FBXO3 Downregulation Attenuates Noise-induced Hearing Loss by Suppressing ATG10 Degradation and Activating Autophagy

Fan Ye
First Affiliated Hospital of Wenzhou Medical University

Bi Lin
First Affiliated Hospital of Wenzhou Medical University

Lian Fang (✉ FangLian@wzhospital.cn)
Department of Otolaryngology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China

Research Article

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Abstract

Noise induced hearing loss (NIHL) is a kind of hearing impairment, which is next to the age-related hearing loss. More and more evidences have verified that overproduction of reactive oxygen species is a common pathologic phenomenon of different inner ear injury including NIHL, and autophagy contributes to attenuate NIHL by reducing oxidative stress. However, the underlying mechanism by which noise exposure causes autophagy activation remains unclear. In this study, we found that NIHL was accompanied by autophagy in the rat cochlea. Furthermore, twelve common genes were found at the GEO datasets GSE85290 and GSE8342, and E3 ubiquitination ligase FBXO3 was confirmed significantly reduced in NIHL rat cochlea. Next, we demonstrated that FBXO3 can directly binding with autophagy-related protein 10 (ATG10), which is necessary for the initiation of autophagy, and mediate its degradation. In vivo animal model treatment with rapamycin, an autophagy activator, significantly reduced the NIHL. Based on these data, we confirmed that FBXO3 played an important role in autophagy caused by NIHL, may be a potential target of NIHL treatment.

Introduction

According to statistics from the World Health Organization, more than 5% of the world’s population, 466 million people, suffer from disabling hearing loss and noise is one of the causes that cannot be ignored [1, 2]. Noise induced hearing loss (NIHL) refers to the progressive hearing loss caused by long-term exposure to a noisy environment, also known as chronic acoustic trauma [3]. Regarding the causes of the NIHL, most scholars agree with the theory of mechanical damage and the theory of metabolic damage [4]. Since then, some researchers have found that the mechanism of oxygen free radical damage, the genetic susceptibility mechanism, and ion regulation disorder mechanisms are related to NIHL [5]. Despite these findings, the underlying mechanism of NIHL has not been fully elucidated. The high prevalence of NIHL is a huge burden to society. Therefore, more and more researchers are interested in mitigating and preventing NIHL, and there has been great effort to understand the molecular and biochemical mechanisms underlying NIHL.

Exposure to noise promotes the production of reactive oxygen species (ROS) in the cochlea, which exerts as a crucial role in NIHL [6]. Oxidative stress is caused by the imbalance between ROS production and antioxidant ability, and potentially leading to oxidative stress injury [7, 8]. Mounting evidence has demonstrated that ROS overproduction is a common pathologic phenomenon of different inner ear injury including ototoxic drug treatment [9] and noise exposure [10]. ROS could induce lipid peroxidation, resulting in the apoptosis of sensory hair cells and weakening cochlear blood flow [11]. In an addition, ROS promotes the production of pro-inflammatory cytokines in the cochlea that further exacerbate injury [12].

Recently, the interaction between ROS and autophagy has been verified in some pathological conditions including cancer [13], ischemic brain damage [14], and NIHL [6]. Noise exposure-induced oxidative stress contributes to the activation autophagy. In turn, autophagy activation results in a marked decrease in
oxidative stress injury by degradating oxidized substance [6, 15]. Autophagy, as a regulatory mechanism that can play the role of a "scavenger", can degrade ineffective proteins and damaged cells to complete the removal of waste and maintenance of cellular functions [16]. Yuan et al., demonstrated that autophagy activator treatment significantly represses 4-HNE and 3-NT levels, and attenuates noise-induced hair cell loss, and results in subsequent decrease in NIHL [6]. Autophagy could serve in an antioxidative capacity and could possess the potential to treat sensorineural hearing loss (SNHL) by acting as an antioxidative role [17].

The ubiquitin proteasome system (UPS) is involved in a variety of cellular processes and Ubiquitin E3 ligase plays a central role in the process through ubiquitination of cellular proteins [18]. Ubiquitination has been proposed as a signal for selective autophagy, and autophagy receptor proteins (such as p62 and NBR1) are associated with ubiquitin and autophagosome-specific Atg8 family protein LC3 (microtubule-associated protein light chain 3)/GABARAP interact to promote autophagy [19]. Here, we found that E3 ubiquitination ligase FBXO3 can directly binding with autophagy-related protein 10 (ATG10), played an important role in autophagy caused by NIHL, may be a potential target of NIHL treatment.

