Published in final edited form as:

Neuroreport. 2019 February 06; 30(3): 145–150. doi:10.1097/WNR.0000000000001173.

Chemical chaperone 4-phenylbutyrate prevents hearing loss and cochlear hair cell death in *Cdh23^{erl/erl}* mutant mice

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Abstract

We previously developed *Cdh23^{erl/erl}* mutant mice (*erl* mice) as a model of hearing loss for otoprotective drug evaluation and showed that the *erl* mutation leads to hearing loss related to endoplasmic reticulum (ER) stress-induced cochlear hair cell apoptosis. Small molecular chemical chaperones, 4-phenylbutyrate (4PBA), targeting ER stress have a neuroprotective effect. To evaluate whether 4PBA has an otoprotective effect, we intraperitoneally injected *erl* mice with 4PBA daily from postnatal age day 7 up to 12 weeks. Our results showed that treatment with 4PBA significantly alleviated hearing loss and suppressed hair cell death in *erl* mice. Additionally, ER stress-related proteins were downregulated via 4PBA treatment. Our study demonstrated that 4PBA has an otoprotective effect, which provides the potential to repurpose the drug for otoprotection.

Keywords

4-phenylbutyrate; Endoplasmic reticulum stress; Age-related hearing loss

Introduction

Hearing loss has been associated with hair cell loss and the induction of apoptosis [1]. Mutations in the *Cadherin 23* (*Cdh23*) gene may cause hearing loss because they cause the disruption of the inner-ear hair cells during late embryonic/early postnatal development [2,3]. In hair cells, CDH23 is localized in the tip link, the extracellular filament proposed to gate the mechanotransduction channel [4]. Usher syndrome type 1D (USH1D), autosomal

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Conflict of interest

The authors declare no conflicts of interest.

recessive deafness (DFNB12) and certain types of age-related hearing loss (AHL) involve mutations of the *Cdh23* gene in humans and mouse models.

One novel recessive mutation *erl*ong (*erl* is the symbol for the mutation; ERL is the symbol for the mutated protein) of the *Cdh23* gene is characterized by progressive hearing loss beginning on postnatal day 27 (P27) [5]. The short interval from normal hearing to deafness (P27-P90) makes this model ideal for screening and validating otoprotective drugs. It was reported that some early-onset hearing-loss patients had *CDH23* mutations that had similar phenotypes to the *erl* mutants [6], thereby highlighting the importance of studying this mouse model.

Recently, numerous studies have demonstrated that ER stress is involved in neurodegenerative disorders [7,8]. ER stress is defined as the accumulation of unfolded or misfolded proteins in the ER. The unfolded protein response (UPR) comprises a set of cellular signaling pathways present in mammalian cells that detect unfolded proteins in the ER and direct the protective and/or apoptotic actions that the cell takes [9]. The UPR processes increase expression of glucose-regulated protein 78 (GRP78/BiP) and CCAAT/enhancer-binding protein-homologous protein (CHOP) [10]. The up-regulation of BiP is commonly used as an ER stress marker. In addition, CHOP, an apoptotic transcriptional factor induced in response to ER stress, is also a popular marker for the assessment of ER stress.

ER stress may also play a role in hearing impairment [11]. Recently, we revealed that *erl* mutation leads to hearing loss related to ER stress-induced hair cell apoptosis [12]. Thus, protein-folding errors that are triggered by genetic mutations can be considered the earliest therapeutic target in some genetic hearing disorders. Many anti-ER stress drugs, including chemical chaperones, can be repurposed for otoprotection. In this study, we chose one of the best-characterized chemical chaperones, 4-phenylbutyrate (4PBA), which the United States Food and Drug Administration (US FDA) has approved, and which has a good safety profile in humans. We report that 4PBA reduces hearing threshold shifts and hair cell loss in *erl* mice.

