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Klotho modulates the stress response in human senescent endothelial cells

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ABSTRACT

Lack of Klotho expression in mice leads to premature aging and age-related diseases, including vascular diseases. The aim of this study was to determine how endothelial cell line senescence affects Klotho expression and whether intra- or extracellular Klotho has any effect on the response of senescent cells to oxidative stress.

The study was performed using human endothelial cells (HUVEC); cell aging was obtained by prolongation of cell division to 42 population doublings (PD). Senescence was also obtained by exposure to TNFα, which causes cell changes resembling cellular senescence. The decline in Klotho preceded the manifestations of cell ageing: telomere shortening and β-galactosidase expression. Klotho was also reduced in cells exposed to the proinflammatory cytokine TNFα. The addition of exogenous Klotho to aging cells did not modify the proportion of cells with short telomeres or any other feature of cell aging; however, exogenous Klotho prevented the changes resembling premature cellular senescence associated with TNFα, such as the decrease in telomere length and the increase in β-galactosidase-positive cells. Likewise exogenous Klotho prevented the increases in reactive oxygen species (ROS) activity, mitochondrial potential and cell apoptosis induced by TNFα.

1. Introduction

Klotho is recognized as an anti-aging protein. Lack of Klotho expression in mice produces premature aging and age-related diseases including vascular diseases. Animals that over-express Klotho live longer and do not present age-related diseases (Kuro-o et al., 1997; Takahashi et al., 2000).

Klotho encodes a single-pass transmembrane protein (α-Klotho) with a long extracellular domain (130 kDa), and a short cytoplasmic tail (Kuro-o et al., 1997; Shiraki-Iida et al., 1998). This protein has a restricted distribution and is predominantly expressed in the distal convoluted tubules of kidneys, parathyroid cells and in the choroid plexus in the brain. Another form of Klotho protein, the secreted Klotho protein, has been described as an alternative mRNA splicing of Klotho derived from the type I protein, and has a molecular weight of approximately 65–70 kDa. Soluble Klotho plays an important role in inhibiting insulin/IGF-1 signals and inducing resistance to oxidative stress; this suggests that soluble Klotho may act as an "anti-aging" hormone (Shiraki-Iida et al., 1998; Li et al., 2004; Hayashi et al., 2007). A third Klotho protein is not present in the cell surface, but is expressed in the cytoplasm; its intracellular distribution mostly overlaps the endoplasmic reticulum and Golgi apparatus (Li et al., 2004; Hayashi et al., 2007), whose expression has been linked to the ability of cells to withstand stress conditions.

Replicative cellular senescence can be studied in vitro and it serves as a model which helps to understand the mechanisms involved in cell aging (Cristofalo et al., 2004). In culture, the ability of normal human cells to divide is finite, and after a limited number of cycles of replication, cells enter a terminal state of arrested growth called replicative senescence. Furthermore, cells exposed to stressors that raise levels of reactive oxygen species (ROS), may undergo premature senescence via a process called stress-induced premature senescence (SIPS) (Toussaint et al., 2000; Serrano and Blasco, 2001). There are analogies between cells undergoing replicative aging and SIPS; some of the genes regulating the response against DNA damage are modified similarly under both conditions, cells express β-galactosidase (β-gal) activity and they

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become large, more granular, and stop their cell division although they remain metabolically active. While replicative senescence (cell aging) is attributed to telomere attrition, SIPS may not be associated with telomere shortening (Toussaint et al., 2000; Serrano and Blasco, 2001; Chen et al., 2001). Previous reports have demonstrated that Klotho secretion induces the generation of nitric oxide in endothelial cells, which enables these cells to withstand oxidative stress (Ikushima et al., 2006; Rakugi et al., 2007). Furthermore, a recent study by Kusaba et al. (2010) demonstrated that Klotho proteins interact with the VEGFR-2/transient receptor potential canonical-1 (TRPC-1) complex on the surface of endothelial cells. This molecular complex is incorporated into cells and is involved in stabilizing the entry of Ca^{2+} in order to maintain the integrity of the endothelial tissue. Soluble Klotho thus protects endothelial cells against SIPS; however, whether Klotho interferes with endothelial cell aging is not known (Ikushima et al., 2006; Rakugi et al., 2007; Kusaba et al., 2010; Saito et al., 1998; Nagai et al., 2000).

