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Mechanisms of Rapid Sensory Hair-Cell Death Following Co-administration of Gentamicin and Ethacrynic Acid

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Abstract

Concurrent administration of a high dose of gentamicin (GM; 125 mg/kg IM) and ethacrynic acid (EA; 40 mg/kg IV) results in rapid destruction of virtually all cochlear hair cells; however, the cell death signaling pathways underlying this rapid form of hair-cell degeneration are unclear. To elucidate the mechanisms underlying GM/EA-mediated cell death, several key cell death markers were assessed in the chinchilla cochlea during the early stages of degeneration. In the middle and basal turns of the cochlea, massive hair-cell loss including destruction of the stereocilia and cuticular plate occurred 12 h after GM/EA treatment. Condensation and fragmentation of outer hair-cell nuclei, morphological features of apoptosis, were first observed 5-6 h post-treatment in the basal turn of the cochlea. Metabolic function, reflected by succinate dehydrogenase histochemistry and mitochondrial staining, decreased significantly in the basal turn 4 h following GM/EA treatment; these early changes were accompanied by the release of cytochrome c from the mitochondria into the cytosol and intense expression of initiator caspase-9 and effector caspase-3. GM/EA failed to induce expression of extrinsic initiator caspase-8. These results suggest that the rapid loss of hair cells following GM/EA treatment involves cell death pathways mediated by mitochondrial dysfunction leading to the release of cytochrome c, activation of initiator caspase-9 and effector caspase-3.

Keywords

Hair cells; gentamicin; ethacrynic acid; apoptosis; cytochrome C; caspase-9; caspase-8; caspase-3

INTRODUCTION

Ototoxic drugs, such as the aminoglycoside antibiotics, platinum-based anticancer agents and loop inhibiting diuretics are frequently used to selectively damage specific cell types within the inner ear in order to assess the physiological consequences of a particular type of lesion or to investigate the effects of acute or chronic cochlear pathology. Aminoglycoside antibiotics such as gentamicin (GM) preferentially damage the hair cells. Outer hair cells (OHC) are more vulnerable than inner hair cells (IHC) (Dallos et al., 1978; Schmiedt et al., 1980) and hair-cell damage begins in the base of the cochlea and spreads towards the apex as the dose or duration of treatment increases (Forge et al., 2000). The base-to-apex damage gradient has been linked

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to intrinsic differences in the level of antioxidant enzymes (Sha et al., 2001) and also differences in aminoglycoside uptake (Dai et al., 2008; Dai et al., 2006). The uptake of GM into hair cells begins in the base and spreads to the apex. Uptake is first seen in OHC, initially in the third row, after two days of treatment and then in IHC after 8 day of treatment. Despite the early entry of the drug, functional hearing impairment and hair-cell loss are only observed 10-14 days after the start of daily treatments (Hiel et al., 1993). Aminoglycosides enter hair cell near the apical pole and accumulate in lysosomes and mitochondria until a cytotoxic level is reached (Ding et al., 1995a; Hashino et al., 1997; Hiel et al., 1993). In cases where the dose is nonlethal, clearance of GM from hair cells is extremely slow, extending out to a year.

The antibacterial effects of aminoglycosides result from their binding to the 16s RNA of the 30 S ribosomal subunit and the formation of inert 70 S ribosome; this inhibits protein synthesis in bacteria and also the mitochondria of mammalian cells (Mehta et al., 2002; Michel-Briand, 1978).

The ototoxic effects of aminoglycoside antibiotics can be greatly enhanced by co-administration with loop inhibiting diuretics such as ethacrynic acid (EA) (Brummett et al., 1975; McFadden et al., 2002; Nourski et al., 2004; Prazma et al., 1974; Tran Ba Huy et al., 1983). Combined treatments of aminoglycoside antibiotics and (EA) have been used to create animal models with massive IHC and OHC loss. Such models have been used to study the effects of electrical stimulation on surviving spiral ganglion neurons, the time course of neural degeneration in the absence of trophic support from hair cells or hair cell regeneration induced by viral-mediated gene transfer (Izumikawa et al., 2005; Matsushima et al., 1991; McFadden et al., 2004). The massive hair-cell loss that occurs shortly after GM/EA treatment occurs because EA accelerates the influx of GM from the stria vascularis into endolymph followed by entry through the apical surface of the hair cell into the cytoplasm (Ding et al., 1995a; Ding et al., 2003; Hiel et al., 1992; Tran Ba Huy et al., 1983).