**Results**

**NIHL was accompanied by autophagy activation in the rat cochlea**

Emerging studies have demonstrated that noise exposures induce hearing loss [20], and autophagy activation contributes to attenuate NIHL by decreasing oxidative stress [6, 17]. However, the underlying mechanism of autophagy activation during noise exposures remains unclear. In the study, exposure of Wistar rats to 6-32kHz noise for 1d was carried out, and hearing loss and autophagy activation in cochlea was first assessed. Auditory Brainstem Responses (ABRs) before and 1 days after noise exposure were recorded. In the experimental setting, loss of auditory function was assessed by threshold shift, which means the difference value of auditory threshold at a given frequency between the pre-noise and post-noise exposure. As shown in Figure 1A, one day of noise exposure, the threshold shifts of low, mid and high frequencies was elevated of about 13 dB, 31 dB and 22 dB respectively, with the greatest hearing loss occurring in the 16-24 kHz range. To further characterize the NIHL, we investigated the latency and amplitude curves of ABR waveforms, which contribute to the transmission times along auditory pathway and neural synchrony, at 20 KHz. Figure 1B and C showed the increased latency and decreased amplitude, indicating the significant impairment of auditory function after noise exposure.

To investigate the correlation between autophagy and NIHL, the expression of LC3, a marker of autophagy, was assessed using Immunohistochemistry (IHC) and Western blot analysis. As shown in Figure 1D, the expression of LC3 in rat cochlea was up-regulated by noise exposure. Consistent with the findings in IHC, the Western-blot data shown the upregulation of LC3 in rat cochlea after noise exposure (Figure 1E and F). These results suggested that NIHL was accompanied with the activation of autophagy.

**FBXO3 was down-regulated in rat cochlea after noise exposure**
To explore the molecular mechanism of NIHL, the data of expression profiling of NIHL were gained from GEO Datasets GSE85290 and GSE8342. Twelve common differentially expressed genes were contained using Venn analysis in both GSE85290 and GSE8342 (Figure 2A). qPCR array data showed that after exposure of rats to 20kHz noise for 1d, 5 genes were significantly dys-regulated (fold change>2, p<0.05) in NIHL tissue compared with control group (Figure 2B). To further confirm the results, Western-blot analysis was used to investigate the protein expression, only FBXO3 shown a similar downregulated expression in rat cochlea at noise exposure group (Figure 2C and D).

**FBXO3 knockdown impaired autophagy in HEI-OC1 cells**

To validate the hypothesis that the FBXO3 down-regulation is associated with the activation of autophagy in NIHL, the HEI-OC1 cells widely used to elucidate the pathological pathway of hair cells were transfected with recombinant lentivirus containing FBXO3 shRNA (Lv-shFBXO3). The gene silencing efficacy of Lv-shFBXO3 was evaluated using Western blot, and as expected, the FBXO3 protein expression was significantly decreased in the FBXO3 shRNA transduced HEI-OC1 cells treated with Lv-shFBXO3 compared with control (Figure 3A and B). The autophagy activation was then assessed in HEI-OC1 after FBXO3 knockdown. As shown in Figure 3C and D, FBXO3 knockdown increased LC3 expression and decreased P62 expression compared with that following transduction with the control shRNA. In addition, Immunofluorescence analysis was used to further confirm the expression of LC3 and showed similarly dysregulated, the expression of LC3 upregulated after FBXO3 knockdown. These data demonstrated that FBXO3 inhibition contributed to activate autophagy in hair cells.

**FBXO3 regulated autophagy via ATG10**

In order to further assess the effect of FBXO3 in autophagy, we investigated the substrate of FBXO3 via UbiBrowser, a bioinformatics platform for investigating the E3 ubiquitin ligase substrate interaction network, and found there are two autophagy genes ATG7 and ATG10 in the FBXO3 substrate with predicted score greater than 0.6 (Figure 4A). Further Co-IP analysis showed that only ATG10 can binding with FBXO3 in HEI-OC1 cells (Figure 4B and C). To clarify the mechanism of FBXO3 regulated the expression of ATG10, we used a cycloheximide chase assay to analyze how the ATG10 expression changed over time in HEI-OC1 cells transfected with FBXO3. Data shown that the half-life of ATG10 was markedly reduced in HEI-OC1 cells after FBXO3 overexpression (Figure 4D). These results suggested that FBXO3 regulated autophagy through ubiquitination of ATG10.

**Autophagy activation attenuated NIHL**

To evaluate an impact of autophagy in NIHL, we then examined the effects of rapamycin, an autophagy activator, on relieving hearing loss in response to noise exposure. As shown in Figure 5A, the ABR threshold shifts at 1-day post-exposure (temporary threshold shift) were significantly decreased after treatment with rapamycin. Similarly, ABR threshold shifts at 7 days post-exposure (permanent threshold shift) were also decreased after treatment with rapamycin (Figure 5B). These data demonstrated that
downregulated FBXO3 may attenuate NIHL by suppressing ATG10 degradation and activating autophagy.

