αKlotho deficiency in acute kidney injury contributes to lung damage

Priya Ravikumar,1,4 Liping Li,4 Jianfeng Ye,4 Mingjun Shi,4 Masatomo Taniguchi,1,4 Jianning Zhang,1,4 Makoto Kuro-o,3,4,5 Ming Chang Hu,1,4 Orson W. Moe,1,2,4 and Connie C. W. Hsia1

1Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas; 2Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas; 3Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas; 4Charles and Jane Pak Center of Mineral Metabolism and Clinical Research, University of Texas Southwestern Medical Center, Dallas, Texas; and 5Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan

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Ravikumar P, Li L, Ye J, Shi M, Taniguchi M, Zhang J, Kuro-o M, Hu MC, Moe OW, Hsia CCW. αKlotho deficiency in acute kidney injury contributes to lung damage. J Appl Physiol 120: 723–732, 2016. First published December 30, 2015; doi:10.1152/japplphysiol.00792.2015.—αKlotho is a circulating protein that originates predominantly from the kidney and exerts cytoprotective effects in distant sites. We previously showed in rodents that the lung is particularly vulnerable to αKlotho deficiency. Because acute lung injury is a common and serious complication of acute kidney injury (AKI), we hypothesized that αKlotho deficiency in AKI contributes to lung injury. To test this hypothesis, we created AKI by renal artery ischemia-reperfusion in rats and observed the development of alveolar interstitial edema and increased pulmonary oxidative damage to DNA, protein, and lipids. Administration of αKlotho-containing conditioned media 6 h post-AKI did not alter plasma creatinine but improved recovery of endogenous αKlotho production 3 days post-AKI, reduced lung edema and oxidative damage, and increased endogenous antioxidative capacity in the lung. Intravenously injected αKlotho rapidly exits alveolar capillaries as a macromolecule, suggesting transcytosis and direct access to the epithelium. To explore the epithelial action of αKlotho, we simulated oxidative stress in vitro by adding hydrogen peroxide to cultured A549 lung epithelial cells. Purified recombinant αKlotho directly protected cells at 20 pM with half-maximal effects at 40-50 pM, which is compatible with circulating αKlotho levels. Addition of recombinant αKlotho activated an antioxidant response element reporter and increased the levels of target proteins of the nuclear factor erythroid-derived 2 related factor system. In summary, αKlotho deficiency in AKI contributes to acute lung injury by reducing endogenous antioxidative capacity and increasing oxidative damage in the lung. αKlotho replacement partially reversed these abnormalities and mitigated pulmonary complications in AKI.

oxidative stress; total antioxidant capacity; cytoprotection; antioxidant response element; nuclear factor erythroid-derived 2 transcription factor.

ACUTE LUNG INJURY culminating in acute respiratory distress syndrome (ARDS) is a life-threatening complication of systemic organ failure such as acute kidney injury (AKI). In general, pulmonary dysfunction in AKI is underappreciated and its complex multifactorial pathophysiology remains incompletely understood. There are a host of “reno-pulmonary” pathways in clinical and experimental settings that can compromise lung function in AKI (8, 10, 42, 47, 55). Septic AKI patients with multiorgan failure are especially prone to developing ARDS. In nonseptic patients, impaired cardiac contractility, metabolic acidosis, and volume overload (26, 33, 34) can all contribute to respiratory failure and a high mortality. Independent from these septic and cardiovascular issues, there are tissue factors such as apoptosis, macrophage-derived mediators, chemokines, and ion channel derangements (12, 28, 29, 44) that have been implicated in the pathogenesis of alveolar hyperpermeability leading to the “inflamed, leaky, and wet” lung with low-pressure pulmonary edema. Patients with AKI develop significant functional impairment of diffusing capacity, forced vital capacity, and maximal ventilation (8, 10, 42, 47, 55). The occurrence of ARDS requiring ventilator support drastically increases AKI mortality from 29% to 81% (6). There is a dire need for strategies to protect the lung against secondary injury in the presence of extrapulmonary organ failure such as AKI.

