Chapter 10
Aminoglycoside-Induced Oxidative Stress: Pathways and Protection

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10.1 Introduction

Drug-induced damage to the vestibular and cochlear structures of the inner ear has probably occurred since antiquity, arising from use of herbs and other remedies that harbor potential to damage the auditory system. With introduction of the aminoglycoside class of antibiotics, however, the prevalence and severity of such damage escalated dramatically. The discovery of streptomycin heralded a new era in antimicrobial chemotherapy against Mycobacterium tuberculosis and allowed for life-saving treatment of Gram-negative infections; but, the destructive potential of this drug became evident concomitantly. A growing awareness of potential detrimental effects of aminoglycosides on hearing and balance provided the impetus for research into the root causes of these injuries and their prevention. A critical observation was that antioxidants could significantly attenuate morphological and functional findings of drug-induced ototoxicity in animals, suggesting a pivotal role for oxidative stress in mediating damage to the inner ear.

The past decade has witnessed unprecedented progress in translating scientific advances in the ototoxicity research from bench-to-bedside (Chen et al. 2007; Sha et al. 2006). Much of this work has pertained to improved understanding of the role of reactive oxygen species (ROS). The link between oxidative stress and injury in

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the ear has provided the basis for both mechanistic investigations and development of a wide array of protective approaches. The overproduction of ROS often results in redox imbalance that overwhelms the cellular antioxidant system. This formation of ROS appears to be a common mechanism by which diverse cochlear and vestibular insults converge on apoptotic and inflammatory pathways that culminate in hair cell death. This chapter provides a translational perspective on aminoglycoside ototoxicity, reviewing the clinical perspective, body of experimental work on oxidative stress in the inner ear, and promising clinical strategies for protection.

10.2 Clinical Perspective

The hearing loss seen with aminoglycoside ototoxicity is usually bilateral and irreversible, often continuing to progress long after antibiotic therapy has been discontinued. The latter phenomenon is thought to be due to retention of the drug within the cochlea. Whereas aminoglycoside-induced renal injury is often reversible, most ototoxicity is permanent due to lack of regenerative capacity of the inner ear's hair cells. Despite these drawbacks, aminoglycosides remain among the most commonly used antibiotics in the world and are the therapy of choice in many cases. For example, they are first-line therapy for life-threatening neonatal sepsis (Grohskopf et al. 2005; Pong and Bradley 2005) and are part of the World Health Organization's recommended treatment regimen for multidrug-resistant tuberculosis.

The reported incidence of gentamicin-induced hearing loss ranges from 3 to 33 % (Govaerts et al. 1990; Rybak and Ramkumar 2007). Children are particularly susceptible to ototoxicity (Forge and Schacht 2000; Gatell et al. 1987), and arguably more severely impaired by it. Most children develop much of their speech through interaction with other children or female caregivers whose speech is in the higher frequencies. Even mild loss of hearing at this critical developmental stage hampers speech, cognition, and social development (Davis et al. 1986; Elfenbein et al. 1994). In one study, 31 % of children with 20 dB or greater hearing loss failed at least one grade. The collective costs of therapy, education, and rehabilitation for each child with hearing loss have been estimated at $417,000 (Centers for Disease and Prevention 2004).

A reasonable question then is: why do aminoglycosides continue to be used given the risk of permanent, bilateral hearing loss, balance disturbance, tinnitus, and nephrotoxicity? The answer is that aminoglycosides continue to offer several distinct advantages over other drug candidates, including broad spectrum bactericidal activity, low rates of resistance, and infrequent allergic reactions. Despite potential for toxicity, aminoglycosides remain vital in treatment of resistant Gram-negative infections, such as in patients with cystic fibrosis who suffer intractable pseudomonal infections or for highly resistant enterococcal infections. Aminoglycosides are inexpensive to produce and widely available, making them widely used in third world countries. Furthermore, aminoglycosides now also have an expanding role in premature stop codon diseases, where these drugs can promote readthrough translation of otherwise truncated proteins. Far from being abandoned, it has been suggested that aminoglycosides are entering a "renaissance," with new indications and

a growing role in resistant infections (Houghton et al. 2010; Xie et al. 2011). These observations make the need for effective strategies for protection all the more urgent.

10.2.1 History

Streptomycin, the first member of the aminoglycoside class of drugs to be discovered, was isolated in 1943 by Albert Schatz, a graduate student in the laboratory of Selman Abraham Waksman at Rutgers University (Comroe 1978). The advent of the aminoglycosides was a defining event in medicine; the Nobel Prize in Physiology or Medicine was awarded to Selman A. Waksman in 1952 for the discovery of streptomycin as the first antibiotic effective against tuberculosis. Streptomycin was shown curative in a randomized, double-blind, and placebo-controlled study (Metcalfe 2011). Aminoglycosides also had remarkable efficacy against Gram-negative bacteria (Schatz et al. 1944). Early trials revealed ototoxicity and nephrotoxicity, however, with the initial report describing one case of deafness and three cases of vestibular impairment after large streptomycin doses (Hinshaw and Feldman 1945). Many additional aminoglycosides were developed during the ensuing years, providing much-needed additions to the medical armamentarium but increasing the number of ototoxic drugs in clinical use. Waksman and Lechavalier discovered that neomycin was produced by *Streptomyces fradiae* in 1949 (Waksman and Lechavalier 1949). Kanamycin was developed in Japan in 1957 (Takeuchi et al. 1957). Gentamicin and netilmicin were released for clinical use in the early 1960s (Weinstein et al. 1963; Matz 1993). Tobramycin was discovered in 1967 (Thompson et al. 1967). Amikacin is a semisynthetic aminoglycoside discovered in the early 1970s (Kawaguchi et al. 1972). There seems to be little evidence that inhaled tobramycin as a solution or as a powder have significant ototoxicity (Konstan et al. 2011; Hennig et al. 2014). These drugs showed varying predilections for cochlear or vestibular toxicity, but none were free of potential for injury.

