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An Ototoxic Antibiotic Gentamicin Can Increase PKA-caveolin-1 Signaling Pathway in Differentiated Vestibular Cell Line (UB/UE-1)

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Abstract

Caveolin proteins are mediators of cell death or the survival of injured cells, and they are inhibitors of various signaling pathways. The expression of caveolin-, which is involved in the protein kinase A (PKA) signaling pathway, was examined in the differentiated mouse vestibular cell line UB/UE-1 after gentamicin ototoxicity. Caveolae in the vestibular hair cell of healthy guinea pigs were observed through an electron microscope. UB/UE-1 cells were cultured at 95% CO_2 with 5% O_2 at 33°C for 48 hours and at 95% CO_2 with 5% O_2 at 39°C for 24 hours for differentiation. Cells were treated with 1 mM gentamicin, 0.02 mM H89 (PKA inhibitor), and then incubated for 24 hours. Caveolin-1 expression was examined by western blotting and PKA activity by a PepTag® assay. Caveolae were observed in the vestibular hair cells of healthy guinea pigs by electron microscopy. Caveolin-1 was expressed spontaneously in differentiated UB/UE-1 cells and increased after gentamicin treatment. PKA was also over-activated by gentamicin treatment. Both gentamicin-induced caveolin-1 expression and PKA over-activation were inhibited by H89. These results indicate that gentamicin-induced caveolin-1 expression is mediated by the PKA signaling pathway. We conclude that caveolae/ caveolin activity, induced via a PKA signaling pathway, may be one of the mechanisms of gentamicin-induced ototoxicity.

Keywords: Gentamicin, Caveolin, PKA, Vestibular cell, Ototoxicity

Aminoglycosides are the most commonly used antibiotics worldwide because of their effectiveness against gram negative bacterial infections, their high efficacy, and low cost. However, they can also have serious side effects such as hearing impairment and disequilibrium. Although the mechanism of aminoglycoside-induced ototoxicity has not yet been determined, reactive oxygen species[19], the excitotoxic process of the cochlear N-methyl D-aspartate (NMDA) receptor[20,21], and mitochondrial dysfunction[22] are possible mechanisms of aminoglycoside-induced ototoxicity.

Caveolae were defined as membrane invaginated vesicle of 50-100 nm in size that were first observed by electron microscopy[1-5]. Further investigations showed that caveolae not only exist in vesicle detached from the plasma membrane proper. But also in various forms such as grape-like clusters or rosettes, and even infused in form as elongated tubules or transcellular channels (Figure 1).

Caveolin is a structural protein of caveolae and plays an important role in transforming plasma membranes which are invaginated by caveolae, forming vesicles[2,6,7]. Using the special qualities of these structures, caveolae/caveolin appear to mediate the selective uptake and transport of several molecules via different processes. In transcytosis, caveolae transport proteins from the luminal side of the endothelial cell to the interstitial compartment for subsequent uptake by underlying tissues[2,8]. In caveolae-mediated endocytosis (distinct from that of clathrin-coated pits), caveolae bud from the plasma membrane and fuse with various intracellular compartments[9-13]. In potocytosis, caveolae mediate the uptake of small solutes less than 1 kDa by pinching off, but remaining associated with the plasma membrane[13,14]. Caveolae/caveolin is a regulator of various intracellular signal transduction pathways it can co-localize with signaling molecules (H-ras, G-protein α subunit, Src family tyrosine kinase, Neu, and eNos) and bind to sequence motifs of target

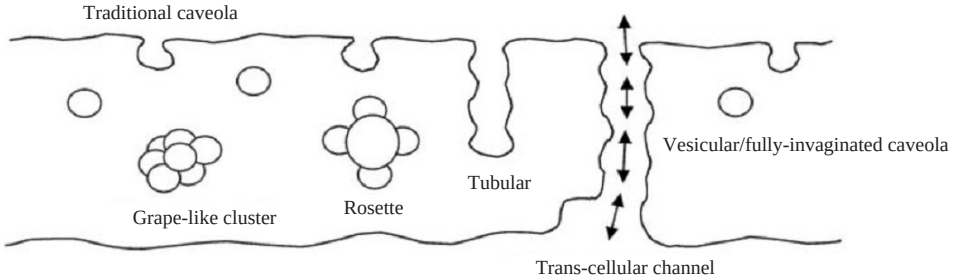

Figure 1. Caveolae can exist in many alternate forms than the traditional membrane invaginated vaiety. They can be found in vesicular form or in aggregates such as grape-like clusters, rosettes, and even elongated tubules[11].