Materials and Methods

Mice

The C57BL/6J (B6) mice and *erl* mutant mice originally were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Studies were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals as well as the Institute of Laboratory Animal Resources (protocol 2014–0155). The *erl* mice were intraperitoneally injected with 4PBA daily (50 mg/kg, Sigma, dissolved in DMSO, diluted in phosphate-buffered saline, ratio is 2:5). The vehicle group mice were intraperitoneally injected with equal volume solvent solution. All of the treatments started from postnatal age day 7 to 12 weeks. Also, the untreated group was a group that received no treatment which acted as a second control.

Immunostaining

The inner ears from B6 mice and *erl* mutant mice were fixed and decalcified. After being dehydrated in sucrose and embedded in tissue O.C.T. compound freeze medium (Tissue-Tek, Sakura Finetek, Japan) at -20°C , the inner ears were sectioned at $5\text{ }\mu\text{m}$. Sections were stained with anti-CDH23, anti-BiP or anti-CHOP antibodies. Sample mounts were observed under fluorescence microscope and read out by LAS X software (Leica DM4500 B, Leica Microsystems Inc., Buffalo Grove, IL, USA). The immunofluorescence intensity was analyzed by Image J software as described previously [13].

Surface preparation and hair cell counting

The surface preparation was performed as described previously [14]. The surface preparations were stained for F-actin with Alexa Fluor 488-conjugated Phalloidin (Invitrogen, CA, USA) to show hair bundles and DAPI (Invitrogen, CA, USA) was used to stain the nucleus. Immunofluorescent signals were examined with a fluorescence microscope (Leica DM4500 B, Germany).

Auditory brainstem response (ABR) and distortion product oto-acoustic emission (DPOAE) testing

A computer-aided evoked potential system (Intelligent Hearing Systems, IHS3.30, Miami, FL) was used as described previously [12]. Briefly, click and 8-, 16-, and 32-kHz tone bursts were channeled through an inserted earphone. The ABR threshold was identified as the lowest stimulus level at which clear and repeatable waveforms were recognized. The DPOAE measurement was conducted for pure tones at frequencies ranging from 4.4 to 20.3 kHz by using the IHS3.30 System. Frequencies were acquired with the F2:F1 ratio of 1.22 and with primary stimulus of 65/55 dB SPL.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic hair cells were detected by TUNEL assay according to the manufacturer's protocol. The numbers of TUNEL-positive cells were counted from three random microscope views from the basal cochlear turns for each animal, and the percentage of apoptotic cells was calculated.

Statistical analysis

All values are expressed as the mean $\pm$ S.E.M. The student *t*-test and one-way ANOVA was used for data analysis using the Statistical Package for the Social Sciences (version 17, SPSS). A value of $P < 0.05$ was considered significant.

Results

CDH23-ERL proteins were retained in the perinuclear cytosol area in the OHC of *erl* mice

We first examined CDH23 protein localization in the inner-ear cryosections of B6 mice and *erl* mice at P6. B6 mice exhibited a CDH23 protein signal at the top of the OHCs (asterisks) (Fig. 1a). Instead, *erl* mice exhibited a striking redistribution of CDH23 with an enriched signal in the cytoplasm moving in the direction of the hair bundles. Anti-CDH23 and anti-

BiP were used to co-stain the inner-ear cryosections of *erl* mice at P12. OHCs were heavily stained for CDH23 and BiP (Fig. 1b), whereas the ampulla of the vestibular was not stained as heavily (Fig. 1c). This may explain why CDH23-ERL affects hearing but not the vestibular function.