The biological events that contribute to the rapid destruction of hair cells following GM/EA treatment are largely unknown. Some in vitro studies with chronic kanamycin treatment suggest that hair-cell death may occur by necrosis (Jiang et al., 2006). On the other hand, hair-cell death from aminoglycoside antibiotics applied in vivo and in vitro is thought to occur by apoptosis (Nakagawa et al., 1998; Ylikoski et al., 2002). Aminoglycoside induced cell death leads to the loss of mitochondrial membrane potential and activation of caspase-3; activation of caspase-3 was blocked by X-linked inhibitor of apoptosis (XIAP) (Dehne et al., 2002; Tabuchi et al., 2007). Aminoglycosides also upregulate Harakiri, a proapoptotic factor, and the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (MAPK) (Kalinec et al., 2005) EA depletes glutathione S-transferase, a cellular antioxidant, and increases the production of reactive oxygen species (Babson et al., 1994; Khadir et al., 1999; Wang et al., 2007). The biological mechanisms that lead to the rapid and massive loss of IHC and OHC following GM/EA treatment are poorly understood; but previous studies suggest that cell death is likely mediated by caspases, a family of aspartate-specific cysteine proteases (Ding et al., 2007). Caspase-8 mediated cell death is initiated through the extrinsic pathway involving cell death receptors on cell membrane while caspase-9 mediated cell death is triggered by intrinsic cell death pathways involving the mitochondria. We recently reported that rapid hair-cell death resulting from co-administration of EA and cisplatin was initiated by caspase-8 (Ding et al., 2007); however, it is unclear if GM/EA induced cell death is mediated in the same manner. To address this question, we treated chinchillas with a dose of GM/EA that destroys all of the hair cells within 24 h and then used several anatomical and biological markers to determine if cell loss was initiated by caspase-8 and/or caspase-9. In contrast to hair-cell death mediated by EA and cisplatin, our results show for the first time that GM/EA-mediated cell death is initiated through the caspase-9 pathway.

METHODS

Subjects

Thirty normal chinchillas, 1-3 years of age and weighing between 560 and 670 g were used in this study. Twenty-four animals were treated with concurrent administration of GM (gentamicin sulfate, Sigma, 125 mg/kg, i.m.) and EA (Sodium Edocrin®, Merck, 40 mg/kg, i.v., right jugular vein) as described previously (Ding et al., 2003; McFadden et al., 2004). As noted in our previous studies, co-administration of this dose of GM and EA abolishes cochlear function 15-30 minutes post-treatment and 100% of the hair cells are missing 24 h post-treatment (Ding et al., 2003; McFadden et al., 2004). The 48 cochleas from the GM/EA treated animals were evaluated with various staining techniques as indicated in Table 1. After the GM/EA injections, animals were sacrificed at 4, 5, 6, 12, or 24 h. The remaining six chinchillas (12 cochleas) were used as controls for various staining techniques. The numbers of ears evaluated by each labeling technique before and at various times following GM-EA treatment are shown in Table 1.

Histology

Chinchillas were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.), decapitated and their cochleas were quickly removed and prepared for histological analysis using several different staining techniques described below. Silver nitrate was used to label the stereocilia on the hair cells as previously described (McFadden et al., 2004). After the cochlea was removed, the oval window was opened, a hole was made at the apex and a 0.5% solution of silver nitrate in distilled water was perfused through the round window three times to exchange the fluid in scala tympani. Afterwards, the cochlea was perfused with distilled water followed by 10% formalin (pH 7.2) and then immersed in fixative for 24 h. The organ of Corti was dissected out, trimmed and mounted in glycerin on glass slides as a flat surface preparation. Slides were exposed to sunlight for approximately 1 h to enhance the brownish-black staining of the stereocilia. Specimens were examined under a light microscope at 400X (Zeiss Axioskop) and photographed (Olympus DP10 or Nikon Coolpix). Images were transferred to a PC computer, processed with Adobe Photoshop (version 5.5).

