Research Square

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Lithium Inhibits Oxidative Stress-Induced Neuronal Senescence Through miR-34a

Kemal Ugur Tufekci
İzmir Democracy University: Izmir Demokrasi Universitesi
Begum Alural
Dokuz Eylul University: Dokuz Eylul Universitesi
Emre Tarakcioglu
Dokuz Eylul University: Dokuz Eylul Universitesi
Tugba San
Dokuz Eylul University: Dokuz Eylul Universitesi
Sermin Genc (sermin.genc@deu.edu.tr)
Izmir Biomedicine and Genome Center https://orcid.org/0000-0001-6126-7460

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Abstract

Neuronal senescence, triggered by telomere shortening, oncogene activation, DNA damage, or oxidative stress, has been associated with neurodegenerative diseases' pathogenesis. Therefore, preventing neuronal senescence could be a novel treatment strategy for neurodegenerative diseases. Lithium (Li), the first-line treatment against bipolar disorder, has been shown to have neuroprotective effects in clinical, pre-clinical, and *in vitro* studies. Lithium can protect cells from senescence, and its effect on neuronal senescence was investigated in our study. Furthermore, we also investigated the effects of lithium on the senescence-associated miR-34a/Sirt1/p53 pathway. In this study, hydrogen peroxide was used as an inducer for the "stress-induced premature senescence" model. In the senescence model, we have assessed Li's effects on senescence by analyzing ß-galactosidase activity, Sudan Black B, and senescence-associated heterochromatin foci (SAHF) stainings, and on cell cycle arrest by BrdU staining. Furthermore, expression levels of senescence and cell cycle arrest-related proteins (p53, p21, p16INK4a, and SIRT1) by western blotting. Finally, the effects of Li on senescence-associated miR-34a levels were measured by quantitative PCR. We show via Sudan Black B staining, β-Gal activity assay, and by detecting SAHF, Li protects against senescence in neuronal cells. Then, lithium's effect on signaling has also been determined on pathways involved in senescence and cell cycle arrest. Moreover, we have observed that Li has a modulatory effect on miR-34a expression. Therefore, we posit that Li suppresses senescence in neuronal cells and that this effect is mediated through miR-34a/Sirt1/p53 axis.

Introduction

Cellular senescence is a state of permanent cell cycle arrest that results in tissue dysfunction and is associated with aging and age-related diseases [1]. The types of senescence by cause are generally addressed in two: replicative senescence, where cells stop dividing due to a DNA damage response (DDR) induced by critical telomere shortening; and premature senescence, where any of several stressors, including mutations in oncogenes or tumor-suppressor genes, DNA damage, endoplasmic reticulum (ER) stress, and oxidative stress causes the cell cycle arrest [2]. Since DNA damage, which is a defining attribute of replicative senescence, can also appear in premature senescence, definitively identifying the type of senescence a cell has undergone can, in some cases, be challenging [3]. Besides the standard features of arrested cell cycle and function loss, it has been reported that some senescent cells acquire an inflammatory phenotype termed as senescent-associated secretory phenotype (SASP), where they engage in paracrine inflammatory signaling, driving chronic inflammatory diseases [4]. Since one function of senescence is oncogenesis prevention, tumor-suppressor proteins' expression or activity tends to be elevated in senescent cells [2]. Tumor-suppressor proteins most commonly used in identifying senescent cells are p53, p21, and p16/INK4A [5]. The causes of senescence are multifactorial, as stated above and often overlap. The two nearly universal senescence factors are DDR, whether caused by telomere attrition, mitochondrial dysfunction/reactive oxygen species (ROS), or ionizing radiation; and elevated activity of the tumor-suppressor proteins p53 or p16/INK4a [6, 7]. Since its discovery, multiple markers of senescence have been identified. A typical senescent cell marker is a change in morphology,

where cells become enlarged and flattened [8]. Though depending on the type of cells or senescence trigger, the morphological change can manifest differently; for example, a morphological alteration that appears with ER stress is increased vacuolization in the cytoplasm [2]. Historically, the first biochemical marker of senescence that is still commonly used is increased activity of the β-galactosidase enzyme, usually termed "senescence-associated β-galactosidase (or SA-β-gal)" [9]. While this was initially shown only in cultured cells' replicative senescence, later in vivo studies have reported β-gal activity in senescent-like cells in animals [10, 11].

