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Expression of Connexin 26 in the Lateral Wall of the Rat Cochlea after Acoustic Trauma

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Objective—Although the mutation in the Gap Junction Beta 2-encoding gap junction protein connexin 26 (Cx26) has been related to hereditary non-syndromic deafness and maturation of cochlear development, the physiological role of Cx26 in the cochlear lateral wall remains unclear. It has been suggested to be responsible for the recycling of K^+ in the endolymph and for maintenance of the endocochlear potential (EP). In noise-induced hearing loss, alterations in the EP and the K^+ concentration in endolymph have been observed. Thus Cx26, which is widely expressed in the cochlear lateral wall, may play a role in the mechanism of acoustic trauma.

Material and Methods—We used a rat model of noise-induced hearing impairment to detect changes in Cx26 expression in the cochlear lateral wall. By means of immunofluorescent staining and Western blotting, we investigated whether Cx26 was involved in the pathophysiological mechanism of acoustic trauma.

Results—The results indicated that abundant Cx26 protein was found on fibrocytes of the spiral ligament in the cochlear lateral wall. Protein extract of cochlear lateral wall expressed Cx26 with a molecular weight of ≈ 21 kDa. After noise exposure, with an increasing threshold of the auditory brainstem response (ABR) of $\approx 54.2 \pm 21.8$ dB SPL, the expression of Cx26 protein increased significantly ($p < 0.05$) as revealed by semi-quantitative analysis from Western blotting.

Conclusion—Cx26 protein was present in the cochlear lateral wall of rats and was upregulated when the ABR threshold shifted after intense noise exposure. Cx26 protein was involved in the pathogenesis of acoustic trauma. *Key words:* acoustic trauma, connexin 26, gap junction protein.

INTRODUCTION

Connexin proteins compose gap junctions which constitute a system of intercellular communication. Gap junctions are responsible for the exchange of electrolytes, secondary messengers and metabolites between adjacent cells (1). Rapid advances in understanding of the molecular biology of the gap junctional proteins, connexins (Cx), have revealed that these proteins are indispensable for various cellular functions. The mutational alteration of Cx genes leads to several human diseases. This provides additional evidence that these proteins possess important but not yet fully understood functions. Mutations in the Gap Junction Beta 2 (GJB2)-encoding gene for gap junction protein connexin 26 (Cx26) have been established as the basis of an autosomal recessive non-syndromic hearing loss, in both Caucasians and Asians (2–5). This was suggested to be a screening target for congenital hearing loss in genetic counseling. Moreover, in the rat and human inner ears, Cx26 expression was widely demonstrated in the basal and intermediate cells of the stria vascularis, the supporting cells, the spiral limbus and the spiral prominence of the cochlea. Cx26 was thought to be related to the maturation of cochlea development (6–10). Disturbance of the gap junction complex of Cx26 would be expected to disrupt the recycling of potassium ions from the synapses at the base of the hair cells through the

supporting cells and fibrocytes of the cochlear lateral wall. This would lead to a decrease in sound-induced cochlear responses, resulting in sensorineural hearing loss (7, 11). In noise-induced hearing impairment, substantial alterations of the endocochlear potential (EP) and significant changes in K^+ concentration in the endolymph have been observed (12, 13). Thus, Cx26 may play a role in the pathophysiological mechanism of acoustic trauma.

To the best of our knowledge, with the exception of the role of connexin mutations in hereditary non-syndromic hearing loss, there have been very few studies in which the functional role of connexin in acquired sensorineural hearing loss has been discussed (8, 14). In this study, we used indirect immunofluorescent staining and Western blotting on tissues of the cochlear lateral wall to elucidate the acute changes in Cx26 protein after intense noise exposure. This may represent a new aspect in the pathogenesis of hearing impairment in acoustic trauma.

MATERIAL AND METHODS

Animals

Ten male Wistar rats (200–250 g) were used. Only animals (20 ears in total) with thresholds of auditory brainstem responses (ABRs) < 30 dB SPL were included. Four rats not subjected to noise exposure were

used as controls. The other six rats subjected to noise exposure formed the experimental group. The treatment of animals was in accordance with the regulations of the Institutional Laboratory Animal Care Committee of our school.

Noise exposure

The animals were kept in a cage inside a sound-proof room and were allowed to move freely during the noise exposure period. White noise with a center frequency of 4 kHz at 115 ± 5 dB was produced by an audiometer (model GSI-61; Grason-Stadler, Milford, NH) and presented through a set of loudspeakers placed on both sides of the cage. The SPL of the noise was measured using a sound level meter (model NA-24; Nion, Tokyo, Japan). As previously established (15, 16), a single, continuous, 48-h period of noise exposure induces a permanent threshold shift in rodents.

