Klotho Prevents NFκB Translocation and Protects Endothelial Cell From Senescence Induced by Uremia

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Abstract

In patients with renal disease, uremia raises oxidative stress and senescence in endothelial cells, which can lead to endothelial dysfunction and cardiovascular disease. Klotho protein is a β-glucuronidase capable of hydrolyzing steroid β-glucuronides. This protein is recognized as an antiaging gene, that modulate both stress-induced senescence and functional response. The aim of the study was to investigate how senescence and oxidative stress induced by uremia in endothelial cells affects Klotho expression and whether intra or extracellular Klotho has effects on the response of these cells. Senescence and oxidative stress was obtained by exposure to uremic serum. Telomere length, the enzyme β-galactosidase, and oxidative stress were studied by flow cytometry. Nuclear factor kappa B activity was determined by electrophoretic mobility shift assay. The expression of Klotho decreased with the uremia and preceded the manifestations of cell aging. Levels of intracellular Klotho decreases associated to endothelial senescence, and exogenous Klotho prevents cellular senescence by inhibiting the increase in oxidative stress induced by uremia and diminished the nuclear factor kappa B–DNA binding ability.

Key Words: Klotho—Uremic serum—Senescence—NFκB
Klotho is now recognized as an antiaging protein which, when it is overexpressed, extends life span and produces a delay in the development of aging-related diseases, but a defect in Klotho expression accelerates aging and associated diseases (10,11). Klotho has a short membrane-spanning domain and two large extracellular domains; it exists as a transmembrane protein but also has two circulating isoforms, one of which is a shed variant of the membrane form (12).

The most fully characterized form of Klotho is the transmembrane form that interacts with the FGF23 receptor, which is involved in maintaining phosphate metabolism. The second form of Klotho, exogenous, is a soluble protein that circulates in the bloodstream and appears to control cellular senescence by interacting with the damage induced by increased oxidative stress through cellular mechanisms that are still poorly characterized. The third form of Klotho is usually expressed diffusely in the cell cytoplasm but not outside the cell membrane, and it has therefore been suggested that it plays an important role in determining the ability of these cells to support stress (13,14).

Klotho protein is a β-glucuronidase capable of hydrolyzing steroid β-glucuronides. Klotho-deficient mice exhibit increased production of vitamin D, and altered mineral–ion homeostasis is suggested to be a cause of the phenotype that was originally considered a “premature aging–like phenotypes.” The findings that the lowering of vitamin D activity reverses the “premature aging–like” phenotypes and prolongs survival in these mutants, it appears that they are due to Klotho-associated vitamin D metabolic abnormalities (hypervitaminosis) (15).

Endothelial cells do not express Klotho on their surface. However, a recent study demonstrated that these cells synthesize messenger RNA (mRNA) for Klotho and express intracellular Klotho proteins (16). Interestingly, in addition to express intracellular Klotho, endothelial cells also respond to signal(s) induced through Klotho protein(s), and it has been demonstrated that extracellular Klotho prevents senescence and damage induced by increased oxidative stress (17,18). Furthermore, increased nuclear factor kappa B (NFκB) activity regulates the activity of both exogenous and intracellular Klotho in endothelial cells, and thus supports the hypothesis that the endothelial response to Klotho is an active process that is associated with the rise in NFκB activity (2,19).

Previous studies have shown that Klotho expression diminished with cell aging, and this decline preceded manifestations of cell aging such as telomere shortening and β-galactosidase (β-gal) expression. The senescent associated to the injury induced by uremia leads a decrease in the telomere length on endothelial cells (20). Intracellular Klotho is also reduced in human umbilical vein endothelial cells (HUVEC) after short exposure to uremic serum (US) (4,21). In humans, cellular senescence is accompanied by a decrease in telomere length as a result of the loss of telomerase activity. However, cellular senescence can be induced prematurely in response to different types of physiological stress, independently of the number of cell divisions. In endothelial cells, the expression of Klotho protein diminishes with cell aging and stress-induced premature senescence (4). In animal models, Klotho-deficient mice manifest a syndrome resembling accelerated human aging and display extensive and accelerated arteriosclerosis (22). Furthermore, Klotho serves as an anti-inflammatory modulator, negatively regulating the production of NFκB-linked inflammatory proteins (23).