Some specimens were labeled with TRITC-conjugated phalloidin or FITC-conjugated phalloidin, which preferentially labels filamentous actin in hair-cell stereocilia, as previously described (Ding et al., 2002a; Zhang et al., 2003). Cochleas were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Afterwards the organ of Corti was microdissected out, rinsed in PBS and immersed into 0.25% Triton-X 100 for 5 min and then placed in TRITC-conjugated (Sigma P1951, 1:200) or FITC-conjugated phalloidin (Sigma 77415, 1:200) in PBS for 30 minutes. After rinsing with PBS, the organ of Corti was mounted on slides in 50% glycerol with 100 mg/ml of 1, 4-diazabicyclo [2.2.2] octane anti-fade compound (Sigma, D2522, DABCO) and coverslipped. Samples were examined under a confocal microscope (BioRad MRC1024) using appropriate filters for FITC (excitation: 494 nm, emission: 518 nm) and TRITC (excitation: 543 nm, emission: 571 nm) fluorescence.

Propidium iodide (PI) (excitation 530 nm, emission 615 nm), which binds to DNA and RNA, was used to label the nuclei in some cochleas. Specimens were fixed, dissected, permeabilized as described above and then immersed in 10 U/ml of RNase for 30 minutes to eliminate background labeling of cytoplasmic RNA (Benbow et al., 2000). Specimens were rinsed in PBS and stained with PI (10 µg/ml for 10 min) to selectively label DNA. Samples were subsequently stained with FITC-labeled phalloidin (Sigma, P5282) for 30 minutes to label the stereocilia bundles and hair-cell cuticular plates, mounted on glass slides and examined under a confocal microscope (BioRad MRC1024) as described above.

In some cases, succinate dehydrogenase (SDH) histochemistry was used to stain mitochondrial SDH that is heavily expressed in hair cells as previously described (Ding et al., 1999; McFadden et al., 2004). The cochlea was rapidly removed, the round window and oval window were opened, and a hole was made at the apex. SDH staining solution (1:1:2 parts 0.2 M Sodium succinate, 0.2 M phosphate buffered saline (pH 7.6), 0.1% tetranitro-blue tetrazolium) was gently perfused through the cochlear openings and then the cochlea was immersed in SDH solution for 45 minutes at 37 °C. Specimens were subsequently fixed in 10% formalin for 24 h and then the cochlear sensory epithelium was microdissected out and mounted in glycerin on glass slides as a surface preparation. Specimens were examined under a light microscope at 400X (Zeiss Axioskop) and photographed (Olympus DP10 or Nikon Coolpix). Images were transferred to a PC computer and processed with Adobe Photoshop (version 5.5).

MitoTracker Green (Molecular Probes M-7514), a cationic fluorescent probe that accumulates in mitochondria was used to stain some specimens. Cochleas were fixed for 15 minutes at 37 °C using freshly made 3.7% paraformaldehyde in Hanks' Balanced Salt Solution (HBSS), rinsed in PBS and then immersed in ice-cold acetone for 10 min. Afterwards, the organ of Corti was dissected out in HBSS, and incubated in 0.5 µM of MitoTracker Green FM for 30-45 minutes. After rinsing in PBS, the tissue was stained with TRITC-labeled phalloidin (Sigma, P1951) for 30 min as described above and examined with a confocal microscope as described above (MitoTracker Green: excitation 490 nm, emission: 516 nm).

