European Journal of Pharmacology 803 (2017) 187–194

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

Calpain inhibitor alleviates permanent hearing loss induced by intense noise by preventing disruption of gap junction-mediated intercellular communication in the cochlear spiral ligament

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ARTICLE INFO

Keywords:
Calpain
Calpain inhibitor
Cochlear spiral ligament
Gap junction-mediated intercellular communication
Hair cell death
Noise-induced hearing loss

ABSTRACT

Our previous studies demonstrated that intense noise-induced hearing loss might be at least in part due to an oxidative stress-induced decrease in the level of gap junction-composing protein connexins in the spiral ligament (SL) of the cochlear lateral wall structures in mice. Further, an *in vivo* exposure of mice to intense noise activates calpain in the cochlear SL. Based on these studies, we sought to determine whether a calpain inhibitor would prevent an intense noise exposure from causing hearing loss, disruption of gap junction-mediated intercellular communication (GJIC) in the SL. An exposure of mice to intense noise (8-Hz octave band noise, 110-dB sound pressure level, 1 h) produced permanent hearing loss and cochlear hair cell death. The results of an *ex vivo* assay using gap-fluorescence recovery after photobleaching of dissected lateral wall structures revealed that the intense noise disrupted GJIC in the cochlear SL at day-7 post exposure. A prior intracochlear injection of the calpain inhibitor PD150606 significantly abolished this noise-induced hearing loss on days 5 and 7 post exposure. Similarly, PD150606 prevented noise-induced hair cell death and the GJIC disruption on day-7 post exposure. The intense noise temporarily enhanced the gene expression of calpain subtypes *Capn1* and *Capn2* immediately after exposure. Taken together, our data suggest that calpain inhibitor alleviated the noise-induced hearing loss, at least in part, by preventing disruption of GJIC in the cochlear SL. It possible that calpain inhibitors would be useful as a candidate of therapeutic drugs for sudden sensorineural hearing loss.

1. Introduction

Noise-induced hearing loss (NIHL) is widely known as one of the irreversible occupational disorders and severely compromises quality of life, as well as leads to accidents and injuries in the workplace through problems in workers' speech communication. Attenuation or prevention of NIHL is highly desired and urgent goal for maintaining quality of life. Animal models of NIHL are useful for elucidation and development of therapeutic strategies for sensorineural hearing impairment (Christie and Eberl, 2014; Moser and Starr, 2016). Thus, in the present study we examined a candidate of therapeutic drugs for developing therapeutic strategies for such impairment.

Accumulating evidence demonstrated that exposure to intense noise produces excess reactive oxygen species resulting in oxidative stress in the cochlea, which stress plays a fundamental role in NIHL (Kopke et al., 1999; Yamashita et al., 2004; Henderson et al., 2006). Excess reactive oxygen species produced by oxidative stress can react with biological macromolecules, producing lipid peroxidation, DNA damage, and enzyme inactivation, and therey by triggering multiple cell death pathways (Evans and Halliwell, 1999). Indeed, we showed earlier that exposure of mice to intense noise produces oxidative stress-induced events, such as increased levels of lipid peroxidation product 4-hydroxynonenal-adducted proteins (Nagashima et al., 2010), enhanced expression of transcription factors including activator protein-1 and nuclear factor-κB (Ogita et al., 2000; Matsunobu et al., 2004; Masuda et al., 2006), as well as activation of the c-Jun N-terminal kinase signaling pathway (Nagashima et al., 2010) in the cochlear lateral wall structures and organ of Corti. In addition, an earlier study of ours demonstrated that free-radical scavengers alleviate NIHL, as well as abolish these oxidative stress-induced events (Nagashima et al., 2010).

Evidence for the possible involvement of a disrupted ion-trafficking system in the cochlear spiral ligament (SL) in permanent hearing loss came from *in vivo* experiments using noise-exposed mice and from *in vitro* experiments using primary cultures of fibrocytes derived from the SL of the cochlear lateral wall structures in mice. Exposure of mice to

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http://dx.doi.org/10.1016/j.ejphar.2017.03.058
Received 24 September 2016; Received in revised form 27 February 2017; Accepted 29 March 2017
Available online 31 March 2017

intense noise produces disruption of ion-trafficking systems including a decrease in the connexin level and Na^+, K^+-ATPase activity, as well as an increase in 4-hydroxynonenal-adducted proteins, in the SL (Yamaguchi et al., 2014). Exposure of cultured fibrocytes to 4-hydroxynonenal leads to activation of calpain, disruption of gap junction-mediated intercellular communication (GJIC), and intracellular translocation and degradation of connexins (Yamaguchi et al., 2014, 2015). The 4-hydroxynonenal-induced degradation of connexins and GJIC disruption were almost completely abolished by the calpain inhibitor. These findings led to a proposal that NIHL is at least in part due to excessive oxidative stress (4-hydroxynonenal)-induced calpain activation, resulting in the disruption of GJIC in the SL. To date, however, no direct evidence has been reported regarding a beneficial *in vivo* effect of calpain inhibitors on NIHL and disruption of GJIC in the SL. To this end, we sought to assess the beneficial effect of the selective non-peptide cell-permeable calpain inhibitor PD150606 (Wang et al., 1996) on NIHL and noise-induced disruption of GJIC in the SL of mice.

2. Materials and methods

2.1. Animals

All experiments used here met the guidelines of the Japanese Society for Pharmacology and were approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Adult male Std-ddY mice (SHIMIZU Laboratories Supplies Co., Ltd, Kyoto, Japan), weighing 22–26 g, were housed in breeding cages in a room with a light-dark cycle of 12–12 h and a humidity of 55% at 23 °C and given free access to food and water. To remove animals with natural auditory impairment, we measured their hearing ability before use and selected those animals with normal hearing in the present study.