The αKlotho gene was discovered when its serendipitous disruption led to premature multiorgan failure in mice (31). αKlotho is a single-pass transmembrane protein produced predominantly by the kidney that functions as an obligate coreceptor for fibroblast growth factor 23, which is crucial for mineral metabolism (22). The extracellular domain of αKlotho is released via cleavage by secretases into blood, urine, and cerebrospinal fluid as endocrine-soluble αKlotho (14, 24, 36) exerting multiple effects, including antioxidation, antiapoptosis, and antiﬁbrosis, on distant organs (15, 22). αKlotho is not normally expressed in the lung, but the alveolar capillary bed is constantly exposed to circulating αKlotho via perfusion by the entire cardiac output. Hemizygous genetic αKlotho haploinsufﬁcient mice are normal in all organs at baseline except the lung, which exhibits age-exacerbated degenerative changes such as air space enlargement and increased compliance (46, 50), suggesting particularly high sensitivity of the lung to αKlotho deficiency. We have shown that administration of conditioned media containing recombinant αKlotho protein protects the lung and lung epithelial cells against hyperoxic damage by increasing endogenous antioxidative capacity in vivo and in vitro (46).

Because circulating soluble αKlotho originates mostly from the kidney, AKI is a state of transient systemic αKlotho deficiency (20, 43). AKI per se is also a state of heightened systemic oxidative stress (40). These observations beg the question of whether αKlotho deficiency in AKI contributes to the development of acute lung injury. We hypothesized that αKlotho deficiency is a mediator of pulmonary damage and dysfunction in AKI. To test this hypothesis, we used a rodent renal ischemia-reperfusion injury (IRI) model of AKI and its associated αKlotho deficiency to characterize and quantify lung injury. To investigate causality, we replenished serum αKlotho in AKI with exogenous αKlotho-containing condi-
tioned medium and queried whether pulmonary abnormalities are alleviated. In addition, we visualized the in vivo transit of circulating αKlotho, a 130-kDa macromolecule, from the alveolar capillary to the interstitium and epithelium. Finally, we simulated oxidative stress in vitro by adding hydrogen peroxide (H₂O₂) to cultured lung epithelial cells, and tested the antioxidative cytoprotective mechanisms of αKlotho cytoprotection.

METHODS

Animal Models

All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals by The National Institutes of Health. The Institutional Animal Care and Use Committee at University of Texas Southwestern approved all study protocols. An established IRI model was conducted as previously described (20). Briefly, male Sprague-Dawley rats (250–350 g) were housed in a 12:12-h light-dark cycle with free access to rodent chow and water. Under anesthesia, renal arteries were clamped with arterial clips (30 min), and occlusion was visually verified as blanching of the entire kidney surface. After clips were removed, the kidneys were observed for at least 5 min to ensure reperfusion. Control (Sham) (30 min), and occlusion was visually verified as blanching of the kidneys without arterial clamping. At 3 days following surgery, blood was drawn and stored at −80°C for further processing. Plasma and urine chemistry was analyzed using a Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis, Rochester, NY). Serum αKlotho concentration was measured by immunoprecipitation-immunoblot (1).

To examine the effects of αKlotho replacement in AKI, the rats received intraperitoneal injections of either control medium (100 μl), or αKlotho-containing conditioned medium (100 μl, 60–100 pM, n = 8) 6 h after induction of IRI (see Conditioned αKlotho-Containing Medium and Recombinant Soluble αKlotho for preparation of conditioned medium). Seven to eight animals per group were used. Three days after IRI, rats were killed by intraperitoneal overdose injection of Euthasol. The left lung was perfused with sterile PBS and snap-frozen in liquid nitrogen for the assays described later. The right lung was fixed by tracheal instillation of 4% paraformaldehyde at 25 cmH₂O pressure. Two control mice received injection of vehicle (saline).

Conditioned αKlotho-Containing Medium and Recombinant Soluble αKlotho

Two types of αKlotho preparations were used. Conditioned media containing αKlotho were prepared as previously described (46) for use in animal experiments. Briefly, Chinese hamster ovary (CHO) cells were stably transfected with soluble αKlotho or empty vector. Serum-free DMEM was added 16 h posttransfection and, after another 16 h, media were collected and stored at −80°C until further processing. The conditioned medium contains recombinant soluble αKlotho along with other secreted proteins from the CHO cells. The control media were obtained from CHO cells transfected with only the expression plasmid.

