Gentamicin affects connexin 26 expression in the cochlear lateral wall

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Abstract. Gentamicin affects connexin 26 expression in the cochlear lateral wall. Objectives/Hypothesis: Aminoglycosides may decrease the expression of some proteins participating in ion-exchange in the cochlear lateral wall. Connexin 26 expression in the lateral wall may play a role in acquired hearing loss by maintaining the endocochlear potential and potassium concentration in the endolymph. We examined the effects of gentamicin on the expression of connexin 26 to obtain a better understanding of aminoglycoside ototoxicity.

Methods: We detected changes in connexin 26 protein and mRNA expression in the cochlear lateral wall using immunohistochemistry staining, western blotting, and real-time PCR in rats after administration of a single dose of gentamicin.

Results: The expression of connexin 26 increased over time in type III fibrocytes after gentamicin administration. Elevated protein levels were detected 3 h after the single injection of gentamicin; while, mRNA levels increased after 24 h.

Conclusion: Connexin 26 plays an important role in the acute effects of high-dose gentamicin and is probably involved in the pathogenesis of ototoxic deafness.

Introduction

Aminoglycosides are clinically important agents for treating gram-negative bacterial infections. They are efficacious but can result in irreversible ototoxicity. The pathophysiology of aminoglycoside ototoxicity is unclear and the damage to the ear occurs both in a dose-dependent and an inherited idiosyncratic fashion. Aminoglycosides exert their toxic effects by affecting DNA, RNA, and protein synthesis; energy metabolism and ion transport; synthesis or degradation of prostaglandins, gangliosides, mucopolysaccharides, and lipids; and synthesis of cochleotoxic metabolites such as free radicals.1 Systemically administered aminoglycosides are trafficked from strial capillaries into marginal cells and are cleared into the endolymph following the electrophysiological gradient. Aminoglycosides enter inner ear hair cells across their apical membranes via endocytosis, or through mechanoelectrical transduction channels in vitro.2 The mechanisms for this trans-strial trafficking of aminoglycosides are not yet unidentified. Current strategies to prevent the ototoxicity of aminoglycosides are mainly based on the molecular mechanisms involved in the accumulation of aminoglycosides in the inner ear and the generation of cochleotoxic metabolites.

The cochlear lateral wall is composed of the stria vascularis (SV) and spiral ligament (SPL). Fibrocytes in the SPL are classified into five major subtypes on the basis of their distribution, morphology, and expression of specific protein markers1 (Figure 1A). The different localization and immunostaining profiles, which mediate or energize ion transport, including Na/K-ATPase, carbonic anhydrase (CA), and creatine kinase, suggest that the fibroblasts play different roles in the hypothesized lateral K+ recycling pathway, which starts with an efflux of potassium from the basolateral membrane of outer hair cells and ends with the secretion of potassium back into the endolymph by strial marginal cells. The lateral pathway involves the supporting cells adjacent to hair cells within the organ of Corti, which recycle K+ through the SPL and SV back into the scala media. Potassium recycling requires a network of iron channels and transporters, including gap junctions assembled by connexins, the anion transporter pendrin, and potassium channels.4 Connexin mediated intercellular communication has been hypothesized to play a crucial role in the generation and maintenance of high potassium concentration in the endolymph.

We considered there might be distinct cell-specific distributions of
Previous studies showed hearing loss induced by experimental endolymphatic hydrops is associated with dysfunction of Cx26 in the cochlear lateral wall,\textsuperscript{5} which is involved in the pathogenesis of acoustic trauma, and expression of Cx26 is upregulated when the ABR threshold shifts.\textsuperscript{6} Thus, Cx26 may play a role in sensorineural hearing loss. Cx26 in the cochlear lateral wall\textsuperscript{7} is important for generating a positive endocochlear potential (EP) and high potassium concentration in the endolymph. Recent research found aminoglycosides may decrease the expression of proteins important for ion-exchange such as Na-K-2Cl cotransporter-1 (NKCC1)\textsuperscript{8} and CA\textsuperscript{9} in the lateral wall, but the change in EP is slight\textsuperscript{10} and does not match the change in protein expression. Therefore, Cx26 may be involved in ototoxic deafness.