In human, further studies are needed to elucidate the potential biological mechanisms by which circulating Klotho could affect longevity but a recent study has shown that in older community-dwelling adults, plasma Klotho is an independent predictor of all-cause mortality (24). Several studies indicated that in human Klotho is mainly produced in the kidney (25). Reduced Klotho expression is a crucial feature in patients with chronic kidney disease (CKD), and it has therefore been suggested that Klotho is a relevant biomarker of kidney failure. However, as far as we know, the possibility that Klotho plays a role in the increased endothelial damage observed in CKD patients has not been studied (26).

Therefore, in this study we analyzed: (i) whether uremia affects endothelial levels of intracellular Klotho; (ii) whether exogenous Klotho can protect endothelial cells from senescence induced by uremia; characterizing intracellular pathways involved in uremia-induced endothelial senescence that may be modulated by Klotho. Particularly we will study if the intracellular signaling modulated by EPO in endothelial cells, that previously we have demonstrated that prevent endothelial damage induced by uremia, also modify Klotho activity in endothelial cells.

**Methods**

**Ureemic Serum**

Serum samples were obtained from 26 CKD stage-5 patients, who were undergoing conservative treatment (14 females and 12 males), with a mean age of 73.7 years (range: 45–79 years). Renal function was calculated in accordance with the Modification of Diet in Renal Disease 7 (MDR-D7). Creatinine clearance was 21.3 ± 5.1 mL/min (range: 14–24). The criterion for patient selection was the absence of inflammatory disease, acute or chronic infection, autoimmune diseases, hepatic insufficiency, diabetes, and malignancy. None of the patients or controls smoked. In preliminary experiments, 10% was the highest nontoxic concentration of US. In order to minimize minor differences between patients, all serum samples were pooled. Informed consent was obtained from all patients after approval by the ethics committee of Reina Sofia University Hospital.

**Cell Culture**

HUVEC were obtained from Lonza (Walkersville, MD). The cell line was cultured at 37°C, 5% CO₂ in standard endothelial cell basal medium (EBM; Lonza), plus endothelial cell growth medium supplements (EGM; Lonza): 10% fetal bovine serum, 0.4% bovine brain extract, 0.1% human epidermal growth factor, 0.1% hydrocortisone and 0.1% GA-1000, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 mmol/L l-glutamine.

**In Vitro Treatments**

HUVEC cells were cultured at 37°C for 36 h in EBM in the presence or absence of 10% US or 10% human autologous serum (normal AB serum; Lonza). The optimal concentration of serum and culture times was based on preliminary results from concentration and time-dependence curves (see Supplementary Figure S1).

In order to determine whether exogenous Klotho prevented senescence induced by uremia, endothelial cells were cultured in the presence of normal serum (NS), US, or Klotho peptide (PT), a synthetic peptide derived from within residues 150–250 of Human Klotho (ab75022; Abcam, Cambridge, UK). The endothelial cells were pretreated with the peptide for 2 hours before the US was added. Klotho peptide was used at a concentration of 0.1 mg/mL. A dose–response curve was generated to identify the optimal dilutions of Klotho peptide (see Figure 3).
In order to compare the effect of exogenous Klotho and EPO, endothelial cells were treated with Darbepoetin alfa (1 µg/mL, Aranesp; Amgen Europe B.V., Breda, The Netherlands) 1 hour before the US was added. Five independent cultures of each experiment set were treated.

**Acidic β-Gal Staining**

To detect senescent cells, we used a cellular senescence kit that quantifies β-galactosidase expression (DetectaGene Green CMFDA lacZ Gene Expression Kit; Molecular Probes, Inc., Eugene, OR). Briefly, the cells were resuspended in 1 mL fresh medium to a concentration no higher than 1 x 10^6 cells per mL and then centrifuged, and the supernatant was discarded. Hypertonic loading medium that contained 1 mM CMFDA was added to the cells. After 10 minutes, 1 mL hypertonic lysis medium was added to the suspension and incubated for 1.5 minutes at 37°C. The cells were centrifuged, and the supernatant was removed. After staining, the cells were washed, counted, and analyzed on a flow cytometer (FACSCalibur; Becton Dickinson). The results were expressed as the percentage of β-gal-positive cells.

**Assessment of Telomere Length by Fluorescence In Situ Hybridization (Flow-FISH)**

Telomere length was measured by flow cytometry using the Dako Telomere probe nucleic acid kit/fluorescin isothiocyanate (FITC/ Dako Cytomation, Ely, UK). Cells (1 x 10^6) were resuspended in 300 mL of hybridization solution containing 70% formamide, with no probe ( unstained control) or with a FITC-conjugated telomere PNA probe. These cells were heated for 10 minutes at 82°C to denature the DNA.

