Downregulation of Inwardly Rectifying Potassium Channel 5.1 Expression in C57BL/6J Cochlear Lateral Wall

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Summary: Age-related hearing loss (AHL) is one of the most common sensory disorders among elderly persons. The inwardly rectifying potassium channel 5.1 (Kir5.1) plays a vital role in regulating cochlear K+ circulation which is necessary for normal hearing. The distribution of Kir5.1 in C57BL/6J mice cochlea, and the relationship between the expression of Kir5.1 and the etiology of AHL were investigated. Forty C57BL/6J mice were randomly divided into four groups at 4, 12, 24 and 52 weeks of age respectively. The location of Kir5.1 was detected by immunofluorescence technique. The mRNA and protein expression of Kir5.1 was evaluated in mice cochlea using real-time polymerase-chain reactions (RT-PCR) and Western blotting respectively. Kir5.1 was detected in the type II and IV fibrocytes of the spiral ligament in the cochlear lateral wall of C57BL/6J mice. The expression levels of Kir5.1 mRNA and protein in the cochlea of aging C57BL/6J mice were down-regulated. It was suggested that the age-related decreased expression of Kir5.1 in the lateral wall of C57BL/6J mice was associated with hearing loss. Our results indicated that Kir5.1 may play an important role in the pathogenesis of AHL.

Key words: presbycusis; Kir5.1; cochlea; C57BL/6J

1 MATERIALS AND METHODS

1.1 Animals

Forty C57BL/6J mice were originally purchased from the Model Animal Research Center of Wuhan University (China). The mice were bred and maintained in a low-noise environment and provided with adequate clean food and water. None of the mice had a history of noise exposure, otitis media or administration of any ototoxic drug. They were randomly divided into four groups of 10 mice each as follows: group A, 4 weeks old; group B, 14 weeks old; group C, 24 weeks old, and group D, 52 weeks old. The care and experimental treatment of the animals were approved by the Animal Research Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

1.2 Tissue Preparations

All of the right cochleae were fixed in 4% paraformaldehyde in 0.1 mmol/L phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C. The cochleae...
were decalcified using 10% sodium ethylenediaminetraacetic acid (EDTA; pH 7.3–7.4) for 7 days followed by an overnight incubation in 30% sucrose. The cochleae were then embedded in the embedding medium for frozen tissue specimens to ensure optimal cutting temperature (OCT) (Sakura Finetek Inc., USA) [12].

1.3 Immunofluorescence Technique

Serial cryosections of 10 μm were cut horizontally along the cochlear axis and collected onto polylysine-coated glass slides. Cryosections of right cochlea were prepared and washed with 0.01 mol/L phosphate buffered saline (PBS) for 5 min, blocked with 5% bovine serum albumin (BSA; Boster, China) for 30 min, and incubated with rabbit anti-Kir5.1 polyclonal antibody (1:500; Sigma-Aldrich Biotechnology, USA) at 4°C overnight. After rinsing three times for 5 min each time with PBS, the sections were incubated with DyLight 594 AffiniPure-conjugated goat anti-rabbit antibody (1:200; Abbkine, USA) for 1 h at room temperature, the sections were washed again with PBS, incubated with 1 mg/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich Biotechnology, USA) overnight at 4°C. After rinsing again with PBS and mounted on glass slides in 50% glycerol for observation under a fluorescence microscope (Olympus BX 51; Olympus, Japan). The images were recorded at the same time of exposure.

1.4 Quantitative Real-time PCR

The lateral wall of each left cochlea was separated under a dissecting microscope and collected in RNase-free D-Hanks’ solution. RNA extraction from half cochlear lateral wall tissue was carried out using TRIzol (Invitrogen, USA), and cDNA was synthesized with ReverTraAce (Toyobo, Japan). Quantitative real-time-PCR was performed using SYBR Green (Sigma-Aldrich Biotechnology, USA) for 10 min, then washed again with PBS and mounted on glass slides in 50% glycerol for observation under a fluorescence microscope (Olympus BX 51; Olympus, Japan). The images were recorded at the same time of exposure.

