Neuroprotective action of α-Klotho against LPS- activated glia conditioned medium in primary neuronal culture.

Vinicius Nakao[1], Caio Mazucanti[1], Larissa de Sá Lima[1], Paloma de Mello[1], Paula Kinoshita[1], Jacqueline Leite[2], Elisa Kawamoto[1], and Cristoforo Scavone[1]

[1]University of São Paulo Institute of Biomedical Sciences
[2]Federal University of Goias

April 8, 2022

Abstract

The α-Klotho is an anti-aging protein that when overexpressed extends the life span in humans and mice. It has an anti-inflammatory and protective action on renal cells by inhibiting NF-ϰB activation and production of inflammatory cytokines in response to TNF-α. Furthermore, studies have shown the neuroprotective effect of α- α-Klotho against neuroinflammation on different conditions, such as aging, animal models of neurodegenerative diseases, and ischemic brain injury. This work aimed to evaluate the effects of α- α-Klotho protein on primary glial cell culture against the proinflammatory challenge with LPS and how this could interfere in neuronal health. Cortical mixed glial cells and purified astrocytes were pretreated with α- α-Klotho and stimulated with LPS followed by TNFα, IL-1β, IL-6, IFN-γ levels and NF-ϰB activity analysis. Conditioned medium from cortical mixed glia culture treated with LPS (glia conditioned medium (GCM) was used to induce neuronal death of primary cortical neuronal culture and evaluate if GCM-KL (GCM-KL: medium from glia culture pretreated with α- α-Klotho followed by LPS stimulation) can reverse this effect. LPS treatment in glial cells induced an increase in proinflammatory mediators such as TNF-α, IL-1β, IL-6, and IFN-γ, and activation of astrocyte NF-ϰB. GCM treated-cortical neuronal culture induced a concentration-dependent neuronal death. Pretreatment with α-Klotho decreased TNF-α and IL-6 production, revert NF-ϰB activation and blocked neuronal death induced by GCM. These data suggest an anti-inflammatory and neuroprotective effect of α-Klotho protein in the CNS. This work demonstrated the therapeutic potential of α-Klotho in pathological processes which envolve a neuroinflammatory component.

1 Introduction

α-Klotho protein gene was discovered randomly in 1997 by Kuro-o and colleagues (Kuro-o, Matsumura et al., 1997). The α- α-Klotho protein can be found in two forms, a transmembrane and a soluble one, the latter composed of both the cleaved α-Klotho and the secreted α-Klotho acting in the central nervous system (CNS) (Kuro-o, Matsumura et al., 1997; Ohyama, Kurabayashi et al., 1998; Wang & Sun, 2009). The transmembrane α-Klotho has a short intracellular domain and two extracellular domains, known as KL1 and KL2 and the secreted α-Klotho is a result of a alternative splicing composed of only KL1 (Kuro-o, Matsumura et al., 1997). These extracellular domains can be cleaved by the proteases disintegrin A and metalloproteinase 10 (ADAM10), disintegrin A and metalloproteinase 17 (ADAM17), and β-secretase 1 (BACE1). These cleavage fragments are present in the blood, urine, and cerebrospinal fluid acting as a humoral factor (Bloch, Sineshchekova et al., 2009; Chen, Podvin et al., 2007; Imura, Iwano et al., 2004).

Evidence suggest that α-Klotho expression is predominant circumscribed to the kidneys and the CNS (Kuro-o, Matsumura et al., 1997). This protein could be found in the choroid plexus (Nabeshima, 2002), in neurons, oligodendrocytes, in the cortical layers, in the hippocampal formation (Clinton, Glover et al., 2013), and Purkinje cells (German, Khobahy et al., 2012). The membrane-bound α-Klotho complexes with several

fibroblast growth factor (FGF) receptors isoforms (Kuro-o, Matsumura et al., 1997), modulating kidney phosphate reabsorption and 1,25-dihidroxicholicalciferol (vitamin D) production for a systemic regulation of phosphate homeostasis (Erben, 2016; Razzaque, 2009). The soluble forms of α-Klotho act as a humoral factor and can be found in extracellular fluids such as blood, urine, and cerebral spinal fluid (CSF) (Akimoto, Yoshizawa et al., 2012; Imura, Iwano et al., 2004; Li, Watanabe et al., 2004).

Physiological and pathological processes influence the expression of α-Klotho. Rats with spontaneous hypertension, 5/6 nephrectomized, and type 1 diabetes had their α-Klotho mRNA levels decreased (Aizawa, Saito et al., 1998) and endogenous factors such as insulin and glutamate modulate α-Klotho expression in mouse neurons (Mazucanti, Kawamoto et al., 2019). The α-Klotho's expression increases significantly after birth and adulthood (Clinton, Glover et al., 2013; Ohyama, Kurabayashi et al., 1998) and there is a decrease during aging (Duce, Podvin et al., 2008; King, Rosene et al., 2012; Xiao, Zhang et al., 2004).

In aging, there is a low-grade chronic systemic inflammation, called inflammaging (Franceschi, Bonafè et al., 2000). Deregulation of inflammation in the brain is associated not only with cognitive deficit related to aging (Ownby, 2010) but also in the pathogenesis and progression of neurodegenerative diseases (Kempuraj, Thangavel et al., 2016).

Lipopolysaccharides (LPS)-treated glial cells (a model of neuroinflammation) activates pathways involving TLR4 and the nuclear transcription factor kappa (NF-kB). The NF-ϰB activity plays an important role in modulating proteins and cytokines (Kinoshita, Yshii et al., 2017). This nuclear factor is constitutively expressed in the cytoplasm, where is binded to the inhibitor ϰB (IϰB) protein which masks its nuclear localization signal, thus retaining it in the cytoplasm (Ghosh, May et al., 1998). Cytokines and other pro-inflammatory mediators are involved in hippocampal neuronal functions (Kim & Diamond, 2002), but they can also cause damage in hippocampal working memory consolidation and LTP (Kim & Diamond, 2002; Liu, Wu et al., 2012; Thomson & Sutherland, 2005). The present study investigated the role of α-Klotho and the activity of NF-kB in glial cells challenged with LPS and determine the ability of this protein to revert the neurotoxicity on neuronal culture cells caused by the GCM.