Purified recombinant αKlotho was used in αKlotho tracking and in cell culture experiments. Murine soluble αKlotho was cloned into pEF1/Myc-His/A vector (Invitrogen) and transfected into suspension of Free-Style 293-F cells (Invitrogen). To establish stable cell lines that expressed recombinant protein, cells were selected in 500 μg/ml G418 (Gibco, Grand Island, NY). Single clones of stable αKlotho-expressing cells were cultured in serum-free medium (Invitrogen) with 100 μg/ml G418 and 10% Pluronic F-68. The condition media were collected at suspension cell density ~300 × 10⁴ and centrifuged at 15,000 g for 30 min at 4°C to remove cell debris. The supernatant was mixed with Ni-NTA resin (Thermo, Waltham, MA) that was pre-equilibrated with 20 mM phosphate, pH 7.4, 300 mM NaCl. After incubation at 1 h at room temperature, the Ni-NTA resin was packed into the Econo column and washed with the above buffer (35× column volume). Bound αKlotho was eluted by 50–250 mM imidazole. The highest concentration αKlotho protein was eluted at the second fraction by 50 mM imidazole. αKlotho protein quality was checked by SDS-PAGE, Coomassie blue staining of a single band, and immunoblot with specific αKlotho antibodies (data not shown).

Lung Edema Estimation (Na⁺ to Dry Lung Weight Ratio)

Lung tissue (~100 mg) was weighed and transferred to a platinum ashing crucible and placed on a hot plate set at 100°C under a heat lamp for 2 h. The dried-sample-containing crucible was weighed to determine the sample dry weight. The crucible was placed overnight in the ash oven set at 600°C, removed, and weighed to determine the ash weight. The ash was dissolved in 2 ml of HCl, and the Na⁺ content was measured by flame photometry.

Cell Culture

Human A549 lung epithelial cells (American Type Culture Collection, Manassas, VA) were grown in DMEM-nutrient mixture F12 (Life Technologies, Grand Island, NY) with 1% l-glutamine. An in vitro oxidative stress model using H₂O₂, modified from one employed by Panesso et al. (43) in kidney cells, was used to study the cytoprotective effects of αKlotho. Triplicate experiments and assays were performed.

Biochemical Assays

Cell injury. A lactate dehydrogenase (LDH) cytopotoxicity detection kit (Clontech Laboratories, Mountain View, CA) was used to measure release of cytoplasmic LDH into the culture supernatant. LDH generates NADH from lactate, which reacts with diaphorase to generate formazan dye products (absorbance 490 nm).

Total antioxidant capacity. Both copper- and iron-based assays were performed. Copper-reducing equivalents were measured using a colorimetric assay (OxiSelect; Cell BioLabs, San Diego, CA). The Trolox antioxidant assay (Sigma-Aldrich) is based on formation of a radical cation, a soluble chromogen (absorbance 520 nm). The oxidized form of Trolox is less soluble and has lower absorbance. Both copper- and iron-based assays were performed. Copper-reducing equivalents were measured using a colorimetric assay (OxiSelect; Cell BioLabs, San Diego, CA). The Trolox antioxidant assay (Sigma-Aldrich) is based on formation of a radical cation, a soluble chromogen (absorbance 520 nm). The oxidized form of Trolox is less soluble and has lower absorbance.
Antioxidant reporter. A dual-luciferase assay (Cignal; SA Biosciences, Valencia, CA) was used to measure the activity of Nrf-1 and -2 (nuclear factor E2-related factors 1 and 2) transcription factor activation of tandem antioxidant response element (ARE) regulating genes that protect cells from oxidative damage. The ARE reporter contains an inducible antioxidant responsive firefly luciferase construct and a constitutively expressed Renilla luciferase construct. Upon transfection with the ARE reporter, the activities of the firefly and Renilla luciferases quantify transcriptional activities of the antioxidant response pathway.

Oxidative damage. 8-Hydroxydeoxyguanosine (8-OHdG) was used as a marker of oxidative DNA injury. DNA was extracted using DNAzol (Life Technologies), precipitated in 100% ethanol, washed with 70% ethanol, and suspended in 8 mM NaOH, and the 8-OHdG concentration was determined by ELISA (OxiSelect; Cell BioLabs) compared against a 8-OHdG standard curve. Protein carbonyl was measured by ELISA (OxiSelect; Cell BioLabs) as a surrogate for protein oxidation against a known reduced/oxidized BSA standard curve. 8-Isoprostane was measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as a marker for lipid oxidation, based on the competitive binding between 8-isoprostane and an 8-isoprostane acetylcholinesterase conjugate (8-isoprostane tracer) for limited specific binding sites.

