Neuroscience Letters 661 (2017) 51–56

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

miR-34a/Bcl-2 signaling pathway contributes to age-related hearing loss by modulating hair cell apoptosis

Qiuhong Huang[a,b], Yiqing Zheng[a,b,*], Yongkang Ou[a,b], Hao Xiong[a,b], Haidi Yang[a,b], Zhigang Zhang[a,b], Suijun Chen[a,b], Yongyi Ye[a,b]

[a] Department of Otolaryngology-HNS, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen university, 107 Yanjiang West Rd, Guangzhou 510120, China
[b] Institute of Hearing and Speech-Language Science, Sun Yat-sen University, China

HIGHLIGHTS

- miR-34a expression increases with age in the cochlea of a hearing loss mouse model.
- Overexpression of miR-34a inhibits Bcl-2 in hair cells.
- Suppression of Bcl-2 by miR-34a increases age-induced apoptosis in hair cells.

ARTICLE INFO

Article history:
Received 31 October 2016
Received in revised form 24 July 2017
Accepted 25 July 2017
Available online 26 July 2017

Keywords:
Apoptosis
Bcl-2
Cochlea
miR-34a
Presbycusis

ABSTRACT

MicroRNAs, such as miR-34, have been reported to influence age-related diseases. In this study, we explored the role of the miR-34a/Bcl-2 signaling pathway in age-related hearing loss (AHL). Using an AHL mouse model (C57BL/6), we found that the expression of miR-34a in the cochlea increased with age, whereas expression of Bcl-2 decreased. Increasing the amount of a miR-34a mimetic in a mouse auditory cell line (HEI-OC1) inhibited Bcl-2, leading to enhanced apoptosis; in contrast, miR-34a inhibition produced the opposite effect. Our results support a link between age-related cochlear hair cell apoptosis and miR-34a/Bcl-2 signaling. The latter may thus serve as a potential target for AHL therapy.

1. Introduction

Age-related hearing loss (AHL), also known as presbycusis, is a multifactorial disease involving genetic and environmental factors [1,2]. Previous studies on aging in humans and in animal models have linked AHL with the irreversible loss of cochlear hair cells in the inner ear [3–5].

Micro RNAs (miRNAs) are endogenous non-coding small RNAs involved in post-transcriptional regulation of gene expression. They have been reported to play an important mechanistic role in AHL. However, to date, their influence on hair cells during aging has not been fully elucidated. The miR-34 family (miR-34a, miR-34b, and miR-34c) is involved in development, cell proliferation and differentiation, oncogenesis, and apoptosis [6]. Xiong et al. [7] discovered that miR-34a expression increased during aging in the cochlea of an AHL mouse model. The Bcl-2 family of proteins plays a crucial role in the regulation of apoptosis in the nervous system. Bcl-2, the first gene shown to be involved in apoptosis, encodes a 26-kDa intracellular membrane-associated protein. Bcl-2 is present in the outer mitochondrial membrane and in some parts of the endoplasmic reticulum and the nuclear membrane. Bcl-2 is a potent regulator of neuronal survival, serving mainly as a repressor of apoptosis. p53 has been found to induce miR-34a, which then acts directly on the Bcl-2 mRNA 3′-UTR, inhibiting its synthesis and leading to cell apoptosis [8]. A previous study [9] has shown that the Bcl-2 family is regulated in the cochlear organ in response to noise-induced hearing loss. In addition, Someya et al. [10,11] have reported that Bak, a Bcl-2 family member, protects against AHL.

We hypothesized that the miR-34a/Bcl-2 signaling pathway might play a crucial role in hair cell apoptosis during aging. In

* Corresponding author at: Department of Otolaryngology-HNS, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen university, 107 Yanjiang West Rd, Guangzhou 510120, China.
E-mail addresses: hqh_sysu@126.com (Q. Huang), yiqingzheng@hotmail.com (Y. Zheng), yongkang_ou@yahoo.com (Y. Ou), drxionghao@gmail.com (H. Xiong), yanghaidi1978@163.com (H. Yang), zhangzz0369@126.com (Z. Zhang), chensuijun@hotmail.com (S. Chen), 381297751@qq.com (Y. Ye).

http://dx.doi.org/10.1016/j.neulet.2017.07.044
0304-3940/

this study, cochlear miR-34a/Bcl-2 expression and activation were examined in a C57BL/6 mouse model of AHL. Additionally, miR-34a/Bcl-2 activation was also assessed in a mouse auditory cell line (HEI-OC1).

2. Materials and methods

2.1. Animals

Fifty-seven C57BL/6 mice were obtained from the Laboratory Animal Center of Sun Yat-sen University. They were divided into two groups: young (male, aged 3–6 weeks, 24 animals) and old (male, aged 12–15 months, 33 animals). Animal care and experimental treatment were approved by the Animal Research Committee of Sun Yat-sen University.

2.2. Auditory brainstem response (ABR)

Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine mixture, after which ABR measurements were performed. A computer-aided evoked potential system (Intelligent Hearing Systems, Miami, FL, USA) was used to test the evoked ABR thresholds, as described previously [12]. Mice were presented with broadband clicks and 8-, 16-, or 32-kHz pure tone bursts.