2. Materials and Methods

Chemicals and kits

Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Protein assay kit was purchased from Bio-Rad (Hercules, CA, U.S.A.). TNF-α, IL-10, and IL-1β immunoassay kits were purchased from eBioscience (San Diego, CA, U.S.A.). The kits were used according to manufacturer´s instructions. Routine reagents and LPS from*Escherichia coli* (O111:B4, L2630) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) and recombinant α-Klotho from R&D Systems (1819-KL-050, Minnesota, MN, U.S.A.). All solutions were prepared immediately before use.

Primary cell culture

Primary mixed cortical glia culture was prepared as previously described (Kinoshita, Yshii et al., 2017). Brifely, cortex from newborn C57BL/6J mice (postnatal days 1-4) was dissected in ice-cold Hanks' balanced salt solution (HBSS) under a microscope and their meninges removed. Small pieces of cortices were incubated in a trypsin solution (GIBCO) for dissociation at 37°C for 20 minutes. DMEM (complemented with glutamine, 10% Hyclone FetalLOne III serum, GE Healthcare and 1% Penicillin/ Streptomycin) was added to the solution to inhibit trypsin action, and cells were dissociated with a Pasteur pipette. The cells were passed through Cell Strainer, and cells were counted in a Neubauer chamber, and each Flask T75 (Sarstedt) was plated with $1x10^6$ cells. The medium was changed every three days and kept for 10-14 days. The culture was plated in a 6-well plate or 24-well plates for experiments. The culture used for the experiments was considered an astroglial-enriched culture based on previous data showing 91,2% astrocytes labeled with GFAP (Kinoshita, Yshii et al., 2017). To obtain astrocyte-pure cell cultures (>98%) for NF-kB experiments, flasks were incubated in an orbital shaker at 37°C , 180 r/min, for 15 h as previously described (Mazucanti, Kawamoto et al., 2019).

Primary cortical neuronal culture was prepared from both male and female postnatal (P1-3) mice C57BL/6 (Mus musculus). The meninges were separated from the brain, and the cortex were cut into small pieces and incubated in trypsin solution (2 mg/mL) for 20 min in a 37 [?]C, 5% CO_2 incubator. After removal of trypsin solution, tissue was washed twicce with HBSS.Tissue was dissociated in HBSS containing 0.1 mg/mLDNAse by mechanical trituration with glass pipette. Cells were counted and plate ($1x10^6$ cells) in polyethylene (Sigma-Aldrich) pre-coated dishes. Neuons were maintained for wwo weks in Neurobasal medium (GIBCO) supplement with B27 (GIBCO), 2mM gluatamine, 100 U/mL penicillin,100 mg/mL streptomycin and 0.25mg/mL amphotericin B

Both cell culture preparation (glial cells and neurons) were conducted in accordance with The Ethical Principle in Animal Research adopted by the Brazilian College of Animal Experimentation (CONCEA) and were approved by the Ethical Committee for Animal Research (CEEA) of the Biomedical Sciences Institute of the Universidade de São Paulo, São Paulo, São Paulo State, Brazil. The protocol was registered under number 12/2016 CEEA of animals used for experimentation.

α-Κλοτηο ανδ ΛΠΣ τρεατμεντ

On the 15^{th} day, the cells were treated with only DMEM without fetal bovine serum (FBS), LPS, α-Klotho (AA 35-982), or LPS + α-Klotho for 24 hours. A dose-response of recombinant α-Klotho and LPS treatments were made with different concentrations (α-Klotho 0.1 nM – 4.0 nM) and LPS (0,01μg/mL-100μg/mL) for 24 hours to observe cell viability. After this first screening, only α-Klotho concentrations (0.1 - 2.0 nM) were tested at different time points (1, 4, and 24 hours) to evaluate the α-Klotho effect in LPS (1μg/mL /8 hours) -induced changes in TNF-α levels. Based on these data, the concentration of 1nM of α-Klotho and 1μg/mL to LPS for 4 and 8 hours was used to evaluate changes in NF-kB activity and IL-1β, IL-6 and IFN-γ levels, respectively.

For the experiments involving Glial conditioned Medium (GCM), on the 15^{th} day, cell culture was pretreated with DMEM without FBS in the absence (control) or in the presence of α-Klotho (1 nM) for 24 hours, and then challenged with 1μg/mL LPS for 8 hours. The media were collected and named GCM or GCM-KL. Primary neuronal culture cells were challenged on the 10^{th} day, by changing normal conditioned medium by 25% or 50% of GCM or GCM-KL for 24 hours.

Cell viability by LDH and MTT

Cell viability was estimated by Cytotox 96 non-radioactive assay (Promega). The assay was performed according to the Manufacturer's instructions. Lactate dehydrogenase (LDH) release was assayed after 24 hours of treatment with α-Klotho or LPS by removing 50μl supernatant from each well into a 96-well plate and incubating with cytotox 96 reagents for 30 minutes covered with aluminum foil at room temperature. The stop solution was added, and the absorbance was measured at 490 nm. The percentage of LDH activity was measured by the ratio : (Absorbance of the sample/ Absorbance of maximum activity) x 100%.

The cells were incubated with filtered MTT in DMEM without FBS at 37 °C for 2 hours and 30 minutes. The supernatant was removed from the plate, and DMSO was added. The new supernatant was plated on a 96-well plate and measured at 570nm. MTT % related to control was calculated by the ratio: (absorbance of the sample - absorbance of DMSO)/ (absorbance of the control- absorbance of DMSO) x100%.