Immunoblot. Whole cell lysates from A549 cells after treatment were prepared using 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 and protease inhibitors and cleared by centrifugation (14,000 \( g \), 4°C, 30 min), and protein content was determined by the method of Bradford. Protein samples (30 \( \mu \)g) were loaded onto SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% nonfat milk, PVDF membranes were probed with primary antibodies against MT (Metallothionein, Santa Cruz Biotechnology, San Diego, CA, sc-11377, 1:500), HOX1 (Heme Oxygenase 1, Abcam, Cambridge, MA, ab13248, 1:500), HOX2 (Heme Oxygenase 2, Abcam, ab90492, 1:1000), and PXDN (Peroxidasin homolog, Abcam, Cambridge, MA, ab90492, 1:1000), and IgG-HC, immunoglobulin heavy chain.

Fig. 1. \( \alpha \)Klotho deficiency and repletion in acute kidney injury (AKI). Sprague-Dawley rats underwent bilateral ischemia-reperfusion injury (IRI), and lungs were harvested 3 days later. The control group underwent general anesthesia and laparotomy with manual manipulation of the kidneys but without IRI (Sham). Rats received intraperitoneal injection of either \( \alpha \)Klotho-containing or control conditioned media 6 h after surgery. A: severity of AKI was measured by serum creatinine level in the animals 3 days after IRI. B: serum \( \alpha \)Klotho level was measured by immunoprecipitation-immunoblot 3 days after IRI at the time of lung harvest. Representative blots are shown. IgG-HC, immunoglobulin heavy chain. C: representative lung histology (Trichrome stain). D: Na\(^+\) to dry weight ratio as a measure of interstitial edema. Means ± SD (n = 7–8 animals per group). Statistical significance was assessed by ANOVA.
Acute Lung Injury in AKI

The established model of renal ischemia-reperfusion injury markedly increased serum creatinine, which typically peaks at five to eight times normal control levels at 1–2 days as reported by Hu et al. (20), then declined to two to three times control levels by Day 3 (Fig. 1A), and reduced circulating αKlotho levels by ~60% (Fig. 1B). In this AKI setting, lung histology showed small crowded distal air spaces, thicker alveolar walls, and cellular edudation into the air spaces (Fig. 1C). The Na⁺ to dry lung weight ratio was elevated, indicating tissue edema (Fig. 1D). The lungs from AKI animals showed generalized oxidative damage of DNA, protein, and lipids compared with control (sham AKI) lungs (Fig. 2A), which is consistent with heightened oxidative stress to the lungs in AKI in a manner similar to that previously described by Ravikumar et al. (46) in direct lung injury caused by acute hyperoxia exposure. Total antioxidant capacity measured by copper-reducing equivalents and Trolox (a water-soluble vitamin E analog) equivalent assays showed diminished antioxidant capacity with AKI. These results show an association between circulating αKlotho deficiency in AKI and lung damage, which may reflect depletion of endogenous antioxidants in AKI (Fig. 2B).

Role of αKlotho in Lung Protection

αKlotho replacement given before or within 1 h after IRI alleviates AKI and accelerates recovery (20), but αKlotho repletion given 4 h or later post-IRI does not affect the peak plasma creatinine (vehicle vs. αKlotho-treated in AKI in a separate cohort of 3 animals each: Day 1: 1.35 ± 0.17 vs. 1.27 ± 0.09 mg/dl; Day 2: 1.25 ± 0.26 vs. 1.13 ± 0.15 mg/dl, means ± SD). Therefore, in this study, by administering αKlotho 6 h after AKI induction by IRI, we did not change the course of AKI as defined by serum creatinine levels on Day 3 of AKI that were similar to that in the untreated group (Fig. 1A). However, exogenous αKlotho administration improved serum endogenous αKlotho levels 3 days after AKI (Fig. 1B). The higher αKlotho levels 3 days post-IRI are unlikely to be attributed to the exogenous αKlotho, because the half-life of αKlotho even in the complete anephric state is only 24 h (21); rather, this observation reflects improved endogenous αKlotho production. Exogenous αKlotho also increases native renal αKlotho expression after two forms of AKI including bilateral IRI (48).