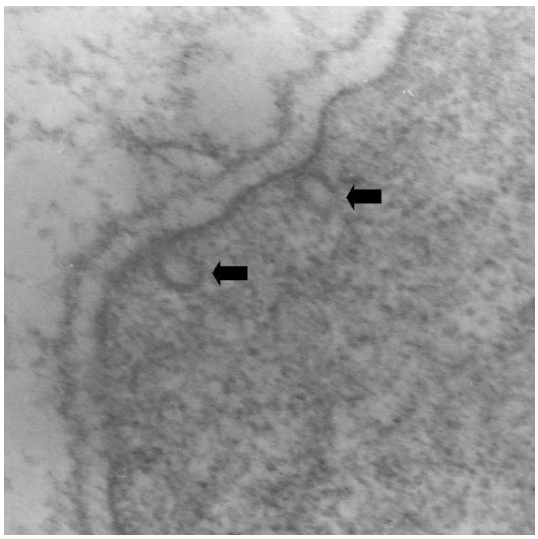

Figure 2. Electron micrograph of a vestibular hair cell of a normal guinea pig was taken at a mignification of 16,000, caveolae shaped of vesicular invagination were observed (arrows).

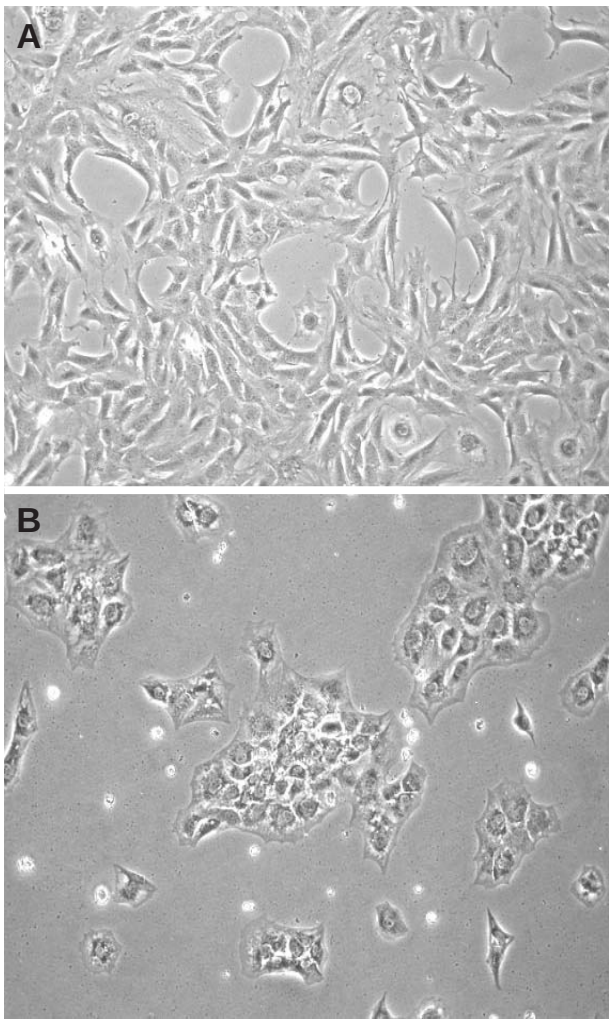

Figure 3. A: UB/UE-1 cells were cultured at 33˚C, 95% CO_2, 5% O_2. Undifferentiated spindle shaped UB/UE-1 cells were observed by inverted microscope. B: UB/UE-1 cells were cultured at 39˚C, 95% CO_2, 5% O_2 for differentiation. The cells are more symmetrical, and flattened.

proteins resulting in inactivation of these proteins[2,15-17]. Because of its functional characteristics, studies to determine caveolae/caveolin's relationship with diseases are in progress, and a few relationships have already been elucidated[1,18]. We hypothesized that if caveolin exists in the inner hair cells, it may account for several mechanisms that are related to diseases in the inner hair cell. However, there were no previous studies that examined the relationship between caveolin and ototoxicity.