10.2.2 Mechanisms of Therapeutic Action

Aminoglycoside antibiotics exert their antibacterial effects through inhibition of protein synthesis, targeting the A-translational site of the 16S rRNA of the 30S ribosomal subunit of prokaryotic bacterial cells. The binding causes misreading of the mRNA, which results in dysfunctional proteins synthesis, impaired cellular homeostasis, and death (Shulman et al. 2014). The mechanism is thought to involve mismatches of amino acids, altered tRNA interactions, and aberrant protein elongation. Aminoglycosides differ from one another in their interactions with the ribosome, allowing for diversity in therapeutic effect. In contrast to penicillin, which is bacteriostatic, aminoglycosides are bactericidal. Bacterial resistance to aminoglycosides results from acetylation, phosphorylation, or adenylation by bacterial enzymes. Mammalian mitochondria contain subunits similar to prokaryotes, potentially providing a target for host injury by aminoglycosides. In addition, despite the

high selectivity of aminoglycosides for prokaryotic ribosomes, aminoglycosides do interact with mammalian ribosomes to a limited degree (Xie et al. 2011).

Aminoglycoside-induced misreading in mammalian ribosomes is used to advantage in premature stop codon suppression therapy. The feasibility of this strategy is borne out by clinical trials involving patients with Duchenne muscular dystrophy (Malik et al. 2010; Wagner et al. 2001). The underlying concept is that many human genetic disorders involve point mutations that cause premature stop codons. Examples include some forms of cystic fibrosis, Hurler syndrome, and Duchenne muscular dystrophy (Kaufman 1999). Aminoglycosides induce partial misreading of (inappropriate) stop codons, such that some full-length transcripts are synthesized and the disease severity alleviated. As with antimicrobial therapy, the challenge is in titrating therapeutic efficacy against the risk of toxicity. Newer aminoglycosides may maximize stop codon suppression while keeping untoward side effects to a minimum (Nudelman et al. 2009).

10.2.3 Clinical Manifestation of Side Effects

Aminoglycoside-induced hearing loss is usually bilateral, beginning at high frequencies, and then extending to lower frequencies with prolonged treatment. As hearing loss progresses to lower frequencies, corresponding to important speech range, communication skills are increasingly impacted. Symptoms usually manifest days to weeks after initiating therapy and may continue to progress well after treatment is discontinued. In a subset of patients with an underlying genetic predisposition to aminoglycoside ototoxicity, hearing loss can occur from a single dose of drug. These patients usually harbor a mitochondrial mutation (for additional discussion of the A1555G mutation, see Chap. 18 by Green and Raphael).

Vestibulotoxicity follows a variable pattern. The timing of onset of vestibular ototoxicity is variable and does not correlate well with cumulative dose of aminoglycoside (Rybak and Brenner 2014). The toxicity is usually manifested in impaired balance, particularly in the dark where visual cues are lacking. Symptoms range from imbalance and staggering to the inability to walk without assistance. Older patients with limited proprioceptive or other compensatory mechanisms are more vulnerable and experience greater impairment. A characteristic finding is imbalance and unstable/uncoordinated gait, which is dramatically worsened by motion. Patients usually transition from normal to symptomatic over a 24–48 h period.

Aminoglycosides differ not only in the degree of ototoxicity but also in predilection for affecting the cochlea, vestibular system, or both. Neomycin is highly ototoxic; gentamicin, kanamycin, and tobramycin are moderately ototoxic; and amikacin and netilmicin are less ototoxic. Some aminoglycosides are preferentially toxic to vestibular versus cochlear structures (Dulon et al. 1986). Amikacin and neomycin affect the cochlea primarily, whereas gentamicin affects both hearing and balance, but is more vestibulotoxic, providing the rationale for treatment of intractable Menière's disease with intratympanic gentamicin. Interestingly, streptomycin is primarily vestibulotoxic whereas dihydrostreptomycin is primarily cochleotoxic, reflecting the marked influence of minute structural differences in the drug class.

Aminoglycosides also induce nephrotoxicity, which is observed in approximately 20 % of patients. The drug accumulates in the proximal tubules of the kidney, potentially due to the glycoprotein transporter megalin (Kawai et al. 2005; Moestrup et al. 1995). Cell death is evident in the proximal and distal tubules and in the loop of Henle, which is responsible for risk of acute renal failure. Decreased kidney perfusion, which can threaten organ function, arises from tubular obstruction and reduced glomerular filtration (Lopez-Novoa et al. 2011).