An antibody against cytochrome-c, a protein that translocates from the mitochondria to the cytosol during the early stages of apoptosis, was used to localize cytochrome-c in some specimens. Cochleas were quickly removed, opened and fixed with 10 % formalin in PBS for 4 h. Afterwards, the organ of Corti was carefully dissected out in 0.1 M PBS, transferred into blocking solution (Blocker™ Casein in PBS, Pierce Chemical Co.) for 60 min (22 °C), rinsed in 0.1 M PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min. Samples were rinsed in PBS, incubated 1 h (22 °C) in mouse primary antibody (anti-cytochrome c, 6H2.B4; BD, Pharmingen, 2.5 µg/ml) in the blocking solution. After rinsing in PBS, specimens were incubated with secondary antibody (Cy2-conjugated goat anti-mouse IgG, 1: 200, Jackson ImmunoResearch Laboratories) for 1 h. Afterwards, the specimens were labeled with TRITC-conjugated phalloidin and evaluated under a confocal microscope as described above.

Some cochleas were labeled in vivo with fluorescently-labeled caspase-3 inhibitor (CaspaTag 3, 1:30 in solvent, Intergen), caspase-8 inhibitor (CaspaTag 8, 1:30 in solvent, Intergen) and caspase-9 inhibitor (CaspaTag 9, 1:30 in solvent, Intergen) (Zhang et al., 2003). Chinchillas were anesthetized (ketamine, 60 mg/kg, i.m.; acepromazine, 0.5 mg/kg, i.m.) and the middle ear space surgically opened through the posterior bulla. The stapes was removed and 10 µl of CaspaTag 3, or 8, or 9 was carefully perfused through a small opening made in the round window membrane. Caspase staining continued for 1 h after which the cochlea was perfused with 10% formalin, then carefully removed and placed in fixative for 3 h. The organ of Corti was carefully dissected out as a flat surface preparation, stained with TRITC-labeled phalloidin and mounted in anti-fade solution on glass slides as described above and examined under a confocal microscope as described above (Green carboxyfluorescein of activated caspases: excitation 488 nm, emission 529 nm).

All procedures regarding the use and care of animals in this study were approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

RESULTS

Stereocilia and Cuticular Plate

Silver nitrate was used to study the degeneration of the stereocilia and cuticular plate following GM/EA treatment. Figure 1A shows the condition of the hair cells in the lower-middle turn of the cochlea 6 h after GM/EA treatment. The surface of the organ of Corti appeared normal at this time. The stereocilia on the three rows of OHC were arranged in a W-shape while those on IHC formed a gently curving arc similar to those seen in normal ears. However, at 12 h (Figure 1B) there was massive damage to the stereocilia and cuticular plate of OHC and IHC in the lower-middle turn of the cochlea. The stereocilia and cuticular plate were absent or severely damaged on both OHC and IHC and cellular debris was evident in the IHC region. OHC and IHC damage decreased from base to apex at this time (data not shown) consistent with our earlier results (McFadden et al., 2004). These results indicate that the IHC and OHC in the lower-middle turn of the cochlea are morphologically intact 6 h post-GM/EA, but at 12 h post-treatment there is massive damage to the stereocilia and cuticular plate of the hair cells in the lower-middle turn of the cochlea. As reported previously, 100% of the hair cells are missing 24 h following this dose of GM/EA treatment (Ding et al., 2003; McFadden et al., 2004).

Hair-cell Nuclei

Nuclear condensation and fragmentation are anatomical hallmarks of apoptosis. To determine if the rapid destruction of hair cells following GM/EA was occurring through necrosis or apoptosis, specimens were stained with PI to label nuclei, counterstained with FITC-conjugated phalloidin and evaluated by confocal microscopy to visualize the hair-cell nuclei and cuticular plates. Figure 2A shows surface preparation of the organ of Corti from the lower-middle turn of the cochlea of a normal animal. The PI-labeled OHC nuclei were large, round, evenly stained and arranged in three orderly rows consistent with previous results (Hu et al., 2002). The green phalloidin staining shows the intense, thin green ring at the boundary between the OHC and adjacent supporting cells and the pale green cuticular plate. In contrast, OHC nuclei in the lower-middle turn of the cochlea 12 h after GM/EA treatment were shrunken, condensed, fragmented (arrows) and in some cases completely missing (arrowheads) (Figure 2B). The thin, green band of actin surrounds the OHC, but there is little or no phalloidin labeling of the apical surface of the OHC due to the damage of the cuticular plate as noted in Figure 1B. These morphological changes in the nuclei clearly indicate that hair cells were undergoing apoptosis following GM/EA treatment (Figure 1B).