Exogenous αKlotho administration significantly ameliorated alveolar septal crowding and exudation of cells and debris into the air space (Fig. 1B); alveolar edema (Fig. 1C); and oxidative damage to DNA, protein, and lipids in the lungs of animals with AKI (Fig. 2A). The reduction of oxidative damage with αKlotho repletion was associated with a higher endogenous oxidative capacity measured by two independent assays (Fig. 2B). These effects occurred with no change in serum creatinine (Fig. 1A), thus dissociating the benefit on the lung from the improvement of renal function by αKlotho. In control (sham) animals, exogenous αKlotho modestly increased total antioxidant capacity measured by copper-reducing equivalents but had no effect on Trolox total antioxidant capacity or any of the oxidative damage parameters. These interventional data strongly sup-

**Fig. 2.** Acute lung injury in AKI. Sprague-Dawley rats underwent bilateral IRI and lungs were harvested 3 days later. The control group underwent general anesthesia and laparotomy with manual manipulation of the kidneys but without IRI (Sham). Rats received intraperitoneal injection with either αKlotho-containing or control conditioned media 6 h after surgery, and lungs were harvested 3 days later. A: markers of DNA (8-hydroxy-2’-deoxyguanosine; 8OHdG), protein (protein carbonyl), and lipid (8-isoprostane) oxidative damage. B: markers for endogenous antioxidative capacity were assessed by a Cu-based (left) or a Fe-based (right) assay. Bars and error bars are mean and SD (n = 7–8 animals per group). Statistical significance was assessed by ANOVA.
port αKlotho deficiency as a pathogenic factor in the development of acute lung injury in the setting of AKI.

Circulating αKlotho Can Directly Access Pulmonary Epithelial Cells

While the animal experiments offer important proof-of-principle data that αKlotho deficiency exacerbates and αKlotho repletion ameliorates lung injury, the complexities of the intact organism preclude examination of the direct actions of αKlotho on pulmonary epithelia. To understand the mechanisms of αKlotho cytoprotection, we first tracked whether this 130-kDa macromolecule can exit the pulmonary vasculature to access the alveolar epithelium. In the kidney it is known that αKlotho cannot traverse the glomerular capillary, but it can selectively cross the peritubular capillary freely, to be taken up by the tubular cells in a surprisingly short time (21). To test whether rapid transport occurs in pulmonary capillaries, we injected FLAG-tagged αKlotho intravenously in mice. Within 20–30 min after injection, the FLAG label was seen inside alveolar capillaries (Fig. 3, A and B), and also clearly detectable in the septal interstitial space with extracapillary cellular uptake, including possibly by alveolar type II cells (Fig. 3B). Thus circulating αKlotho can rapidly exit the capillary to access and directly act on the alveolar epithelium.

Cytoprotection by αKlotho via Antioxidation

To examine whether αKlotho ameliorates cellular oxidative damage, H$_2$O$_2$ was added to cultured A549 human lung epithelial cells to simulate the post-AKI milieu of oxidative stress. We observed that H$_2$O$_2$ induced cellular damage measured by LDH release in a dose-dependent fashion (Fig. 4A). We chose a H$_2$O$_2$ concentration of 200 nM to induce moderate oxidative stress with measurable cell harm to enable the evaluation of cytoprotection. Previously, we found that αKlotho transfection and the addition of αKlotho-conditioned media prevented A549 cell death from hyperoxic insult (46), but that system did not permit deter-
mination of the αKlotho concentration required for cytoprotection and whether it is comparable to the circulating αKlotho concentration. Here we used purified recombinant αKlotho protein to define the dose range of αKlotho required for cytoprotection. A decline in H$_2$O$_2$-induced LDH release was detectable at a αKlotho concentration of 25 pM, and maximal response was attained at ~200 pM (Fig. 4B). The range where cytoprotective efficacy is observed is in the same order of magnitude as the circulating αKlotho concentration in humans and rodents (1, 21). Consistent with its effect on LDH release, αKlotho addition ameliorated the increase in oxidative damage to DNA, protein, and lipids in a dose-dependent fashion in A549 cells exposed to H$_2$O$_2$ (Fig. 5, A–C).