The aminoglycoside, gentamicin, can be observed inside the outside hair cells of cochlea (OHCs) as soon as 24 h after injection and is eliminated in two phases: one rapid and one slow with approximate half-lives of 2 days and 5-6 months, respectively.\textsuperscript{11} In this study, we measured the expression of Cx26 in the cochlear lateral wall during the 2 day half-life of ADME processes (absorption, distribution, metabolism, excretion) after a single-dose of gentamicin was administered by intraperitoneal injection. We used immunohistochemistry staining, western blotting, and real-time PCR on tissues of the cochlear lateral wall to elucidate acute changes in Cx26 in gentamicin-treated rats and examine the pathogenesis of aminoglycoside ototoxicity.

**Figure 1**

A: The approximate location of the different subtypes of fibrocytes in the spiral ligament (SPL) (I–V). B-F: Immunoreactivity to connexin 26 (1:300) in the lateral wall of the cochlea. Fibrocytes in the SPL show a differential staining pattern. In the control (0 h, B) and 1 h group (C), type I fibrocytes exhibited intense staining and moderate staining was seen in cells of the stria vascularis and type II and V fibrocytes. Type III and IV fibrocytes were lightly immunoreactive. In the 3 h, 24 h, and 48 h groups (D-F), the expression of connexin 26 was increased over time in type III fibrocytes.
Materials and methods

Animal procedures

Experiments were carried out with male SD rats (280-320 g) with thresholds of auditory brainstem responses (ABRs) < 30 dB SPL and signs of middle ear infection under otoscopic exam. Animals were divided into 7 groups of 5 animals each. The animals in the 6 experimental groups were sacrificed 30 min, 1 h, 3 h, 6 h, 1 day, and 2 days, respectively, after a single intraperitoneal (i.p.) injection of high-dose gentamicin sulfate (200 mg/kg). The control group (0 h group) was sacrificed after a single injection of the same volume of sterile saline (50 ml/kg). All procedures were performed following approved protocols for the care and use of animals by the Institutional Laboratory Animal Care Committee of our school.

ABRs

All animals were assessed for their auditory function just before sacrifice. The animals were anesthetized with an i.p. injection of pentobarbital sodium (35 mg/kg). The ABRs were recorded between a subcutaneous stainless-steel needle electrode at the vertex and an electrode behind the ipsilateral ear, with a ground electrode on the neck. Stimuli were presented at a repetition rate of 10/s. Around threshold, responses were averaged over 500 stimuli at each intensity level, which varied in steps of 5 dB sound pressure levels.

Tissue collection

Immediately after the final ABR measurement, the animals were decapitated. The temporal bones were quickly removed and opened to expose the otic capsule. For immunohistochemistry staining, the isolated cochlea was fixed with 4% paraformaldehyde for 30 min and then decalcified in 10% EDTA at room temperature with gentle agitation for 6-8 days, with solution changes every 2 days. The specimens were dehydrated through graded concentrations of alcohol and then embedded in paraffin blocks and sectioned into 4 μm thick slices.

Chemicals and reagents

Polyclonal rabbit anti-connexin 26 antibody (BA1591), peroxidase-conjugated goat anti-rabbit IgG, diaminobenzidine (DAB), and immunohistochemistry kits (SA1022) were from Boster Biological Technology, Ltd, China. Trizol reagent was from Invitrogen Corporation, Carlsbad, California, USA and SYBR Green PCR Master Mix was from TaKaRa Biotechnology Limited Company, Dalian, China. The Total Protein Extraction Kit was produced by ProMab Biotechnologies, St. Albany, California, USA. The bicinchoninic acid protein assay kit was produced by Pierce Biotechnology, Rockford, USA.

Immunohistochemistry staining

After deparaffinization and rehydration, sections were micro waved in 10 mM citrate acid (pH 6.8) for antigen retrieval and incubated in 0.3% H2O2 for 10 min to block endogenous peroxidase. Then, the samples were incubated with diluted normal goat serum for 20 min to suppress nonspecific binding of immunoglobulin, incubated with rabbit anti-connexin 26 antibody (1:300 dilution) overnight, and then with goat anti-rabbit IgG peroxidase conjugate (1:10 dilution). The immunostaining signal was visualized after 1-5 min incubation with DAB, and sections were also counterstained with hematoxylin. In negative con-
controls, the rabbit anti-cx26 antibody was replaced with normal rabbit serum in the reaction. Then, sections were dehydrated and coverslipped with Permount. The immunohistochemical expression was evaluated with Imag Pro Plus 6.0 to detect the photo density.