Discussion

In the current study, we investigated the role of FBXO3 in regulating autophagy and NIHL. The present data demonstrated that (i) NIHL was accompanied by autophagy activation in the rat cochlea, (ii) FBXO3 was down-regulated in rat cochlea after noise exposure, (iii) FBXO3 knockdown impaired autophagy in HEI-OC1 cells, (iv) FBXO3 regulated autophagy via ATG10, (v) Autophagy activation attenuated NIHL. These results identified the important role of FBXO3/autophagy pathway in NIHL and indicated that pharmacological intervention for FBXO3/autophagy pathway might be an effective way to treat NIHL.

Autophagy plays a vital role in maintaining cell homeostasis and is closely related to the occurrence of various human diseases, such as tumors, cardiovascular diseases, and neurodegenerative diseases [21, 22]. Since the 1990s, genetic studies of yeast have identified a series of autophagy-related (ATG) genes, of which 15 ATG genes are called "core" ATG genes because they are required for the formation of autophagosomes [23]. Acting as a core autophagy protein, the evolutionary conserved ATG10 plays a key role in autophagy and appears to link autophagy with the UPS. Autophagy is known to occur in response to various environmental stresses, such as lack of nutrition, lack of growth factors, and so on. Autophagy can eliminate these damages. NIHL, as a common sensorineural deafness, is greatly affected by the environment, and the role of autophagy in NIHL is of great research significance.

In the present study, we used a mouse model to demonstrate that FBXO3 played an important role in NIHL by suppressing ATG10 degradation and activating autophagy.

In this study, based on the expression of autophagy marker LC3, we found that FBXO3 is accompanied by activation of autophagy in noise-induced hearing loss. It further confirms that ATG10 plays a key role in autophagy above, linking autophagy to UPS. Next, we show here that FBXO3 is activated in NIHL with autophagy flux. In HEI-OC1 cells transfected with FBXO3 shRNA lentivirus, FBXO3 knockdown increased LC3 II expression and decreased p62 expression, demonstrating the hypothesis that FBXO3 down-regulation is associated with autophagy activation in NIHL. We believe that the role of FBXO3 in autophagy is related to ATG10.

With the help of the biological information platform of the E3 ubiquitin ligase substrate interaction network, We show here that ATG10 present in FBXO3 substrate can bind to HEI-OC1 cells, further clarifying FBXO3 to regulate the mechanism of ATG10 expression, we used the cycloheximide chase analysis method to analyze the change of ATG10 in HEI-OC1 cells transfected with FBXO3 over time, proving that FBXO3 indeed regulates autophagy through the ubiquitination of Atg10. FBXO3 binds to the substrate by recognizing the binding site on the substrate, and is ubiquitin-degraded by E3 ligase, which plays an important role in the NIHL process. A complete cellular autophagy process includes autophagy induction, autophagosome formation, and autophagolysosome degradation. LC3 is involved in the formation of autophagosomes, and p62 is involved in the degradation of autophagosomes. When
autophagy occurs, the level of p62 decreases. Finally, we investigated the effect of the autophagosome activator rapamycin on the formation of autophagosomes.

In vivo animal model treatment with rapamycin can significantly reduce NIHL. Based on the above experimental results, we confirmed for the first time that FBXO3 plays an important role in autophagy caused by NIHL, and may be a potential target for NIHL treatment.

### Materials And Methods

#### Experimental Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University. Care and use of animals followed the guidelines in the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize pain. After comprehensive consideration of various factors such as noise sensitivity, auditory threshold and aging hearing loss, Wistar rats were used as experimental animals in this experiment. Wistar rats (female; 3 months; n=10) were provided by the animal facility of the Shanghai SLAC Laboratory Animal Co.,Ltd (Shanghai, China), and were housed in standard cages and maintained on a 12/12 h light/dark cycle at a controlled temperature (20-22 °C) and humidity (50 ± 5%). Rats had free access to standard rodent chow and filtered water at all times. After adapting to the environment for one week, Wistar rats were randomly divided into 2 groups (n=5 of each group): noise exposed group (NIHL group) and non-exposed group, and the auditory brainstem response before and after noise exposure was recorded.

#### Noise exposure

Rats were exposed to low-frequency, mid-frequency, and high-frequency noise for one day in a custom-made sound exposure room (Acoustical Laboratory, Naval Medical Research Institute, Shanghai, China) equipped with an internal light source and ventilation system, and compared with the unexposed group. Animals were conscious, unrestrained and had free access to food and water throughout the noise exposure period.