Multiplex analysis of cytokines and chemokines

Concentrations of TNF-α, IL-1β, IL-6, IL-18, and INF-γ were simultaneously measured in 25 μL of medium from homogenized cells using a Milliplex M.A.P. kit Mice Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) by following manufacturer's instructions. Antibody immobilized beads were detected on a Luminex 100 xMAP technology machine (Austin, TX). Standard curves were generated for each cytokine/chemokine using standards included in the kit for serum samples. The median fluorescence intensity for each analyte was calculated using a five-point logistic parameter curve, and normalized to the amount of protein in each sample.

Immunofluorescence

For evaluate the effects of α-Klotho on NF-κB activation by LPS, after purification, astrocytes were treated for 24 hours with vehicle (control) (PBS) or 1 nM α-Klotho. Inflammatory stimulation with LPS 1 μg/ml was then performed and the cells fixed with methanol (10 min) 4 hours later and washed with PBS three times for 5 min. The fixed cells were incubated with serum blocking (5% normal donkey serum in triton X-100 0.01%) for one hour and incubated overnight with primary antibodies GFAP (1:300) (3670; Cell Signaling, and RelA (p65 (1:100) (ab7970; ABCAM, USA). The primary antibody was removed, and the plate was washed with serum blocking three times for 10 minutes. The cells were incubated with secondary antibody (rabbit or mouse anti donkey- Alexa 594 or 488, Thermo Fischer Scientific,1:1000), diluted in PBS with Triton X-100 0.01% for 2 hours, protected from the light. The coverslips were washed five times with PBS for 5 min and incubated with DAPI (4'-6-Diamidino-2- phenylindole; Sigma) for 1 minute in dilution 1:10.000. Slices were transferred to glass slides and analyzed in Fluorescence microscope Nikon Eclipse 80i (Nikon, Tokyo, Japan) with a camera system, Nikon Digital Camera DXM 1200C.

Protein extraction - nucleus and cytoplasm

Culture media were removed from 6-well plate, and the cells were scraped in cold PBS with 0.5mM PMSF and centrifuged at 4°C for 2 min at 13,000g. Pellet was resuspended in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.1 mM EDTA, 2 μg/mL leupeptin, 2 μg/mL antipain, 30 mM NaF, 3 mM sodium orthovanadate, 20 mM sodium pyrophosphate and 5 mM BG-P) and incubated on ice for 15 min. NP-40 was added, and the samples were homogenized and centrifuged for the 30s at 13,000g at 4°C. Supernatants were used for Western blotting assay and pellets were resuspended in extraction buffer (1.5 mM $MgCl_2$, 20 mM HEPES, pH 7.9, 25% glycerol, 300 mM NaCl, 0.5 mM PMSF, 0.25 mM EDTA, 2 μg/mL leupeptin, 2 μg/mL antipain, 3 mM sodium orthovanadate, 30 mM NaF, 20 mM sodium pyrophosphate and 5mM BG-P) and kept on ice for 20 minutes. Samples were centrifuged for 20 min at 13,000g at 4°C and supernatants were aliquoted as nuclear extract used for EMSA assay. Protein concentration was determined using the Bradford protein reagent (BioRad) .

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from control or treated cells were prepared as previously described (Kinoshita, Yshii et al., 2017). Doublestranded oligonucleotide containing the NF-κB consensus sequence from Promega (5'-AGTTGAGGGGACTTTCCCAGGC-3') was end labelled using T4 polynucleotide kinase (Promega) in the presence of γ-32P dATP. Nuclear extracts (2.5 μg) were incubated with 32P-labelled NF-κB probe. The binding reaction was performed at room temperature for 30 min in a reaction buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM MgCl2, 2.5 mM EDTA, 20 % glycerol, 0.25 μg/μL of poly (dI-dC) and 2.5 mM dithiothreitol. DNA protein complexes were separated by electrophoresis through a 6 % acrylamide:bis-acrylamide (37.5:1) gel in TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA) for 2 h at 150 V. Gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at -80 degC. For competition assays, nuclear extract was incubated with specific competitor (unlabelled double-stranded NF-κB consensus oligonucleotide) or a non-specific competitor (unlabelled transcription initiation factor IID [TFIID]). For supershift assay, antibodies against subunits of NF-κB (p50 and p65,1:20) (Santa Cruz Biotechnology) were added into the binding reactions. Autoradiographs were visualized using a photodocumentation system DP-001-FDC and quantified with ImageJ (NIH) software 70.

Western Blotting

Electrophoresis was performed using 10% polyacrylamide gel and the Bio-Rad mini-Protean III apparatus (Bio-Rad, Hercules, CA, U.S.A.). In brief, the proteins present in cytosolic and nuclear fractions were size-separated in 10% SDS-PAGE (90 V). The immunoblotting was performed as described previously (Kawamoto, Lepsch et al., 2008)The proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.) and incubated with the specific antibody: RelA (p65) (1:1000, sc-0372; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1:2000 (58169; Cell Signaling, U.S.A.) and after with secondary antibody (Rabbit). Proteins recognized by antibodies were revealed by an electrochemiluminescence

(ECL) technique, following the Manufacturer's instructions (Amersham Biosciences, Amersham, U.K.). To standardize and quantify the immunoblots, we used the photo documentation system DP-001-FDC (Vilber-Lourmat, Torcy, France) and N.I.H. ImageJ software (*http://rsb.info.nih.gov/ij*). Several exposure times were analyzed to ensure the linearity of the band intensities.