Human umbilical vein endothelial cells (HUVEC) are widely used as an in vitro model of vascular endothelium. These cells undergo replicative senescence so they can be used to investigate the mechanisms of endothelial aging (Levine and Mueller, 1979). Aging HUVEC show telomere attrition and express "senescent markers" such as β-galactosidase (Hastings et al., 2004).

The aim of this study was to determine: (1) whether HUVEC express Klotho and whether Klotho expression decreases with cell age; (2) whether intra- or extra-cellular Klotho modulates the response of senescent cells to oxidative stress.

2. Materials and methods

2.1. Cell culture and reagents

A human umbilical vein endothelial cell (HUVEC) line (from Cell Systems/Clonetics, Solingen, Germany) was cultured at 37 °C, 5% CO_2 in standard endothelial cell basal medium (EBM, CAMBREX Bio Science, Walkersville, MD, USA), plus endothelial cell-growth medium supplements (EGM, CAMBREX) and 10% fetal calf serum (FCS, Invitrogen – Molecular Probes, Eugene, OR, USA). First passage cryopreserved HUVEC were grown and serially passaged. The number of population doublings (PD) was calculated using the formula PD = (ln [number of cells harvested] − ln [number of cells seeded])/ln 2. When HUVEC reached PD 2, PD 26 or PD 42, were determined both cellular senescence and the changes induced by TNFα. To analyze changes induce by Tumor Necrosis Factor-alpha (TNFα), cells were exposed to TNFα (TNFα, Sigma Chemical Co., St. Louis, MO, USA) (10 ng/ml) for a 24 h period at passages PD 2, PD 26 and PD 42. The optimal concentration and time of stimulation with TNFα was established on the basis of preliminary results obtained from concentration and time-dependent curves. Treatments were performed on five independent cultures. Synthetic peptide derived from within residues 150–250 of Human Klotho (Klotho peptide ab75022, Abcam, Cambridge, UK) was used at a concentration of 0.1 mg/ml for 1 h prior to the addition of TNFα, and was maintained during the following 24 h. In some experiment, an anti-Human Klotho polyclonal antibody (pAb) (ab75023, 5 μg/ml Abcam) was added to the cultures simultaneously with the peptide of Klotho. A dose–response curve was performed to obtain the optimal dilutions of both antibody and Klotho peptide used in this study.

2.2. Animals

Two groups of male Wistar rats were evaluated: young rats (4 months old, $n = 16$) and old rats (12 months old, $n = 16$). Rats from both groups received either lipopolysaccharide (LPS) or vehicle. LPS (250 μg/kg) (LPS from *Escherichia coli*, Sigma Chemical Co.) was given i.p. 48 and 12 h before being sacrificed by aortic puncture and exsanguination under general anesthesia (intraperitoneal sodium thiopental, Abbott Laboratories, Redwood City, CA, USA) at 50 mg/kg of body weight. Control rats receive normal saline. Thoracic aortas were harvested immediately and processed. The experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba, and all rats received humane care in accordance with the Principles of Laboratory Animal Care of the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Science.

References and further reading may be available for this article. To view references and further reading you must purchase this article.

Extraction of vascular cells: aortic endothelial cells were obtained as described by Nogueras et al. (2008). Aortas were sliced (<1 mm), and cells were dislodged mechanically under constant saline flow without enzymes for 15 min. The resulting cell suspension was centrifuged three times at 1800 rpm for 5 min with physiological saline, and the cell pellet was resuspended in 5 ml phosphate-buffered saline (PBS; Invitrogen) with 0.1% bovine serum albumin (BSA; Sigma Chemical Co.).

Cells from the aorta were quantified by flow cytometry. Endothelial cells (EC) were identified as cells expressing rat endothelial cell antigen-1 (RECA-I, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The proportion of endothelial cells (RECA-1+) recovered from aortas of control animals was 2.3 ± 1.2%.

