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TAUROURSODEOXYCHOLIC ACID PREVENTS HEARING LOSS AND HAIR CELL DEATH IN *Cdh23*$^{erl/erl}$ MICE

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Abstract—Sensorineural hearing loss has long been the subject of experimental and clinical research for many years. The recently identified novel mutation of the *Cadherin23* (*Cdh23*) gene, *Cdh23*$^{erl/erl}$, was proven to be a mouse model of human autosomal recessive nonsyndromic deafness (DFNB12). Tauroursodeoxycholic acid (TUDCA), a taurine-conjugated bile acid, has been used in experimental research and clinical applications related to liver disease, diabetes, neurodegenerative diseases, and other diseases associated with apoptosis. Because hair cell apoptosis was implied to be the cellular mechanism leading to hearing loss in *Cdh23*$^{erl/erl}$ mice (*erl* mice), this study investigated TUDCA's otoprotective effects in *erl* mice: preventing hearing impairment and protecting against hair cell death. Our results showed that systemic treatment with TUDCA significantly alleviated hearing loss and suppressed hair cell death in *erl* mice. Additionally, TUDCA inhibited apoptotic genes and caspase-3 activation in *erl* mouse cochleae. The data suggest that TUDCA could be a potential therapeutic agent for human DFNB12.

Key words: *Cdh23*, apoptosis, hearing loss, tauroursodeoxycholic acid (TUDCA), otoprotection.

INTRODUCTION

Hearing loss is one of the most common sensory impairments in humans, affecting about 1.3 billion people worldwide (Vos et al., 2013). At least 50% of deafness presenting before speech development is genetic, and approximately 70% of hereditary deafness is nonsyndromic (no other anomaly exists) (Smith et al., 2014). The different gene loci for autosomal recessive nonsyndromic deafness are presented by DFNB (Genetic Evaluation of Congenital Hearing Loss Expert Panel, 2002). More than 70 DFNB loci have been identified (Smith et al., 2014). In previous research studies of mouse models of DFNB, the mice were either deaf at birth or had very late-onset deafness with slow progression. In our recent study, a novel point mutation (T208C) of the *Cadherin23* (*Cdh23*) gene in mice was identified and named *erl* (Han et al., 2012). The *Cdh23*$^{erl/erl}$ mutant mice (*erl* mice) showed progressive hearing loss beginning at postnatal day (P) 27 and developed prolonged deafness by P100. This mutation is considered to be a novel mouse model for DFNB12. Because of the time interval from hearing loss initiation to total deafness, the *erl* mice are an ideal tool for testing otoprotective drugs and screening potential therapies. We demonstrated that hair cell apoptosis was the pathological mechanism through which *erl* mutation led to hearing loss. Furthermore, treatment with erythropoietin and the apoptosis inhibitor Z-VAD-FMK could significantly preserve cochlear hair cells and prevent hearing loss in *erl* mutants (Han et al., 2012, 2013a).

Tauroursodeoxycholic acid (TUDCA) is a taurine-conjugated bile acid derived from ursodeoxycholic acid (UDCA). Under natural physiological conditions, UDCA is present at a low concentration in human bile. For the past thousand years, UDCA has been isolated from dried black bear gallbladders and used in the treatment of several illnesses in traditional Chinese medicine (Beuers, 2006). Nowadays, TUDCA has been chemically synthesized and is widely used in clinical and experimental research to treat liver disease, diabetes, and neurodegenerative diseases (Momose et al., 1997; Keene et al., 2002; Rodrigues et al., 2003; Green and Kroemer, 2004; Ozcan et al., 2006; Kars et al., 2010; Ceylan-Isik et al., 2011; Laukens et al., 2014). Previous research revealed that TUDCA functioned by modulating the apoptotic threshold in various cell types (Rodrigues et al., 2003; Amaral et al., 2009; Ramalho et al., 2013). Given TUDCA's cell-protective effects in disorders associated with apoptosis and its clinical safety, we measured its

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Abbreviations: ABR, auditory-evoked brainstem response; *Cdh23*, *Cadherin23*; DFNB, autosomal recessive nonsyndromic deafness; DPOAE, distortion product oto-acoustic emission; FDA, Food and Drug Administration; OHC, outer hair cell; PBS, phosphate-buffered saline; SEM, scanning electron microscope; s.e.m, standard error of the mean; SPL, sound pressure level; TNF, transforming growth factor; TUDCA, tauroursodeoxycholic acid; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UDCA, ursodeoxycholic acid; USH1D, Usher syndrome type 1D.

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hearing protective effects using *erl* mice as an animal model for screening new otoprotective drugs.

In the present study, we found that TUDCA showed protective effects against hearing loss and hair cell apoptosis in *erl* mice. To the best of our knowledge, this is the first *in vivo* study about TUDCA's otoprotective effects in the mouse model of *Cdh23* mutations. These data suggest that TUDCA is a potential therapeutic agent for human DFNB12.