4PBA prevents hearing loss in *erl* mice

CDH23-ERL protein retention in the ER decreased the migration of this protein to the tip links. We hypothesized that the unfolded or misfolded CDH23-ERL protein may induce ER stress and UPR in the hair cells of *erl* mice. We used 4PBA, a chemical chaperone, and an ER stress inhibitor to test whether 4PBA could have an otoprotection effect in *erl* mice. For the *erl* mice in the untreated, vehicle, and 4PBA groups, ABR and DPOAE were measured (Fig. 2a and b). The ABR thresholds in the 4PBA group were significantly lower than those in the vehicle group or the untreated group for click, 8-, 16-, and 32-kHz stimulus frequencies at the ages of 6, 8, and 12 weeks (Fig. 2a). DPOAE amplitudes in the 4PBA group at high frequencies were significantly higher than those of the vehicle group and the untreated group at 12 weeks (Fig. 2b) but not at 4, 6, or 8 weeks (Supplementary Fig. 1a).

4PBA protects against haircell loss and apoptosis in *erl* mice

OHC loss was observed in the apex, middle and basal turns of the cochleae in the vehicle group at 10 weeks. In the 4PBA group, only a small amount of OHC loss was seen in the apex, middle and basal turns (Fig. 3a). The mean percentage of OHC loss in the apex, middle and basal turns of the cochleae was significantly lower in the 4PBA group than in the vehicle group. We then applied TUNEL staining in the vehicle and 4PBA groups at 6 weeks (Fig. 3b). In the basal turns of the cochleae, the vehicle group showed more TUNEL-positive OHCs than it did in the 4PBA group.

4PBA downregulated ER stress-associated proteins in *erl* mice

To investigate ER stress-associated proteins *in situ* in *erl* mice, and to determine whether 4PBA could suppress ER stress in this mouse model, we stained the *erl* mice tissue section with BiP and CHOP antibody in the vehicle and 4PBA groups at P30 (Fig. 4a and b). The results showed that the BiP and CHOP signals in the OHCs in the 4PBA group were significantly decreased compared with the vehicle group. Therefore, ER stress-related proteins were downregulated after the 4PBA treatment.

Discussion

Hair cell loss in *erl* mice involves apoptosis, as hearing is protected up to 30 dB with anti-apoptotic drugs [5,15]. Our results demonstrate that the *erl* mutation causes the mutant CDH23-ERL protein to accumulate in the ER, which then triggers the UPR. If the UPR is unable to handle the accumulation (or overproduction) of the mutant CDH23-ERL, it switches to ER stress and then induces apoptosis.

The ER organization is well developed and specialized in the OHCs. The ER system in the OHCs includes apical cistern, subsurface cistern, Hensen's body, and subsynaptic cistern [16]. OHCs contain these membranous organelles that may contribute to mechanoelectrical

transduction [17]. Impaired ER function in the OHCs may influence some molecular machinery, for instance, protein folding.

In this study, we provided direct in vivo morphological evidence of protein accumulation related to ER stress causing hearing loss in a mammalian model of USH1D or DFNB12. This opens a preventive time window targeted at an upstream pathway leading to apoptosis in mammals. In this study, only cochlear hair cells and not vestibular hair cells were affected. This explains why the *erl* mice had only hearing loss without balance defects, representing normal vestibular function. The reason why it only affects cochlear hair cells warrants further studies.

The major function of 4PBA is to scavenge for ammonia and glutamine. The US FDA has approved 4PBA to treat urea cycle disorders, which are genetic diseases in which nitrogen waste builds up in the blood plasma as ammonia glutamine [18]. 4PBA is supposed to interact with the hydrophobic domains of misfolded proteins and thus prevent their aggregation, thereby reducing ER stress. 4PBA may stabilize the CDH23-ERL mutant protein in the ER, allowing it to reach the tip link to perform normal functions at younger ages.

4PBA may potentially be used to treat neurologic diseases because it goes through the blood-brain barrier, and it shows significant neuroprotective effects in mouse models of neurodegenerative diseases [19,20]. In our study, 4PBA may cross the blood-labyrinth barrier, a barrier between the blood and the inner-ear fluids, to enter hair cells, attenuate ER stress, and facilitate the migration of the CDH23-ERL mutant protein in the ER, allowing it to form the tip link to perform normal functions and thus protect hearing loss.