The senescence of neurons and other central nervous system cells contributes to neurodegenerative diseases [12, 13]. Although senescence in postmitotic cells like neurons may sound counterintuitive, several in vivo and in vitro studies have reported senescent-like states identified by other markers of senescence in neurons and glia [12, 11, 13]. Premature senescence, especially SASP, has been implicated in various age-related diseases, including diabetes, osteoarthritis, atherosclerosis, cancer, Alzheimer's disease, and Parkinson's disease [12, 1]. As poor health brought about by aging creates a substantial economic burden [14], it is advantageous to reduce the rate of degenerative aging and increase global health.

Lithium, the first-line treatment against bipolar disorder, has been shown to have neuroprotective effects in clinical, pre-clinical, and in vitro studies [15]. Mechanisms of lithium that might help exert its protective role include reducing pro-apoptotic/pro-senescence p53 and increasing anti-apoptotic Bcl-2 and brain-derived neurotrophic factor (BDNF) [16]. The effect of lithium in senescence induction seems to depend on cell type, the senescence trigger, and the strength of pro-apoptotic vs. pro-survival signals [17]. The primary anti-senescence action of lithium is the inhibition of glycogen synthase kinase 3 (GSK3) [18], a protein that helps p53 to exert its role [19]. A study of replicative senescence in human fibroblasts shows that lithium, by its anti-GSK3 action, lets late-passage cells enter a reversible quiescence state instead of senescence [20]. Lithium also reverses the rise in p53 and p21 protein expression and β-gal activity in late passage fibroblasts [20]. In endothelial cells, lithium has a pro-senescence effect that is independent of its GSK3 inhibition, where it upregulates the expression of matrix metalloproteinase 1 (MMP1) [21], an enzyme often secreted by SASP cells [1]. Similar results have also been observed in rat nucleus pulposus cells, where lithium again induced senescence and a rise in matrix metalloproteinases [22]. A recent study on human iPSC-derived astrocytes (in a model without a senescence trigger) shows that low-dose lithium can prevent senescence and SASP markers, including β-gal activity and mRNA expression of p16, p21, and IL-1β [23].

MicroRNAs (miRNAs) are 22-nt members of short non-coding RNA species that regulate expression by binding to 3’-UTR of their target genes. Naturally, miRNAs that target pathways suppressing senescence tend to be upregulated in senescent cells [24, 25]. A well-studied senescence-associated miRNA is miR-34a [26], which suppresses telomerase activity and expression of SIRT1, a histone deacetylase that promotes cell survival [27]. Another biomarker of senescence –primarily observed in oncogene-induced senescence– is the condensation of repressive epigenetic marks in chromatin into multiple focal points, termed senescence-associated heterochromatic foci (SAHF), which can be visually detected by DAPI

staining or antibodies for methylations common in senescence such as H3K9me3 [28]. As none of the senescence markers have been specific enough to identify the senescent state exclusively, most studies use multiple markers in combination [7].

In this study, to gain insight into how lithium can prevent the occurrence or ameliorate effects of premature senescence in neurons, we have used an H_2O_2-induced (i.e., ROS-mediated) premature senescence model, together with lithium treatment on the human neuroblastoma cell line SH-SY5Y. We show via Sudan Black B staining, β-Gal activity assay, and by detecting SAHFs, that lithium protects against senescence in SH-SY5Y cells. Moreover, we have observed that lithium has a modulatory effect on miR-34a-5p, a miRNA that is associated with aging [29, 30] and targets –among others- the mRNA of SIRT1 [31], a longevity associated protein that boosts mitochondrial function [32]. Therefore, this study aims to examine Li's effects on oxidative stress-induced neuronal senescence and unravel the related signaling mechanisms.

Materials And Methods

Cell culture and treatments

In this study, SH-SY5Y human neuron-like cells were used. Cells were cultured in DMEM: F/12 culture medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in an incubator containing 5% CO_2 (Thermo Scientific, USA).