Hybridization was performed overnight at room temperature in the dark. After washing, the cells were resuspended in 0.5 mL of Dako DNA-staining solution and incubated at 4°C for 2 hours in the dark. Each sample was then analyzed on a FACSCalibur flow cytometer (Becton Dickinson); using the logarithmic scale FL1-H for probe fluorescence and the linear scale FL3-H for DNA staining. FL1-H and FL3-H data were used to calculate the relative telomere length of sample cells as compared with control cells. According to the manufacturer’s instructions: relative telomere length (%) = (mean FL1-H of cells with probe – mean FL1-H of cells without probe) x DNA index control cells (x2) x DNA index cells (x1) / 100 x (mean FL1-H of control cells with probe – mean FL1-H of control cells without probe). The telomere length was determined by comparing the molecular equivalents of soluble fluorochrome units values of the test sample with those obtained in three cell lines (K562, U937, and Daudi) that are known to have different telomere lengths, which served as standard. The intraassay coefficient of variation was 4.6%.

**Detection of Reactive Oxygen Species**

Intracellular superoxide levels were detected by quantification of fluorescence of the oxidation of hydroethidine (2 µM; Molecular Probes), a substance that is oxidized by reactive oxygen species (ROS) to become ethidium (Eth) and to emit red fluorescence. Briefly, cultured HUVECs were treated with NS or US for 24 hours. The treatment was halted by washing cells three times with phosphate-buffered saline. Two milliliters of hydroethidine (2 µM) was added into each well of six-well plates and incubated for 15 minutes at room temperature. Staining was stopped by washing cells three times with phosphate-buffered saline. The cells were fixed with 2% paraformaldehyde and analyzed by FACSCalibur flow cytometer (Becton Dickinson).

**Flow Cytometry Staining for Klotho Quantification**

Intracellular Klotho expression was assessed by indirect immunofluorescence. HUVEC (10^6/mL) were permeabilized using FACS Permeabilization Solution (Becton Dickinson). For each sample, 100 µL of cells were incubated with the permeabilization solution. After vortexing, cells were incubated for 5 minutes at room temperature in the dark. Cells were then washed (5 minutes at 1,500 rpm) in buffer (phosphate-buffered saline + 0.1% NaN_3 + 10% autologous serum) and incubated with an affinity-purified rabbit polyclonal antibody raised against a peptide mapping within an internal region of Klotho of human origin (ab75023, 5 µg/mL; Abcam). After 60 minutes of incubation at 4°C, cells were washed and incubated with goat polyclonal secondary antibody to rabbit IgG-H&L-FITC (Abcam) at a 1:1,000 dilution for 1 hour at 4°C in the dark. The cells were then washed and resuspended in 0.5 mL 1% formaldehyde and stored at 4°C until analysis by flow cytometry. A sample without secondary antibody was included as autofluorescence control. We simultaneously performed the same staining protocol but using nonpermeabilized samples. Samples were analyzed on a FACSCalibur System (Becton Dickinson). To detect senescent cells, we quantified β-gal and Klotho expression in the same cell. In those cases, the polyclonal antibody used for Klotho staining was labeled in PE (Abcam).

**Klotho RNA Measurement**

Total RNA Isolation: Total RNA was isolated from 2 x 10^6 HUVEC using an easy-spin Total RNA Extraction Kit (Cat. No. 17221, Intron Biotechnology, Inc., Gyeonggi-Do, Korea), and was immediately converted to complementary DNA, using the Improm-II Reverse Transcription System (Promega Corporation, Madison, WI). Polymerase chain reactions to detect the products of KLOTHO and β-actin genes were performed using the PCR Core System (Promega) reagents and thermostable Taq polymerase. The following pairs of primers were used: sense 5'-GTGTCCATGGCCTAAG-3', antisense 5'-CTCTGGAGTAGTACC-3' (GenBank accession number: NM_004795); and for β-actin gene: sense 5'-GACCTTCAGCTTCTCC-3', antisense 5'-ATCACAATGCTGGAAAGTT-3' (GenBank accession number: NM_001101). Both products were amplified using Stratagene MX3005P real time PCR system (Promega Corporation) and the following reaction conditions: initial denaturation 94°C, 10 minutes; 30 amplification cycles containing melting for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and elongation 30 seconds at 72°C; after final cycle, termination for 10 minutes at 72°C followed by cooling and storage at 4°C. Polymerase chain reaction products were resolved in standard 2% agarose gel with ethidium bromide and band fluorescence was visualized using the GelPrinter Plus Image Acquisition and Analysis System and LabWorks software (both from TDI Products). Kidney RNA (human) obtained from biopsy tissues was used as internal positive control.