ABRs

An evoked-potential detection system (Spirit; Nicolet, Madison, WI) was used to measure the ABR thresholds in all animals. Click sounds at a rate of 57.7/s were given to evoke the ABR. The intensity of the stimulus was varied in 5-dB stepwise increments. The response signals were recorded by means of subcutaneous needle electrodes. The active electrodes were placed in the vertex and the ipsilateral retroauricular region, with a ground electrode on the neck. Animals in the non-exposed control group were painlessly sacrificed after measurement of ABR thresholds. In the noise-exposed group, ABR thresholds were measured immediately after noise exposure and just before sacrifice. Changes in the ABR thresholds in the experimental group were assessed pre- and post-noise exposure.

Collection of lateral wall specimens

Immediately after the final ABR measurement, the animals were lethally anesthetized with an i.p. injection of pentobarbital sodium (35 mg/kg) and decapitated. The temporal bones were quickly removed and opened to expose the otic capsule and the tissue samples were then collected by microdissection. The lateral wall, including the spiral ligament and the stria vascularis of the cochlea, was obtained and immersed in clean, ice-cold L-15 medium until use. Care was taken to avoid collecting other cochlear tissues, such as the organ of Corti or Reissner's membrane. For immunofluorescent staining, the tissue samples were buried in Cryomatrix and freshly frozen in liquid nitrogen. For Western blotting, the tissues were lysed in 200 μ l of 10% sodium dodecyl sulfate (SDS) extraction buffer for 2 cochleae, homogenized by ultrasonication (VC-600; Sonics and Materials,

Danbury, CT) and then immediately stored at -70°C until assayed.

Antibodies

Rabbit polyclonal anti-26 antibody was used (Zymed, San Francisco, CA) as the primary antibody for immunofluorescent staining and Western blotting. Fluorescein isothiocyanate (FITC)-conjugated and horseradish peroxidase-conjugated anti-rabbit IgG antibody were obtained from Sigma (St. Louis, MO) and used for staining and Western blotting, respectively.

Immunofluorescence

Sections (5- μ m thick) were cut in a cryostat and placed on a lysine-treated glass slide. Indirect immunofluorescent staining was performed to localize Cx26 at the light microscopic level. The tissue sections were fixed in methanol at -20°C for 10 min. After fixation, tissues were incubated in blocking solution (Immunotech, Marseille, France) for 5 min at room temperature; they were then incubated in primary antibody (diluted 1:200 in PBS) at 37°C for 1 h. They were then washed 3 times with PBS and incubated with FITC-conjugated secondary antibody (1:200) for 1 h at 37°C . Negative controls of immunoreactivity were obtained by omitting the primary antibody or replacing the primary antibody by blocking solution. Rat liver sections were used as positive controls. For visualization, cover slips were mounted on slides in 1% p-phenylenediamine in 33% glycerol:PBS (10:1). Tissue sections were visualized with a Nikon inverted microscope equipped with fluorescence optics and a $\times 40$ objective lens. Fluorescence was detected with a cool charge-coupled device and digitized photographs were recorded with Image-Pro software (Media Cybernetics, Silver Spring, MD).

Protein determination

Protein was determined using the bicinchoninic acid (BCA) method (17). Bovine serum albumin (BSA) was used as the standard. The linearity of the standard curve was confirmed. Homogenates (1 μ l) of the lateral wall of the cochleae were incubated for 30 min at $37 \pm 0.5^\circ\text{C}$ in BCA working reagent. Optical density was determined at 570 nm using an ELISA reader (MR-7000; Dynatech, Ashford, UK). Total protein (in micrograms) was determined versus a BSA standard curve.

Western blotting

Tissue proteins (20 μ g per lane) were applied to SDS – 12% polyacrylamide gel, electrophoresed and transferred onto Hybond-C super membranes (Amersham). The blots were pre-incubated with blocking

solution (10 mM Tris-HCl, pH 7.4, 0.9% NaCl and 4% skimmed milk) for 1 h and reacted with primary antibody (1:300) at room temperature for 1 h. The blots were then washed 3 times with washing buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl and 0.2% Tween-20), followed by incubation with diluted secondary antibody (1:500) at room temperature for 1 h. After washing three times with washing buffer, the blots were developed with freshly prepared Enhanced Chemi-Luminescence (ECL) substrate (Amersham). Luminescence was detected by X-ray exposure. For detection of β -actin protein, a mouse monoclonal antibody (Sigma) was used as the primary antibody, and goat anti-mouse IgG as the secondary antibody.