SDH

SDH, a mitochondrial enzyme involved in aerobic metabolism, is heavily expressed in OHC and IHC, but is largely absent from the surrounding supporting cells in the organ of Corti (Ding et al., 2001). Consistent with previous reports, intense, dark blue SDH labeling was observed in IHC and OHC of normal animals (Figure 3A). SDH staining was fairly uniform throughout the cytoplasm. To determine if GM/EA treatment would alter aerobic metabolism, SDH staining was monitored at several times following GM/EA treatment. At 4 h post GM/EA, SDH labeling was completely absent or greatly reduced in many OHC (Figure 3B), but nearly normal in some OHC. In generally, SDH staining tended to be less intense than normal in IHC 4 h after GM/EA treatment. The decrease in SDH staining suggests that OHC metabolism is greatly reduced 4 h post-treatment.

Mitochondrial Membrane Potential

The rapid decline in the SDH staining suggested that mitochondrial function was deteriorating during the early stages of GM/EA treatment. To evaluate this possibility, specimens were

stained with Mito-Tracker M-7514, a cell permeant, fluorescent probe that concentrates in functionally active mitochondria and is retained in the mitochondria after fixation plus TRITC-conjugated phalloidin. In the normal cochlea, bright Mito-Tracker labeling (green; yellow overlap of TRITC and MitoTracker) was observed throughout the cytoplasm of OHC and IHC (Figure 4A). However, 4 h after GM/EA treatment, the green/yellow fluorescence (overlap of Mito Tracker plus TRITC) was greatly reduced in the OHC. Green fluorescent Mito-Tracker labeling was still present in the IHC at 4 h post-treatment, but labeling tended to be less intense or absent in some IHC (Figure 4B).

Cytochrome c

The rapid decline in mitochondrial membrane potential suggested by Mito-Tracker staining (Figure 4B) could trigger the release of cytochrome c into the cytoplasm of the hair cells. To evaluate this hypothesis, cochleas from normal and GM/EA treated chinchillas were immunolabeled with an antibody against cytochrome c. In normal ears, cytochrome c immunolabeling was heaviest along the lateral wall of the OHC where many mitochondria are located (Ikeda et al., 1993; Lim, 1986) while in IHC, labeling was diffuse and dispersed within the cytoplasm (Figure 5A). Cytochrome c labeling was relatively weak in pillar cells and supporting cells lateral to the OHC. Four h after GM/EA treatment, there was a notable increase in cytochrome c labeling in the OHC; labeling tended to be more granular in appearance at this time and was dispersed throughout the cytoplasm (Figure 5B). Cytochrome c labeling in the IHC region tended to be more dispersed and granular 4 h after GM/EA treatment. Occasionally a few OHC were seen with necrotic-like features characterized by swollen cytoplasm filled with cytochrome c; however the nuclei in some of these cells were shrunken, indicative of apoptosis (Figure 5B, center).