Statistical analysis

Results are expressed as mean ± S.E.M. of the indicated number of experiments. Statistical comparisons for α-Klotho-induced changes in cytokines, Western blotting, and cell viability were performed by one-way analysis of variance (ANOVA), followed by the Tukey post-test. All analyses were performed using a Prism 9 software package (GraphPad Software, San Diego, CA, U.S.A.). P -values < 0.05. were considered to reflect a statistically significant difference.

3 Results

3.1. Effect of α-Klotho and LPS treatments on cell viability and cytotoxicity of primary glia culture

The different α-Klotho concentrations (0.1nM - 4 nM) did not alter cell viability or show toxicity to glial cells in both MTT and LDH assays (Fig. 1A and 1B). Regarding the LPS challenge, concentrations between 0.01 to 100 ug/mL caused no effect in MTT assay, but in the LDH assay, only concentrations of 10 and 100 ug/mL showed toxicity to glial cells when compared to the control group (Fig. 1C and 1D). Based on these data, the concentration of 1 μg/mL of LPS was chosen for the subsequent experiments.

3.2. Effect of α-Klotho on LPS-induced cytokines secretion in primary mouse glia culture

After analyzing cell viability and toxicity of different concentrations of α-Klotho in primary glia culture, time-course and concentration of α-Klotho effects on LPS - induced TNF-α secretion was evaluated. This cytokine was chosen based on previous studies in glial cells showing an increase of this cytokine after LPS treatment (Yshii, Denadai-Souza et al., 2015). Thus, concentrations ranging from 0.1 – 2 nM of α-Klotho and different time points of treatment (1,4 and 24 hours) were used followed by a LPS challenge (1 μg /mL for 8 hours) and TNF-α levels were determined by ELISA. Data showed that LPS induced increase in TNF-α levels compared to the control group and this effect was blocked by α-Klotho protein pretreatment at concentrations of 2 nM for 1 hour, and 1-2 nM for 4 and 24 hours (Fig. 2A, 2B, and 2C).

Based on these data, the concentration of 1 nM for 24 hours was chosen for evaluating the influence of α-Klotho on LPS (1 μg / mL for 8 hours) induced change in proinflammatory cytokines. In addition to TNF-α, LPS increased the production and secretion of other pro-inflammatory cytokines, such as IL-1β, IL6, and IFN-γ (Fig 3A, 3B, and 3C). However, α-Klotho treatment (1 nM for 24 hours) only reversed the LPS -induced increase of IL-6 levels (Fig. 3B) but not of IL-1β and IFN-γ (Fig. 3A and 3C).

3.3. Effect of α-Klotho on LPS-induced NΦ-κB activation in astrocytes primary mouse glia culture

To evaluate the influence of α-Klotho on NF-κB signaling we performed experiments in astrocytes primary culture based on previous evidence that secrected neuronal α-Klotho modulates astrocytic metabolic activity (Mazucanti, Kawamoto et al., 2019). For immunofluorescence experiments, we used staining of RelA (p65), which is a NF-kB subunit that is activated during LPS-induced inflammation (Fig. 4). Pre-treatment of cells with concentrations of 1 nM α-Klotho for 24h, followed by challenge with LPS at 4 hours (Fig. 4A,B) confirmed increased RelA nuclear translocation by LPS *vs* control. RelA nuclear translocation induced by LPS was completely revert by recombinant α-Klotho treatment (Fig. 4A,B). For a quantitative assessment, cells extract from cytosolic and nuclear fractions were used to evaluate the RelA (p65) content in each compartment by Western Blotting (Fig.4C,D). Results confirmed immunofluorescence data as the RelA (p65) subunit translocation subunit induced by LPS is inhibited by recombinant α-Klotho (Fig.4C,D). Finally, these groups were submitted to an EMSA assay to more precisely detect whether the NF-kB that translocated to

the nucleus was active and bound to its specific sequence in DNA (Fig. 4E,F). EMSA data confirmed both previous data as the binding of the nuclear extract to the 32P-labeled probe was much higher in LPS-treated astrocytes and pre-treatment with α-Klotho was able to inhibit the activation of this transcription factor. A super-shift assay was also performed (Fig. 4G) to clarify that NF-kB subunits are involved in this activation. Data confirmed that RelA (p65) and p50 subunits are involved, which typically occurs following activation by LPS (Glezer, Munhoz et al., 2003) .

3.4. Effects of α-Klotho on GCM -induced cytotoxicity of mouse primary neuronal culture

Primary glia culture was pretreated with serum-free medium (GCM) or with 1nM α-Klotho (GCM-KL) for 24 hours, and then challenged with 1 μg/mL LPS for 24 hours. Initially, it was necessary to determine the amount of GCM coming from LPS-challenged glia cell (GCM group) which caused neuronal death. Thus, we replaced 10, 25, and 50% of the cultured medium of the neuronal culture by GCM. The results showed that when the concentration of 25 and 50% of the neuronal culture medium were switched to GCM, there was an increase in neuronal toxicity when compared to the control group (Fig.5).

After determining the concentration of GCM that induced neurotoxicity, we investigated whether the GCM from α-Klotho pretreated glial culture (1nM) and stimulated with LPS (GCM-KL) would be able to decrease the neuronal death when compared to GCM at both 25 and 50% concentrations (Fig.6). The results showed that 25% of GCM-KL reversed the increase in neuronal death caused by GCM (Fig.6A), but α-Klotho pretreatment was not able to induce any effect to a high concentration (50% of GCM-KL) (Fig.6B).