10.2.4 Incidence of Ototoxicity

The severity of ototoxic side effects related to aminoglycoside therapy roughly correlates with dose and duration of treatment, although in families or geographic regions where the 1555 mutation is endemic, the risk of aminoglycoside ototoxicity is increased (Estivill et al. 1998, full citation is below). Taken collectively, aminoglycosides have roughly a 15–20 % incidence of auditory side effects and vestibular disturbances after short periods of treatment (Fee 1980; Moore et al. 1984). However, these estimates are heavily influenced by the testing paradigm. For example, testing higher frequencies (>8 kHz) reveals an incidence of injury approaching 50 % (Fausti et al. 1992). A variety of factors contribute to inconsistent reporting of ototoxicity: lack of pre-/post-treatment audiology evaluation, the tendency of high-frequency losses to spare communication (and hence evade detection), and inconsistent criteria for hearing loss or inadequate clinical follow-up to capture late effects. In tuberculosis patients requiring prolonged treatment with aminoglycosides (amikacin or kanamycin), measurable hearing loss occurred. 18.75 % of the patients developed sensorineural hearing loss involving higher frequencies while 6.25 % had involvement of speech frequencies also. All patients were seen again approximately 1 year after aminoglycoside discontinuation and all hearing losses were permanent with no threshold improvement (Duggal and Sarkar 2007).

10.2.5 Risk Factors and Genetic Predisposition

Dietary factors can influence ototoxicity, at least in experimental studies. Guinea pigs received dietary supplementation to their normal diet with β-carotene, vitamins C and E, and magnesium. Significant decreases in gentamicin-induced hearing loss at 12 kHz and below were reported, with protection of both inner and outer hair cells. These findings provide a rationale for future studies in patients (Le Prell et al. 2014).

Experiments in a guinea pig model of gentamicin ototoxicity have demonstrated that low protein intake increases vulnerability to gentamicin insult (Lautermann et al. 1995). Guinea pigs on a regular (18 %) protein diet or deficient (7 %) protein diet received 100 mg/kg/day of gentamicin for 15 days. Animals on the regular protein diet had an average hearing loss of 9–42 dB at 3–18 kHz with gentamicin, whereas guinea pigs on a 7 % protein suffered a more severe average hearing loss of 52–74 dB

across the same 3–18 kHz range with gentamicin. Glutathione levels in the cochlear sensory epithelium were diminished in animals in the low protein diet condition. In contrast, in animals on a regular protein diet, dietary glutathione had no effect on glutathione levels in the cochlear sensory epithelium or on hearing loss. The authors note that nutritional status is often impaired in critically ill patients receiving aminoglycosides and concluded that compounds with potential protection against gentamicin ototoxicity may be more rigorously assessed in animal models with deficient nutritional states, where endogenous detoxifying mechanisms are compromised.

A variety of other factors can influence risk for aminoglycoside ototoxicity (Garetz et al. 1994). The uptake, excretion, metabolism, and detoxification of drug all affect drug efficacy and ototoxicity. Concomitant treatment with ethacrynic acid, furosemide, or other loop diuretics during aminoglycoside therapy can cause a sudden and devastating hearing loss (Mathog and Klein 1969). Intense noise exposure may also exacerbate ototoxicity (Li et al. 2011). It is uncertain whether neonates are more susceptible to aminoglycoside ototoxicity, but animal studies suggest increased sensitivity during cochlear differentiation during gestation (Raphael et al. 1983).

Gentamicin and gentamicin-Texas Red (GTTR) were found to have rapid uptake in a bullfrog saccular explant, particularly in peripheral hair cells and preferentially in hair bundles (Steyger et al. 2003). Subsequent work investigated uptake in guinea pig, mouse, chick, and bullfrog. Vertebrate inner ear cells were found to take up and retain GTTR in the inner ear in vivo following injection, although cellular damage death was not detected in this acute model. The authors observed a baso-apical gradient of intracellular GTTR uptake in guinea pig cochleae at early time points (<3 h). The uptake of GTTR also resembled the pattern of aminoglycoside-induced hair cell death in bullfrogs and chicks (Dai et al. 2006). Clinical aminoglycoside exposure may at an early age occur along with noise in neonatal intensive care units, leading to synergistic ototoxic effects early in life (Li and Steyger 2009). The increased susceptibility in this situation may result more from the combined insults rather than young age, however.

Mutations in the mitochondrial genome also play a critical role in increasing susceptibility to ototoxicity in patients with predisposing genetic makeup. The best described mutation involves the A1555G mutation in the 12S ribosomal RNA, where patients may develop profound deafness after a single injection of aminoglycoside (Prezant et al. 1993). The vestibular system is not involved in this hypersusceptibility to aminoglycosides (Tono et al. 2001). Mitochondrial mutations are thought to be present in approximately 20 % of patients who suffer hearing loss after aminoglycoside therapy.

10.3 Experimental Models

Advances in understanding, preventing, and treating aminoglycoside ototoxicity are predicated on experimental models that reliably mirror the morphologic and functional features of injury in human patients. Although human temporal bones afford a valuable resource for study of aminoglycoside ototoxicity, available tissue is limited and the

practical obstacles to design and execution of randomized, blinded clinical trials are formidable. In studies of the inner ear, animal models are critical due to the relative inaccessibility of the vestibulocochlear structures, the paucity of tissue that make up the sensory epithelium, and need to probe nuances of anatomy, physiology, and function in research. A wide range of animal models have been developed, each with inherent strengths and limitations. This section reviews the range of injury models, pathological correlates, and considerations of cellular uptake and pharmacokinetics.