Caspase-3, 8 and 9

To determine which caspase-mediated cell death pathways were involved in GM/EA induced hair-cell death, we evaluated the time course of expression of initiator caspase-8 and caspase-9 and executioner caspase-3 using fluorogenic caspase inhibitors specific to each protease and also stained the organ of Corti with PI to evaluate the status of the nuclei. Normal cochleas were devoid of caspase-8, caspase-9 and caspase-3 fluorescence (data not shown). However, at 5 h post-GM/EA, intense, caspase-9 fluorescence was evident in nearly all OHC (Figure 6A) in the basal turn of the cochlea. The nuclei of the caspase-9 positive OHC were condensed and considerably smaller than those in normal ears. In contrast, caspase-8 staining was completely absent from both OHC and IHC (Figure 6B) despite the fact that the nuclei of the OHC were condensed and in some cases fragmented. Caspase-3 fluorescence was also present in most OHC (Figure 6C). The PI labeled nuclei of these caspase-3 positive OHC were condensed or fragmented. In contrast, the IHC nuclei were large and round and the cytoplasm was devoid of caspase-3 labeling at this time.

DISCUSSION

Our light microscopic observations show that there was massive OHC and IHC loss and/or damage in the lower-middle and upper-basal turn of the cochlea 12 h after GM/EA treatment (Figure 1) consistent with earlier observations (Brummett et al., 1975;McFadden et al., 2002;Nourski et al., 2004;Prazma et al., 1974;Tran Ba Huy et al., 1983). During the early phase of damage, OHC loss preceded IHC loss, consistent with previous reports. Hair-cell loss induced by GM/EA treatment begins near the base of the cochlea and spreads rapidly toward the apex so that by 24 h all of the hair cells are missing as noted in our earlier report (Ding et al., 2007).

Caspase-9 Mediated Apoptosis

The results presented here extend earlier observations by providing new insights on the biological events that give rise to the rapid and widespread hair-cell death in the chinchilla cochlea. While most of our results were from the lower-middle turn of the cochlea, the changes seen in this region were fundamentally the same as those seen more basally or apically albeit at slightly different times. The results presented here clearly indicate that GM/EA-mediated hair-cell death in the chinchilla occurs primarily by apoptosis, similar to what occurs with aminoglycosides alone (Dehne et al., 2002; Tabuchi et al., 2007). Whether these same mechanisms apply to other species in which GM/EA is used to induce rapid and widespread hair-cell loss remains to be determined (Russell et al., 1979; Shepherd et al., 1994; Xu et al., 1993). The initial stage of programmed hair-cell death is associated with reduced expression of SDH, a metabolic enzyme associated with mitochondria. This is accompanied by a decline in the mitochondrial membrane potential, increased release of cytochrome c from mitochondria into the cytoplasm; these effects are similar to those seen with aminoglycosides alone (Dehne et al., 2002). Cytochrome c binds to the cytosolic protein Apaf-1 along with dATP (Acehan et al., 2002). This leads to the activation of intrinsic, initiator caspase-9, which subsequently cleaves and activates caspase-3 (Lenaz et al., 1999; Samali et al., 1999). The activation of caspase-3 results in the proteolytic cleavage of a range of cellular targets that ultimately leads to cell death. These biochemical changes, which begins in the OHC in the base of the cochlea 4-6 h following GM/EA treatment, occur several hours prior to the destruction of the stereocilia and cuticular plate on OHC (Figure 1).

The pattern of hair-cell loss and the cell death program induced by GM/EA treatment differ from those seen with EA plus cisplatin (Cis) in a number of important respects (Ding et al., 2007). OHC loss induced by Cis/EA treatment followed a base-to-apex gradient similar to that seen with GM/EA; however, IHC lesions by contrast were relatively uniform along the length of the cochlea. Hair-cell death following Cis/EA treatment also occurred by apoptosis, but cell death was initiated by membrane death receptors and initiator caspase-8 in contrast to GM/EA which begins with caspase-9. The mechanisms responsible for these differences are poorly understood, but most likely are related the main target of GM versus Cis damage. High levels of tritium labeled aminoglycosides have been observed in hair-cell mitochondria following treatment suggesting that mitochondria are a target of EA/GM (Ding et al., 1995a; Ding et al., 1997). Cis, on the other hand has been shown to trigger apoptosis through membrane death receptors (Nagane et al., 2000; Tsuruya et al., 2003).