4 Discussion

Neuroinflammation is a key feature of the aging process, neurodegenerative diseases and injuries that affect the CNS, and is characterized by the activation of microglia and astrocytes. These cells have a fundamental role in the regulation of neuroinflammation, depending on the nature of their activation, they can lead to the production of pro and / or anti-inflammatory mediators and have both beneficial and detrimental effects on neurons (Kotas & Medzhitov, 2015; Medzhitov, 2008). In this work, we sought to evaluate the role of α-Klotho in the modulation of inflammatory processes induced by LPS in glial cells. It is well known that activation by LPS in those cells leads to increased production and secretion of pro-inflammatory cytokines (including TNF-α, IL-6, IL-1, IL-12). In addition, LPS increases expression of other pro-inflammatory mediators, such as chemokines (like CXCL8, CCL5, and CCL2), complement system proteins (like C3, C3aR, C5a5, and factor B) and enzymes (like cyclooxygenase type 2 (COX-2) and induced nitric oxide synthase (iNOS) (Nazem, Sankowski et al., 2015; Rivest, 2009). Our data confirmed these data since LPS increased the secretion of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and IFN-γ.

Pro-inflammatory mediators, such as TNF-α, IL-1β, and IL-6, when produced in excess and/or in a chronic manner by glial cells can lead to neuronal death (Allan & Rothwell, 2001; Heneka, Kummer et al., 2014). Studies have already shown that the application of GCM of microglia and mixed glial culture activated by LPS in neurons leads to neuronal death (Dai, Yuan et al., 2019; Guadagno, Xu et al., 2013; Sun, Shen et al., 2018; Yshii, Denadai-Souza et al., 2015). According to these data, our data showed that the application of GCM from LPS-activated GCM in neurons led to a dose-concentration dependent increase in neuronal death. As the GCM was used, it is not possible to say which molecule is leading to neuronal death. Probably not just one, but a set of mediators, including the proinflammatory cytokines that were elevated after LPS stimulation such as TNF-α, as we previously demonstrated (Yshii, Denadai-Souza et al., 2015).

The activation of glial cells by LPS leads to an increase in the production of proinflammatory mediators in glial cells and these mediators are capable of inducing neuronal death (D'Angelo, Astarita et al., 2017). Therefore, we investigated whether α-Klotho would be able to decrease the effects induced by LPS in glial cells, since protective and anti-inflammatory activity of α-Klotho protein has already been seen in the renal, vascular and pulmonary systems (Kuro-o, 2019). However, the protective effect of α-Klotho protein in neuroinflammation has been poorly studied. α-Klotho has been shown to decrease NF-κB activation and reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, and IL-1β, *in vivo* and *in*

vitro in models of cardiac inflammation (Guo, Zhuang et al., 2018; Hui, Zhai et al., 2017), kidney disease (Zhao, Banerjee et al., 2011) and lung disease (Krick, Baumlin et al., 2017; Li, Wang et al., 2015). For example, in human kidney embryonic cells (HEK293), Zhao *et al.* demonstrated that pretreatment with 200 pM of α-Klotho for 45 minutes was able to decrease NF-κB activation by approximately 70% and reduce the expression of IL-8, MCP-1, RANTES, and IL-6 after the addition of TNF-α (Zhao, Banerjee et al., 2011). In HUVECs cells, the same pretreatment for 6 hours has been shown to decrease NF-κB activation and expression of adhesion molecules, intercellular adhesion molecule 1 (ICAM-1) and adhesion molecule of vascular cell 1 (VCAM-1), induced by TNF-α (Maekawa, Ishikawa et al., 2009).

Recent studies have demonstrated the protective and anti-inflammatory effect of α-Klotho in the CNS. α-Klotho overexpression in the mouse choroid plexus improved behavioral deficit and increased the number of live neurons after cerebral hypoperfusion, accompanied by a decrease in translocation p65 from the cytoplasm to the nucleus, production of proinflammatory cytokines and activation of astrocytes and microglia (Zhou, Li et al., 2017). A recently published study showed that α-Klotho's systemic overexpression in an experimental model of amyotrophic sclerosis (mouse transgenic for superoxide dismutase 1), led to later onset and progression of the disease and increased survival of these animals (Zeldich, Chen et al., 2019). Still, it was observed that α-Klotho decreased the expression of inflammatory markers and prevented neuronal death. In addition, a reduction in the secretion of TNF-α, IL-6, and the expression of iNOS and COX-2 induced by LPS / IFN-γ in mouse microglia culture that overexpressed α-Klotho (Zeldich, Chen et al., 2019).

In our study, α-Klotho was able to decrease the secretion of pro-inflammatory cytokines, TNF-α and IL-6. Pretreatment with 1 nM α-Klotho for 4 and 24 hours and 2 nM for 1, 4, and 24 hours decreased LPS-induced TNF-α secretion in glial cells. Also, pretreatment with 1 nM α-Klotho for 24 hours reversed the increase in IL-6 secretion induced by LPS.

Interestingly, experiments in astrocytes purified glial cells reforce the anti-inflammatory action of α-Klotho as pretreatment with this recombinant protein significantly reduce nuclear translocation of Rel A (p65) subunit of NF-κB induced by LPS as revealed by immunofluorescence and Westering blotting experiments. In addition, EMSA data confirmed that α-Klotho was able to revert the p65/p50 NF-κB activation induced by LPS suggesting a putative neuroprotective action of this protein. The present data reforces the importance of understanding the roles of astrocytes and microglia in the neurodegenerative diseases to develop effective therapies to neurodegenerative diseases (Kwon & Koh, 2020). Considering that insulin and glutamate - induced neuronal secreted α-Klotho plays a important role in brain metabolism and neuroinflammation by modulating neuronastrocyte coupling (Mazucanti, Kawamoto et al., 2019) it would be important to consider it an important player in the complex activated glial cells during degenerative diseases.

In fact, when the conditioned medium of glial cells was preincubated with α-Klotho (1 nM) for 24 hours before they being challenged with 1μg/mL LPS for 8 hours (GCM-KL), the neuronal death caused by the GCM of glial cells treated only with LPS was rescued in a lower concentration of GCM (25%), reinforcing, the therapeutic potential of α-Klotho in the CNS shown in other studies.