10.3.1 Pathology of the Inner Ear After Aminoglycoside Therapy

Human temporal bones are the primary basis for current understanding of the inner ear pathology associated with aminoglycoside-induced ototoxicity. Studies in mice, rats, chinchillas, gerbils, and hamsters have all expanded upon the findings observed in human temporal bones. It is generally accepted that the sensory hair cells are the primary targets of ototoxicity, correlating closely with the extent of cochlear and vestibular functional loss (Ruedi et al. 1952). The ototoxic injury has distinct features in each portion of the inner ear.

10.3.2 Pathology of the Vestibular System

Streptomycin, the first aminoglycoside used clinically, had preferential vestibular toxicity. The pathology is first apparent in the apex of the cristae and the striolar regions of the maculi (Lindeman 1969). Impairment of vestibular function is primarily due to the loss of type I and type II hair cells, however. Hair cell loss moves peripherally, with type I hair cells affected initially, followed by the type II hair cells. The otoconial membrane and otolith also may be injured by aminoglycosides. Although regeneration of vestibular hair cells has been observed in mammals (Forge et al. 1993, 1998), it is uncertain whether such regeneration takes place in humans.

10.3.3 Pathology of the Cochlea

Aminoglycoside cochleotoxicity involves a gradient of injury that begins in the hook region and progresses through the basal turn into the cochlear apex. The aminoglycosides also induce a more subtle lateral injury gradient as well, with injury or loss of hair cells in the innermost position preceding hair cell death in the second and third rows. With intensive or prolonged aminoglycoside therapy, the injury involves other parts of the organ of Corti, eventually replacing the organ with a flat epithelium—a layer of epithelial scar tissue devoid of the distinctive architecture and sensory components that make up the normal hearing organ (Hawkins 1976). Aminoglycosides also have effects on other structures, including thinning of the stria vascularis (Ruedi et al. 1952) with fewer marginal cells (Hawkins 1973). More recent studies suggest that the ribbon synapses of inner hair cells are particularly

susceptible to aminoglycosides and may be an early target of these drugs (Liu et al. 2013). We are not aware of studies showing decreased auditory brainstem response amplitude in the absence of threshold shift at low doses or at low test frequencies in animal studies with aminoglycosides.

10.3.4 Pathology of the Spiral Ganglia

The effects of aminoglycosides on the spiral ganglia remain a source of some contention. It has generally been assumed that degeneration of spiral ganglion cells after aminoglycoside therapy was secondary to loss of hair cells (Hawkins 1976); nonetheless, other work indicates that degeneration of the spiral ganglia may occur without hair cell loss (Hinojosa and Lerner 1987; Sone et al. 1998). In some subjects rendered profoundly deaf by aminoglycosides, the spiral ganglia remain intact (Nadol 1997). Spiral ganglia degeneration may be ongoing, long after therapy has been discontinued (Leake and Hradek 1988; Webster and Webster 1981). In a guinea pig model using a combination of aminoglycoside and a loop diuretic to destroy hair cells, the loss of auditory neurons that otherwise occurs after the loss of auditory hair cells can be prevented by in vivo neurotrophin therapy with either NT-3 or BDNF (Staecker et al. 1986).

10.3.5 Animal Models of Ototoxicity

A variety of experimental animal models of aminoglycoside ototoxicity have been developed, including mice, rats, chinchillas, gerbils, and hamsters. The inner ear pathology is similar to that observed in humans, involving a base to apex gradient in loss of outer hair cells of the cochlea. As with humans, the auditory deficit initially affects high frequencies and then progresses to lower frequencies with either higher doses or longer courses of aminoglycoside treatment.

A variety of physiological and functional changes accompany these pathological changes. There is progressive hearing loss beginning in the high-frequency range, elevation of compound action potential and cochlear microphonic output (van Ruijven et al. 2005), and deterioration of distortion product otoacoustic emissions, which are indicative of outer hair cell dysfunction. During early treatment, aminoglycosides seldom induce changes in endocochlear potentials (Komune et al. 1987), but prolonged treatment is associated with thinning of the stria vascularis. Strial changes can also occur without hair cell damage (Forge and Fradis 1985).

Although the guinea pig and Mongolian gerbil were formerly the cardinal animal models for studying aminoglycoside-induced hearing loss, the mouse has become a particularly valuable research model with advances in molecular biology. Initial efforts in developing the murine model were plagued by difficulty in inducing auditory or vestibular deficits without necrosis or nephrotoxicity. High doses of aminoglycoside were necessary in mice but eventually yielded a typical pattern of base to apex loss of hair cells and high-frequency threshold (Blakley et al. 2008; Poirrier

et al. 2010; Taylor et al. 2008; Wu et al. 2001). Despite the requirement for high dosing, serum levels of drug were similar to that in the guinea pig, suggesting more rapid elimination of drug. Experimental models that combine aminoglycosides with loop diuretics are also used to allow for models of complete ablation of outer hair cells. Animal models frequently use very high doses of aminoglycosides in order to obtain hearing loss and cochlear damage, and there are significant differences in species, susceptibility to aminoglycoside ototoxicity. For example, gentamicin produced less hearing loss in mice, even though the dose utilized was well above the lethal dose for humans (Blakley et al. 2008). An unresolved question is whether rodent models for ototoxicity are translatable to humans.

