Research report

Lysosomal augmentation during aminoglycoside uptake in cochlear hair cells

Eri Hashino*, Marlene Shero, Richard J. Salvi

Center for Hearing and Deafness, State University of New York at Buffalo, Buffalo, NY 14214, USA

Accepted 19 September 2000

Abstract

Aminoglycoside antibiotics, such as kanamycin, have ototoxic side effects, which often result in degeneration of cochlear and vestibular hair cells in the inner ear. Cytotoxic effects of aminoglycosides, however, do not appear immediately after cellular uptake of aminoglycosides. In order to understand the mechanisms responsible for the delayed emergence of aminoglycoside ototoxicity, changes in lysosomal activities in cochlear hair cells were evaluated during a repeated administration of kanamycin by two methods. Electron microscopic localization of acid phosphatase (AcPase) revealed that AcPase started to accumulate in vesicles 27 h after the start of kanamycin administration. In addition, the number and size of AcPase-filled vesicles increased with repeated kanamycin doses. Confocal microscopic localization of the LysoTracker probe, a vital lysosomal marker, showed an increase in the size of lysosomes in hair cells that were treated with kanamycin. The temporal changes in the augmentation of lysosomes paralleled those in intracellular kanamycin levels. These results suggest that the intralysosomal compartments can accumulate extensive amounts of aminoglycosides, which might lead to lysosomal swelling and subsequent rupture.

Ó 2000 Elsevier Science B.V. All rights reserved.

Theme: Sensory systems

Topic: Auditory, vestibular, and lateral line: periphery

Keywords: Hair cell; Lysosome; Aminoglycoside; Ototoxicity; Chicken

1. Introduction

Aminoglycoside antibiotics, such as kanamycin (KM) and gentamicin (GM), have long been used in antibacterial therapy of severe gram-negative infection. Despite high antibacterial efficacy, aminoglycosides have prominent ototoxic side effects, causing the destruction of sensory hair cells in the inner ear when administered at high doses. Although evidence clearly indicates that the intracellular accumulation of aminoglycosides is associated with hair cell damage [12,24,28], the cellular and molecular mechanisms responsible for the aminoglycoside-induced hair cell degeneration remain poorly elucidated. One intriguing aspect of aminoglycoside ototoxicity is that the drugs do not exert cytotoxic effects upon cellular uptake. A considerable delay of several days to weeks has been reported between the first sign of drug uptake and that of cell death [13,15]. The delayed cytotoxic effects suggest that hair cells can live for a substantial period of time containing high levels of intracellular aminoglycosides.

Recent studies have uncovered several important aspects of aminoglycoside cellular uptake mechanisms. Our previous studies, using immunogold electron microscopy, demonstrated that aminoglycosides are internalized into hair cells by receptor-mediated endocytosis, primarily through the apical cell surface [11,24]. Mutant hair cells with defects in apical endocytosis were resistant to aminoglycoside toxicity, strongly supporting our assumption of endocytotic drug uptake [25]. The drugs are subsequently transported into lysosomes via a vesicle-mediated process [7,13]. These observations are consistent with evidence from kidney epithelial cells, another preferential target of aminoglycoside toxicity. It has been shown that gentamicin is taken up into cultured kidney epithelial cell lines or renal proximal tubular cells by an energy-dependent endocytotic process and that the drug subsequently ac-
cumulates in lysosomes [1,2,8,16]. In addition, the entry of aminoglycosides into renal proximal tubular cells was shown to be mediated by a cell surface receptor [22]. Collectively, the currently available data suggest that aminoglycosides are transported into lysosomes of target cells after their endocytotic uptake. Since little is known about the subsequent processes that lead to the slow progressive degeneration of hair cells, the present study investigated the temporal changes in lysosomal activities in hair cells and related them to the temporal changes in intracellular KM levels [13]. Parts of this study have been reported in an abstract form [14].

2. Materials and methods

2.1. Kanamycin administration

A total of 45 White Leghorn chicks (7–15 day old) were used as subjects. One group of animals was given a single intramuscular injection of kanamycin monosulfate (Sigma, 400 mg/kg) and sacrificed either 12 h (n=10) or 27 h (n=10) post-injection. A second group of animals (n=15) received daily injections of KM (400 mg/kg/day) for 5 consecutive days and was sacrificed 24 h after the last injection. A third group (n=10) was not given KM and served as controls.

