 was also significantly abolished in cells lacking Klotho (Fig. 6d), indicating that Klotho restoration by Rhein effectively mitigates LPS-induced inflammatory responses in cells.

Klotho is crucial for Rhein protection against acute renal inflammation and kidney injury

To further explore the in vivo relevance of Klotho restoration by Rhein, we tested the effects of Klotho knockdown by small interfering RNA (siRNA) on Rhein anti-inflammation and renoprotection in a mouse model of LPS-induced AKI. We injected mice with either siRNA-control or siRNA-Klotho, subjected mice to Rhein, LPS or Rhein plus LPS treatment for 24 h, and then examined the kidney pathohistological changes and renal pro-inflammatory cytokine expressions. Klotho knockdown noticeably caused the increase of LPS-induced renal tubule injuries, judged by renal tubule dilatation, renal cell flattening or vacuolization (Fig. 7a, b), E-cadherin and IκBα reductions, TLR4 accumulation (Fig. 7c), and enhanced expressions of TNFα, IL-1β, and NOS2 (Fig. 7d); however, Rhein treatment markedly reduced the renal tubule injuries (Fig. 7a, b), attenuated the abnormal expressions of TLR4, E-cadherin, and IκBα (Fig. 7c, d), and alleviated the inductions of TNFα, NOS2, and IL-1β (Fig. 7d) in control mice, but the beneficial effects were largely abolished in mice injected with siRNA-Klotho (Fig. 7a–d). Thus, Klotho restoration by Rhein not only inhibits TLR4