Aminoglycosides and Cell Death

The rapid hair-cell destruction seen after GM/EA treatment stands in marked contrast to the delayed and protracted hair-cell loss that occurs when aminoglycosides are administered systemically for several weeks. After the second day of daily treatments, aminoglycosides first appear in OHC in the base of the cochlea. Labeling was localized mainly below the cuticular plate and on stereocilia, the basal body and nuclei (Ding et al., 1995b; Hiel et al., 1992). Aminoglycosides continue to accumulate in hair cells, particularly in lysosomes and mitochondria, but it is not until 10 days later that significant hearing loss and OHC loss are observed (de Groot et al., 1990; Ding et al., 1997; Ding et al., 1991; Hashino et al., 2000; Hiel et al., 1993; Tran Ba Huy et al., 1986; Trautwein et al., 1998; Voldrich, 1965; Zhu et al., 1993). The biological events that give rise to the delayed hair-cell death resulting from chronic systemic administration of aminoglycosides remain controversial. Some results suggest that hair-cell loss from systemic administration of aminoglycosides occurs by apoptosis, while others suggest a non-apoptotic form of cell death mediated by calpains (Jiang et al., 2006; Nakagawa et al., 1998).

EA, Strial Damage and Apoptosis

Hearing loss from intravenous administration of EA and other loop inhibiting diuretics, unlike aminoglycosides, begins shortly after drug treatment and the primary target is the stria vascularis (Bosher, 1980; Conlon et al., 1998; Ding et al., 1996; Ding et al., 1988; Ding et al., 2002b; Rybak, 1985; Syka et al., 1981). EA and furosemide cause severe stria edema, damage the marginal and intermediate cells of the stria vascularis, diminishes blood flow through the capillary beds in the stria (Akiyoshi, 1981; Ding et al., 2002b; Forge, 1981; Kasajima et al., 1978; Naito et al., 1997), and alter the ionic composition of the endolymph. The structural changes in the stria are associated with a rapid decline in the endolymphatic potential (EP) as well as the cochlear microphonic and compound action potential which depend on the EP for their generation. The cochlear potentials and stria pathology begin to recover after 5-6 h and full recovery of function may require several days (Ding et al., 2002b; Kuzel et al., 1990).

The rapid induction of hair-cell loss from GM/EA treatment is believed to be due to the ability of EA to disrupt the blood-inner ear barrier thereby facilitating the entry of gentamicin into the cochlear fluids during periods of reduced blood flow following EA treatment (Conlon et al., 1998; Ding et al., 2003; Tran Ba Huy et al., 1983). Although EA facilitates the entry of GM into the cochlear fluid, GM must eventually be taken up into hair cells for cell death to occur (Hashino et al., 1997; Hiel et al., 1993). When GM was applied to isolated OHC at concentrations as high as 5 mM, fluorescently tagged GM was excluded from the cytoplasm and hair cells remained viable for at least 6 h (Dulon et al., 1989). However, when GM and EA were administered together in vivo, GM uptake into hair cells was greatly accelerated (Hayashida et al., 1989). This suggests that EA also enhances GM entry into hair cells thereby accelerating cell killing. EA is also known to deplete glutathione antioxidants, increase reactive oxygen species, decrease the mitochondrial transmembrane potential and ATP levels, increase lipid peroxidation and change membrane permeability (Babson et al., 1994; Khadir et al., 1999; Wang et al., 2007). Collectively, these results suggest that EA may accelerate GM induced hair-cell loss through multiple mechanisms.

Acknowledgments

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List of Abbreviations

GM	Gentamicin
EA	Ethacrynic acid
EP	Endolymphatic potential
Cis	Cisplatin
HC	Hensen cells
HBSS	Hank's balanced salt solution
IHC	Inner hair cell
MAPK	Mitogen activated protein kinase
OHC	Outer hair cell
PC	Pillar cell
PI	Propidium iodide
SDH	Succinate dehydrogenase

XIAP X-linked inhibitor of apoptosis

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Figure 1. Surface preparations from the lower-middle turn of the cochlea stained with silver nitrate. (A) Six h after GM/EA treatment the stereocilia bundles and apical surface of the organ of Corti appear relatively normal. Scale bar = 20 μ M. (B) The stereocilia and cuticular plate of most OHC are missing or severely damaged 12 h after GM/EA treatment.