αKlotho-conditioned media contain many cell-derived substances that can potentially activate the Nrf2 pathway (46). Here we used purified recombinant αKlotho to show that it activates Nrf2 in a dose-dependent fashion in A549 cells challenged with H$_2$O$_2$ (Fig. 6A). We screened an array of downstream effectors of Nrf2 and found that αKlotho increased the protein levels of methalothionine (MTA), heme-oxygenase 1 and 2 (HOX1 and HOX2), and peroxidasin (PXDN; Fig. 6B).

**DISCUSSION**

**Summary of the Rationale and Major Findings**

This is the first report to examine the role of αKlotho in acute lung injury resulting from extrapulmonary organ failure. We previously showed that the lung exhibits heightened oxidative damage in mice with genetic αKlotho insufficiency and that exogenous αKlotho-containing conditioned media protect the lung against hyperoxia-induced acute damage; protection is at least partly mediated via increasing endogenous antioxidant capacity (46). We also previously showed that the rodent IRI model represents a state of acute systemic αKlotho deficiency (20), while the reperfusion phase post-renal ischemia is a state of high oxidative stress (51). Thus the AKI model represents double oxidative insults on the lung and provides the rationale for our hypothesis that the acute αKlotho deficiency in AKI contributes to lung damage.

Our findings support the following hypothesis: 1) acute lung injury characterized by interstitial edema and tissue oxidative damage is detectable in this rodent model of endogenous αKlotho deficiency caused by AKI; 2) repletion of αKlotho 6 h post-AKI does not alter the severity of AKI as measured by serum creatinine levels but significantly reduces the severity of acute lung injury; 3) circulating αKlotho exits the alveolar microvasculature rapidly to reach the alveolar interstitium and epithelium; and 4) αKlotho directly alleviates oxidative damage of pulmonary epithelia in cell culture by augmenting endogenous antioxidative capacity at least partly via activation of Nrf2 effectors. In concert, these findings support the notion that endogenous αKlotho deficiency in AKI contributes to development of acute lung injury and suggest the potential for therapeutic replacement.

**Cytoprotection by αKlotho**

αKlotho is the first member of the three-member KLOTHO gene family (22). αKlotho was touted as an antiaging protein by 2003 (Fig. 5, A–C).

αKlotho deficiency in AKI contributes to lung damage. Our findings support the following hypothesis: 1) acute lung injury characterized by interstitial edema and tissue oxidative damage is detectable in this rodent model of endogenous αKlotho deficiency caused by AKI; 2) repletion of αKlotho 6 h post-AKI does not alter the severity of AKI as measured by serum creatinine levels but significantly reduces the severity of acute lung injury; 3) circulating αKlotho exits the alveolar microvasculature rapidly to reach the alveolar interstitium and epithelium; and 4) αKlotho directly alleviates oxidative damage of pulmonary epithelia in cell culture by augmenting endogenous antioxidative capacity at least partly via activation of Nrf2 effectors. In concert, these findings support the notion that endogenous αKlotho deficiency in AKI contributes to development of acute lung injury and suggest the potential for therapeutic replacement.

**Cytoprotection by αKlotho**

αKlotho is the first member of the three-member KLOTHO gene family (22). αKlotho was touted as an antiaging protein due to the premature multiorgan failure that results from its serendipitous disruption (31) and the life span extension by its ectopic overexpression (32). The extracellular domain of αKlotho is proteolytically cleaved and released into the circulation, cerebrospinal fluid, and urine (4, 5, 18, 24), and exerts a wide array of effects on many target organs (15, 22). The kidney expresses the highest level of αKlotho, and the circulating (soluble) αKlotho comes primarily from the kidney (21, 35). It is not surprising that circulating levels of αKlotho plummets in AKI, with trough levels usually reached around Day 1 (20, 21, 43). The potential diagnostic, prognostic, and
Unlike most organs that manifest no pathology at baseline and are particularly susceptible to oxidative stress (46); Klotho deficiency in rodents (20, 43); Klotho is protective against oxidative stress in general (16). In general, the cytoprotective effects of Klotho have been attributed to its antiapoptotic, antifibrotic, and antioxidative properties (22, 30).