2.2. Acid phosphatase cytochemistry

Acid phosphatase cytochemistry was based on modification of published protocols [3,20]. Briefly, the basilar papilla was dissected out of surrounding bone and immersed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. The tissue was washed and placed in 0.1 M cacodylate buffer overnight. Thereafter, the specimen was placed in the incubation medium consisting of 8 mM sodium glycerophosphate, 2.4 mM lead nitrate and 7% sucrose in 40 mM Tris-maleate buffer (pH 5.2) for 1 h at 37°C. Cytochemical controls were carried out in the same media either devoid of the substrate or the same media supplemented with NaF (10 mM) to inhibit AcPase activity. After being washed in tris-maleate buffer containing 7% sucrose, the tissue was postfixed with 1% osmium tetroxide for 1 h, dehydrated in ethanol and embedded in Epon/Araldite. Ultrathin sections were cut at a position approximately 10% of the distance from the basal end of the cochlea and stained with 2% uranyl acetate. The specimen was examined on a transmission electron microscope at an accelerating voltage of 80 kV. Transmission electron micrographs that contained the entire hair cell region with a nucleus were scanned and digitized with a Hewlett-Packard ScanJet II.

The total area with positive AcPase reactivity in each hair cell was estimated using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MA). The area of the largest vesicle showing AcPase reactivity in a hair cell was measured from photomicrographs. A Student’s t-test or, in cases where a normality test or an equal variance test failed, a Mann–Whitney Rank Sum test was used to evaluate any statistically significant difference in the AcPase-positive area or the total area of a hair cell between groups.

2.3. Immunogold electron microscopy

Some of the inner ear tissues treated with KM for 5 days were processed for immunogold electron microscopy according to previously described procedures [13]. Briefly, the inner ear tissues were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C overnight. After being dissected out of the surrounding bone, specimens were embedded in LR White resin (Ted Pella, Redding, CA). Ultrathin sections were cut at a position 10% of the distance from the basal end of the cochlea and collected on thin bar nickel grids. Post-embedding immunostaining was carried out first by treating the sections with protein A (Sigma, 100 μg/ml), 5% bovine serum albumin (BSA) and 1% Tween 20 in tris buffered saline (TBS) for 30 min at 37°C. Then, the sections were incubated with anti-KM serum (1:40, Western Chemical Research Co., Fort Collins, CO) for 5 h, followed with protein A-gold (10 nm, 1:40, Ted Pella) for 30 min. Finally, the sections were stained with uranyl acetate and examined on a transmission electron microscope.

2.4. Vital staining of lysosomes

Some of the animals treated with KM for 5 days and untreated control animals were used for in vitro labeling with the LysoTracker Probe. The animals were killed by CO₂ asphyxiation and their cochleas were removed and placed in Medium-199 containing Hank’s salt and 25 mM Hepes (Life Technologies, Grand Island, NY). Microscopic dissection was carried out to remove the tectum vasculosum and tectorial membrane. The dissected basilar papilla was incubated in Medium-199 supplemented with 25 mM Hepes, 26 mM sodium bicarbonate, 0.69 mM L-glutamine (Life Technologies) and 10% normal calf serum (Life Technologies) for 4 h at 37°C. Thereafter, the LysoTracker Red (0.1 μM, Molecular Probes, Eugene, OR) was added to the culture medium and incubation continued for another 2 h. The specimens were washed with Medium-199 to remove surface-bound LysoTracker Red and then fixed with 4% paraformaldehyde for 4 h. After being counterstained with BODIPY-phalloidin (1:50 in PBS, Molecular Probes, Eugene, OR) and mounted in the SlowFade mounting medium (Molecular Probes), the specimens were examined on a Bio-Rad 1024 confocal microscope. A series of images were collected along the Z-axis at 1 μm steps, with two sweeps averaged at each step, over a depth of 15 μm. The Z-series were...
projected as a single image using Bio-Rad software. X–Z sections were also obtained from the frame to visualize the localization of LysoTracker staining and Phalloidin staining along the vertical axis of the hair cell. Quantitative analysis of the LysoTracker staining was performed with the Image-Pro Plus software. LysoTracker-positive puncta were detected and their areas were automatically calculated with the software.

3. Results

3.1. AcPase histochemistry

AcPase histochemistry has long been used as a convenient and reliable marker for lysosomal structures in many cell types [3,20]. Localization of acid hydrolases, the major hydrolytic enzymes in lysosomes, can be visualized by precipitation with their specific substrate. In control animals that were not treated with KM, the AcPase activity was observed in a dispersed pattern over the cytoplasmic region of a hair cell (Fig. 1). Small puncta of AcPase activity were more often observed in the apical half rather than the basal half of a hair cell, because a large portion of the basal region is occupied by the nucleus. There was no sign of accumulation of AcPase activity in any discrete region of a hair cell. The average AcPase positive area per hair cell was 0.06±0.01 μm².