RESULTS

Hearing thresholds of ABR

The average ABR thresholds were 23.1 ± 5.5 and 22.9 ± 6.8 dB SPL for the control and noise-exposure groups, respectively and this difference was not statistically significant ($p = 0.959$; Student's *t*-test). In the noise-exposure group, the ABR thresholds increased to 77.1 ± 15.8 dB SPL ($p < 0.001$; paired *t*-test). The difference in mean ABR thresholds before and after noise exposure was 54.2 ± 21.8 dB SPL (95% CI 38.1–70.2 dB SPL).

Indirect immunofluorescent staining (Fig. 1)

At high magnification, positive fluorescent staining for Cx26 protein was abundantly distributed in the spiral ligament from the lateral wall of the cochlea (Fig. 1A), occurring as punctated spots in the intercellular area. Negative control staining was shown at the same magnification (Fig. 1B) when the primary antibody was omitted for comparison of background intensity.

Fig. 1. Immunofluorescent staining of the cochlear lateral wall with anti-Cx26 antibody. (A) Arrows indicate some positive staining of Cx26 protein. Cx26 was abundantly distributed in the lateral wall of the rat cochlea and appeared as punctated spots in the intercellular area. (B) Negative control showing no staining. Bar = 50 μ m.

Fig. 2. Western blotting of Cx26 protein in the lateral wall of the rat cochlea. Upper panel: Cx26; lower panel: internal control (β -actin). S = control (crude extract of rat liver); C₁, C₂ = protein extracts from rats not exposed to noise; N₁, N₂ = protein extracts from rats exposed to noise.

Western blotting (Fig. 2)

Protein extract from tissues of the cochlear lateral wall expressed Cx26. The molecular weight of rat Cx26, calculated using the amino acid sequence obtained from www.ca.expasy.org, was 21 kDa. This differed from that of other species such as the guinea pig or mouse (26 kDa). Semi-quantitative analysis of Cx26 was performed using the Cx26: β -actin ratio estimated densitometrically. The result indicated that the amount of Cx26 increased significantly ($p < 0.05$; Student's *t*-test) after acoustic trauma resulting from noise exposure (Fig. 3).

DISCUSSION

Mutations in Cx26 are the commonest cause of autosomal recessive non-syndromic forms of deafness

Fig. 3. Semi-quantitative analysis of the change in Cx26 after noise exposure.

worldwide. This gene is thought to be involved in half of all cases of hereditary deafness (18). Because of the known properties of gap junctions, which are constructed from connexin proteins, Cx26 was suggested to play an important role in the ion transport mechanism, most likely involving the pathway for recirculation of K^+ in the cochlea (6). According to a recent hypothesis (19), Cx26 protein is essential for maintaining a high K^+ concentration in the endolymph of the inner ear. When sound propagates via the ossicular chain and causes vibration of the basilar membrane through the oval window, K^+ ions enter the hair cells and thus mechanical signals are converted into neural signals. The system is regenerated by release of K^+ ions from hair cells to the supporting cells; the K^+ ions are then passed from cell to cell of the lateral cochlear wall via gap junctions and eventually recycled into the endolymph.

In noise-induced hearing loss, a significant decrease in the sound-induced cochlear responses (summing potentials and action potentials) together with substantial alterations of the EP and significant changes in endolymphatic ion concentrations were immediately observed in animals exposed to intense noise (12, 13, 20, 21). Following this line of reasoning, gap junctions composed of Cx26 in the cochlear lateral wall might be changed after intense noise exposure. In this study, we have shown that Cx26 protein in the cochlear lateral wall was significantly upregulated in an animal model. This phenomenon demonstrated that an acute change in the K^+ concentration in the endolymph after noise exposure may send a message to the K^+ recycling system. In order to compensate, Cx26 proteins may be produced according to the signal in order to maintain the intercellular recycling efficiency of K^+ ions. Thus, recovery of the EP may result after acoustic trauma in the inner ear.

In our previous studies (15, 16) of the ion-exchange enzyme systems (Na^+ , K^+ -ATPase and Ca^{2+} -ATPase) in the cochlear lateral walls after acoustic trauma, both enzyme activities were found to be significantly decreased. In comparison, Cx26 proteins were increased during the acute stage of acoustic trauma. Although the interaction between these enzymes and the expression of Cx remains unclear, we may expect that changes in the gap junction system in the cochlear lateral wall would contribute to K^+ recirculation, cochlear potential changes and the noise-induced hearing threshold shift. Further studies, such as those involving chronological changes in Cx26 expression and functional aspects of gap junctions composed of Cx26, should be performed to elucidate the role of Cx26 protein in the metabolic signal transduction of the cochlear lateral wall after noise exposure.

In conclusion, an increase in Cx26 protein in the cochlear lateral wall contributes to the hearing threshold shift observed during the acute stage of acoustic trauma. The intercellular communication system of gap junctions in the cochlear lateral wall may be a crucial determinant of noise-induced sensorineural hearing loss.

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