**RNA extraction and cDNA preparation**

Tissue specimens were directly homogenized in 1 ml Trizol reagent and total RNA was isolated. After two chloroform extraction steps, RNA was precipitated with isopropanol and the pellet was washed twice in 70% ethanol. After air drying, RNA was resuspended in DEPC-treated water and the concentration of each sample was determined by measuring the absorbance at 260 nm. Residual DNA was removed using DNase I, in accordance with the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed to cDNA according to the manufacturer's instructions.

**Real-time RT-PCR**

According to the manufacturer's protocol (Applied Biosystems), real-time RT-PCR was performed using 4 μl of cDNA and 1 μl of SYBR Green PCR Master Mix. Samples from reverse transcription reactions that did not contain SuperScript III enzyme were used as negative controls. The cDNA abundance for GAPDH was also determined as an internal control. Real-time PCR was carried out on an Eppendorf Master Realplex2 PCR machine (Brinkmann Instruments, Westbury, NY) using the SYBR Green method, with previously published primers as follows: for Cx26 (GenBank accession no: NM_001004099), 5-ACGTTGGCCTTTTGGTTATG-3 (sense) and 5-TGTTGCGGGCTGACTCAG-3 (antisense) and for glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 5-TTGTGAAGGTGCTGTGAAC-3 (sense) and 5-AGGGGTCGT-TGATGGCAACA-3 (antisense). The expected sizes of the amplicons obtained using these pairs of primers were 204 and 197 bp, respectively.

All PCR assays were performed as follows: after heating at 95°C for 2 min to inactivate the reverse transcriptase, cycling conditions were 95°C for 15 sec, 56°C for 30 sec, and 72°C for 5 min. Dissociation curves of PCR products were obtained by heating samples from 60°C to 95°C, to attest to the primers' specificities.

**Western blot**

Total proteins from the cochlear lateral wall were extracted using a total protein extraction kit, following the manufacturer's instructions. Protein concentrations were measured using a bicinchoninic acid protein assay kit. Proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Equal amounts of protein (20 μg) were loaded in each lane of the SDS gel. After transfer to nitrocellulose membrane, bands of Cx26 were detected using the primary antibody against Cx26 (1:200). The amount of loading in each lane was verified by detection of GAPDH, using a mouse monoclonal antibody (ProMab) as the primary antibody. Secondary antibodies used in the western blots were goat anti-rabbit and antimouse IgG horseradish peroxidase (HRP) conjugate. Blots were developed using CL-Xposure x-ray film (Pierce, Rockford, IL). The developed x-ray films were scanned using a flat-bed scanner and the density of images was measured with NIH Image Software after calibration to a 20-panel OD595 grayscale density control, supplied by Kodak (Kodak, Rochester, NY).

**Data and statistical analysis**

After real-time PCR, gene amplification was quantified by determining the cycle threshold (CT) based on the fluorescence detected within the geometric region of the semi-log view of the amplification plot. Data were analyzed according to the 2\(^{-\Delta\Delta C T}\) method. The \(\Delta C T\) value was determined by subtracting the target CT of each sample from its respective GAPDH CT value. Calculation of \(\Delta\Delta C T\) involved using the mean \(\Delta C T\) value of the control group as an arbitrary constant that was subtracted from all other \(\Delta C T\) mean values. Fold changes in gene expression of the target gene were individually calculated. All values were analyzed by one-way ANOVA at each time point for each treatment, with significance set at 5%.

**Results**

**Hearing thresholds of ABR**

There were no significant differences between the thresholds of ABRs in the six experimental groups and control group (\(p>0.05\), Student’s t-test).

**Connexin 26 expression in the cochlear lateral wall** (Figure 1)

Cx26 immunolabeling was detected throughout the SV and the SPL. Immunoreactivity was found to be differentially distributed in
Gentamin and connexin 26 in cochlea

The lateral wall of the cochlea. In the control and 1 h group, type I fibrocytes exhibited intense staining, and moderate staining was seen in cells of the SV and type II and V fibrocytes. Type III and IV fibrocytes were lightly immunoreactive. In the 3 h group, the expression of Cx26 was increased over time in type III fibrocytes. There was no immunoreactivity present in preabsorption controls.

Changes in cx26 protein expression (Figure 3)

Semi-quantitative analysis of Cx26 was performed using the Cx26:GAPDH ratio, which was estimated densitometrically and indicated the amount of Cx26 increased significantly 3 h after intraperitoneal injection of gentamicin (P < 0.01); afterwards, there were no significant changes.