To induce senescence SH-SY5Y cells, they were incubated with 25 µM H_2O_2 for 1 hour, then rested for 72 hours in DMEM:F12 medium containing 10% FBS. At the end of the incubation periods, senescence markers in the cells were analyzed. To test Li's effects on senescence, cells were incubated with lithium at a dose of 2 mM for 24 hours before H2O2 treatment.

β-Galactosidase (β-Gal) activity assay

The β-Gal activity was measured using Cellular Senescence Activity Assay (Enzo Life Sciences, USA) according to the manufacturer's recommendations. Briefly, at the end of treatments, cells were washed once with PBS. Then the cells that were lysed for 5 minutes at 4°C with lysis solution, which is supplied with the kit, were centrifuged, and 50 µl of each sample was taken into an empty 96-well plate for analysis, and the incubation buffer was added and kept at 37°C for 2 hours. Finally, fluorescence reading was carried out at 355nm (excitation) and 460nm (emission). The increase in cell aging activity is given as a "relative fluorescence unit" (RFU) [33].

Sudan Black B staining

The lipophilic dye Sudan Black B (SBB) detects a biochemical marker of senescence called lipofuscin, an aggregate of oxidized substances that accumulate in aged tissue or cells [34]. Following the treatment of cells with H2O2 with or without lithium, the medium was replaced with 70% ethanol and then incubated with a saturated SBB solution (in 70% Ethanol) for 8 minutes [35]. Following SBB incubation, slides were

rinsed 50% ethanol and placed in distilled water. Afterward, a 0.1% Nuclear Fast Red stain solution was applied and incubated for 2 minutes. Finally, sections were washed and mounted in 50% glycerol solution in distilled water. Stained cells were visualized by light microscope (IX71, Olympus, Japan).

BrdU incorporation assay

According to the manufacturer, the effect of cell aging on the cell cycle was examined with the commercially purchased BrdU (5-Bromo-2-deoxyuridine) detection kit to determine proliferation (Cell Signaling, USA) 's instructions. Briefly, at the end of H_2O_2 and Lithium treatments, the cells were fixed and permeabilized by adding 100 µl 1X fixation solution at room temperature. Then, the fixation solution was removed, and the detection antibody solution was added to each well and incubated for 1 hour at room temperature. At the end of the incubation, 100 µl/well of 1X HRP-conjugated secondary antibody solution was applied at room temperature for 30 minutes. Following final washing steps after secondary antibody 1:1 mixture of Luminol / Enhancer Solution and Stable Peroxide solution was applied to each well, and luminometric measurement was performed using Centro LB 960 microplate luminometer (Berthold Technologies, Switzerland).

Quantitative PCR

RNA was isolated by the miRNeasy kit protocol (Qiagen, Germany) using TRIzol reagents. cDNA synthesis was done with the miScript II RT Kit (Qiagen, Germany). For real-time PCR, primary, precursor, and mature forms of miR-34a were assessed with specific primers and control RNA primers (U6 and SNORD_95). Relative expression changes were analyzed according to the ΔΔCt method [36].

Western blotting

Cells were collected by centrifugation at the end of the incubation period for protein extraction. The RIPA lysis solution containing protease inhibitors was added to the pellet for 15 minutes on ice and occasionally vortexed to facilitate lysis. Afterward, the protein was obtained by 15 minutes centrifugation at 15000 x g. Protein concentration was determined by BCA protein assay. Protein samples were separated with 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membrane and blocked in 5% milk solution. After this procedure, samples were incubated overnight at 4°C with p53, ac-p53 (Lys 120), p-p53 (Ser 15), p21, p16INK4a, Sirt1, and β-actin antibodies. Then the blots were incubated with HRP conjugated secondary antibody, were visualized with the ECL kit based on the membrane chemiluminescence method, and the images were obtained using the Vilber Lourmat chemiluminescence documentation system. Band densities were evaluated densitometrically with Image J software.