**Western Blotting of Klotho**

Cellular extracts from HUVEC were prepared according to standard protocols. Cytoplasmic extracts (50 µg) were resolved in sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Klotho protein levels were determined using anti-Klotho Rabbit pAb (Cat#423500, Calbiochem; Merck Chemicals Ltd., Nottingham, UK). This antibody recognizes...
the ~130 kDa Klotho protein. Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL Plus Western Blotting Detection Reagents (GE Healthcare Bio-Sciences, Uppsala, Sweden). Protein levels were quantified using Intelligent Quantifier image analysis software, version 2.1.1 (Bio Image, Ann Arbor, MI). Results were calculated in terms of integrated optical density and expressed in arbitrary units.

siRNA Knockdown of Klotho Expression

A small interfering RNA (siRNA) was generated against Klotho. siRNA for knocking down Klotho was synthesized by Qiagen (Foster City, CA). The DNA target sequence of the annealed double strand siRNA that we used was: 5′-ATGGGCATAGGTGATCGTAAA-3′. siRNA, with a nonsilencing oligonucleotide sequence (nonsilencing siRNA) that does not recognize any known homology to mammalian genes, was also generated as a negative control.

Cells (HUVECs) were seeded at a density of 2×10⁵ cells/well in 6-well plates and grown in EBM containing 20% fetal bovine serum and antibiotics. One day after seeding, cells were transfected with siRNA using siRNA Reagent System (Santa Cruz Biotechnology Inc.) according to the manufacturer’s instructions. Briefly, siRNA (40 pmol) was mixed with transfection medium (100 μL) to which transfection reagent (6 μL plus transfection medium (100 μL) was added. After mixing (45 minutes), the formulation was added dropwise onto the cells. Control cells were exposed to transfactant in the absence of siRNA. Eighteen hours after transfection, media was changed to EBM containing 20% fetal bovine serum and antibiotics. Part of the cells was lysed after 24 hours, and gene silencing was monitored at the mRNA level.

Figure 1. Klotho expression in HUVEC. (A) Representative Western blot and mRNA gel (RT-PCR products) from HUVEC cells incubated for 24 h with normal serum (NS) vs uremic serum (US). Klotho mRNA is normalized for β-actin mRNA. Data presented are mean ± standard deviation of ratio between Klotho/β-actin mRNA levels (n = 5). Protein quantification expressed as the ratio of protein levels Klotho/β-actin, data presented are mean ± standard deviation (n = 5). (B) Flow cytometry of Klotho in HUVEC cells: (a) Representative histogram (n = 5) of the distribution of the endothelial cells, using the forward scatter-height (FSC-height)/side scatter-height (SSC-height), (b) Intracellular expression of Klotho and (c) membrane expression of Klotho. HUVEC = human umbilical vein endothelial cells; mRNA = messenger RNA; RT-PCR = reverse transcription-polymerase chain reaction.

Figure 2. Effect of senescence induced by uremic serum in Klotho expression. β-gal expression and intracellular Klotho were studied simultaneously by flow cytometry in the same HUVEC cells. More than 90% of cells with low Klotho expression were β-gal–positive cells. Data presented a representative dot plot of the distribution of these cells (n = 5). β-gal = β-galactosidase; HUVEC = human umbilical vein endothelial cells.
level by reverse transcription-polymerase chain reaction and the other part was used to analyze the telomere length as described previously.

Western Blot of FGF-R and MAPK Pathway
For Western blot, equal amounts of protein were electrophoresed in 8% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. The membranes were blocked with 2% bovine serum albumin for 1 hour at room temperature and then incubated with primary antibody for 2 hours at room temperature. Primary antibodies used were rabbit polyclonal FGF-R1 antibody (ref. no. #3472; Cell Signaling Technology, Danvers, MA), rabbit anti-pERK1/2 (sc-7383; Santa Cruz Biotechnology Inc.), mouse anti-MAPK (ref. no. 02/2009, Cell Signaling Technology), and mouse anti-Actin (sc-8432; Santa Cruz Biotechnology Inc.). Blots were immunolabeled using a horseradish peroxidase-conjugated secondary antibody and developed on autoradiographic film using the ECL Plus Western blot detection system (GE Healthcare Bio-Sciences). Specific bands were quantified by densitometric analyses with Quantity One 4.4.0 software (Bio-Rad Laboratories) and were normalized to Actin levels.