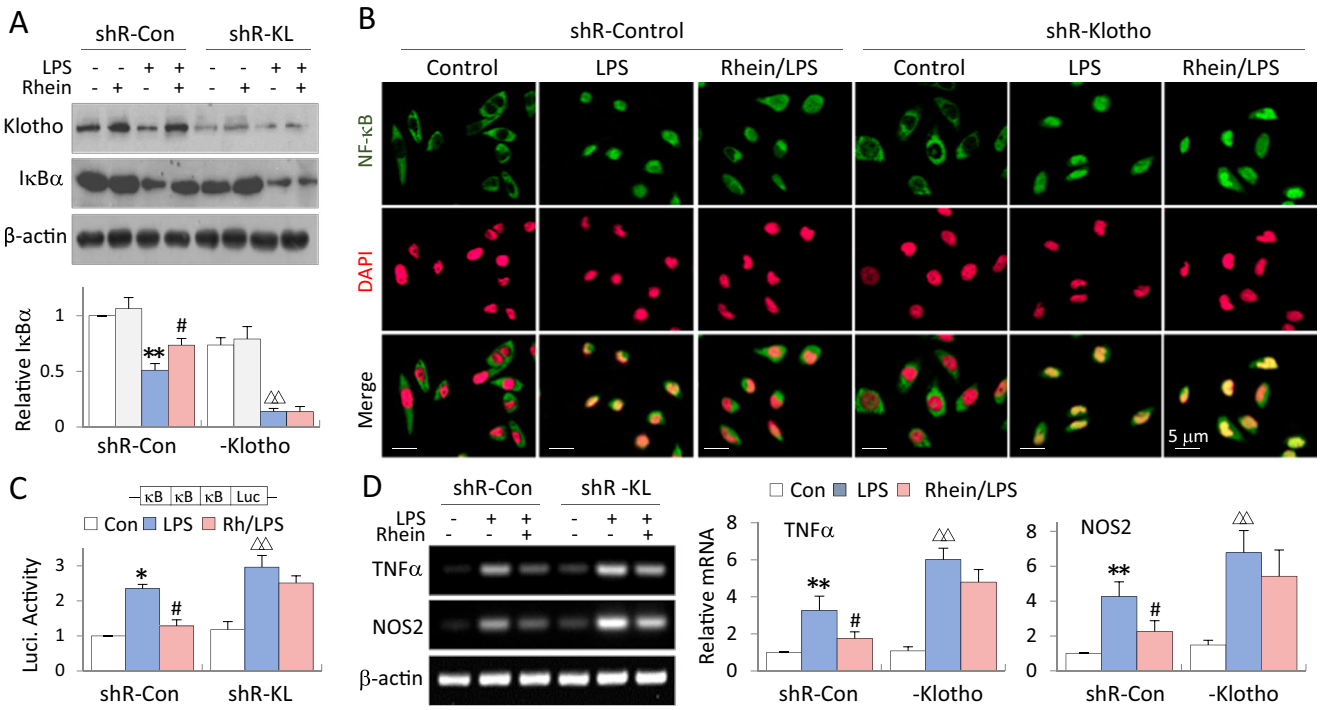

Fig. 6 Rhein restoration of Klotho inhibits LPS-induced NF-κB activation and inflammatory cytokine expression in macrophages. **a** HK2 cells were first transfected with control or shRNA-Klotho plasmid for 24 h, respectively, and then treated with LPS (100 ng/ml) and/or Rhein (10 μg/ml) for 30 min. The expressions of Klotho and IκBα were examined by Western blotting. Quantification of IκBα in Fig. 6a was underneath the figure. **b** Immunofluorescent staining of NF-κB nuclear translocation. HK2 cells were transfected with either shRNA-control or shRNA-Klotho for 24 h, then treated with Rhein (10 μg/m) in presence or absence of LPS (100 ng/ml) for 30 min. The cells were stained with anti-NF-κB antibody (top panel), counter-stained with DAPI (middle panel), and the figures were merged (lower panel). The original blue nuclear DAPI staining was converted to red so that the nuclear green NF-κB staining was shown in orange for partial or in yellow for complete co-localization with nuclear red DAPI staining for easy recognition. **c** NF-κB reporter luciferase assay. HK2 cells were first co-transfected with control or Klotho specific shRNA plasmid plus NF-κB reporter and a control renilla luciferase plasmid, then treated with LPS (100 ng/ml) and/or Rhein (10 μg/ml) for 24 h. The NF-κB luciferase activities were normalized with renilla control and represented as fold changes. **d** Pro-inflammatory cytokine expression. HK2 cells transfected and treated as in Fig. 6a for 24 h were tested for TNFα and NOS2 mRNA levels by RT-PCR. Bar graphs on the right: quantifications of Fig. 6d. The statistics were based on at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control in control plasmid-transfected cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS treatment in control plasmid-transfected cells; $^{\triangle\triangle}P < 0.01$ vs. control in shR-Klotho-transfected cells

signaling and the associated inflammation in cells, but also attenuates LPS-induced acute inflammatory kidney injuries.

Discussion

Klotho's anti-inflammation property is emerging as a critical part of its anti-aging functions. We have presented several novel findings in this study: (1) Klotho is a known renal tubule cell-enriched protein and its substantial expressions in human and mouse mono-macrophages indicate that Klotho directly influences local and systemic inflammations; (2) Klotho suppresses inflammation mainly via decreasing TLR4 through a deglycosylation-associated proteolytic process, which interrupts the subsequent inflammatory responses and attenuates LPS-induced AKI; (3) Rhein can effectively preserve Klotho after LPS insult, providing strong evidence that Klotho loss after acute inflammation can be prevented, which beneficially changes the course of inflammatory responses and contributes significantly to its renal and extrarenal protective functions (Fig. 8).

Klotho repression can be caused by a number of cellular stressors and inflammatory mediators such as IFN-γ (interferon-γ), IL-1β, TNFα, and TGFβ [17, 42, 43], as well as epigenetic modifications of aberrant protein acetylation [42] and DNA methylation [43]. Klotho promoter contains functional NF-κB binding site [42] and a long stretch of GC islands [35, 43]. Since acute inflammatory stimuli and the associated signaling tend to be temporal, it is likely that early and sustained Klotho repressions observed in various acute and chronic renal injuries involve both a transcription factor-mediated early and epigenetic modification-associated prolonged suppression phases. Several recent studies reported that up-regulation of endogenous Klotho by resveratrol, testosterone, statins, or rapamycin exerted impressive renal protective effects in both cell assays and in animal studies of renal diseases [44–47]. We previously showed that Klotho preservation by Rhein prevented Klotho loss from obstructive and chronic renal