2.3. Assessment of telomere length by fluorescence in situ hybridization (flow-FISH)

Telomere length was measured by flow cytometry using the Dako Telomere peptide nucleic acid kit/fluorescein isothiocyanate (FITC/Dako Cytomation, Ely, UK). Cells (5×10^5) 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 min 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 h in the dark. Each sample was then analyzed on a FACSCalibur flow cytometer (Becton Dickinson Company, Mountain View, CA, USA) 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 (RTL) of sample cells as compared with control cells. According to the manufacturer's instructions: RTL (%) = (mean FL1-H of cells with probe − mean FL1-H of cells without probe) × DNA index control cells (=2) × DNA index cells (=1) × 100/(mean FL1-H of control cells with probe − mean FL1-H of control cells without probe). RTL was calculated using a standard curve that had been obtained from cell lines (K562, U937, Daudi, and 1301).

2.4. Acidic β-galactosidase staining

Acidic β-gal staining was used to identify senescent cells. β-gal was identified by using a kit (SA-ß-gal Staining Cat. No CBA-230, Cell Biolabs Inc., San Diego, CA, USA). Briefly, cells were washed twice with 3 ml PBS and resuspended in 2 ml of fixing solution for 5 min at room temperature. They were then washed three times in 2 ml PBS and immersed in freshly prepared cell-staining solution for 4 h in the dark. After staining, cells were washed and counted under a light microscope (Eclipse Ti-S, Nikon Co., Tokyo, Japan). The results, obtained from at least 100 cells that were counted by light microscopy, were expressed as the percentage of β-gal-positive cells (blue-stained cells).

2.5. Klotho measurement

Total RNA isolation: total RNA was isolated from 2×10^6 HUVEC using an easy-spin™ Total RNA Extraction Kit (Cat. No 17221, Intron Biotechnology, Inc., Gyeonggi-Do, Korea), and immediately converted to cDNA, using the Improm-II Reverse Transcription System (Promega, Madison, WI, USA). PCRs 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′-GTGTCCATTGCCCTAAG-3′, antisense 5′-CTCTCGGGA-TAGTCACC-3′ (GenBank accession number: NM_004795); and for β-actin: sense 5′-GCACTCTTCCAGCCTTCCTT-3′, antisense 5′-ATCCACATCTGCTGGAAGGT-3′ (GenBank accession number: NM_001101). In the design of the primers we used the PRIMER 3 (v.0.4.0). Amplification of both products was performed using the Stratagene MX3005P and the following reaction conditions: initial denaturation 94 °C, 10 min; 30 amplification cycles, comprising melting for 30 s at 94 °C, annealing 30 s at 60 °C, and elongation 30 s at 72 °C; after termination of the final cycle, 10 min at 72 °C followed by cooling and storage at 4 °C. PCR products were resolved in standard 2% agarose gel with ethidium bromide and their 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.

2.6. Real-time PCR quantification of Klotho expression

The same primers used for RT-PCR were also utilized for real-time PCR. Products of Klotho gene were amplified by PCR as described above in order to prepare the standard curves. Stratagene MX3005P and QuantiTect SYBR Green PCR kit (Qiagen, Germantown, MD, USA) was used for realtime PCR. Raw data were analyzed using the Stratagene Software. The reaction was performed using 10-min activation at 95 °C, followed by 40 cycles of 10 s at 95 °C; 5 s at 60 °C and 10 s at 72 °C each, followed by 30 s cooling at 40 °C. Results for Klotho gene product were presented as the proportion of the amount of accumulated gene product relative to that of β-actin gene product. Each RNA sample was analyzed in triplicate, and each experiment was performed at least three times. The 2ΔΔCT method described by Livak and Schmittgen (2001) was used to analyze the data.

2.7. Western blotting of Klotho

Cellular extracts from HUVEC were prepared according to standard protocols (Andrews and Faller, 1991). Cytoplasmic extracts (50 μg) were resolved in sodium