EXPERIMENTAL PROCEDURES

Mice and treatment

All experiments were approved by the Animal Research Committee of the Case Western Reserve University School of Medicine (protocol R01DC009246). All mice were housed in the same environment, and received treatments by intraperitoneal injection. The *erl* mutant mouse model was developed from the C57BL/6J (B6) genetic background, which was homozygous for *Cdh23*^{ahl} mutation (Han et al., 2012, 2013a). Thus we chose B6 mice as controls to test whether TUDCA had any toxic effect in the ear. A total of 14 B6 mice and 85 *erl* mice were used in this study. The B6 mice were randomized into two groups with both genders: a TUDCA-treated group (treated with TUDCA, 100 mg/kg, EMD Chemicals Inc. Catalog No. 580549, diluted in 1 × PBS, phosphate-buffered saline), and an untreated group. The *erl* mice were randomized into three groups with both genders: a test group (treated with the same dosage of TUDCA), a vehicle group (treated with an equal volume of PBS), and a control group (untreated). All treatments started on P7, with subsequent injections given every other day for the first eight weeks. The injections were then continued once weekly for the duration of the experiments. The starting time point was selected to prevent caspase upregulation, which was detected at P14 in untreated *erl* mice in our previous work. The TUDCA dosage was selected from preliminary experiments that showed it to be a good balance between safety and effectiveness, and with reference to previous reports (Rodrigues et al., 2003; Drack et al., 2012).

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

A computer-aided evoked potential system (Intelligent Hearing Systems, the Smart-EP software) was used in ABR testing, as previously described (Zheng et al., 1999). Mice were anesthetized, and the body temperature was maintained at 37 °C. Subdermal needle electrodes were used; the recording electrode was inserted at the vertex of the skull, the ground electrode was inserted in the apex of the nose, and the reference electrodes were fixed near each ear. Clicks and tone bursts of 8 kHz, 16 kHz, and 32 kHz were channeled through an inserted earphone. ABR thresholds were identified as the lowest stimulus (sound pressure level, SPL) at which clear and repeatable ABR waveforms could be recognized. As previously reported, mice showing ABR thresholds above 55-dB SPL (for the click stimulus), 40-dB SPL (for 8-kHz tone bursts), 35-dB SPL (for 16 kHz), or 60-dB SPL (for 32 kHz) were considered to be hearing impaired (Han et al., 2013a).

The Intelligent Hearing System (Smart EP 3.30 Software) was used in DPOAE measurements. The test was conducted for pure tones at frequencies ranging from 4.4 to 20.3 kHz. Frequencies were acquired with an F2:F1 ratio of 1.22 and with primary stimulus of 65/55-dB SPL. The test model started from the lowest frequency and increased to the highest. The distortion product (DP) level (2F1−F2) of DPOAE amplitudes (in dB SPL) was extracted from the averaged spectra along with the noise floor.

Surface preparation and hair cell counting

The surface preparation was performed by a modified method as described previously (Han et al., 2012). Briefly, the mice were euthanized, and the temporal bones were collected. The surface preparations were performed using a modified protocol. The temporal bones were fixed in 4% paraformaldehyde overnight. The organ of Corti was then carefully micro-dissected out and was cut into three separate segments: apical, middle, and basal turn. The surface preparations were permeabilized in 0.2% Triton X-100, stained for F-actin with Alexa Fluor 568 phalloidin (Invitrogen), and finally observed with a fluorescence microscope (Leica). Hair cells were counted as present if cell bodies and V-shaped hair bundles were intact. The outer hair cells (OHCs) were counted in three discontinuous microscope views (×40 magnification) of each segment, and the average percentage of missing cells was obtained.

Scanning electron microscope (SEM)

The SEM analysis was performed as previously described (Furness et al., 2013). Briefly, after cardiac perfusion with 1% PBS and then with 2.5% glutaraldehyde, the entire cochleae was dissected. The bony capsule, spiral ligament, and Reissner's membrane were carefully removed from the apical turn to the basal turn so as to expose the whole organ of Corti. Afterward, the specimens were incubated in 1% osmium tetroxide (O) three times for one hour each, and in 1% thiocarbohydrazide (T) twice for one hour each (the OTOTO technique). The specimens were dehydrated in a gradient ethanol series, critical point dried using carbon dioxide (CO_2), and finally coated in gold and then palladium. The samples were then viewed under a high-resolution SEM (Helios Nano Lab 650).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic hair cells were detected by TUNEL assay according to the manufacturer's protocol. Briefly, the inner ears were fixed in 4% paraformaldehyde overnight. The organ of Corti was microdissected, as described above. The surface preparations were permeabilized in 0.2% Triton X-100. The specimens