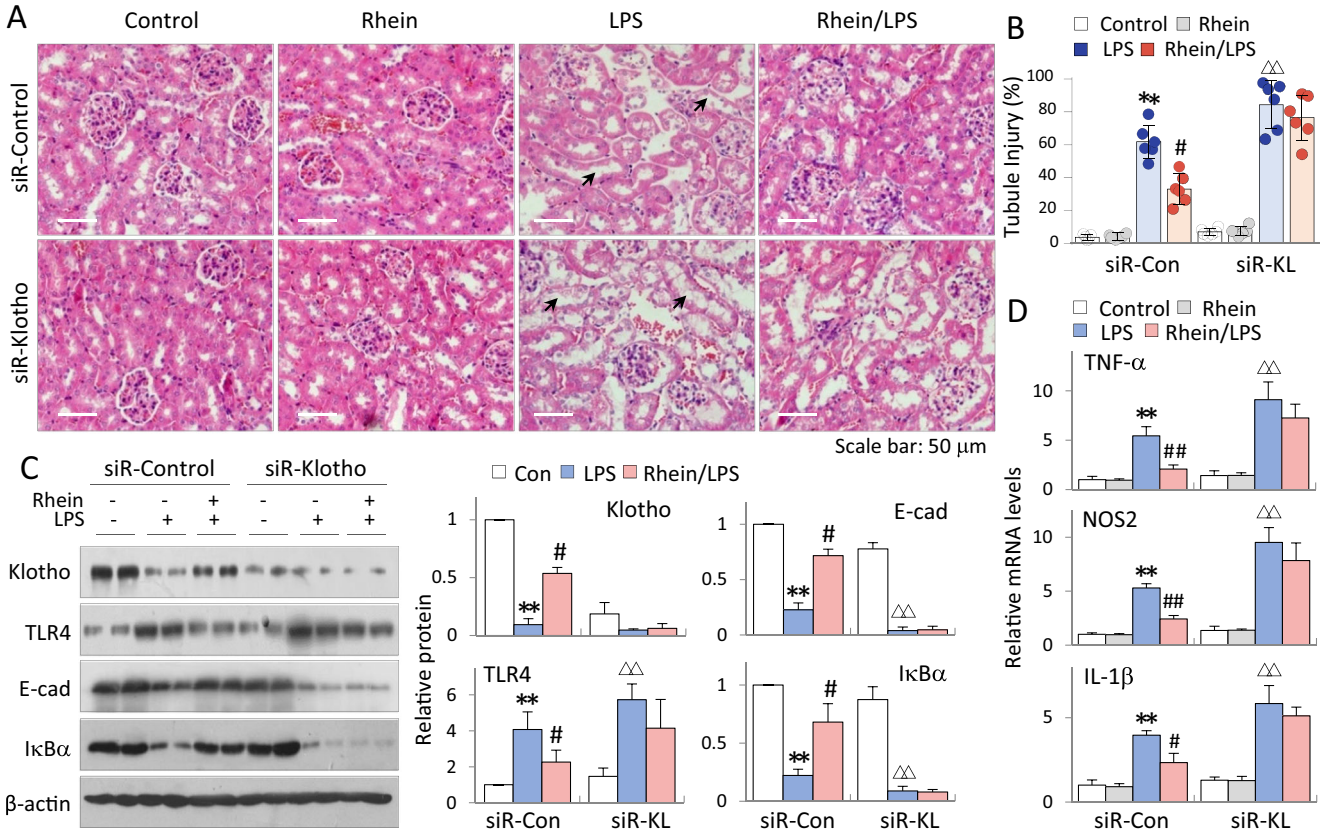

Fig. 7 Klotho is crucial for Rhein protection against acute renal inflammation and kidney injury. Mice injected with either siRNA-control (siR-Con) or siRNA Klotho (siR-KL) were treated with Rhein in presence or absence of LPS for 24 h. **a** Representative photomicrographs of H&E-stained kidney sections. Tubular damage, including tubular dilatation and tubular cell flattening or vacuolization (indicated by dark arrows), was observed in some local regions of renal cortex in mouse kidney, which were exacerbated in mice lacking Klotho (lower panel). **b** Kidney tubule injury scores of Fig. 7a. Data are presented as the ratio of damaged renal tubule including tubular dilatation, tubule cell flattening, or vacuolization over the total visible tubules in the cortex area ($n = 6$ in each group). **c** Renal expressions of Klotho, TLR4, E-cadherin (E-cad), and IκBα were assayed by Western blotting (two randomly selected samples from each group were shown). The bar graph on right: quantifications of Fig. 7c based on all animals in each group. **d** Renal mRNA levels of TNFα, NOS2, and IL-1β from mice in Fig. 7a were assayed by qRT-PCR and quantified as fold changes ($n = 6$ in each group). Data were presented as mean ± SD. $^{**}P < 0.01$ vs. control in siR-control mice; $^{\#}P < 0.05$ vs. LPS treatment in siRNA-control mice; $^{\triangle}P < 0.05$ or $^{\triangle\triangle}P < 0.01$ vs. control in siRNA-Klotho mice

injuries through an epigenetic demethylation mechanism [20, 21]. Now, our study demonstrates that Rhein also effectively prevents Klotho loss from LPS-induced acute inflammatory process, supporting the scenario that both early and sustained Klotho losses can be targeted with beneficial renoprotective consequences.