Investigators can adapt animal models along any of several parameters to achieve the desired level of severity of injury. For example, injury models may vary the amount of drug given per dose (mg/kg dosing), dosing schedule (once per day versus more often), duration of therapy (single dose versus multiple days), route of delivery (subcutaneous, intramuscular, or intraperitoneal), and possible combination with other drugs (such as use of loop diuretics to potentiate aminoglycoside ototoxicity). The approach selected depends on the experimental question. In some models, a complete loss of sensory hair cells is desired, whereas a less severe, gradient-type injury is often useful for studies of otoprotection. The combination of loop diuretic with aminoglycoside can induce catastrophic hair cell loss (Schacht et al. 2012). In some cases, animals receive higher doses or longer duration of therapy in order to achieve maximal damage, but the model depends on the goals of the investigator. The extent of hearing and vestibular impairment observed in human subjects after aminoglycosides spans a wide range, due to different aspects of dosing, duration, schedule, and individual susceptibility (Rybak and Brenner 2014).

Recently, the zebrafish has attracted interest as a research model of understanding hair cell biology and for drug screening. The zebrafish possesses mechanosensory hair cells in the neuromasts of its lateral line that are susceptible to aminoglycosides, similar to mammalian auditory hair cells (Ou et al. 2007; Williams and Holder 2000). The hair cells provide the fish information from water movement around the body, with water motion inducing either depolarization or repolarization. Although the different functional role of the neuromast hair cells raises some questions about applicability to the inner ear, the zebrafish's small size, ease of breeding, and external location of hair cells all make it highly conducive to studies of hair cell injury, protection, and regeneration. Recently, this model was used in drug screening experiments, demonstrating that quinolone ring derivatives confer protection against aminoglycoside-induced hair cell death in the zebrafish model (Ou et al. 2012).

10.3.6 Cellular Uptake and Pharmacokinetics

Aminoglycosides achieve peak levels in the serum at approximately 60 min with a half-life of 2–6 h. They exhibit minimal serum protein binding and undergo renal excretion with negligible modification. Aminoglycosides are detectible in the inner ear fluids within minutes after systemic administration (Tran Ba Huy et al. 1986), reaching levels

about 10 % of peak serum concentrations (Henley and Schacht 1988). Aminoglycosides undergo rapid uptake into the cochlea, primarily in the hair cells (Dai et al. 2006), although aminoglycosides also accumulate into other cells of the ear (Imamura and Adams 2003). The drugs have delayed, biphasic, clearance (Tran Ba Huy et al. 1986). Gentamicin has been demonstrated in hair cells at 11 months after treatment (Dulon et al. 1993). There is little correlation between aminoglycoside uptake/accumulation and extent of toxicity, however. Hair cells may have an inherent increased sensitivity, particularly outer hair cells in the basal turn of the cochlea (Sha et al. 2001).

Much research has focused on uptake of aminoglycosides into hair cells, and the prevailing notion is that multiple mechanisms occur simultaneously. Early studies in the guinea pig with kanamycin showed lysosomal accumulation in the subcuticular region, suggesting endocytosis at the hair cell apices (Darrouzet and Guilhaume 1974). Subsequent studies confirmed drug initially present at the hair cell apex (de Groot et al. 1990; Hashino et al. 1997; Hiel et al. 1992), which was further corroborated by studies of myosin VIIA mutants, which do not take up aminoglycosides in organ of Corti preparations (Richardson et al. 1997). Other possible modes of entry are polyamine-like transport (Williams et al. 1987) or receptor-mediated drug transport (Lim 1986). Megalin, a glycoprotein involved in renal transport (Moestrup et al. 1995), has been investigated but expression of megalin may be absent in hair cells; in general, the cochlear expression does not match known patterns of drug uptake and toxicity (Mizuta et al. 1999).

Several studies have focused on ion channels, often using fluorescently tagged aminoglycosides (Arbuzova et al. 2000; Dulon et al. 1989) to investigate the entry and localization of drug over time. Such studies demonstrate swift uptake into sensory cells (Dai et al. 2006). Interestingly, the pattern of a differential gradient for basal to apical uptake did not persist beyond 3 h. More recently, attention has focused on ion channels in hair cells (Marcotti et al. 2005). Transient Receptor Potential channels (trpv1, trpv4, trpa1, and trpp1) are nonselective cation channels that appear to permit aminoglycoside entry (Stepanyan et al. 2011). Mechanoelectrical transduction (MET) channels are necessary for aminoglycoside ototoxicity (Alharazneh et al. 2011). A link between onset of mechanoelectrical transduction and susceptibility to aminoglycoside toxicity has been postulated, although zebrafish lateral line experiments indicate they are distinct (Santos et al. 2006). Potassium channel inhibition has also been postulated to play a role in toxicity, whereby aminoglycosides induce ototoxicity by depleting phosphoinositides (Leitner et al. 2011). Aminoglycoside antibiotics have long been known to bind phosphoinositides strongly (Schacht 1979), to deplete phosphatidylinositol trisphosphate in murine hair cells (Jiang et al. 2006b).