SAHF Staining

After treatments, cells were fixed with 4% paraformaldehyde for 10 minutes. The cells were then washed with PBS and incubated with 0.2% Triton X-100 / PBS for 10 minutes to permeabilize. Subsequently, staining was performed with the H3K9me3 antibody (Abcam, USA) and Alexa Fluor 594 conjugated secondary antibody (Jackson Immunoresearch, USA). Finally, DNA was stained with DAPI (Sigma Aldrich,

Germany) for 1 minute. At the end of staining, cells were washed with PBS and imaged with a confocal microscope (Zeiss LSM 880, Germany) to examine heterochromatin formation [37].

Results

Effects of Lithium and Oxidative stress on cell proliferation

To determine whether H2O2 or lithium affected cell proliferation, we pre-treated SH-SY5Y cells with 2 mM lithium for 24 hours, then 25 μM H2O2 for 1 hour, after which the cells were incubated for 72 hours in the standard growth medium. At the end of the incubation period, we performed a BrdU incorporation assay to assess the proliferation rate. We observed that lithium promotes cell proliferation when given alone and restores a slightly decreased proliferation rate of the H2O2 administered cells (Fig. 1).

Lithium attenuated oxidative stress-induced effects of cell morphology and cellular senescence

To confirm H2O2-induced senescence and look into the effects of lithium treatment on H2O2-induced senescence, the cells were visualized at the end of their post-treatment incubation with phase-contrast microscopy to observe changes in cellular morphology. A portion of the H2O2 administered cells acquired a flat and enlarged shape, which is typical in cellular senescence, while cells with the lithium + H2O2 treatment had a reduced number of these cells (Fig. 2A). In another instance of the same experiment, we looked at lipofuscin aggregation as a measure of senescence; and observed a moderate rise in lipofuscin deposition in the H2O2 group and a reversal of the effect in the lithium + H2O2 group (Fig. 2B). We also performed a β-galactosidase activity assay as a third measure of senescence and witnessed a 39% rise in β-galactosidase activity in the H2O2 group, which decreased 32% with lithium pretreatment (Fig. 2C).

Effects of Lithium and Oxidative stress on epigenetic markers of senescence

To further probe for senescence signs, we stained the SH-SY5Y cells for SAHF on their chromatin, using antibodies against the common methylation marker H3K9me3. The imaging indicated that H_2O_2 administration induces SAHF formation, while lithium treatment downregulated this effect (Fig. 3).

Lithium treatment reversed oxidative stress induced expression alterations of cell cycle arrest and longevity related genes

To detect whether tumor suppressor pathways were also activated in our model, we explored the change in protein levels of senescence-associated tumor suppressors p53, p21, and p16/INK4A. While the expression of total p53 was mostly unchanged, the protein levels of acetylated and phosphorylated p53, p21, and p16/INK4A were increased with H2O2 administration and reverted closer to basal levels with lithium treatment. Similarly, levels of the anti-senescence/pro-survival protein SIRT1 fell slightly with H2O2 and rose back to basal levels with lithium. (Fig. 4).

Lithium affects senescence associated expression of miR-34a

In order to gain insight into how lithium might be protecting SH-SY5Y cells from senescence, we analyzed the expression levels of the senescence-associated microRNA miR-34a, which decreases with lithium treatment [38]. The qPCR results indicate that mature miR-34a and its pri- and pre- forms are downregulated with lithium alone (-20% for mature miRNA) and upregulated in the senescence model (+ 63% for mature miRNA). Here too, lithium acts protectively, inhibiting the rise in miR-34a in H2O2 administered cells, albeit slightly (-10.5% for mature miRNA) (Fig. 5).

Discussion

In our study, we show by multiple techniques that lithium protects against H_2O_2-induced senescence in SH-SY5Y cells. Moreover, we have observed that lithium has a modulatory effect on miR-34a-5p, associated with aging, and targets –among others- the mRNA of SIRT1, a longevity-associated protein. Therefore, we suggest that lithium suppresses senescence in neuroblastoma cells and that this effect is mediated at least partially through miR-34a-5p.