Electrophoretic Mobility Shift Assay
Nuclear extracts (20 μg) were tested for NFκB-binding activity in electrophoretic mobility shift assay analyses (consensus oligonucleotides: 5′-AGTTGAGGGGACTTCCAGGC-3′ and 3′-TCAAC TCCCTGAAAGGTCCG-5′) using the digoxigenin electrophoretic mobility shift assay kit from Roche Diagnostics (Basel, Switzerland) according to the manufacturer’s recommendations. Supershift analysis was carried out to examine the composition of the protein complexes binding to the consensus probe (using p65/p50 antibodies).

Enzyme-Linked Immunosorbent Assay
Determination of Supernatant IL-6 and TNFα
A commercial enzyme-linked immunosorbent assay was used to evaluate the interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) production levels by endothelial cells, in four independent sets of experiments. The supernatant from the cultured cells was collected and centrifuged at 1,500 rpm, 10 minutes, 4°C. The concentration of IL-6 and TNFα was determined using the corresponding Instant ELISA kit (eBioscience, Bender MedSystems GMbH, Vienna, Austria). The optical density in each kit well was detected using a Power Wave XS microplate reader (BioTek Instruments, Inc., Winooski, VT) and the levels of both cytokines were deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

Statistical Analysis
Mean values were compared by Student’s t test for paired or unpaired data. Nonparametric data were analyzed by the Wilcoxon test or Mann–Whitney U test for paired and unpaired comparisons, respectively. Differences were considered to be significant at values of p < .05.

Results
Endothelial Damage Induced by US Is Associated With Changes in Intracellular Klotho
We demonstrated the presence of Klotho in HUVEC by reverse transcription-polymerase chain reaction analysis and immunoblotting. As shown in the representative study of Figure 1A, high levels of Klotho mRNA or protein expression in endothelial cells were detected. These levels were significantly reduced in presence of US (p < .05).

Flow cytometry was used to determine whether anti-Klotho antibodies recognized the intracellular protein or also recognized the expression of this protein on the surface of HUVEC cells. As Figure 1B shows, when the cells were not permeabilized, the anti-Klotho polyclonal-Ab not recognized proteins on the surface of endothelial cells. However, when cells were permeabilized, the primary antibody used recognized a protein expressed in the endothelial cytoplasm. These data serve as a control and allow us to compare the expression of Klotho in basal conditions and in the presence of US.

Following the induction of senescence by US, the level of expression of intracellular Klotho decreased. In order to confirm that the fall in Klotho occurred in senescent endothelial cells, β-gal and intracellular Klotho expression were studied simultaneously in the same cells. Figure 2 shows that more than 90% of cells with low Klotho expression, were β-gal–positive cells. The number of cells with low Klotho expression correlations to cells that shows increased β-gal activity.

Knockdown of Klotho Induces Senescence in HUVEC Cells
To confirm the specific role of Klotho in the regulation of cellular senescence, HUVEC were transfected to knockdown Klotho

Figure 3. siRNA inhibition of Klotho induces senescence. (A) Relative mRNA expression of HUVEC transfected with siRNA of Klotho 24 h after transfection, wild-type HUVEC were included as controls, p < .05 vs controls. (B) HUVEC transfected with siRNA of Klotho shows short telomeres than wild-type cells (p < .05). Bars are the mean ± standard deviation of five independent experiments. HUVEC = human umbilical vein endothelial cells; mRNA = messenger RNA; siRNA = small interfering RNA.
expression. Klotho mRNA expression was 50% reduced \((p < .05)\) in HUVEC transfected with siRNA versus wild-type cells (Figure 3A). Moreover, as Figure 3B shows, cells with Klotho knockdown exhibit shorter telomeres (66.67 ± 3.21% cells with short telomeres) versus wild-type cells (10.67 ± 1.53% cells with short telomeres), \((p < .05)\).

Exogenous Klotho Prevents Increased ROS and Senescence Induced by US

Endothelial senescence assessed according to telomere length and the expression of the \(\beta\)-gal was determined in endothelial cells. To establish the model and validate the doses of Klotho peptide, the dose dependence of the peptide prevention of senescence was performed (Figure 4A).