A similar distribution of AcPase-positive areas was observed in hair cells 12 h after a single KM injection. The total area showing AcPase reactivity per hair cell (0.05±0.008 μm²) was not significantly different from that in control hair cells (t(22)=0.799, P=0.433, Fig. 2A). Notable changes in the area and distribution of AcPase activity were first observed in hair cells 27 h after a single KM injection. An intense AcPase staining was observed in one or two smooth vesicles in each hair cell (Fig. 3). These vesicles were located either beneath the apical plasma membrane or in the subcuticular region of a hair cell. The total area with positive AcPase reactivity per hair cell was 0.24±0.05 μm², which was significantly greater than that in control hair cells (t(13,14)=253.5, P<0.001, Fig. 2A).

In hair cells obtained from animals that were given KM for 5 consecutive days, a notable increase in the AcPase-positive area was observed. The hair cells showed a 5-fold increase in the total AcPase-positive area per hair cell (1.34±0.33 μm², Fig. 2A) compared to hair cells 27 h post-injection. This increase in the AcPase-positive area can be attributable to an increase in the number of large vesicles filled with AcPase. The majority of hair cells contained more than two vesicles that showed AcPase reactivity (Fig. 4A, B). The average number of AcPase-positive vesicles per hair cell was 4.1 compared to 1.9 at 27 h post-injection. In addition, the size of the largest vesicle in a hair cell was larger after 5 days of KM than after 1 day of KM (27 h) (t(28)=−4.43, P<0.001, Fig. 2B).

Immunogold electron microscopy was carried out to
3.2. LysoTracker staining

To verify the observations with AcPase histochemistry and to obtain data from the entire hair cell region, lysosomal structures in hair cells were labeled in vitro with LysoTracker probe and examined on a confocal microscope. LysoTracker, a newly developed acidotropic probe, selectively accumulates in cellular compartments with low pH and can be used to label lysosomes in living cells [6,11]. In addition, acidic compartments of cells stained with the LysoTracker probe retain their staining pattern following fixation, which enables us to double-label the cells with another marker. LysoTracker staining was dispersed in small puncta throughout the cytoplasm of control hair cells (Fig. 5A), whereas significantly larger vesicles accumulating the LysoTracker probe were observed in hair cells that were treated with KM for 5 days (Fig. 5B).

The number of LysoTracker-positive puncta (>0.005 μm²) in a sampled region (30 μm × 30 μm) was 608 and 226 in a control and KM-treated basilar papilla, respectively. Of all the stained puncta, vesicles equal to or larger than 0.05 μm² were further analyzed. The number of these vesicles (>0.05 μm²) in the control and KM-treated basilar papilla was 68 and 78, respectively. A histogram showing the size distribution of the vesicles for control and KM-treated hair cells is shown in Fig. 6. The vesicle size of most control hair cells was less than 0.2 μm², whereas the number of larger vesicles (>0.2 μm²) increased dramatically after KM treatment (Fig. 6). The average size of the vesicles in KM-treated hair cells (0.56 μm²) was significantly larger than that in control hair cells (0.14 μm², t(68, 76)=2950.5; P<0.01). The largest vesicle in KM-treated hair cells was 5.2 μm², compared to 0.5 μm² in control hair cells.

4. Discussion

Lysosomes in untreated control hair cells were seen as small puncta scattered throughout the cytoplasmic region (Fig. 1), as was previously shown at the light microscopic level [18]. Similar dot patterns were observed for the LysoTracker in control hair cells in vitro (Fig. 5), verifying that the scattered distribution of AcPase is not an artifact during tissue fixation or embedding procedures. An increase in the size of AcPase-positive vesicles was first observed 27 h post-injection, at which time the cellular accumulation of KM was first detected by immunogold electron microscopy [13]. AcPase was localized in vesicles, the size, shape and location of which coincide with the vesicles containing KM (Figs. 3, 4). This supports our assumption that aminoglycosides are transported into lysosomes after their cellular uptake [13]. After repeated KM doses, the total area with AcPase-positive staining
increased due to an increase in the size of large lysosomes (Fig. 2). These observations agree with our previous results with immunogold electronmicroscopy, where the number and diameter of vesicles containing KM increased for several days with repeated KM doses [13]. In vitro labeling of hair cells with the LysoTracker probe is consistent with our observations with the AcPase histochemistry. Intense labeling with LysoTracker Red was seen in large vesicles in hair cells that were treated with KM, but not in untreated control hair cells (Fig. 5). Collectively, our data from both in vivo and in vitro experiments clearly demonstrate that intralysosomal compartments become larger in size while accumulating KM. It should be noted, however, the vesicle size estimated by AcPase histochemistry is not comparable to that estimated by the LysoTracker probe due to shrinkage of tissues during embedding for EM.