In fact, it is unclear which mediators were leading to neuronal death as well as to the neuroprotective effect. It may have been due to the decrease in TNF-α and IL-6 levels, which were observed in this study, as well as the decrease in other inflammatory mediators not evaluated or by inducing an adaptive response linked to an increase in protective factors, such as BDNF, or GNFT. In addition, α-Klotho may have had this protective effect due to the secretion of anti-inflammatory mediators and /or neuroprotective factors. Studies that have demonstrated an anti-inflammatory effect of α-Klotho observed a decrease in NF-κB activation (Maekawa, Ishikawa et al., 2009; Zhao, Banerjee et al., 2011). This modulation of NF-κB activation was also observed in the CNS (Zhou, Li et al., 2017). Our data confirmed that at least part of the mechanism by which α-Klotho could be leading to this anti-inflammatory and neuroprotective effect is mediated by NF-κB. The pretreatment with α-Klotho led to a decrease in the activation of NF-κB in astrocytes and, consequently, resulted in a decrease in the production and secretion of proinflammatory cytokines. It is interesting that previous studies from our laboratory showed that α-Klotho has a strong influence in the astrocytic metabolis stimulating aerobic glycoliysis and lactate release mediated by FGFR1 and Erk1/2 activation (Mazucanti,

Kawamoto et al., 2019). The ability of α-Klotho to modulate the FGFR and NF-ϰB signaling in astrocytes is consistent with results in other cell types (Wang, Liu et al., 2019) and suggest that in CNS α-Klotho can modulating neuroinflammation by neuron-astrocyte coupling action.

5 Discussion

In conclusion, our work demonstrated for the first time in primary cortical neuronal culture an anti-inflammatory and neuroprotective effect of the α-Klotho protein as the citotoxiciyty of GCM could be rescued by α-Klotho pretreatment (GCM-KL). This neuroprotective α-Klotho effect is at least partially mediated by astrocyte NF-ϰB modulation of neuroinflammation. These data suggest that α-Klotho can act not just in the metabolic coupling between neurons and astrocytes (Mazucanti, Kawamoto et al., 2019), but it is also an important player in modulating neuroinflammation which is important to integrate insulin and glutamate action in neurons. Thus, α-Klotho's therapeutic potential is evidenced in pathological processes that have a neuroinflammatory component.

Figures and Legends

Figure 1. Effect of α-Klotho (A,B) and LPS (C,D) treatment on cell viability (A,C) and cytotoxicity (B,D) of mouse glial cells. The primary culture of glial cells was subjected to 24-hour treatment with LPS in different concentrations. Cell viability and cytotoxicity were assessed by MTT and LDH assays, respectively. One-way ANOVA analysis, followed by Tukey's post-test, * $p < 0.05$. Results are presented as mean ± SEM of 7 independent experiments.

Figure 2. Effect of α-Klotho on LPS-induced TNF-α secretion in mouse glial cells. Glial culture was pre-treated with serum-free medium (control) or with α-Klotho protein in different concentrations (0.1, 0.5, 1 and 2 nM) and times, 1 (A), 4 (B) and 24 (C) hours, and then challenged with 1 μg/mL / mL LPS for 8 hours. The supernatant was collected to measure TNF-α levels. One-way ANOVA analysis, followed by Tukey's post-test, * $p < 0.05$, ** $p < 0.01$. Results are presented as mean ± SEM of 7 independent experiments.

Figure 3. Effect of α-Klotho on IL-1β, IL-6 and IFN-γ levels in mouse cell glia challenged with LPS. Glial culture was pretreated with serum-free medium (control) or with 1 nM α-Klotho for 24 hours, and then challenged with 1 μg/mL / mL LPS for 8 hours. The supernatant was collected to measure the levels of IL-1β (A), IL-6 (B), IFN-γ (C). One-way ANOVA analysis, followed by Tukey's post-test, * $p < 0.05$, ** $p < 0.01$, **** < 0.0001. Results are presented as mean ± SEM of 7 independent experiments.

Figure 4. α-Klotho rescue the NF-ϰB activation induced by LPS in nuclear fraction. (**A**) Purified astrocytes from cortical glial cells were treated 24h with vehicle (control) (PBS) or 1 nM α-Klotho. Inflammatory stimulation with LPS 1 μg/ml was then performed and the cells fixed with methanol 4 hours later stained with RelA antibody and DAPI. (**B**) The RelA (p65)-positive nuclei were counted and divided by the total number of nuclei, and the graph shows the comparison between the LPS group and for α-Klotho + LPS group expressed by the ratioin abitray units of RelA (p65) translocated to the nucleus over the total amount of RelA (p65) (n = 5). One-way ANOVA analysis, followed by Tukey's post-test, **p<0,01; ***p<0,001; ****p<0,0001. (**C,D**) Effect of α-Klotho on p65 subunit NFkB translocation in astrocytes cells.Nuclear (15 mg) and cytosol (20 mg) proteins were extracted from primary cultured cells: (C) Representative Western blotting autoradiographs of RELA (p65) nuclear and cytosolic and b-actin; (D) Densitometric analysis (arbitrary units, A.U.) of p65 nuclear/b-actin and p65 cytolosic/b-actin ratios of groups presented in C panel (n = 5). One-way ANOVA analysis, followed by Tukey's post-test, **p<0,01; ***p<0,001; ****p<0,0001. (E) Nuclear fraction was used to perform the EMSA assay to measure NF-kB activity. (**F**) Densitometric analysis comparing NF-ϰB activity of contro, α-Klotho, LPS and α-Klotho – LPS groups (n = 5). One-way ANOVA analysis, followed by Tukey's post-test,***p<0,0001.(**G**) A super-shift was also performed to show which NF-ϰB subunits are involved in this phenomenon.