2.2. Exposure to intense noise

The sound was generated and amplified as previously described (Nagashima et al., 2010). The mice were exposed to an octave-band noise, centered at 8 kHz, at a 110-dB sound pressure level (SPL) for 1 h within a sound chamber. Each animal was placed in a cage. The sound chamber was fitted with a speaker 300HT (FOSTEX, Tokyo, Japan) driven by a noise generator SF-06 (RION, Tokyo, Japan) and power amplifier DAD-M100proHT (FLYING MOLE, Shizuoka, Japan). To ensure uniformity of the stimulus, we calibrated and measured the sound levels with a sound level meter, NL-26 (RION, Tokyo, Japan). The meter was positioned at the level of the animal's head. As a control, naïve animals were placed in the same cage without the noise.

2.3. Drug application

Drugs were applied to the inner ear by injection into the posterior semicircular canal, according to previously reported methods (Nakagawa et al., 2003; Okano et al., 2006) with several modifications. Briefly, a retroauricular incision was made in the left ear to expose the posterior semicircular canal under anesthesia with chloral hydrate (500 mg/kg, i.p.). Under microscopy, a small hole was made in the bony wall of the posterior semicircular canal, and a fused silica glass needle (EiCOM, Kyoto, Japan) was then inserted into the perilymphatic duct of the posterior semicircular canal to apply the calpain inhibitor PD150606 (Sigma-Aldrich Co., St. Louis, MO, USA) at the injection rate of 1 μl/min for 5 min *via* a micro syringe pump, CX07200 (ISIS Co., Ltd, Osaka, Japan).

2.4. Auditory brainstem response (ABR) recording

To determine hearing ability of the animals, we measured their ABR under anesthesia with chloral hydrate (500 mg/kg, i.p.), according to a previous report (Nagashima et al., 2010). Briefly, stainless steel needle electrodes were inserted subcutaneously at the ventrolateral aspect and vertex of the left and right ears. Electroencephalogram recordings were performed with an extracellular amplifier Digital Bioamp system BAL-1 (Tucker-Davis Technologies, FL, USA), and waveform storing and stimulus control were performed by using Scope software of the Power Lab system Power Lab 2/20 (AD Instruments, Castle Hill, Australia). Sound stimuli were produced by a coupler-type speaker ES1spc (Bioresearch Center, Nagoya, Japan) inserted into the external auditory canal of the mouse. Tone burst stimuli, 0.1 ms rise/fall time (cosine gate) and 1-ms flat segment, were generated by using a Real-Time Processor RP2.1 (Tucker-Davis Technologies, FL, USA), and the amplitudes were specified by a Programmable Attenuator PA5 (Tucker-Davis Technologies, FL, USA). Sound levels were calibrated with a sound-level meter, Type 6224 (ACO Co., Ltd.). ABR waveforms were recorded for 12.8 ms at a sampling rate of 20,000 Hz by using 100–10000 Hz band bypass filter settings. Waveforms from more than 500 stimuli were averaged. For recordings, animals were anesthetized by isoflurane inhalation. The threshold of ABR was determined before and at selected days after noise exposure at the frequencies of 4, 12, 20 kHz, by using a 5-dB SPL minimum size step-down from the maximum amplitude. The hearing threshold was defined as the lowest stimulus intensity that produced a reliable wave I of the ABR. Because the test tones were set to SPLs of less than 100 dB at all frequency, the thresholds were recorded as 100 dB for the calculation of the threshold shift value when there was no response due to profound hearing impairment.

2.5. Gap-fluorescence recovery after photobleaching (FRAP) assay

Animals were exposed or not to the intense noise for 1 h, and the SL tissues of the basal/middle turn were then taken from the animals on day-7 post exposure. The tissues were then put on poly-L-lysine-coated dishes containing Dulbecco's modified Eagle medium supplemented 10% fetal bovine serum and incubated at 37 °C in a 5% CO_2/95% air-humidified incubator for 4 h. For the gap-FRAP assay, the tissues were preloaded with calcein-AM (Nacalai Tesque, Inc., Kyoto, Japan) for 20 min. Baseline fluorescence emission at 535 nm was recorded for 6 s, followed by focal laser irradiation at 405 nm for 15 s to bleach intracellular calcein. Thereafter, FRAP was monitored each 2 s up to 180 s. For analysis, we delineated a region of interest inside the bleached area for each time point.

2.6. Quantitative assessment of hair cell loss

The bone near the apex was first removed, and then the round and oval windows of the inner ear were opened, followed by gentle local perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were obtained and preserved more than 4 h and then washed 3 times in phosphate-buffered saline (PBS), washed again with, and then incubated in 4% EDTA solution at least for 2 days. Under a dissecting microscope, the cochlear tissues were dissected in PBS by removing the bony capsule to separate the lateral wall tissues and the epithelium of the organ of Corti from the bony modiolus. To visualize hair cells, we incubated the epithelium of the organ of Corti of the basal and middle turns with a solution containing 0.3% Triton X-100 and Alexa-Fluor 568-conjugated phalloidin (1:100 dilution; Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature protected from light. After having been washed 3 times with PBS, the stained specimens were mounted onto slides with an antifade mounting medium (VECTASHIELD; Vector Laboratories, Burlingame, CA, USA) and then observed under a confocal fluorescence microscope using the FV1000D system (Olympus, Tokyo, Japan) to count the numbers of missing hair cells in the base and mid part of the cochleae. The ratio of missing-to-whole hair cells was expressed as a percentage.