**Pulmonary Involvement and Mechanisms of Acute Lung Injury in AKI**

Pulmonary impairment in AKI is usually attributed to extrapulmonary factors such as sepsis, cardiac dysfunction, volume overload, and metabolic acidosis (8, 10, 42, 55). Of the twelve comorbid variables examined in one study, severe pulmonary involvement requiring mechanical ventilation conferred the worst prognosis elevating mortality from 29% to 81% even after multivariate adjustment (6). Because current treatment modalities for AKI cannot alter the natural course of kidney injury, the prevention or mitigation of complications associated with AKI should be prime therapeutic targets, as they will likely improve clinical outcomes. The possibility that AKI depletes a natural cytoprotective factor thereby predisposing to lung injury had not been examined.

The mechanism of acute lung injury in AKI is complex and multifactorial. αKlotho deficiency is one potentially important pathogenetic factor. Circumstantial observations prompted us to formulate the following hypothesis that αKlotho deficiency contributes to pulmonary dysfunction in AKI: 1) AKI is accompanied by systemic αKlotho deficiency in rodents (20, 43); 2) AKI is a state of heightened oxidative stress (51); 3) the lung is particularly susceptible to αKlotho deficiency and relies on αKlotho to maintain its native antioxidative capacity (46); and 4) αKlotho is protective against oxidative stress in general (30). Unlike most organs that manifest no pathology at baseline in heterozygous hypomorphic αKlotho haplo-insufficient mice, the lungs show age-related morphological abnormalities and increased oxidative DNA damage when the circulating αKlotho level is ~50% of normal (46). If one lowers the circulating αKlotho level further and contemporaneously increases the oxidative stress challenge such as in AKI, it is conceivable that the lungs would be rendered even more susceptible to damage. Using an interventional approach, we showed that post-AKI restoration of circulating αKlotho levels partially reversed the lung damage without altering peak AKI severity. These results support our hypothesis.

**Mechanisms of αKlotho Protection in the Lung**

The finding of partial reversal of lung damage by repletion of circulating αKlotho agrees with earlier findings where αKlotho prevents and ameliorates ischemic and nephrotoxic kidney damage (17, 20, 43). Soluble αKlotho functions as an endocrine substance, although a cognate receptor in its target organs has not been identified. One enigma is how this 130-kDa protein transits through the vasculature and reaches its targets. In AKI, pulmonary capillaries are leaky (2, 28), which can theoretically enhance the exit of macromolecules, but circulating levels of αKlotho are low in AKI (20) so increased permeability alone will not suffice. In the kidney, exogenously injected αKlotho crosses the peritubular capillary readily and reaches the urinary tubular lumen via tandem transcytosis across the peritubular capillary and tubular epithelium, but αKlotho does not traverse the glomerular capillary, indicating selective permeability and suggesting differential endothelial transcytosis (21). Now we demonstrate that αKlotho exits the pulmonary capillary to reach the interstitium and alveolar

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**Fig. 6. Activation of nuclear-factor erythroid derived 2 transcription factor (Nrf2) pathway in lung epithelial cells by αKlotho.** A: A549 cells were transfected with an antioxidant responsive element (ARE)-luciferase reporter and were treated with the stated concentration of recombinant αKlotho. Activation of ARE is expressed as firefly luciferase luminescence ratio to the control Renilla. Average of 3 independent experiments (means ± SD). Statistical significance was assessed by ANOVA. *P < 0.0001 vs. untreated cells, †P < 0.001 vs. cells treated with 200 mM H2O2 and 0 pM αKlotho, ‡P < 0.0001 vs. cells treated with 200 nmol/l H2O2 and 25 pM αKlotho, §P < 0.0001 vs. cells treated with 200 nM H2O2 and 50 pM αKlotho, †P < 0.05 vs. cells treated with 200 nM H2O2 and 100 pM αKlotho. B, left: selected antioxidant protein expressions were quantified by immunoblot. MTA, methalothionine; HOX1 and HOX2, heme oxygenase 1 and 2; PXDN, peroxidasin. β-Actin served as loading control. B, right: densitometry of antioxidant levels was normalized to β-actin and expressed as a ratio to control (no H2O2, no αKlotho). Average of 3 independent experiments (means ± SD). Statistical significance was assessed by ANOVA. *P < 0.05 vs. control, †P < 0.05 vs. 200 mM H2O2 and no αKlotho, ‡P = 0.065 compared with 200 mM H2O2 and no αKlotho.
epithelium. The transport of this large protein across the capillary may occur via caveolae- or clathrin-mediated endocytosis in the capillaries (27, 37, 49) or via vesicular vacuolar organelles toward the venules (9, 11); elucidating these mechanisms will require further investigation.