Klotho beneficially regulates many cellular processes through various mechanisms. Soluble Klotho reportedly contains glycosidase activities, through which it stabilizes epithelial Ca(2+) channel TRPV5 (transient receptor potential cation channel subfamily V member 5) by removing its sialic acid residues, allowing galectin-1 binding and its membrane retention [38, 41]. Klotho also β-glucuronidase-dependently increases the abundance of the excitatory amino acid transporters (EAATs) [39]. On the contrary, Klotho deglycosylates sodium and phosphorus transporter NaPi-2a and causes its proteolytic degradation [48]. Therefore, Klotho deglycosylation activities may either positively or negatively affect the stabilities and functions of its substrates. Our results showed that Klotho physically associated with TLR4 and down-regulated TLR4 in an LPS stimulation- and glycosylation inhibition-sensitive manner, strongly indicating that TLR4 is a substrate of Klotho which degrades TLR4 via a deglycosylation process. Further, Klotho has been shown to reduce renal fibrosis via promoting autophagic degradation of collagen I, leading to reduced extracellular matrix deposition [49, 50]. Our results showed that Klotho promotes TLR4 degradation likely through autophagy pathway under AKI condition, suggesting that Klotho-associated aggrephagy, -autophagic clearance of TLR-induced aggregation of signaling proteins, contributes to its anti-inflammatory and renoprotective functions. Our results also suggest that Klotho likely affects other TLR family members in a similar fashion since all TLR family members share the structure and glycosylation similarities, which warrants further investigation.

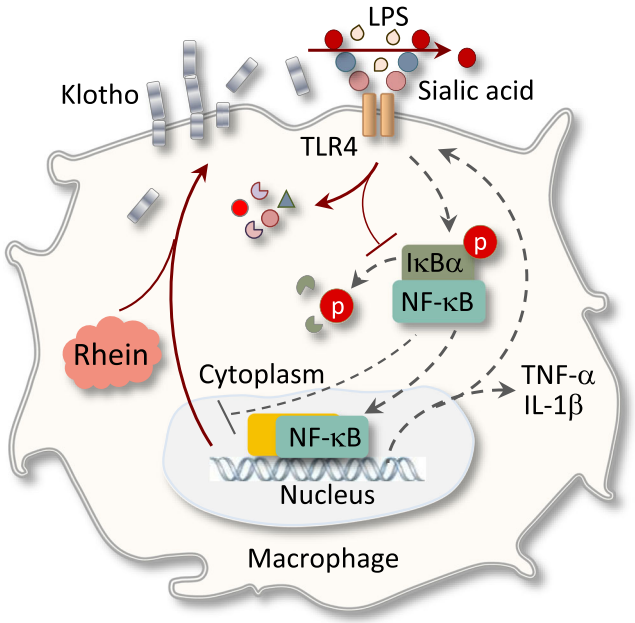

Fig. 8 A schematic diagram of Klotho restoration by Rhein, subsequent TLR4 suppression, and the inhibition of TLR4-associated inflammatory responses. LPS induces TLR4 activation and accumulation in macrophages, which causes IκBα phosphorylation and degradation, subsequent NF-κB nuclear translocation, and the inductions of pro-inflammatory cytokines such as TNFα, NOS2, and IL-1β with concomitant Klotho suppression (dashed lines). Rhein preserves Klotho, which removes sialic acid from glycosylated TLR4 and causes its degradation, leading to the inhibition of TLR4 signaling and pro-inflammatory responses (solid lines)

Glycosylation modifications are pivotal for protein's structural integrity, cellular localization, and proper functions. Protein glycosylation modification modulates enzyme and hormone activity, regulates protein intracellular traffic, controls protein folding, ligand recognition, and cell-cell interactions [51]. In particular, N-glycosylation aids in folding of the nascent polypeptide chain and stabilizes the mature glycoprotein. When it is prevented by glycosidase, the protein will not maturate and undergo subsequent proteasomal or lysosomal degradation [29]. The N-terminal ectodomain of human TLR4 contains at least nine N-linked glycosylation sites which ensure its membrane trafficking and expression after ligand stimulation [52]. TLR4 mutants carrying substitutions in Asn526 or Asn575, the two key N-linked glycosylation sites, failed to be transported to the cell surface [53]. Further, tunicamycin, an N-glycosylation inhibitor, blocked the membrane presence of TLR4 and led to its lysosomal degradation [52]. Thus, Klotho deglycosylation of TLR4 will reduce its stability and promote its degradation, leading to the inflammation inhibition as observed in our study and others. Future structure/glycosylation studies of TLR4 or other members of TLR will reveal the precise mode of deglycosylation patterns mediated by Klotho.

In conclusion, our data demonstrate that Klotho down-regulation of TLR4 via its proteolysis negatively controls TLR-associated inflammatory signaling and Rhein preservation of Klotho effectively suppresses TLR4 and contributes, at least in a significant part, to its anti-inflammatory and renal protective functions. Since Klotho suppressions have been reported in various cancers and aging-related disorders [54, 55], our study also opens up new perspectives for the future exploration of Klotho-targeted interventions by natural or synthetic compounds in the prevention and treatment of renal inflammatory diseases, cancer, and aging-related disorders.

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Compliance with ethical standards

Use of animal and the experimental procedures were in accordance with the animal use guidelines and approved by the Institutional Animal Care and Use Committee of Nanjing University School of Medicine (Nanjing, China).

Conflict of interest The authors declare that they have no conflict of interest.

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