#### Auditory Brainstem Responses (ABR)

ABR analysis for the auditory evaluation was performed in rats used for NIHL study[24]. Wistar Rats were anesthetized with ketamine (50 mg/kg) and xylazine (6 mg/kg) cocktail by intraperitoneal injection and placed on a 37°C thermostatic heating pad. Electodes were placed on the vertex (non-inverting) and on ipsilateral mastoid (inverting) and hind limb (ground). ABRs were collected through Intelligent Hearing Systems (IHS, Miami Florida, USA). ABRs were filtered, amplified and digitized (1024 presentations, 40 kHz sampling rate, 30-3000 Hz, 100X) in response to tone bursts presented at 6, 12, 16, 20, 24 and 32 kHz (1 ms rise/fall, cosine gated, 5 ms duration, 21/s).

#### Cell culture
The cochlear auditory cell line HEI-OC1, purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (CAS; Shanghai, China), were treated with DMEM medium supplemented with 10% FBS. All cells were kept in a 37 °C incubator with 5 % CO₂.

**Construct of lentivirus and cell transfection**

FBXO3 shRNA (shFBXO3) were designed and synthesized from Shanghai Genechem Co., LTD (Shanghai, China). The corresponding sequences were cloned into the lentiviral vector to construct recombinant lentiviral Lv-shFBXO3. Lv-shFBXO3 was used to inhibit FBXO3 expression in HEI-OC1 cells. FBXO3 expression level in selected clones was verified by western blot analysis.

**Western bolt analysis**

HEI-OC1 cells were lysed with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). The protein concentration was determined using a BCA Protein Assay Kit (Abcam, Cambridge, MA, USA). Then, 40 μg protein was separated on 10% SDS-PAGE and transferred onto PVDF membranes (Merck, Darmstadt, Germany). The membranes were blocked with 5% skimmed milk and probed with primary antibodies [FBXO3 (ab224603, 1:1000, Abcam), ATG10 (ab240901, 1:1000, Abcam), LC3 A/B (ab128025, 1:1000, Abcam) and β‐actin (ab179467, 1:5000, Abcam) at 4°C overnight. Next, secondary antibody Goat Anti-Rabbit IgG H&L (HRP) (ab205718, 1:5000, Abcam) was added. The obtained bands were detected using the ECL system, and protein bands were analysed using Quantity One software from Bio-Rad.

**Tissue preparation and immunohistochemistry assay**

The cochleae from NIHL group and non-exposed group rats were fixed with 4% paraformaldehyde (PFA) overnight. Samples were decalcified in 10% EDTA for 5 to 7 days and then dehydrated in 30% sucrose solution for 12 h, sectioned into 10-μm-thick slices across the modiolus, and stored at -80 °C until immunohistochemistry staining.

For the immunohistochemistry assay, the sections were treated with 3% H₂O₂ and then incubated in a blocking buffer with 5% BSA and 0.3% Triton X-100 (Sangon Biotech, Shanghai, China) for 60 min at room temperature (RT). Anti-LC3B antibody (ab128025, 10 μg/ml, Abcam) at 4°C overnight, washed 3 times with PBS for 10 min, and then incubated with secondary antibody (ab205718, 1:5000, Abcam) at RT for 40 min. DAB chromogenic reagent was added and incubated for 10 min. The nuclei were further stained with hematoxylin.

**Immunofluorescence**

HEI-OC1 cells were fixed with 4% PFA for 20 min, and then washed with PBS. The cells were incubated with anti-FBXO3 antibody (1:200; ab224603, Abcam) overnight at 4 °C, and then incubated with the second antibody (1:5000) for 1 h after PBS washing 3 times. The nuclei were stained with DAPI (Sigma-Aldrich) for 20 min. A fluorescence microscope was used to observe and record the images.
Co-IP assay

Co-IP was carried out with a Pierce™ Co-Immunoprecipitation kit (Thermo Fisher Scientific) as per the manufacturer's protocol[25]. In brief, HEI-OC1 cells were lysed on ice with lysis buffer for half an hour, centrifuged for 12 min and the supernatant was discarded. 400 μl supernatant with 1mg protein was incubated with 10 μg anti-FBXO3, anti-ATG10 or anti-IgG antibodies for 12 h at 4°C. Beads were washed, eluted in sample buffer, and boiled for 10 min at 100°C. Finally, immune complexes were stained with Coomassie Blue and analyzed using SDS-PAGE.

Statistical analysis

Data were represented as mean ± SD. The difference between two groups was compared using two-tailed student’s t-test, or one-way analysis of variance (ANOVA) followed by the Scheffé test. A value of p < 0.05 was considered statistically significant. Data statistical analyses was carried out by the SPSS software version 16.

Declarations

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Author contributions

L.F. designed the study. F.Y. performed the biochemical and histological experiments. B.L. analyzed the data. L.F. wrote the main manuscript and all authors reviewed the manuscript.

Competing Interests

We reported no competing interests relevant to this paper.

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