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Activation of miR-34a/SIRT1/p53 signaling contributes to cochlear hair cell apoptosis: implications for age-related hearing loss

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ABSTRACT

The molecular mechanisms underlying age-related hearing loss are not fully understood, and currently, there is no treatment for this disorder. MicroRNAs have recently been reported to be increasingly associated with age-related diseases and are emerging as promising therapeutic targets. In this study, miR-34a/Sirtuin 1 (SIRT1)/p53 signaling was examined in cochlear hair cells during aging. MiR-34a, p53 acetylation, and apoptosis increased in the cochlea of C57BL/6 mice with aging, whereas an age-related decrease in SIRT1 was observed. In the inner ear HEI-OC1 cell line, miR-34a overexpression inhibited SIRT1, leading to an increase in p53 acetylation and apoptosis. Moreover, miR-34a knockdown increased SIRT1 expression and diminished p53 acetylation, and apoptosis. Additionally, resveratrol, an activator of SIRT1, significantly rescued miR-34a overexpression-induced HEI-OC1 cell death and significantly reduced hearing threshold shifts and hair cell loss in C57BL/6 mice after a 2-month administration. Our results support a link between age-related cochlear hair cell apoptosis and miR-34a/SIRT1/p53 signaling, which may serve as a potential target for age-related hearing loss treatment.

1. Introduction

Age-related hearing loss (AHL), also known as presbycusis, is the most common form of hearing loss and the predominant age-related neurodegenerative disease affecting approximately 40% of people by age 65 years (Gates and Mills, 2005). AHL is thought to result from the coactions of genetic predisposition and a lifetime of insults to the ear as a result of aging (Bielefeld et al., 2010; Ohlemiller, 2009). One of the main causes of AHL is thought to be the irreversible loss of cochlear hair cells in the inner ear, with this being well documented in both aging humans and animal models (Kidd Iii and Bao, 2012; Someya et al., 2009; Yamasoba et al., 2013).

MicroRNAs (miRNAs) are noncoding RNAs of approximately 20–24 nucleotides that act posttranscriptionally to regulate messenger RNA (mRNA) stability and ultimately translation (Abe and Bonini, 2013). Recently, miRNAs were observed in the cochlea and suggested to play an important role in AHL (Patel and Hu, 2012; Zhang et al., 2013). Nevertheless, the role of miRNAs in determining hair cell fate during AHL pathogenesis has not been fully elucidated. Of interest is miR-34a, which has been implicated as a key player inducing senescence, cell cycle arrest, and apoptosis (Hermeking, 2010; Liu et al., 2012; Rokhlin et al., 2008). The actions of miR-34a regarding apoptosis are thought to depend on cellular context and miR-34a target protein expression levels (Castro et al., 2013).

Sirtuin 1 (SIRT1) is an NAD-dependent deacetylase that regulates apoptosis in response to oxidative and genotoxic stress (Donmez, 2012; Imai and Guarente, 2014; Longo and Kennedy, 2006; Yamakuchi and Lowenstein, 2009). Recent data indicate that SIRT1 modulates apoptosis through deacetylation of molecular targets that include p53 (Donmez, 2012; Yamakuchi and Lowenstein, 2009). p53 is a regulator of genotoxic stress that plays an important role in DNA damage

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response, DNA repair, cell cycle regulation, and triggering apoptosis after cell injury (Rozan and El-Deiry, 2007). At an organismal level, p53 activity has been implicated in driving tissue degeneration and aging (Reinhardt and Schumacher, 2012; Tyner et al., 2002). Recent evidence shows that p53 expression is increased during aging in the cochlea of AHL mouse models (Tadros et al., 2008). Interestingly, SIRT1 has been confirmed to be a direct miR-34a target (Yamakuchi et al., 2008). Thus, we hypothesize that miR-34a/SIRT1/p53 signaling may play a crucial role during hair cell death and AHL pathogenesis, and that strategies aimed at inhibiting miR-34a activity, or restoring SIRT1 function, may be beneficial in treating AHL. In this study, cochlear miR-34a/SIRT1/p53 expression and activation was examined in C57BL/6 mice, a mouse model of AHL. Additionally, the potential preventive effect of resveratrol, an activator of SIRT1, was also assessed in HEI-OC1 cells and C57BL/6 mice.

2. Methods

2.1. Animals and diets

Ninety-seven C57BL/6 mice (Laboratory Animal Center, Sun Yat-sen University) were divided into 2 groups: a "young" group (1–2 months of age; 47 subjects) and an "old" group (12–16 months of age; 50 subjects). Additionally, 28 mice (2–3 months of age) were randomly divided into a resveratrol treatment (RT) group and a control group (15 and 13 subjects, respectively). The RT group of mice were subjected to dietary supplementation with 400 mg kg^{-1} day^{-1} resveratrol (Sigma-Aldrich, USA) added to the chow for a period of 2 months, and the control group only fed standard chow. After treatment, hearing tests were conducted, and cochlear tissues collected for hair cell counting. Animal care and experimental treatment were approved by the Animal Research Committee, Sun Yat-sen University.