Previous research has confirmed markers of neuronal senescence in brain sections of old mice [11, 39] and long-term cultures of rat cortical neurons [40]. These markers include increased β-galactosidase activity, accumulation of lipofuscin deposits, cytoplasmic enlargement, and a rise in heterochromatic foci. Furthermore, in mice, it was shown that for DNA-damaged neurons to senesce, the presence of p21 was essential [11], so a rise in p21 expression is to be expected in senescence. Accordingly, we have probed for these markers in our H_2O_2 induced senescence model to confirm that H_2O_2 does indeed produce previously established markers of senescence in neurons.

We have used a ROS-mediated senescence model, where administration of H_2O_2 leads to an accumulation of ROS dependent DNA damage [41]. A 2014 study that employs 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) to induce ROS-mediated senescence in SH-SY5Y cells has demonstrated elevated β-galactosidase activity and increased protein levels of p21 and p16/INK4A [42]. Our similar findings indicate that neurons undergoing ROS-mediated senescence are likely to display these markers.

We observe in our H_2O_2 induced senescence model an increase in p21 and p16 levels (and similarly, the activity of p53, the primary inducer of p21, appears to be increased). These proteins are potent cell cycle inhibitors and are cell cycle arrest agents in senescence [43]. Lithium treatment reverses this trend, rescuing cells from cell cycle arrest. It is reported that the longevity-associated deacetylase SIRT1 inhibits the p53/p21 pathway [44] and expression of p16 [45]. In agreement with that, we also see that SIRT1 decreases in the senescent cell group and returns to normal levels in the lithium-treated group. The SIRT1 drop is in line with a previous finding that nuclear SIRT1 declines in senescent-like neurons with lipofuscin deposits in old mice [39]. The H_2O_2-induced decrease in SIRT1 is accompanied by a rise in miR-34a –which targets SIRT1 [31]– in our study. Seeing how the senescence-associated changes in both SIRT1 and miR-34a are reversed by lithium, we suggest that lithium induces a drop in miR-34a expression,

which would result in a restoration of SIRT1 levels and inhibition of p53, p21 and p16 (though further experiments would be required to demonstrate a more evident causation link).

It is also possible that the decrease in miR-34a levels is induced by the reduced activity of p53, as the two molecules are reported to create a positive feedback loop [46]. Thus, lithium might be suppressing p53 activity and accumulation by inhibiting GSK3 [20, 19], leading to a drop in miR-34a levels. However, the relationship between GSK3 and p53 is disputed, as another study indicates inhibition of GSK3 results, conversely, in an increase in transcriptionally active p53 [47]. Cell state (the cell's propensity to undergo senescence, apoptosis, or proliferation) may influence how GSK3 inhibition would affect p53 levels and activity.

The ROS-induced senescence model is not the only model of senescence, and the effect of lithium in other instances of physiological senescence may vary. The effect might also change when translated from cell culture into in vivo. The lithium dose would need to be adjusted for clinical usage as a senostatic (suppressor of senescence). To shed more light on lithium's senescence-related properties, lithium's effects could be studied in other cell types —preferably primary cells, for a better representation of the physiological milieu.

There is also the issue that mere suppression of senescence may not necessarily be beneficial for an organism in the long term [48]. Since in a physiological context, senescent cells arise due to cellular stress or DNA damage and display aberrant function. It would be preferable to dispose of them entirely rather than risk their deleterious influence on their surroundings (as in the case of SASP). Thus, while our study shows that lithium has a senostatic effect, it remains to be shown that this suppression of senescence also cancels the harmful paracrine actions and restores senescent neurons' functionality.

In conclusion, our data establish that H_2O_2 induces senescence efficiently in SH-SY5Y cells. Our neuronal senescence model displays previously confirmed senescence markers that appear with ROS or old age in mammalian neurons. We also provide evidence that lithium protects against ROS-mediated senescence in neuronal cell culture. Our finding that this protection is coupled with a decrease in tumor-suppressor proteins p53, p21, p16/INK4a, a decline in miR-34a, and a rise in SIRT1, suggests that lithium's modulation of these proteins is the primary cause of its senostatic effect.

Declarations

Author contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kemal Ugur Tufekci, Begum Alural, Emre Tarakcioglu, and Tugba San. The first draft of the manuscript was written by Kemal Ugur Tufekci and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethical approval: This study does not require any ethical statement.

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