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Puerarin alleviates noise-induced hearing loss via affecting PKCγ and $GABA_B$ receptor expression

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ABSTRACT

Noise-induced hearing loss (NIHL) often results from prolonged exposure to high levels of noise. Our previous study revealed that during the development of NIHL, the expression of protein kinase C γ subunit (PKCγ) and $GABA_B$ receptor ($GABA_BR$) was changed within the cochlear nuclear complex (CNC), suggesting that these molecules might be the potential targets for the treatment of NIHL. As an extending study, here we focused on puerarin, a major isoflavonoid extracted from *Pueraria lobota*, which has been used in the treatment of cardiovascular and cerebrovascular diseases, and investigated whether it could protect against NIHL by acting on PKCγ and $GABA_BR$. Transgenic GAD67-GFP knock-in mice were subjected to the NIHL model and their auditory functions were evaluated by the auditory brainstem response thresholds and distortion product oto-acoustic emission signals. Our results showed that 200 mg/kg puerarin treatment ameliorated the thresholds of auditory brainstem response of NIHL mice significantly. Triple immunofluorescence staining and electron microscopy results revealed that GFP-positive neurons in the superficial layers of CNC expressed both PKCγ and $GABA_BR1$, and GAD67-positive terminals contacted PKCγ- or $GABA_BR1$-positive neurons. Immunoblotting and RT-PCR results showed that NIHL increased the expression of PKCγ but decreased that of $GABA_BR1$ and $GABA_BR2$ at both protein and mRNA levels in the CNC. Puerarin significantly attenuated the increased expression of PKCγ but elevated the reduced expression of $GABA_BR1$ and $GABA_BR2$ after noise exposure. Thus, we provided the first evidence that puerarin ameliorated the auditory functions of NIHL mice, and this effect may be due to its ability to regulate the expression of PKCγ and $GABA_BR$.

1. Introduction

Increasing numbers of modern people are suffering hearing loss, and undue exposure to noise is one of the important reasons [1]. Unfortunately, to date there are still no effective pharmacological therapies approved by the Food and Drug Administration for the treatment of noise-induced hearing loss (NIHL) [2]. Noise over-exposure induces many changes which may include a series of neuroplasticity changes in the central auditory structures [3]. Cochlear nucleus complex (CNC), the primary acoustic nuclear in central auditory system, is the first relay station in auditory information processing, and is also vulnerable to noise impairment. A body of evidence showed that NIHL was associated with the changes in the expression of PKC gamma subunit (PKCγ) or/and $GABA_B$ receptor ($GABA_BR$) in the CNC [3–5], implying that PKCγ and $GABA_BR$ may be served as the potential targets for the treatment of hearing loss, including NIHL.

Puerarin is a kind of isoflavone glycoside and the major active components of *Pueraria lobata*, a commonly used herb in Chinese medicine [6]. Puerarin has been widely used in traditional oriental medicine for the treatment of various cardiovascular disorders and cerebrovascular diseases [6–10]. Importantly, it was also reported that puerarin had the therapeutic effect on sudden deafness [11]. However, little is known about the effects of puerarin on the NIHL. For this purpose, in the present study we explored the possible effects of puerarin on the mouse model of NIHL, and further investigated whether this action was, at least in part, mediated by regulating the expression of PKCγ and $GABA_BR$ in the CNC.

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2. Experimental procedure

2.1. Animals

C57BL/6J (C57) mice were used in the present study since they are vulnerable to NIHL [12]. The mice were housed in standard conditions (12 h light/dark cycles) with water and food available ad libitum. Six young (2-month-old) male GAD67-GFP transgenic knock-in mice (C57 genetic background), in which GAGBnergic neurons can be visualized by GFP fluorescence [4], were used for immunofluorescent and ultrastructure observation. And forty C57 mice (2-month-old) were randomly distributed into five subgroups: (A) the sham group, (B) the noise group, (C) the sham plus puerarin treatment group, (D) the noise plus puerarin treatment group, and (E) the noise plus vehicle treatment group, with 8 mice in each group. Puerarin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in the vehicle (Cremophor:ethanol:saline = 1:1:4) [6]. At 10 min before noise exposure, mice received a single intraperitoneal injection of 200 mg/kg puerarin [13,14], or equal volume of vehicle. The Animal Care and Use Committees of the Fourth Military Medical University reviewed and approved all protocols.

2.2. NIHL model

Noise exposures were performed in a double-walled soundproof room, according to our previous study [3]. In order to develop a noise-induced lesion, a RadioShack Supertweeter was attached to the top of the cages and driven by a power amplifier (Yamaha AX-500U, Japan) as a loudspeaker. The noise was amplified with noise levels being measured with a sound level meter (Bruel and Kjaer, type 2606). The noise level variation was less than 2 dB within the space available to the animals. Forty C57 mice were randomly placed into two ventilated chambers. Mice had free access to food and water, and were acclimated to the environment for one week. Then the mice were exposed to a noise (an octave band of noise centered at 4 kHz, 110 dB SPL), 8 h per day for 14 days, and those in the shame groups were exposed to the soundproof room for the same time without noise exposure.