2.2. Auditory brainstem response

All mice were anesthetized with an intraperitoneal injection (100 mg/kg ketamine and 10 mg/kg xylazine mixture), and auditory brainstem response (ABR) measurements were performed by inserting subdermal needle electrodes at the vertex (active), under the left ear (reference), and under the right ear (ground). The acoustic signals were generated, and the responses were processed using Tucker-Davis Technologies (TDT System III, Alachua, FL, USA) hardware and software. Ten-millisecond (ms) tone bursts with a 1 ms rise or fall time were presented at 4, 8, 16, and 32 kHz at a rate of 21.1/s. The average response to 1000 stimuli was obtained by reducing the sound intensity at 5 dB intervals near the threshold, which was defined as the lowest stimulation decibel level at which a positive wave in the evoked-response trace was evident.

2.3. Tissue preparation

After ABR recordings, the deeply anesthetized mice were decapitated, and the cochleae removed and fixed by immersion in 4% paraformaldehyde in 0.1 mM phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. The cochleae were decalcified in 4% sodium ethylenediaminetetraacetic acid for 4 days, followed by an overnight incubation in 30% sucrose. The next day, the cochleae were embedded in optimal cutting temperature compound (Sakura, USA), cryosectioned at a 10-μm thickness, and stored at −20 °C. For RNA and protein preparations, cochlear tissues were dissected with small forceps, snap frozen in liquid nitrogen, and stored at −80 °C.

2.4. Nuclear staining and terminal deoxynucleotidyl transferase dUTP nick end labeling

After 4% paraformaldehyde fixation, the organ of Corti was microdissected, rinsed in PBS, immersed in 0.5% Triton X-100 for 15 minutes at room temperature, incubated with 4',6-diamidino-2-phenylindole (DAPI) (10 mg/mL, Sigma, USA) for 10 minutes, and mounted on glass slides in 50% glycerol. Some surface preparations of the organ of Corti were incubated with 100 μL of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture (Roche Diagnostics Corporation, Indianapolis, IN, USA) containing dUTP-FITC and terminal deoxynucleotidyl transferase at 37 °C in the dark for 60 minutes, followed by incubating with propidium iodide (PI)-staining solution (10 mg/mL PI in PBS) for 30 minutes at room temperature. Samples were observed and imaged with an Olympus BX63 microscope. Hair cell counts were performed in 2 locations, at 10%–20% and 65%–70% of the total cochlear duct distance from the apex, which approximately corresponds to the frequency range of 7–8 kHz or 32–36 kHz (Viberg and Canlon, 2004). Hair cells were counted in 6 mice from each group and were considered to be degenerated if the cell nuclei were absent.

2.5. HEI-OC1 cell culture

HEI-OC1 cells (kindly provided by F. Kalinec at the House Ear Institute, Los Angeles, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco) at 33 °C under 10% CO_2 (permissive conditions).

2.6. Transfection of micro RNA mimics and inhibitors

To examine the effect of miR-34a on SIRT1 and p53, HEI-OC1 cells were transfected with a miR-34a mimic, a negative control micro RNA (miRNA) mimic, a miR-34a inhibitor, or a negative control miRNA inhibitor (GenePharma, Shanghai, China) at various concentrations and harvested 48 or 72 hours later. The protective effect of resveratrol was tested in HEI-OC1 cells under different conditions.

2.7. Oxidative stress exposure

To explore whether increased miR-34a expression could alter the deleterious effects of oxidative stress, HEI-OC1 cells were exposed to 50 μM H_2O_2 for 1 hour post transfection in the presence or absence of resveratrol at the indicated concentration.

2.8. Quantitative real-time polymerase chain reaction

Total RNA was isolated using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol, with 1 μg of total RNA reverse-transcribed using a ReverTra-Plus-TM kit (Toyobo, Osaka, Japan). Primer sequences used for amplifications were as follows: SIRT1 forward: 5′-CGGCTACCGAGGTCCATATAC-3′, reverse: 5′-ACAATCTGCCACAGCGTCAT-3′; p53 forward: 5′-AGAGACCGCCGTACAGAAGA-3′, reverse: 5′-GCATGGGCATCCTTTAACTC-3′; glyceraldehyde-3-phosphate dehydrogenase: forward: 5′-TGAACGGGA AGCTCACTGG-3′, reverse: 5′-GCTTCACCACCTTCTTGATGTC-3′. Complementary DNA samples were amplified using SYBR Premix Ex Taq (Tli RNaseH Plus, TaKaRa, Otsu, Japan) and detected with the Roche LightCycler 480 real-time PCR system. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control for SIRT1 and p53 normalization.