As Figure 4B and C show, 67.8 ± 5.15% of cells cultured with US expressed \(\beta\)-gal, while 59.2 ± 3.15% had short telomeres versus the 5.85 ± 2.3% of cells that expressed \(\beta\)-gal or 4.25 ± 1.5% of cells had short telomeres when they were cultured with NS \((p < .05)\). However, only a small percentage of cells cultured with US plus Klotho peptide displayed features indicating senescence. \(\beta\)-Gal expression and cells with short telomeres decrease with the addition of Klotho peptide \((\beta\)-gal: 19.3 ± 3.12%; cells with short telomeres: 16.2 ± 2.3%; \(p < .05)\).

To examine the potential pathways through the peptide is acting we analyzed the expression of FGF-R1 on HUVEC treated with US, Klotho peptide or both treatments combined. Our study shows that cells under baseline conditions exhibit activity of FGF-R1, which is reversed by treatment with US. Klotho peptide protects the cell senescence and oxidative stress induced by US, and promotes the cell exhibiting similar characteristics to those presenting under normal conditions and thus activity FGF-R1 is observed again (Figure 5A).

To determine whether the Klotho peptide reduces the US-induced stress response, we analyzed HUVEC exposed to 10% US with or without Klotho peptide. US increased the expression of pERK1/2 compared with the control group \((p < .05)\). The peptide decreased pERK1/2 protein expression to levels observed in control cells (Figure 5B).

US induced an increase in ROS activity in most endothelial cells (from 10.9 ± 3.3% to 58.5 ± 4.2%; \(p < 0.05)\). To study whether the antisenescent effect of exogenous Klotho is related with their ability to inhibited increased oxidative stress, in cells pretreated with Klotho peptide, ROS activity induced by the uremia was evaluated. As shown in Figure 6, in the presence of Klotho peptide and 10%
of US, the percentage of ROS-positive cells decreased significantly. Furthermore, the ability of exogenous Klotho to prevent increased oxidative stress induced by the US was similar to that observed using the antioxidant agent vitamin C.

The Ability of Exogenous Klotho to Prevent Senescence Is Related to NFκB Activity: The Effect of EPO on NFκB Activity

Many of the stimuli that lead to increased ROS activity and senescence in vascular cells act by regulating the complex NFκB/IκB. As the representative image in Figure 7 shows, senescence induced by US endothelial cells is associated with an increase in NF-κB activity and the degradation of the NFκB/IκB complex.

To determine the functional consequence of the effect of Klotho on NFκB pathway in our model of senescence, we analyzed the levels of two proinflammatory cytokines, such as IL-6 and TNFα, on the cell supernatant. As shown in Figure 8A, after 24 hours of incubation, US achieved a significant increase in IL-6 levels over NS (US: 391.5 ± 32.2 pg/mL vs NS: 148.5 ± 25.9 pg/mL, p < .05). This increase was significantly attenuated when we treated the cells with Klotho peptide before adding US (US: 391.5 ± 32.2 pg/mL vs PT+US: 236.2 ± 15.5 pg/mL, p < .05). Figure 8B shows that similar results were observed with the TNFα levels. TNFα concentrations were significantly higher in the group of US than in NS (US: 62.5 ± 3.3 pg/mL vs NS: 9.3 ± 1.6 pg/mL; p < .05). There were no differences between the PT+US and NS group (PT+US: 15.4 ± 1.0 pg/mL).

We studied whether the antisenescent effect of exogenous Klotho can be associated with inhibition of NFκB–DNA binding ability, so we compared the capability of exogenous Klotho and EPO. We used EPO because we have already shown that this molecule prevents senescence and endothelial damage induced by uremia through an NFκB-dependent pathway. As the representative study in Figure 9A makes clear, exogenous Klotho prevents NFκB induced by US and the efficiency with which Klotho peptide inhibits the degradation of NFκB/IκB complex is similar to that observed with EPO. Furthermore, in order to confirm that changes in intracellular Klotho are a consequence of senescence, we inhibited the endothelial senescence (induced by US), by the addition of EPO. As