Changes in Gjb2 mRNA expression (Figure 4)

Although not statistically significant (30 min, P = 0.100; 3 h, P = 0.253; 6 h, P = 0.118), there was a tendency toward a decrease in Gjb2 mRNA expression, which encodes the Cx26 protein, in the cochlear lateral wall of rats 30 min–6 h after intraperitoneal injection of gentamicin. At 24 h and 48 h after gentamicin injection, Cx26 transcript levels were significantly increased 3.81- and 10.9-fold, respectively (**P < 0.01).

Discussion

The cell-specific distribution of Cx26 provides morphological evidence that it can play different roles in the transport of ions/nutrients in the cochlear lateral wall. No changes in Cx26 immunostaining in the lateral wall were observed 1 h after gentamicin injection. Lower expression was found among inferior fibrocytes (Type III and Type IV) in the control and 1 h group, which is consistent with previous research. Three to 48 h after administration, there was a significant increase in immunostaining of type III fibrocytes in the SPL, which are positive for CA and Aquaporin1(Aqp1), a water transporter protein, but not Na+/K+-ATPase.

Western blot also showed the expression of Cx26 increased in 3 h. CA is an important ion transport-mediating enzyme, which converts H₂O and CO₂ to H⁺ and HCO⁻, which in turn, can be exchanged for Na⁺, K⁺, and Cl⁻. These fibrocytes have been proposed to form different pathways for K⁺-recycling; thus, this peculiar pattern of Cx26 immunoreactivity indicated Cx26 may be involved in ototoxic deafness by playing an important role in the ion balance of cochlear fluid cells in the SPL. Unexpectedly, the Cx26 protein levels were not consistent with mRNA levels. Although protein levels of Cx26 were already significantly elevated at 3 h, a significant increase in mRNA was not observed until 24 h. A possible explanation for this discrepancy is...
that, in the short period (within 3 h) after single-dose gentamicin treatment, the available Cx26 mRNA is rapidly translated, suggesting that gentamicin has a more rapid effect on Cx26 translation/processing than on transcription of the Cx26 gene. This would indicate that the posttranscriptional mechanism was responsible for the appearance of Cx26. Many reports point out and confirm Cx26 mRNA synthesis appears to be controlled transcriptionally, while posttranscriptional regulatory mechanisms determine the relative abundance of connexin mRNA. Further study is necessary to determine if posttranscriptional regulation prevents the Cx26 mRNA, synthesized after treatment with gentamicin, from being translated to protein.

Until recently, there were only a few acute toxic effects reported and no signs of cochlear dysfunction detected after a single injection of an aminoglycoside alone. Jin X et al. found the firing mechanism of the inner ear might be affected by a single dose of gentamicin, indicating the transient ototoxicity induced by gentamicin was caused by administration of a large single dose and, presumably, the mechanism was not related to gentamicin but to the absorption and rapid spread of the drug. Aran et al. confirmed that the transient ototoxicity induced by gentamicin occurred at the medial efferent/outer hair cells synapse, probably via the reversible blockade of calcium channels in vitro. In addition, Zhuravskii et al. demonstrated the protective effect of the calcium channel blocker verapamil in experimental kanamycin-induced acute ototoxicity. Previous research also found that a single, large dose of gentamicin over a 35-day period may have ototoxic effects, as evaluated by ABR, similar to multiple doses of gentamicin. Therefore, there must be a building process of drug-induced deafness and superposition of these effects leads to deafness.

Although the mechanisms are still being unraveled, aminoglycoside ototoxicity likely involves the following steps: 1) trafficking of the cationic, hydrophilic aminoglycosides across the blood-labyrinth barrier into the endolymphatic fluid of the scala media, 2) permeation into hair cells along the electrochemical gradient, and 3) exerting cytotoxic effects and disrupting auditory function. There may be a regulatable barrier for gentamicin entry into the endolymph at the interface between cells of the lateral wall. Aminoglycosides from endothelial cells, basal cells, and fibrocytes could pass into intermediate cells via gap junctions. Once in intermediate cells, aminoglycosides would then be cleared into the intra-strial space and then transported into marginal cells by an unidentified mechanism, where aminoglycosides could enter into the endolymph along the electrochemical gradient. We speculated that Cx26 may play a role in the regulatable permeability of the blood-labyrinth barrier by aminoglycoside-permissive cation channels or ion transport proteins. If so, it’s likely that increased expression of Cx26 could enhance the potential for redundancy in a variety of aminoglycoside-permissive cation channels expressed in the cochlear lateral wall and finally result in a sharp increase in aminoglycosides in the endolymph. Gap junctions composed of Cx26 are believed to be nonspecific porous structures, allowing passage of molecules up to 1.2 kDa, therefore, they may be permeable to gentamicin (575.67 Da) and provide a pathway.