Table 1 Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Kir 5.1-S</td>
<td>5′-AGCCCTCTTGATGCCCCTTG-3′</td>
</tr>
<tr>
<td>Kir 5.1-A</td>
<td>5′-CTCTGGACTGGTGGGATGT-3′</td>
</tr>
<tr>
<td>β-actin-S</td>
<td>5′-GCCAAAGTACTGTGGTGA-3′</td>
</tr>
<tr>
<td>β-actin-A</td>
<td>5′-GAAAGGGGTGAAAAACGCAGC-3′</td>
</tr>
</tbody>
</table>

1.5 Western Blotting

Half cochlear lateral wall tissue was homogenized in lysis buffer, followed by incubation on ice for 30 min. The homogenates were ultrasonicated, followed by centrifugation (Eppendorf model 5417R, Eppendorf, Germany) at 12 000 r/min for 30 min at 4°C. Samples containing equal amount of protein (20 μg) were loaded onto a polyacrylamide gel and separated by electrophoresis at 90 V. Proteins were then transferred onto polyvinylidifluoride membranes (Millipore, USA). Nonspecific binding was blocked in Tris-buffered saline containing 0.2% Tween-20 and 5% BSA for 2 h at room temperature. The membranes were incubated with primary antibody against Kir5.1 (1:2000; Sigma-Aldrich Biotechnology, USA) and β-actin (1:2000; Sigma-Aldrich Biotechnology, USA) overnight at 4°C. The membranes were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000; Sigma-Aldrich Biotechnology, USA). Protein bands were visualized using enhanced chemiluminescence reagents (Amersham Biosci. USA) and a UVP imaging system (EC3-Imaging-System, USA). Imaging signals were digitized and analyzed; the ratio of band intensity to β-actin was obtained for analysis [13, 14]. All the tests were repeated three times.

1.6 Statistical Analysis

All data are presented as x ± s. and were statistically analyzed with SPSS (19.0; SPSS Inc., USA). One-way analysis of variance (ANOVA) and Student’s t test were used to analyze differences between the groups. A P value <0.05 was considered significant.

2 RESULTS

2.1 Distribution of Kir5.1 and Age-related Decreased Expression in Cochleae

Immunofluorescence technique showed the Kir5.1 immunoreactivity appeared in type II and IV fibrocytes but not in type I or III fibrocytes of SL (fig. 1 and 2). The Kir5.1 immunoreactivity in SL weakened remarkably in 24-week-old mice cochleae and no positive immunoreactivity was visualized in 52-week-old mice cochleae (fig. 2). In comparison with group A, the positive immunoreactivity areas in SL were also decreased in groups B, C and D with aging.

![Fig. 1 Photomicrographs of Kir5.1 expression in the cochlea of C57BL/6j mice](image)

A: 5% BSA was used as the first antibody incubated with the cochlea slides in 14-week-old mice for the negative control. B: Kir5.1 expression in the cochlea of 14-week-old mice. Kir5.1 immunoreactivity (red) was detected in type II and IV fibrocytes of the spiral ligament. Nuclei were counterstained with DAPI (blue). The yellow arrows show the positive staining regions. SV: stria vascularis; SL: spiral ligament; HC: hair cells; SGC: spiral ganglion cells. Scale bar: 10 μm

2.2 Age-related Decreased Kir5.1 mRNA and Protein Expression in Cochlear Lateral Wall

In comparison with group A, the expression of Kir5.1 mRNA in the lateral wall of the cochleae was significantly decreased in groups B, C and D (1.00±0.05 vs. 0.81±0.07, 0.72±0.05 and 0.50±0.02 respectively) in an age-related manner (fig. 3, one-way ANOVA, F=16.811, P<0.0001). However, there was no significant difference between group B and group C (Student’s t test, P=0.33).