10.4 Oxidative Stress and Pathways

Oxidative stress plays a pivotal role in modulating cellular homeostasis (for detailed discussion, see Chap. 2 by Leeuwenburgh). ROS are found at low levels in all cell types, neutralizing threats, functioning as chemical signals and messengers, or present as byproducts of metabolism. Aminoglycoside-induced ROS can produce a

destabilizing redox imbalance, however. Such redox imbalance is now believed to underlie the pathogenesis of diverse forms of inner ear trauma including noise, age, and drug-induced hearing loss (for review, see Chap. 4 by Seidman and Shirwany; see also Chap. 7 by Altschuler (noise), Chap. 13 by Someya (age), and Chap. 11 by Laurell (cisplatin)). Preservation of cellular integrity and survival rely upon the delicate balance of ROS and mechanisms for their removal. The mechanism of ROS likely differs, however. For example, calcium dysregulation may be most applicable to noise-induced hearing loss, deletions of mitochondrial DNA to aging, and mitochondrial ribosome targeting in the case of aminoglycosides.

10.4.1 Reactive Oxygen Production by Aminoglycosides

Early studies in the 1950s to 1960s provided initial evidence of the role of oxidative stress in mediating the toxic effects of aminoglycosides, as reflected in the ability of 2,3,-dimercaptopropranol to decrease streptomycin ototoxicity (Schacht et al. 2008). Subsequent work showed that free radical scavengers could confer protection from kanamycin and other aminoglycosides (Clerici et al. 1996; Hirose et al. 1997; Lautermann et al. 1995). A key observation was that gentamicin and iron could form redox-active complexes, resulting in reduction of oxygen to superoxide radicals (Sha and Schacht 1999a). Subsequent work showed that redox-active ternary complexes between Fe 2+/3+, gentamicin, and arachidonic acid are able to produce superoxide radicals, which can be converted to the highly reactive hydroxyl radical in the presence of iron (Lesniak et al. 2005). This, in turn, can result in formation of highly toxic peroxidation products. Reactive nitrogen species may also contribute to impaired homeostasis, although evidence for these reactions in hair cells is lacking.

Enzymatic mechanisms likely introduce additional ROS into the milieu after aminoglycoside therapy. Aminoglycosides activate redox pathways linked to Rho-GTPases (Jiang et al. 2006c). Rac-1 is a member of this Rho-GTPase family whose activity is increased in the presence of aminoglycosides in vivo and activates NADPH oxidase, which in turn increases formation of superoxide radicals. This observation is analogous to the stimulation of the cochlear-specific NADPH oxidase, NOX3, in models of cisplatin ototoxicity. Inducible nitric oxide synthase allows for production of nitric oxide as a second messenger and serves a variety of normal physiological functions. As a free radical, nitric oxide has an intrinsic capacity for damage but can also produce peroxynitrite when combined with superoxide, which is highly reactive and potentially destructive.

Several protective mechanisms exist within cells to prevent injurious oxidative effects, but these can be exhausted or depleted. Among the key protective elements are glutathione, superoxide dismutase, catalase, and peroxidases. The adverse effects of reactive oxygen and nitrogen species are counteracted through a variety of mechanisms. In some cases (e.g., glutathione), the ROS may be scavenged. In other cases, the ROS are kept in check through enzymatic actions. When these defenses are overwhelmed, redox imbalance occurs. Such disruptions may be due to depletion of copper and selenium required for superoxide dismutase and peroxidase function, inactivation of the

enzymes, or depletion of glutathione and NADPH. The resulting loss of homeostasis leads to activation of cell death pathways as described in subsequent sections.

10.4.2 Mitochondrial Factors and Oxidative Stress

Given that protein synthesis inhibition by aminoglycosides is linked to ototoxicity, an important mechanistic question is whether mitochondrial or cytoplasmic protein synthesis inhibition is the primary culprit in injury to the inner ear. Aminoglycosides induce oxidative tissue stress and damage in mammalian cells, with evidence of mitochondrial dysfunction, increased ROS formation, and increased expression of genes involved in antioxidant defense (Kalghatgi et al. 2013). A recent study (Shulman et al. 2014) showed in mammalian subjects that aminoglycosides inhibited mitochondrial protein synthesis, disrupted cellular respiration, and ultimately led to cell death. The underlying mechanism implicated the Fenton reaction, with an increase in superoxide overproduction, oxidative damage of mitochondrial aconitase, and accumulation of free ferrous iron ion. The degree of inhibition of the mitoribosome (and resulting oxidative stress) correlated with injury to inner ear structures in explants and functional impairment in guinea pig in vivo models.

These findings are consistent with the dramatically increased susceptibility to ototoxicity in patients that harbor the A1555G mitochondrial mutations (Prezant et al. 1993) The aminoglycoside-susceptibility A1555G allele alters the morphology of the mitochondrial 12S ribosomal subunit, making it more similar to bacterial ribosomal RNA and hence more susceptible to aminoglycoside-induced protein misreading (Matt et al. 2012). The A1555G mutation is thought to account for approximately 20 % of cases of deafness in patients with aminoglycoside ototoxicity (Fischel-Ghodsian 2005) and is found in all ethnic groups. Other mitochondrial mutations have been identified, although they are much rarer. The aminoglycoside apramycin had low affinity for eukaryotic ribosomes (including ribosomes with the 1555G susceptibility mutation) but high antimicrobial potency. The findings suggest that aminoglycoside ototoxicity is unrelated to NMDA receptor activation, given that apramycin induces NMDA receptor activation similar to neomycin, a far more ototoxic aminoglycoside (Harvey et al. 2000), yet does not induce any hearing loss (Matt et al. 2012).