Many studies have suggested gentamicin-induced ototoxicity is mediated by reactive oxygen species (ROS) and reactive nitrogen species, and overproduction of ROS was suggested to be an initial step in triggering apoptotic pathways that cause cell death. Furthermore, it was also previously demonstrated that expression of Cx26 in the SPL is negatively
regulated by ROS and NO/peroxynitrite generated by acoustic overstimulation. Thus, the change in Cx26 expression may be due, at least in part, to the overproduction of ROS induced by gentamicin. Previous research by Wei Chung Hsu et al. showed Cx26 protein on the cochlear lateral wall was up-regulated when the ABR threshold shifted after intense noise exposure. Thus, it is likely that Cx26 up-regulation is one of the common responses to injury, but not a specific response to gentamicin administration.

Cx26 was hypothesized to play a crucial role in the EP generation and maintenance by maintaining a high K+ concentration in the endolymph of the inner ear. Enhanced expression of Cx26 could, therefore, disrupt this ion circulation, resulting in deafness. It’s reported that kanamycin clearly inhibited the expression of NKCC1 in the SV, and kanamycin destroyed NKCC1 protein possibly via the ROS pathway. NKCC1 is a potassium ion channel protein and plays an important role in cochlear potassium ion recycling, it expresses mainly in the stria marginal cells and type II fibrocytes in the inferior portion of the SPL. Thus, the kanamycin could interfere with the K+ recycling. Consequently, the EP generated by K+ flow decreased and cochlear auditory function was impaired eventually. However, it was reported that gentamicin could reduce EP, but with only marginal significance, and the potassium ion activities in the endolymph and perilymph and the EP did not change appreciably throughout the experimental days in kanamycin-treated animals. The data indicated there must be some unknown mechanisms playing a compensating or antagonistic role in maintaining the stability of EP. In comparison with the decreased expression of NKCC1, expression of Cx26 may increase according to a signal generated during the acute stage of ototoxic deafness. Although the mechanisms by which gentamicin affects Cx26 and the interaction between NKCC1 and the expression of Cx26 remains unclear, our present data suggest that changes in the gap junction system in the cochlear lateral wall contribute to maintaining the intercellular recycling efficiency of K+ and prevent damage from otoxicity.

The present findings showed Cx26 plays a protective role, via maintaining the K+ concentration stability, in aminoglycoside ototoxicity. This indicates mutations of the GJB2 gene, which result in alteration of the Cx26 structure, possibly lead to a weakening and loss of cochlea protection. Thus, we suppose GJB2 mutations are an aggravating factor in the phenotypic expression of the non-syndromic hearing loss associated with aminoglycoside ototoxicity, whether they carry the mitochondrial mutation A1555G or not. The GJB2 gene has been shown to modulate the severity of hearing loss associated with the mitochondrial mutation A1555G, but the mechanism is unclear. Our present findings allow for a new interpretation of this phenomenon and suggest new possibilities, related to potential gene interactions, in the pathogenic mechanism of hearing loss. Thus, the aminoglycoside antibiotics should be rigorously controlled in people with these mutations to prevent hearing loss.

Conclusion

In conclusion, the systematic administration of a single dose of gentamicin can rapidly influence the expression and distribution of Cx26 protein. The Cx26 mRNA also increased but failed to translate to protein, which suggests an unclear regulation at the posttranscriptional level that requires further study. Cx26 is one of the earliest markers appearing in response to gentamicin, before any toxic effect and cochlear dysfunction. Cx26 up-regulation is probably one of the common responses of the cochlear lateral wall to injury, such as acoustic trauma and early stage drug ototoxicity. Cx26 can play a protective role in aminoglycoside ototoxicity via maintaining the K+ concentration stability; thus, changes in the gap junction system in the cochlear lateral wall may contribute to maintaining the intercellular recycling efficiency of K+ and prevent damage from ototoxicity. Since mutations of the GJB2 gene result in alteration of the Cx26 structure, possibly leading to weakening and loss of cochlear protection, the aminoglycoside antibiotics should be rigorously controlled in people with these mutations to prevent hearing loss.

References


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