We previously showed that transfection of either the transmembrane or the extracellular domain of αKlotho increases the endogenous antioxidative capacity of cultured human alveolar type I epithelial cells and A549 lung cell lines (46). Transfection does not replicate the in vivo exposure to circulating αKlotho or allow examination of dose-response relationships. Therefore, we synthesized, purified, and added recombinant αKlotho to cultured A549 cells to test the direct effects conferred by αKlotho and to establish a dose response of αKlotho action. Purified αKlotho exerts protection in concentrations compatible with its circulating levels, although we do not know the intracellular αKlotho concentration in the lung. A single injection of recombinant αKlotho, albeit at a pharmacologic concentration, led rapidly to detectable levels in the pulmonary interstitium. The clearance of αKlotho from the circulation is largely via the kidneys (21), but the mechanism of αKlotho clearance from tissue is unclear. It is conceivable that there may be accumulation of αKlotho in the lung interstitium to levels higher than that in plasma.

The Cap and Collar family of transcription factors comprises four related factors: Nrf1, Nrf2, Nrf3, and p45 NF-E2 (3). Nrf1 is important for counteracting steady-state stress under basal homeostatic states while Nrf2 is crucial for maintaining cell health in response to severe stressors such as reactive oxygen species, inflammatory cytokines, and endoplasmic reticulum stress (7, 41, 53). The ARE binds to Nrf2 and small Maf proteins. The small Maf proteins, originally described in musculoaponeurotic fibrosarcoma, are leucine zipper-containing transcriptional cofactors that function cooperatively with Nrf2 in vivo (38). The AREs are found in the transcriptional regulatory regions of many antioxidant and xenobiotoxin-metabolizing enzyme genes (3, 39) and are thought to account for the concerted upregulation of these genes during oxidative stress. Several lines of evidence support antioxidation via the Nrf2 pathway as a mechanism underlying αKlotho cytoprotection. The stimulation of many antioxidant and detoxification enzyme genes is severely impaired in Nrf2-null mutant mice (25), while αKlotho-overexpressing mice are more resistant to paraquat-induced oxidative stress (54). αKlotho overexpression drives Nrf2 localization to the nucleus in cells (13). Pretreatment of hippocampal neurons with recombinant αKlotho increases expression of the thioredoxin/peroxiredoxin system (56), which is a known downstream effector of the Nrf2 pathway (52). Our findings of increased expression of antioxidant targets of the Nrf2 pathway (MTA, HOX1, HOX2, and PXDN) following αKlotho administration in AKI are consistent with this mechanism of action. In addition to Nrf2, αKlotho also activates the FoxO family of transcriptional factors and induces superoxide dismutase 2 (SOD2) expression (23, 54), and αKlotho-containing conditioned media increase SOD2 expression and nitric oxide production in human umbilical vein endothelial cells (45). Additional mechanisms of αKlotho action such as antipapoptosis may also play a role.

In conclusion, acute lung injury is a life-threatening complication in AKI that significantly escalates morbidity and mortality (6). The pathogenesis of acute lung injury in AKI involves diverse multisystem interactions including cardiac, extracellular fluid volume, metabolic, cytokine, and sometimes sepsis-related factors. We provide evidence that αKlotho secreted from the kidney into the circulation reaches and directly acts on the alveolar epithelia, and its deficiency in AKI is an important contributing factor to the loss of endogenous antioxidative capacity of the pulmonary epithelium. The diminished αKlotho-mediated antioxidative cytoprotection occurs in a setting of heightened oxidative stress, which can compound and amplify the deleterious effects of all the other pathogenic intermediates that contribute to the development of lung injury and ARDS in the setting of AKI. These data suggest that prevention or amelioration of lung damage by αKlotho replacement holds substantial promise as a therapeutic intervention in AKI to prevent or mitigate secondary pulmonary complications. While αKlotho replacement reduced lung damage in the absence of an improvement in serum creatinine, endogenous αKlotho production also improved. Thus the observed cytoprotective effects of αKlotho arose from a combination of direct pulmonary and indirect systemic actions.

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