Figure 5. Effects of GCM -induced cytotoxicity of mouse neurons. The primary embryonic culture of neurons was subjected to 24-hour treatment with GCM in different concentrations (25% and 50%). Cytotoxicity was assessed by the LDH assay. One-way ANOVA analysis, followed by Tukey's post-test, * p<0.05. Results are

presented as mean ± SEM of 5 independent experiments.

Figure 6. Effects of GCM and GCM-KL -induced cytotoxicity of primary cortical mouse neurons. The primary embryonic culture of neurons was submitted to 24-hour treatment with GCM or GCM-KL in different concentrations, 25% (A) and 50% (B). Cytotoxicity was assessed by the LDH assay. One-way ANOVA analysis, followed by Tukey's post-test, * $p < 0.05$, ** $p < 0.01$. Results are presented as mean ± SEM of 5 independent experiments.

Figure 7. Representative schedule of the anti-inflammatory and neuroprotective effect of α-Klotho protein. LPS induces GCM to produce pro-inflammatory mediators that can lead to neuronal death (A). α-Klotho protein decreases the production of pro-inflammatory mediators induced by LPS in GCM (B), and it can have a protective effect on neurons from the neurotoxic effects of LPS induced by GCM. Figure was "Created with BioRender.com."

7 References

Aizawa H, Saito Y, Nakamura T, Inoue M, Imanari T, Ohyama Y*, et al.* (1998). Downregulation of the Klotho gene in the kidney under sustained circulatory stress in rats. Biochemical and biophysical research communications 249**:** 865-871.

Akimoto T, Yoshizawa H, Watanabe Y, Numata A, Yamazaki T, Takeshima E*, et al.* (2012). Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease. BMC Nephrol 13**:** 155.

Allan SM, & Rothwell NJ (2001). Cytokines and acute neurodegeneration. Nature reviews Neuroscience 2**:** 734-744.

Bloch L, Sineshchekova O, Reichenbach D, Reiss K, Saftig P, Kuro-o M*, et al.* (2009). Klotho is a substrate for alpha-, beta- and gamma-secretase. FEBS letters 583**:** 3221-3224.

Chen CD, Podvin S, Gillespie E, Leeman SE, & Abraham CR (2007). Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Proceedings of the National Academy of Sciences of the United States of America 104**:** 19796-19801.

Clinton SM, Glover ME, Maltare A, Laszczyk AM, Mehi SJ, Simmons RK*, et al.* (2013). Expression of klotho mRNA and protein in rat brain parenchyma from early postnatal development into adulthood. Brain research 1527**:** 1-14.

D'Angelo B, Astarita C, Boffo S, Massaro-Giordano M, Antonella Ianuzzi C, Caporaso A*, et al.* (2017). LPS-induced inflammatory response triggers cell cycle reactivation in murine neuronal cells through retinoblastoma proteins induction. Cell Cycle 16**:** 2330-2336.

Dai D, Yuan J, Wang Y, Xu J, Mao C, & Xiao Y (2019). Peli1 controls the survival of dopaminergic neurons through modulating microglia-mediated neuroinflammation. Scientific reports 9**:** 8034.

Duce JA, Podvin S, Hollander W, Kipling D, Rosene DL, & Abraham CR (2008). Gene profile analysis implicates Klotho as an important contributor to aging changes in brain white matter of the rhesus monkey. Glia 56**:** 106-117.

Erben RG (2016). Update on FGF23 and Klotho signaling. Mol Cell Endocrinol 432**:** 56-65.

Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E*, et al.* (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. Annals of the New York Academy of Sciences 908**:** 244-254.

German DC, Khobahy I, Pastor J, Kuro OM, & Liu X (2012). Nuclear localization of Klotho in brain: an anti-aging protein. Neurobiol Aging 33**:** 1483 e1425-1430.

Ghosh S, May MJ, & Kopp EB (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annual review of immunology 16**:** 225-260.

Glezer I, Munhoz CD, Kawamoto EM, Marcourakis T, Avellar MC, & Scavone C (2003). MK-801 and 7-Ni attenuate the activation of brain NF-kappa B induced by LPS. Neuropharmacology 45**:** 1120-1129.

Guadagno J, Xu X, Karajgikar M, Brown A, & Cregan SP (2013). Microglia-derived TNFα induces apoptosis in neural precursor cells via transcriptional activation of the Bcl-2 family member Puma. Cell death & disease 4**:** e538.

Guo Y, Zhuang X, Huang Z, Zou J, Yang D, Hu X*, et al.* (2018). Klotho protects the heart from hyperglycemia-induced injury by inactivating ROS and NF-ϰB-mediated inflammation both in vitro and in vivo. Biochimica et biophysica acta Molecular basis of disease 1864**:** 238-251.

Heneka MT, Kummer MP, & Latz E (2014). Innate immune activation in neurodegenerative disease. Nature reviews Immunology 14**:**463-477.

Hui H, Zhai Y, Ao L, Cleveland JC, Jr., Liu H, Fullerton DA*, et al.* (2017). Klotho suppresses the inflammatory responses and ameliorates cardiac dysfunction in aging endotoxemic mice. Oncotarget 8**:** 15663-15676.

Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N*, et al.* (2004). Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. FEBS letters 565**:** 143-147.

Kawamoto EM, Lepsch LB, Boaventura MF, Munhoz CD, Lima LS, Yshii LM*, et al.* (2008). Amyloid beta-peptide activates nuclear factor-kappaB through an N-methyl-D-aspartate signaling pathway in cultured cerebellar cells. Journal of neuroscience research 86**:**845-860.

Kempuraj D, Thangavel R, Natteru PA, Selvakumar GP, Saeed D, Zahoor H*, et al.* (2016). Neuroinflammation Induces Neurodegeneration. Journal of neurology, neurosurgery and spine 1.

Kim JJ, & Diamond DM (2002). The stressed hippocampus, synaptic plasticity and lost memories. Nature reviews Neuroscience 3**:**453-462.