2.3. Tissue preparation

After ABR recordings, the deeply anesthetized animals were euthanized. Cochleae were removed immediately and fixed by immersion in 4% paraformaldehyde in 0.1 mM phosphate-buffered saline (PBS), pH 7.4, overnight at 4 °C. Cochleae were decalcified in 4% sodium ethylenediaminetetraacetic acid for 4 days, followed by an overnight incubation in 30% sucrose. The next day, cochleae were embedded in OCT compound (Sakura, Torrance, CA, USA). For immunohistochemistry staining, cochleae were cryosectioned into 10-μm-thick slices, mounted on glass slides, and stored at −20 °C.

For RNA and western blot preparations, cochlear tissues were dissected with small forceps and immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.4. HEI-OC1 cell culture

The immortalized mouse auditory cell line, House Ear Institute-Organ of Corti 1 (HEI-OC1), was kindly provided by Prof. F. Kalinec (House Ear Institute, Los Angeles, CA, USA). HEI-OC1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad. CA, USA) supplemented with 10% fetal bovine serum (Gibco) at 33 °C in 10% CO_2.

2.5. Transfection with a miRNA mimetic and inhibitor

To examine the effect of miR-34a on Bcl-2, HEI-OC1 cells were transfected with a miR-34a mimetic, a negative control miRNA mimetic, a miR-34a inhibitor, or a negative control miRNA inhibitor (GenePharma, Shanghai, China) at various concentrations and harvested 48 or 72 h later.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol; 1 μg was reverse transcribed to cDNA using ReverTra-Plus-TM (TOYOBO, Tokyo, Japan). The sequences of the primers used were as follows: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), forward: 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′; and *Bcl-2*, forward: 5′-GTCGCTACCGTCGTGACTTC-3′, reverse: 5′-CAGACATGCACCTACCCAGC-3′. cDNA samples were amplified using SYBR Green premix Ex TagTM (Tli RNaseH Plus, TaKaRa, Shiga, Japan) and detected with a Roche LightCycler 480 RT-PCR system (Roche Diagnostics, Basel, Switzerland). Thermocycling conditions were as follows: denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, and extension for 10 s at 72 °C. *Bcl-2* levels were normalized to the housekeeping gene *GAPDH*. The PCR products were also separated on a 2% agarose gel and visualized by ethidium bromide staining.

For miR-34a expression analysis, enriched small RNAs were isolated from cochlear tissues using TRIzol Reagent. RNA (500 ng) was reverse transcribed using specific miRNA stem primers and a PrimeScript RT reagent kit (TaKaRa). Mature miRNA expression was measured with TaKaRa Taq Version 2.0 plus dye according to the manufacturer's instructions, and miRNA levels were normalized to U6 small nuclear RNA expression.

2.7. Immunohistochemistry

Cochlear sections were thawed at room temperature and washed in PBS. Next, they were incubated in 0.5% Triton X-100 for 15 min at room temperature, washed with PBS, and incubated with blocking solution consisting of 0.5% bovine serum albumin and 5% goat serum in PBS with 0.5% Triton X-100. Sections were washed and incubated overnight at 4 °C with primary antibodies (1:100) against Bcl-2 and Myosin VIIA (both purchased from Santa Cruz Biotechnology, Dallas, TX, USA). Negative control sections were incubated in PBS without primary antibodies. Following several washes, tissues were incubated with secondary antibodies (Alexa Flour 488 and 594, 1:200; Invitrogen, Carlsbad, CA, USA) at 4 °C for 1 h. The slides were counterstained with 4′,6-diamidino-2-phenylindole and covered with glass cover slips. Sections were viewed using an Olympus BX63 microscope (Olympus, Tokyo, Japan).

2.8. Western blot

Dissected tissues were homogenized with a rotor tissue homogenizer in RIPA buffer supplemented with 1 mM phenylmethane sulfonyl fluoride and a mixture of additional protease inhibitors (Halt Proteinase Inhibitor Cocktail; Thermo Scientific, Rockford, IL, USA). Total tissue lysates were centrifuged at 14000 rpm for 20 min at 4 °C. Protein concentration in the resulting supernatant was determined using the BCA protein assay reagent (Sigma-Aldrich, St. Louis, MO, USA). Lysates from each tissue (50 μg per lane) were separated by 7.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). These were blocked for 1 h at room temperature in 5% non-fat milk in Tris-buffered saline-Tween (TBST). Membranes were incubated with an anti-Bcl-2 rabbit polyclonal antibody (1:200) at 4 °C overnight. After washing with TBST, membranes were incubated for 1 h at room temperature with a secondary antibody (IRDye 680 goat anti-rabbit IgG, 1:2000; LI-COR Biosciences, Lincoln, NE, USA).

2.9. Apoptosis analysis

HEI-OC1 cells were transfected with a miR-34a mimetic or a miR-34a inhibitor. After incubation for 48 or 72 h, cells were harvested, stained with propidium iodide and anti-annexin V antibody (eBioscience, San Diego, CA, USA), and analyzed by fluorescence-activated cell sorting (FACS). In brief, viable cells do not stain with propidium iodide and Annexin V. In contrast, early apoptotic cells can be stained with Annexin V, and nonviable cells can be stained with both.