2.3. Auditory brainstem response

After NIHL model was completed, the mice were subjected to auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) tests at different time points (0, 7, 14, 21 d). ABR was performed as previously described [15,16]. A previous study confirmed that after noise exposure for 14 days, the mice were suffered from noise injury [17]. Briefly, mice were anesthetized with an injection of pentobarbital sodium (5 mg/100 g, i.p.), then the body temperature was maintained at 37 °C with a warm pad. The needle electrodes were inserted subcutaneously behind the pinna of the measured ear (active), at the vertex (reference) and in the back (ground). The stimulus signal was generated through Intelligent Hearing Systems (Bio-logic Systems, USA) which was controlled by computer and delivered by a Telephonics earphone (TDH 39, USA). Evoked responses to the ABR "click" stimuli (2–4 kHz) were recorded and the thresholds were obtained for two ears. Potentials were sent to a computer where the average waveform in response to 1024 sweeps was displayed. Each tone frequency was presented in descending intensity steps beginning at 80 dB sound pressure level (SPL) with steps of 5 dB and ending when a visually discernible ABR waveform could no longer be detected [17]. Thresholds were then defined as the lowest intensity level at which a clear waveform was visible in the evoked trace.

2.4. Distortion product otoacoustic emissions

After ABR test, the mice were subjected to DPOAE measure, according to a previous study [3]. In brief, 2f1–f2 was assessed for the recordings of DPOAE (Capella Cochlear Emissions Analyzer, GN Otometrics A/S Denmark), which was recorded in left ear at f2 frequencies (750, 1000, 2000, 4000, and 8000 Hz). These f2 frequencies produced the most complete input/output (I/O) functions (from low to high stimulus levels) and were well within the usable range of hearing in mice [18]. The f2:f1 ratio was 1.25 and L1 was greater than L2 by 10 dB. After calibration of the probe in the ear canal, a visual determination of DPOAE "threshold" was made. Responses were recorded to stimuli presented 5 dB higher than the visual threshold and to f2 stimuli up to and including 75 dB SPL in 5 dB steps in order to construct I/O functions, consisting of at least eight different stimulus levels. As described previously, the I/O functions for the switched and continuous conditions were interleaved [19]. That is, responses to both recording conditions were recorded for a particular stimulus level before the intensities of the primaries were increased for collection of data at the next level. The entire switched and continuous I/O functions for one f2 frequency took approximately 25-min.

2.5. Triple immunofluorescence staining

Mice were anesthetized and then perfused with 4% (*w*/*v*) formaldehyde. The brainstems were obtained and stored in 30% (*w*/*v*) sucrose solution in 0.05 M phosphate-buffered saline (PBS, pH 7.4) overnight

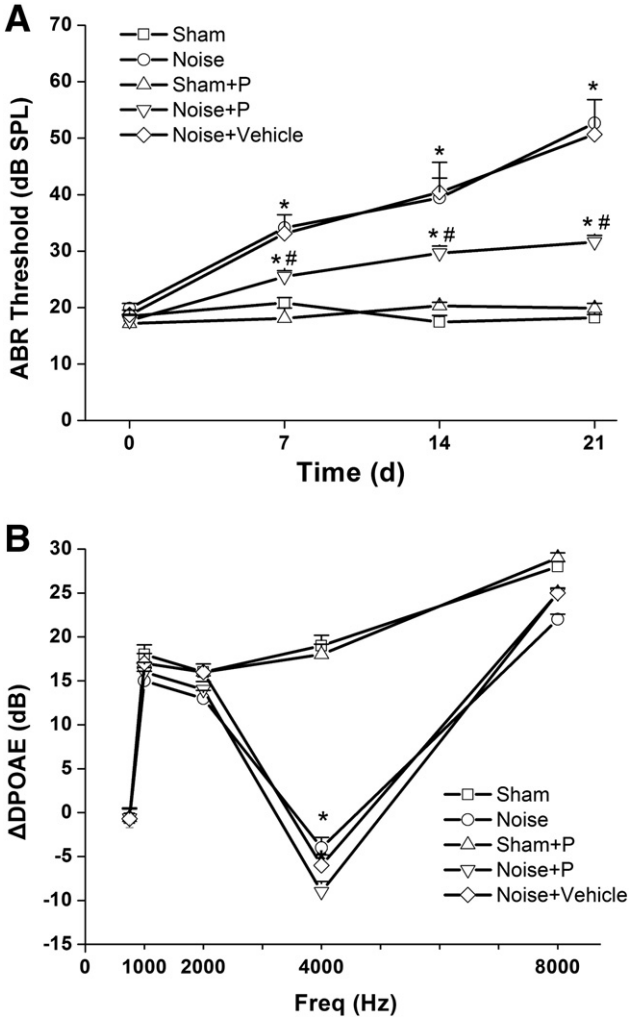

Fig. 1. Puerarin attenuates NIHL-impaired auditory functions in mice. (A) The changes in auditory brainstem response (ABR) in sham, noise, sham plus puerarin (P), noise plus puerarin, and noise plus vehicle groups at different time points (0, 7, 14, 21 d) after noise exposure. (B) The changes in distortion product otoacoustic emissions (DPOAE) magnitudes evoked by different characteristic frequencies (750, 1000, 2000 4000, 8000 Hz) in different groups. * $p < 0.05$, vs sham or sham plus puerarin group; # $p < 0.05$, vs noise or noise plus puerarin group.