King GD, Rosene DL, & Abraham CR (2012). Promoter methylation and age-related downregulation of Klotho in rhesus monkey. Age (Dordrecht, Netherlands) 34**:** 1405-1419.

Kinoshita PF, Yshii LM, Orellana AMM, Paixão AG, Vasconcelos AR, Lima LdS*, et al.* (2017). Alpha 2 Na+,K+-ATPase silencing induces loss of inflammatory response and ouabain protection in glial cells. Scientific reports 7**:** 4894.

Kotas ME, & Medzhitov R (2015). Homeostasis, inflammation, and disease susceptibility. Cell 160**:** 816-827.

Krick S, Baumlin N, Aller SP, Aguiar C, Grabner A, Sailland J*, et al.* (2017). Klotho Inhibits Interleukin-8 Secretion from Cystic Fibrosis Airway Epithelia. Scientific reports 7**:** 14388.

Kuro-o M (2019). The Klotho proteins in health and disease. Nature Reviews Nephrology 15**:** 27-44.

Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T*, et al.* (1997). Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390**:** 45-51.

Kwon HS, & Koh S-H (2020). Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. Translational Neurodegeneration 9**:** 42.

Li L, Wang Y, Gao W, Yuan C, Zhang S, Zhou H*, et al.* (2015). Klotho Reduction in Alveolar Macrophages Contributes to Cigarette Smoke Extract-induced Inflammation in Chronic Obstructive Pulmonary Disease. The Journal of biological chemistry 290**:** 27890-27900.

Li SA, Watanabe M, Yamada H, Nagai A, Kinuta M, & Takei K (2004). Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. Cell Struct Funct 29**:** 91-99.

Liu X, Wu Z, Hayashi Y, & Nakanishi H (2012). Age-dependent neuroinflammatory responses and deficits in long-term potentiation in the hippocampus during systemic inflammation. Neuroscience 216**:**133-142.

Maekawa Y, Ishikawa K, Yasuda O, Oguro R, Hanasaki H, Kida I*, et al.* (2009). Klotho suppresses TNF-alpha-induced expression of adhesion molecules in the endothelium and attenuates NF-kappaB activation. Endocrine 35**:** 341-346.

Mazucanti CH, Kawamoto EM, Mattson MP, Scavone C, & Camandola S (2019). Activity-dependent neuronal Klotho enhances astrocytic aerobic glycolysis. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 39**:** 1544-1556.

Medzhitov R (2008). Origin and physiological roles of inflammation. Nature 454**:** 428-435.

Nabeshima Y (2002). Klotho: a fundamental regulator of aging. Ageing Res Rev 1**:** 627-638.

Nazem A, Sankowski R, Bacher M, & Al-Abed Y (2015). Rodent models of neuroinflammation for Alzheimer's disease. Journal of neuroinflammation 12**:** 74.

Ohyama Y, Kurabayashi M, Masuda H, Nakamura T, Aihara Y, Kaname T*, et al.* (1998). Molecular cloning of rat klotho cDNA: markedly decreased expression of klotho by acute inflammatory stress. Biochemical and biophysical research communications 251**:** 920-925.

Ownby RL (2010). Neuroinflammation and cognitive aging. Current psychiatry reports 12**:** 39-45.

Razzaque MS (2009). The FGF23-Klotho axis: endocrine regulation of phosphate homeostasis. Nat Rev Endocrinol 5**:** 611-619.

Rivest S (2009). Regulation of innate immune responses in the brain. Nature reviews Immunology 9**:** 429-439.

Sun L, Shen R, Agnihotri SK, Chen Y, Huang Z, & Büeler H (2018). Lack of PINK1 alters glia innate immune responses and enhances inflammation-induced, nitric oxide-mediated neuron death. Scientific reports 8**:** 383.

Thomson LM, & Sutherland RJ (2005). Systemic administration of lipopolysaccharide and interleukin-1beta have different effects on memory consolidation. Brain research bulletin 67**:** 24-29.

Wang C, Liu Z, Ke Y, & Wang F (2019). Intrinsic FGFR2 and Ectopic FGFR1 Signaling in the Prostate and Prostate Cancer. Frontiers in Genetics 10.

Wang Y, & Sun Z (2009). Current understanding of klotho. Ageing research reviews 8**:** 43-51.

Xiao NM, Zhang YM, Zheng Q, & Gu J (2004). Klotho is a serum factor related to human aging. Chinese medical journal 117**:** 742-747.

Yshii LM, Denadai-Souza A, Vasconcelos AR, Avellar MC, & Scavone C (2015). Suppression of MAPK attenuates neuronal cell death induced by activated glia-conditioned medium in alpha-synuclein overexpressing SH-SY5Y cells. Journal of neuroinflammation 12**:** 193.

Zeldich E, Chen CD, Boden E, Howat B, Nasse JS, Zeldich D*, et al.*(2019). Klotho Is Neuroprotective in the Superoxide Dismutase (SOD1(G93A)) Mouse Model of ALS. Journal of molecular neuroscience : MN 69**:** 264-285.

Zhao Y, Banerjee S, Dey N, LeJeune WS, Sarkar PS, Brobey R*, et al.* (2011). Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation. Diabetes 60**:** 1907-1916.

Zhou HJ, Li H, Shi MQ, Mao XN, Liu DL, Chang YR*, et al.* (2017). Protective Effect of Klotho against Ischemic Brain Injury Is Associated with Inhibition of RIG-I/NF-κB Signaling. Frontiers in pharmacology 8**:** 950.

Hosted file

Figure1-7ViniBJP.pdf available at https://authorea.com/users/378268/articles/564587-neuroprotective-action-of-%CE%B1-klotho-against-lps-activated-glia-conditioned-medium-in-primary-neuronal-culture