10.4.3 Pathways of Cell Death

Redox imbalance typically leads either to protective pathways (in the early stages of drug-induced hearing loss), to programmed cell death (Jiang et al. 2005), or to necrosis (Nakagawa et al. 1998). A variety of pathways to cell death have been proposed, including the c-Jun N-terminal kinases (JNK), caspase cascades (Eshraghi et al. 2010; Ylikoski et al. 2002), nuclear translocation of endonuclease G (Endo G), and activation of μ-calpain (Fee 1980; Jiang et al. 2006a).

10.4.4 Caspase Activation, Bcl-2 Family, and p53

Evidence for caspase-mediated pathways in response to aminoglycoside therapy is limited, due to a paucity of in vivo data; but, the bulk of evidence suggests that caspase-9 is the major signal for caspase-mediated apoptosis. The mechanism involves formation of an apoptosome complex with cytochrome C release from the mitochondria. Caspase-3 and caspase-9 inhibition conferred protection. However, caspase inhibitors have not been consistently successful in preventing aminoglycoside-induced cell death (Momiyama et al. 2006; Tabuchi et al. 2007). For example, inhibition of caspase-8 failed to provide protection from cell death (Cunningham et al. 2002; Tabuchi et al. 2007). Caspase-3 activation is the classic criterion for canonical apoptosis, and in vivo evidence for caspase pathways comes from single high-dose gentamicin treatment of the chick basilar papilla or vestibular cultures of the guinea pig vestibular system (Mangiardi et al. 2004; Shimizu et al. 2003), as well as a chronic rat model of caspase-3 inhibition (Ladrech et al. 2004).

The anti-apoptotic protein Bcl-2 and Bcl-X_L protect from aminoglycoside toxicity (Cunningham et al. 2004; Pfannenstiel et al. 2009; Staecker et al. 2007). The Bcl-2 family also includes pro-apoptotic factors from the Bax and Bak subfamily and the BH-3 group. This finding supports a role of mitochondria in aminoglycoside cell death pathways. When mitochondrial permeability transition pores form, cytochrome c is released (Mangiardi et al. 2004; Matsui et al. 2004). Cyclosporin A inhibits mitochondrial permeability transition pore formation and thereby mitigates aminoglycoside toxicity in vitro (Dehne et al. 2002). The role of tumor suppressor p53 in aminoglycoside-induced cell death has not been well-characterized, but its role is better delineated in cisplatin models. In cisplatin models, deletion of the p53 gene prevents cytochrome c translocation, caspase-3 activation, and hair cell death (Cheng et al. 2005). P53-mediated cell death appears mediated by STAT-1 in cisplatin ototoxicity (Schmitt et al. 2009). The p53 protein can upregulate Bax. Alternatively, p53 can translocate to the mitochondria resulting in more direct injury with loss of membrane potential and cell death.

10.4.5 Caspase-Independent Pathways

In aminoglycoside ototoxicity, apoptosis and necrosis may also occur through caspase-independent pathways, particularly in chronic models of treatment that are similar to those used in human patients. In experimental models, the onset of hearing loss is typically delayed (>1 week after initiation of therapy) and may progress after cessation of therapy. The events include endonuclease G translocation to the nucleus with activation of μ-calpain. Lysosomal destabilization and rupture results in release of cathepsins into cytoplasm, leading to cleavage of the DNA repair enzyme PARP1 and necrotic cell death. In utricle explants, calpain inhibition with leupeptin decreased hair cell loss (Ding et al. 2002).

10.4.6 JNK Pathways

The JNK/mitogen-activated protein kinase (MAPK) pathway has also been implicated in cell death, primarily based on studies in organ of Corti culture. Aminoglycoside-induced cellular stress induces activation of c-Jun and other transcription factors of the JNK pathway (Maroney et al. 1998; Ylikoski et al. 2002), with partial attenuation of ototoxicity with JNK inhibition in the guinea pig in vivo (Ylikoski et al. 2002) and in vitro (Bodmer et al. 2002; Eshraghi et al. 2010; Pirvola et al. 2000; Wang et al. 2003). The JNK pathway may be induced by G-proteins Rho, and Ras; Ras inhibitors reduce activation of c-Jun and attenuate gentamicin-induced toxicity (Battaglia et al. 2003). Inhibition of Rac, Rho, and Cdc42 with *Clostridium difficile* toxin B is also protective (Bodmer et al. 2002).

10.4.7 Epigenetic Factors

Influences may also arise from influencing the relative accessibility of DNA for expression, for example, by altering acetylation of histone core proteins (Mohtat and Susztak 2010), or other post-translational modifications such as methylation, ubiquitination, and sumoylation. With aminoglycoside treatment, histone acetylation decreases in outer hair cells due to an increase in histone deacetylase levels, and histone deacetylase inhibitors may protect hair cells (Chen et al. 2009).