In this study, to confirm the basic expression of caveolae in vestibular hair cells *in vivo*, vestibular neurosensory epithelia were dissected from healthy guinea pigs and caveolae were observed through electron microscopy. In addition, the effect of gentamicin, one of the aminoglycosides that cause toxicity in vestibular hair cells, was investigated *in vitro* through examination of expression of caveolin-1 and protein kinase A (PKA) activity in UB/UE-1 cells (vestibular sensory cell line) before and after gentamicin treatment. Although aminoglycoside toxicity is known to be concentration-dependent, we only focused on the

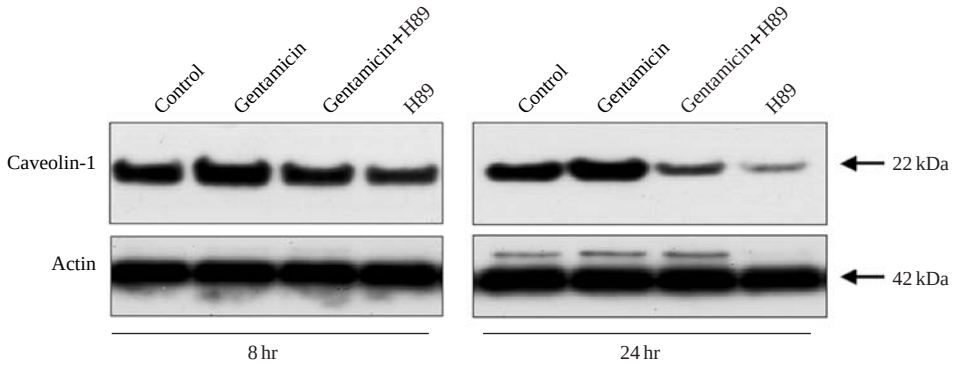

Figure 4. The expression of caveolin-1 was detected by western blot. In differentiated UB/UE-1 cells, caveolin-1 was increased by gentamicin treatment. The baseline caveolin-1 expression and the gentamicin-induced overexpression in their levels are inhibited by H89.

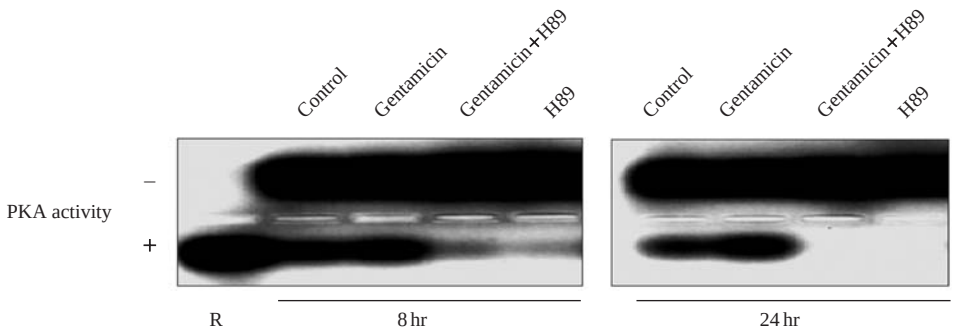

Figure 5. PKA activity was detected by PepTag® assay. In differentiated UB/UE-1 cells, PKA activity was increased by gentamicin treatment, and decreased by H89 treatment. The suppression of PKA activity by H89 was more certain in 24 hours. R: positive control for PKA activity.

toxicity itself and not concentration threshold of aminoglycoside toxicity.

Caveolae in Healthy Guinea Pigs

Vesicular-shaped caveolae were observed in the vestibular hair cells of healthy guinea pigs by electron microscopy (Figure 2).