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**Figure 5.** Expression of FGF-R1 and pERK1/2 on HUVEC. (A) Representative Western blot of HUVEC treated with US, Klotho peptide, or both treatments combined. Klotho peptide protects the cell senescence and oxidative stress induced by US, and promotes the cell exhibiting normal activity of FGF-R1. (B) Levels of pERK1/2 in HUVEC exposed to US, Klotho peptide, or both treatments combined. Klotho peptide regulates the expression of pERK1/2 on HUVEC cells treated with US. Bars are the mean ± standard deviation of five independent experiments. HUVEC = human umbilical vein endothelial cells; US = uremic serum.
the representative image in Figure 9B shows, no decrease in intracellular Klotho was observed in cells cultured with US+EPO. In addition, as expected, EPO also inhibited senescence in endothelial cells. Unfortunately, we could not use the Klotho peptide to study intracellular Klotho. Although we have also observed that in the presence of Klotho peptide no decrease in intracellular Klotho occurs, anti-Klotho antibodies may recognize the Klotho peptide that may be attached to the cell.

**Discussion**

The prevention of endothelial senescence may be an effective mechanism for preventing cardiovascular disease. US induces premature senescence in endothelial cells, which would explain the early development of cardiovascular disease in CKD patients (1,2). Understanding the mechanisms whereby uremia causes accelerate endothelial senescence and identifying potential therapeutic targets that might prevent such endothelial damage and the subsequent cardiovascular disease has therefore become a priority for clinicians treating CKD patients (27). In this study we have shown that uremia induced increased oxidative stress and accelerate senescence in endothelial is associated to a decrease in expression of intracellular Klotho in these cells. Addition of exogenous Klotho prevents both increased oxidative stress and senescence induced by US. This anti-senescent effect of exogenous Klotho appears to be related to their ability to stabilize the NFκB complex, preventing the increase in NFκB induced by the uremia. Furthermore, EPO that inhibits increase in NFκB induced by uremia in endothelial cells, also prevent intracellular Klotho decrease, increased oxidative stress, and accelerates the senescence in these cells.

Previously have demonstrated that uremia induces senescence and injury in endothelial cells (28). Recent studies have shown that US downregulates renal expression of Klotho through production of ROS and activation of NFκB (22), and promotes cell senescence with expression of senescence-related proteins such as p16, p21, p53, and retinoblastoma protein in the kidney of hypertensive rats (29,30). Klotho is considered as a regulator of oxidative stress and senescence (11). Inhibiting Klotho mRNA expression could induce premature senescence of human cells. It is considered that regulating Klotho expression via DNA methylation might have a critical role in the cell senescence process caused by uremic toxins. Several factors associated with CKD, such as uremic toxins, oxidative stress, or increased levels of proinflammatory cytokines, have been shown to decrease Klotho expression in cell culture. Decreased expression of Klotho mRNA and protein are both associated with accelerated aging features (20,28).

In this study we observed that endothelial cell senescence induced by uremia is associated with a decrease in levels of intracellular Klotho. But we have not been able to determine whether the decline in intracellular Klotho is an epiphenomenon associated with senescence or a regulatory mechanism for this process. To demonstrate if intracellular Klotho decrease is a causal or a consequence of cell aging, we performed experiments to knockdown Klotho expression and determine the role of this protein on the development of senescence. We found that the inhibition of 50% of gene expression results in a shortening of telomere length. This suggests that the partial inhibition of Klotho is associated with aging, inducing attrition of telomeres.

Even though the fact that the addition of exogenous Klotho prevent premature senescence induced by uremia in endothelial cells, suggest that at least expression of intracellular Klotho may be used as a marker for endothelial senescence. In previous studies performed by our group, we have used synthetic Klotho peptide to analyze if intra- or extracellular Klotho modulates the response of senescent cells to oxidative stress. In our model, we made some experiments to verify whether blocking the Klotho peptide prevents a rise in the...
level of Klotho, so we added pAb to the test instead of Klotho. Our results demonstrated that blocking the Klotho peptide by means of pAb rather than Klotho reduces intracellular Klotho levels (4). Under basal conditions, when the cell has high levels of Klotho, the peptide has no additional effect. However, the addition of US to the culture induces a reduction of intracellular Klotho that leads to cellular senescence, which is partially reversed by the peptide. The addition of exogenous Klotho did not retard aging of HUVEC but it was able to prevent cell changes induced by US.