Conclusion

Our studies on the *erl* mutation in *Cdh23* mutant mice demonstrated that 4PBA has an otoprotective effect, which provides the potential to repurpose the FDA-approved drug. Through this study, we hope to provide new targets for exploring preventive therapies for hearing loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01DC015111); the National Natural Science Foundation of China (81530030, 81400467, 81500797 and 81600809); the Taishan Scholars Program Foundation of China; and the Foundation of Binzhou Medical University of China (BY2014KYQD19).

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Fig. 1. Retention of CDH23-ERL protein in the OHC cytosol of *erl* mice compared to B6 mice. (a) The inner-ear cryosections of B6 mice and *erl* mice (P6) were stained with CDH23 antibodies ($n = 6$ mice/group). Asterisks and plus symbols indicate the positions of OHC and IHC respectively. Nuclei were labeled with DAPI (blue). CDH23 signal was mostly localized at the tops of hair cells in B6 mice but was relocated to cytoplasm of the hair cells in the *erl* mice. Scale bar, 20 μ m. Line plot of the immunofluorescence intensity along the arrows drawn in the left panel. (b) Anti-CDH23 (red) and anti-BiP (green) were used to co-stain the ear cryosections of B6 and *erl* mice at P12. BiP was highly detected in the OHCs of *erl* mice, and co-localized with CDH23 in the cytoplasm regions. Scale bar, 20 μ m. Line intensity plots of the arrows drawn from the left panel ($n = 6$ mice/group). (c) The left panel showed image of the ampulla region of vestibular in *erl* mice at P12. BiP signal was very faint in the ampulla. Line intensity plots from left panel. The white arrow heads point to the ampulla. Scale bar, 40 μ m.

Fig. 2. 4PBA protected hearing loss in *erl* mice.

(a) ABR thresholds to evaluate hearing in *erl* mice in the untreated, vehicle, and 4PBA groups at 4, 6, 8, and 12 weeks. ABR thresholds were measured at the stimulus frequencies of click, 8-, 16-, and 32-kHz. 4PBA group showed lower ABR thresholds than vehicle group or untreated group at 6, 8 and 12 weeks. Data are presented as mean $\pm$ S.E.M. (* $P < 0.05$, ** $P < 0.001$, $n = 6$ per group, one-way ANOVA) (b) The DPOAE amplitudes in the untreated, vehicle, and 4PBA groups were measured at 12 weeks at different F2 frequencies. 4PBA group showed higher DPOAE amplitudes at high frequencies. Data are presented as mean $\pm$ S.E.M. (* $P < 0.05$, $n = 6$ per group, one-way ANOVA). See also Supplementary Fig. 1.

Fig. 3. 4PBA prevented OHC loss and apoptosis in the *erl* mice.

(a) The whole-mount preparations from the apex, middle and basal turns of the cochleae in the vehicle and 4PBA-treated mice at 10 weeks. 4PBA group showed less OHC loss than in the vehicle group. The mean percentages of OHC loss are shown on the bar graph. * $P < 0.05$, student's t -test. Scale bar, 20 μ m. (b) Apoptotic cells were examined via TUNEL staining in the basal turns of the cochleae in the vehicle and 4PBA groups at 6 weeks. 4PBA group showed less TUNEL-positive OHCs than vehicle group. The mean percentage of the TUNEL-positive OHCs is shown on the bar graph. Data are presented as mean $\pm$ S.E.M. ($n = 6$ mice/group). ** $P < 0.001$, student's t -test. Scale bar, 20 μ m.

Fig. 4. 4PBA downregulated ER stress-associated proteins in *erl* mice.

(a and b) BiP and CHOP signals are shown in the OHC of the vehicle and 4PBA groups. BiP and CHOP signals in the 4PBA group were decreased than the vehicle group. The line intensity analysis along the white arrow is shown in the right panel ($n = 6$ mice/group). Scale bars, 20 μm.