One possible mechanism that may give rise to large lysosomes is to assume that individual lysosomes aggregate or fuse together to produce larger lysosomes. In support for this possibility, several aminoglycosides including KM have been shown to precipitate coat protein, a soluble precursor of coat proteins [17]. Coatmer plays a critical role in membrane trafficking by forming coat protein-coated vesicles at sites where membrane fusion takes place. Aminoglycosides are most likely taken up into hair cells by an energy-dependent endocytotic process that involves membrane fusion and formation of ligand-containing vesicles [11,24,27]. The existence of multiple binding sites on coatmer to aminoglycosides [17] suggests that aminoglycosides cross-link coat, thereby producing large vesicle aggregates. An alternative hypothesis is that individual lysosomes expand by an increase in intralysosomal contents. It was shown that intralysosomal compartments (low pH) can accumulate an extensive amount of polycationic molecules, which results in lysosomal swelling and eventual rupture [5,10]. Likewise, polycationic aminoglycosides could conceivably accumulate in lysosomes over a long period of time. The average size of lysosomes increased in hair cells over time during repeated KM injections (Figs. 2, 5, 6). Overloading of the lysosomal compartments, however, could lead to lysosomal membrane rupture and release of KM along with acidic content of lysosomes into the cytosol. Although we do not
Fig. 4. AcPase histochemistry (A, B) and immunogold labeling of KM (C) in hair cells at 120 h after the first injection of 5 day doses of KM. in KM-treated hair cells. Intense AcPase activity staining is filled in multiple vesicles (A, B). The shape, size and location of the vesicles coincide with vesicles accumulating gold particles (C). Bars: 1 μm (A, B); 0.5 μm (C).

have any direct evidence to show that lysosomes can be ruptured by overaccumulation of aminoglycosides, several reports suggest that the lysosomal membrane can be ruptured by overloading of intralysosomal contents [4,23].

Whether lysosomal rupture triggers the cell death process is another important question that remains to be determined. Several lines of evidence indirectly support this possibility. First, lysosomal membrane rupture was shown to initiate vacuolation that is associated with the initiation of cell lysis [19,21]. Second, prevention of lysosomal rupture in vitro spares the cell from lysis, suggesting lysosomal rupture as a cause of cell death [9]. Third, diffuse staining for aminoglycosides was found in cochlear hair cells that showed necrotic figures [13], suggesting that lysosomal rupture occurs prior to cell death. An alternative possibility suggests that lysosomal rupture could be a consequence, not a cause, of cell death. Expansion of lysosomal structures by accumulating KM, which occupies 25% of the total cytosolic region (except the cuticular plate and nucleus) of the hair cell in an extreme case, could conceivably load other cellular organelles with stress and tension. This could directly trigger
the cell death process, which may accompany lysosomal disintegration.

In summary, this study presents evidence that lysosomes become larger in size in synchrony with the intracellular accumulation of KM in hair cells. These findings support the hypothesis that lysosomal targeting and accumulation of aminoglycosides are key cellular events that are involved in the aminoglycoside-induced progressive hair cell degeneration [26]. The capacity of lysosomes to hold an extensive amount of aminoglycosides can explain, at least partially, why aminoglycoside ototoxicity appears several days to weeks after cellular uptake of the drug. In addition, the close correlation between increases in lysosomal sizes and increases in intracellular KM levels suggests that lysosomal markers (e.g. LysoTracker probe) can be used to estimate the amount of internalized aminoglycosides in hair cells. Further investigation is required to determine whether lysosomal rupture occurs as a consequence of overaccumulation of aminoglycosides and whether lysosomal rupture triggers the process of subsequent hair cell degeneration.

Acknowledgements

This study was supported by National Institutes of Health grants R01 DC03900 (E.H.) and R01 DC01685 (R.J.S). The authors would like to thank Drs. Mark Warchol and Jeffrey Corwin for their guidance at an initial stage of in vitro experiments.

References