Differentiation of UB/UE-1

UB/UE-1 cultured under proliferation-inducing conditions of 33˚C with 95% CO_2 for 48 hours appeared to be spindle-shaped (Figure 3A). In contrast, UB/UE-1cells cultured at 39˚C with 95% CO_2 for 24 hours appeared to be more flattened and symmetrical (Figure 3B). These results are in agreement with the morphological aspects that Holley *et al*. observed when UB/UE-1 cells differentiated to vestibular sensory cells after cultivation at 39˚C[23-25]. Therefore, we regarded UB/UE-1 cells cultured for 24 hours at 39˚C with 95% CO_2 as differentiated vestibular hair cells.

Caveolin-1 Expression of UB/UE-1 after Gentamicin Treatment

Caveolin-1 was spontaneously expressed in both early (8 hours after treatment) and late (24 hours after treatment) control groups and caveolin-1 expression increased with gentamicin treatment. In addition, caveolin-1 expression was clearer in late (24 hours after) groups than early groups (hours after) (Figure 4).

Caveolin-1 expression was decreased by H89 treatment in all groups, and its expression was clearer in the late groups as well (Figure 4).

The Effects on PKA Activity of Gentamicin Treatment

When UB/UE-1 cells were treated with gentamicin, PKA activity increased in both early (8 hours after treatment) and late (24 hours after) groups, and decreased in all groups with H89 treatment. The decline of PKA activity by H89 treatment was more definite in late groups (24 hours after) (Figure 5).

Discussion

Caveolae are invaginations of cellular membranes approximately 50-100 nm in size that are found in differentiated cells, and are characterized by their protein marker, caveolin[1]. They were first described by electron microscopy (Figure 2). According to recent reports, it is now clear that caveolin proteins (caveolin -1, caveolin-2, caveolin-3) serve to modulate many cellular signal transduction pathways as recruitment factors and regulatory factors[26], and play an important

role in the development of various diseases[1,18,27].

In our study, caveolae in the vestibular hair cells of normal guinea pigs were studied, and spontaneous expression of caveolin-1 in UB/UE-1 cells was observed, indicating that caveolae/caveolin acts as a signaling molecule in vestibular hair cells. Over-activation of the PKA signaling pathway and increased caveolin -1 expression was observed in gentamicin-treated UB /UE-1 cells, as well as the decrease of caveolin-1 expression by blocking PKA pathway through H89 co-treatment. These results indicate that gentamicin increases expression of caveolin-1 through the PKA signaling pathway.

If we consider this result in relation to clinical ototoxicity, we can conclude that gentamicin induces ototoxicity, such as hearing impairment and disequilibrium, via the up-regulation of the PKA signaling pathway. It also increases caveolae/caveolin, which maintains inner ear homeostasis.

The exact mechanism of aminoglycoside ototoxicity in vestibular hair cells is not clear. Takumida *et al*. suggested that nitric oxide and iNOS play an essential role in inner ear pathophysiology, including aminoglycoside ototoxicity[28-30]. Nagy *et al*. reported that cellular stress by NMDA receptor excitation could be a mechanism of gentamicin ototoxicity based on experiments using DNA analysis of the gentamicin-exposed organ of Corti[20,21]. These are the two main theories as to how cells are destroyed by the administration of aminoglycosides. Whatever the mechanism, destruction is focused on the hair cells in either the cochlea or the vestibular organ[31]. Previous studies investigating these proposed mechanisms focused on free radicals or receptors. Our study focused on the caveolin protein, which has previously been reported to be related to various intracellular signaling pathways and cell death.

In this study, a relationship between caveolin and gentamicin ototoxicity was identified. Gentamicin regulates caveolin-1 through the PKA signaling pathway rather than directly. Based on this, it we propose that caveolin-1 expression via the PKA signaling pathway results in aminoglycoside ototoxicity. Finally, to reveal a more precise mechanism, we are currently actively researching the functional significance of caveolin, NO, iNOS, and their relationship in the inner ear, and we expect that this research will give us greater insight into the mechanisms of and possible treatment for gentamicin ototoxicity. Although aminoglycoside toxicity is known to be concentration-dependent, we only focused on the toxicity itself and not concentration threshold of aminoglycoside toxicity. In the near future, we are planning the investigation of concentration-dependent change of aminoglycoside toxicity in the inner ear.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), penicillin G (Sigma-Aldrich,st. Louis, MD, USA), and fetal bovine serum (FBS) was purchased from Gilbco BRL (Gland Island, NY, USA). Rabbit polyclonal IgG caveolin-1 antibody and horseradish peroxidase-conjugated rabbit-IgG were purchased from Santa Cruz (Santa Cruz, CA, USA). Monoclonal anti-β-actin was obtained from Sigma (St. Louis, MO, USA), H89 from Calbiochem (Darmstadt, Germany), gentamicin from Kook-jae pharm. (Seoul, Korea), PepTag® Assay from Promega (Madison, WI, USA), and tissue culture dishes from TPP (Trasadingen, Germany).