Our results demonstrated that senescence induced by uremia is dependent on two important intracellular mediators, an increase in oxidative stress and the disruption of the NFκB/IκB complex and the subsequent activation of the NFκB-dependent pathway. Previous studies have shown that uremia induces increased ROS activity (31,32); a cellular response closely related to the induction of senescence in most cells (33). The degree of oxidative stress increased progressively with aging and with stimulus like US. The addition of exogenous Klotho did not affect the process of aging and had no effect in the oxidative stress associated with aging (4). However, our results demonstrated that exogenous Klotho protects HUVEC from the damage of oxidative stress induced by US. The addition of exogenous Klotho prevent the classical features of cell senescence induced by US, thus Klotho levels remain similar to the baseline cell. This favors that Klotho may carry out their function through the regulation of FGF-R (15) and the MAPK pathway (16).

Others intracellular signals that we have observed modified associated to the endothelial senescence induced by the uremia are a disruption of the NFκB/IκB complex. Our results confirm that inflammatory cytokines, such as IL-6 and TNFα, were associated with Klotho low expression through an NFκB-dependent mechanism. These results may partially explain the relationship between inflammation and diseases characterized by accelerated aging, including CKD. Increased NFκB activity have been reported associated to endothelial dysfunction induced by inflammation and oxidative stress (2,34,35), and therefore, have been identified as a therapeutic target in the prevention of vascular diseases. The result obtained in our study support and extend this hypothesis, and identify NFκB activity as a therapeutic target in the accelerate senescence induced by the uremia in endothelial cells. Another important

Figure 7. NFκB activity correlates with the ability of exogenous Klotho to prevent senescence. Representative EMSA of HUVEC treated 24h with NS, US, PT, or PT+US. Senescence induced by US in endothelial cells is associated with increase in NFκB activity and degradation of the IκB protein. Bars are the mean ± standard deviation of five independent experiments. EMSA = electrophoretic mobility shift assay; HUVEC = human umbilical vein endothelial cells; NFκB = nuclear factor kappa B; NS = normal serum; US = uremic serum.
finding of our study is that exogenous Klotho protects endothelial cells from uremia-induced senescence. Klotho is mainly produced by the kidney, and renal failure is associated with a decrease in Klotho expression and low levels of exogenous Klotho \(^{(2)}\). The results of this study suggest that if the decrease in exogenous Klotho also occurs in the intracellular Klotho expressed in endothelial cells, these cells are more susceptible against the stress triggered by uremic toxins and perhaps other factors such as inflammatory cytokines or modified lipoproteins. Exogenous Klotho may prevent the reduction of endogenous Klotho induced by the cytokine and averted the results of such a reduction.

Interestingly, therapeutic agents such as recombinant EPO or angiotensin II antagonist upregulate Klotho expression in kidney \(^{(36,37)}\). We were unable to measure soluble Klotho as we have not been able to identify antibodies that can be used to quantify this protein in serum or plasma. However, soluble exogenous Klotho has been described as a truncated form of the membrane Klotho protein. It would appear to be a reasonable assumption that agents that favor Klotho synthesis in the kidney will also promote an increase of exogenous Klotho. Therapeutic agents such as EPO could therefore have a beneficial effect by protecting the vascular endothelium, in addition to their principal objective.

In conclusion, our results support the hypothesis that Klotho modulates uremia-induced senescence in endothelial cells. Levels of intracellular Klotho decrease with endothelial senescence, and exogenous Klotho prevents cellular senescence by inhibiting the increase in oxidative stress induced by uremia, and by stabilizing NFκB/IκB. Future studies are needed to clarify whether the link between Klotho and uremia-induced senescence can be extended to situations such as aging or other vascular diseases in which endothelial cells undergo senescence. However, our first result make the possibility of protecting the endothelium of patients increasing levels exogenous Klotho by EPO or other therapeutic agents specifically designed to enhance exogenous Klotho at least a tempting hypothesis.

**Supplementary Material**

Supplementary material can be found at: [http://biomedgerontology.oxfordjournals.org/](http://biomedgerontology.oxfordjournals.org/)

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Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the author(s).

References


Figure 9. Effect of EPO on NFκB activity. (A) Representative EMSA of HUVEC cells. Klotho peptide and EPO prevent the NFκB nuclear translocation induced by US and IkB degradation on cellular cytosol. (B) Representative Western blot of HUVEC cells treated with EPO+US during 24 h. In the presence of EPO, a decrease in intracellular Klotho was not observed in cells cultured with US. Bars are the mean ± standard deviation of five independent experiments. EMSA = electrophoretic mobility shift assay; EPO = erythropoietin; HUVEC = human umbilical vein endothelial cells; NFκB = nuclear factor kappa B; US = uremic serum.