10.5 Protection and Prevention: From Bench to Bedside

Several strategies are available to decrease the ototoxic side effects of aminoglycosides. The most common clinical approach is to carefully monitor administration of aminoglycosides and screen for functional impairment. Pharmacokinetic consultations and monitoring of peaks and troughs along with renal function may help detect fluctuations in drug level. High-frequency audiometry is ideal for detecting early auditory toxicity. Such approaches are resource intensive, however, and are logistically challenging to implement. The measures are also of debatable value in cases where drug therapy must continue regardless of the toxicity. Most laboratory efforts have focused on either enhancing protective pathways or attenuating cell death pathways. Selective prevention of entry of drug into susceptible hair cells might protect vestibulocochlear structures while preserving therapeutic efficacy. Most recently, the identification and/or development of aminoglycosides that have decreased mitochondrial affinity have shown promise for optimizing the balance of efficacy relative to potential toxicity.

10.5.1 Antioxidants

Antioxidant therapy is arguably the most clinically investigated strategy for protection from aminoglycoside-induced ototoxicity. One appealing feature of antioxidants is that many such compounds are readily available with favorable safety profiles and

encouraging evidence for protection in in vitro studies, animal experiments, and a limited number of human studies. A key feature is that antioxidants appear not to interfere with the antimicrobial efficacy of aminoglycosides. *N*-Acetylcysteine (NAC) decreased the incidence of hearing loss in hemodialysis patients receiving gentamicin (Feldman et al. 2007). In contrast, vitamin E appeared to protect against aminoglycoside toxicity in guinea pigs (Fetoni et al. 2004) but failed to afford protection in humans (Kharkheli et al. 2007). D-methionine afforded protection in animal studies without affecting gentamicin serum levels (Sha and Schacht 2000). For a current review of gentamicin otoprotection, see Le Prell et al. (2014).

Drawing upon promising demonstration of otoprotection with salicylate in guinea pigs (Sha and Schacht 1999b), the effects of aspirin were investigated in human subjects receiving gentamicin for acute infections. The design was a randomized double-blind placebo-controlled trial (Sha et al. 2006), and found significant protection, with 14 of 106 patients experiencing hearing loss in the placebo group versus 3 of 89 patients in the aspirin group. An independent clinical trial confirmed benefit (Behnoud et al. 2009). Limitations of aspirin include (small) risks of hemorrhage and gastrointestinal symptoms, as well as contraindications in young patients due to risk of Reye's syndrome. Other antioxidant agents explored for protective effects have been vitamins (A,C,K, B-complex) with variable success, as well as other amino acids, steroids, antibiotics, and naturally occurring agents such as methylene blue.

10.5.2 Development and Characterization of Non-ototoxic Aminoglycosides

Development of aminoglycosides that do not induce ototoxicity is an important goal for antimicrobial therapy. Such aminoglycosides would thus induce bacterial ribosomal dysfunction (and death of the prokaryotic bacteria) while sparing the function of the eukaryotic cell's mitochondrial ribosomal function, and hence allowing for high efficacy with low toxicity. In keeping with the endosymbiont hypothesis, the mitochondrial and bacterial ribosomes are structurally similar and thought to share similar lineage. Identifying aminoglycosides that have high affinity for the bacterial ribosome but low affinity for the mitoribosome is a strategic approach to drug design. Apramycin, an aminoglycoside primarily used for veterinary purposes that has broad spectrum antimicrobial activity, has little effect on eukaryotic ribosomes and low ototoxicity but is bactericidal, thus dissociating ototoxicity and antimicrobial efficacy (Matt et al. 2012).

New application of aminoglycoside therapy involves mitigating the devastating effects of certain genetic diseases resulting from nonsense mutations. As previously mentioned, stop codon suppression is particularly attractive in cases where mutations lead to truncated proteins (Hainrichson et al. 2008). These approaches exploit the ability of aminoglycosides to achieve readthrough of premature stop codon mutations such that premature translational termination is decreased. Synthetic aminoglycosides that preferentially inhibited cytoplasmic ribosomes over mitochondrial ribosomes did not show the deleterious effects of aminoglycosides in mammalian cells. The lower affinity

for the mitochondrial ribosome correlated with lower ototoxic potential, both in murine cochlear explants and the guinea pig in vivo, suggesting the usefulness of this approach in developing aminoglycosides for "readthrough therapy" (Shulman et al. 2014).

10.5.3 Additional Approaches on the Horizon

A wide range of other approaches may afford further translational potential. Emerging approaches in inner ear research include use of short-interfering RNAs (siRNA), which may have utility in gene knockdown or use of gene delivery paradigms. Strategies that prevent uptake of drug, delivery via transtympanic routes for localized protective therapy, and agents that provide broad anti-apoptotic effects, such as caspase inhibitors all may have a potential role. In recent years, DNA editing strategies using CRISPR (clustered regularly interspersed short palindromic repeats)—Cas9 system has emerged as a powerful tool that can be used to insert, remove, or otherwise alter DNA sequences and their expression (Barrangou 2014; Horvath and Barrangou 2010; Wiedenheft et al. 2012). The practical applications in the auditory system are just beginning to be explored.

10.6 Conclusion

Reduction of aminoglycoside-induced ototoxicity is feasible, and the key questions pertain to how best to realize this objective. Whereas current approaches emphasize monitoring, the role of antioxidants is expanding. One of the clinical challenges encountered is that the clinical practitioners who administer antibiotics are seldom the same practitioners who treat ototoxic complications, and hence awareness is limiting. The development of aminoglycosides with reduced ototoxic potential is therefore an important strategy. It has been suggested that the science of implementation is at least as challenging as the science of discovery in efforts to improve human health and minimize toxicity.

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