The Expression of Caveolae in Healthy Guinea Pigs

Guinea pigs were fixed by perfusing them with 4% paraformaldehyde for 6 minutes. Temporal bones, including inner ear structures, were obtained by dissecting the skulls using a bone cutter. After dissecting utricles from temporal bones, they were fixed using 4% paraformaldehyde and 1% OSO_4, and were then embedded in Epon 812 (EMS, Washington, USA). After making slides, vestibular hair cells of guinea pigs were observed using a transmission electron microscope (TEM, H-7100, Hitachi, Japan).

Cell Culture and Differentiation of UB/UE-1

The UB/UE-1 cells used in this research are an immortal cell line derived from supporting cells of an immortomouse utricular macula at postnatal day 2, and were given to us by Dr. Matthew. C. Holley[23,24]. UB/UE-1 were plated in 100 mm cell culture dishes with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin G 100 U/mL. They were incubated at 33˚C with 95% CO_2 for 48 hours for proliferation. Following proliferation, the cells were incubated at 39˚C with 95% CO_2 for 24 hours for differentiation, as previously described by Holley *et al*.[24,25]. The differentiation of UB/UE-1 was confirmed using an inverted microscope.

Treatment of Gentamicin and H89

The groups tested consisted of a UB/UE-1 control, a 1 mM gentamicin treatment, a 0.02 mM H89 (PKA inhibitor) treatment, and a 1 mM gentamicin and H89 co-treatment group. The expression of caveolin-1 was monitored by western blot, and PKA activity was as-

sayed using the PepTag® assay in early (8 hours after gentamicin treatment) and late (24 hours after treatment) groups.

Protein Extraction for Western Blot Analysis

UB/UE-1 cells were detached from culture dishes using lysis buffer and then were lysed via ultrasonification. Protein concentration was determined by using the Pierce BCA protein kit (Pierce, Rockford, IL).

Western Blot Analysis

Western blot analysis was used to detect caveolin-1 in UB/UE-1 cells. Proteins samples were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.005% bromophenol blue) and then heated at 100˚C for 10 minutes. The proteins were separated by electrophoresis (73 V) for 2 hours on a 10% SDS polyacrylamide gel and then transfered onto a PVDF membrane (Millipore, Bedford, MA). The membrane was then blocked to suppress non-specific protein interactions using TBS-T buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk at room temperature for 1 hr. The membrane was washed with TBS-T buffer for 5 min five times and then caveolin-1 antibody was applied at room temperature for 1 hr. After washing off the free antibodies with TBS-T buffer, horseradish peroxidase-conjugated rabbit-IgG antibody (1 : 5,000) was bound to the primary antibody at room temperature for 1 hr. After washing the membrane, an enhanced chemiluminescence (ECL) kit (Pierce) was used for detection, and bands were detected using X-ray film.

PKA Activity

The protein samples were mixed with a PKA control enzyme and PKA dilution buffer (PepTag PKA 5× reaction buffer, 0.4 μg/μL Peptide, and PKA 5× activator solution) in the triple distilled water for a total volume of 25 μL. This mixture was then incubated in a 30˚C water bath for 30 minutes. The mixture was heated again at 95˚C for 10 minutes, and then the proteins were separated by electrophosesis on an agarose gel (0.8% agarose in 50 mM Tris-HCl, pH 8.0) for 30 minutes at 80 V. PKA activity was detected using a cAMP-dependent protein kinase assay system (Promega, Madison, WI).

Acknowledgements

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