Figure 2.

Surface preparation from the lower-middle turn of the cochlea labeled with FITC-conjugated phalloidin (green) and PI (red). (A) Normal cochlea showing OHC with large, round PI-labeled nuclei. (B) Twelve h after GM/EA treatment, OHC nuclei in the lower-middle turn of the cochlea were condensed, fragmented (arrows) or completely missing (arrowhead).

Figure 3.

Surface preparations from the upper-basal turn of cochleas stained with SDH. (A) Organ of Corti from normal ear shows intense SDH labeling in IHC and OHC with little or no labeling in surrounding supporting cells. (B) Surface preparation from the upper-basal turn of the cochlea 4 h after GM/EA treatment. SDH staining was absent from many OHC; IHC staining was less intense than in normal ears. Outer hair cells (OHC), pillar cells (PC), and inner hair cells (IHC); scale bar in panel B is 20 μ m.

Mito-Tracker (green) TRITC-phalloidin (red)

Figure 4.

Surface preparations from the middle turn of cochleas stained with Mito-Tracker M-7514 cationic fluorescent probe (green) and TRITC-labeled phalloidin (red). (A) In a control cochlea, green Mito-Tracker labeling was present in the cytoplasm of IHC and OHC (green cytoplasm) and yellow in the cuticular plate region (overlap of Mito-Tracker and TRITC), but not in the Hensen cells (HC). (B) Four h after GM/EA treatment Mito-Tracker labeling intensity was greatly reduced relative to controls in the cytoplasm of OHC (green) and in the cuticular-plate region (yellow, overlap of Mito-Tracker and TRITC); Mito-Tracker labeling in the cytoplasm of IHC (green) and cuticular plate (yellow) was moderately reduced relative to controls. Scale bar: 10 μ m.

Cytochrome-C (green) Propidium Iodide (red)

Figure 5.

Surface preparations from the middle turn of cochleas stained with PI to label the nuclei (red) and an antibody against cytochrome c (green). (A) In control cochlea note distinct band of cytochrome c labeling (green) along the body of the OHC (dotted arrows); cytochrome c shows more diffuse and dispersed labeling in the IHC region. Cytochrome c labeling was largely absent from the PC region. (B) Four h after GM/EA treatment, green cytochrome c labeling was more intense and dispersed within OHC cytoplasm compared to normal controls. OHC nuclei are slightly condensed after GM/EA-treatment relative to normal controls. However, several OHC with necrotic-like features (swollen cytoplasm, displaced soma) were present in row 2 and 3 (arrowheads). Scale bar: 10 μ M.

Figure 6.

Surface preparations from the middle turn at 5 h post GM/EA. Specimens labeled with PI (red) and caspase-9, caspase-8 or caspase-3 (green). Nuclei of most OHC were condensed or fragmented (A-C), a morphological feature of apoptosis. (A) Caspase-9, a member of the intrinsic mitochondrial cell death pathway, was heavily expressed in OHC with condensed and fragmented nuclei. (B) Caspase-8, a member of the extrinsic membrane cell death pathway was absent in OHC with condensed and fragmented nuclei. (C) Caspase-3, a member of the executioner cell death pathway was heavily expressed in OHC with condensed and fragmented nuclei.

Table 1

Number of Samples Used for Each Labeling Technique

Label #1	Silver nitrate	PI	SDH	MitoTracker	Cytochrome c	Caspase 8	Caspase 9	Caspase 3
Label #2		FTTC phalloidin		TRITC phalloidin	TRITC phalloidin	PI	PI	PI
Normal	1	1	1	2	1	2	2	2
4 h post			3	3	3			
5 h post			3			3	3	3
6 h post	2	3	3					
12 h post	2	3	3			3	3	3
24 h post	2							